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Inhibition of ErbB2 and Thymidylate Synthase by a Multi-Targeted Small-Interfering RNA in Human Breast Cancer Cell Lines

Rebecca Stephanie Hunter
Yale University

INHIBITION OF ERBB2 AND THYMIDYLATE SYNTHASE BY A MULTI-TARGETED SMALL-INTERFERING RNA IN HUMAN BREAST CANCER CELL LINES

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by

Rebecca Stephanie Hunter

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List of Abbreviations

AMD	Age-related macular degeneration
ALL	Acute lymphoblastic leukemia
CML	Chronic myelogenous leukemia
DHFR	Dihydrofolate reductase
dsRNA	Double-stranded RNA
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
EGFR	Epidermal growth factor receptor
GIST	Gastrointestinal stromal tumor
MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
MTA	Multi-targeted antifolate
NSCLC	Non-small cell lung cancer
nt	Nucleotide
ORN	Oligoribonucleotide
PDGFR	Platelet-derived growth factor receptor
RISC	RNA-induced silencing complex
RNAi	RNA interference
RTK	Receptor tyrosine kinase
RT-PCR	Reverse-transcriptase PCR
siRNA	Small-interfering RNA
TKI	Tyrosine kinase inhibitor
TS	Thymidylate synthase
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
5-FU	5-Fluorouracil

Abstract

The therapeutic potential of a novel multi-targeted small-interfering RNA (siRNA) was investigated in human breast cancer cells. Previous studies had identified an siRNA that specifically and potently inhibited expression of thymidylate synthase (TS) by directly targeting human TS mRNA. TS is a folate-dependent enzyme that catalyzes the key reaction involved in synthesizing nucleotide precursors for DNA biosynthesis, and as such, it plays a critical role in maintaining cell growth. The goal of this thesis was to design and develop a novel siRNA molecule that targeted TS mRNA as well as a cellular mRNA that encodes a different cellular protein involved in cancer cell growth and proliferation, such as a member of the ErbB family. Gene sequence analysis was performed and identified an overlapping sequence between TS and ErbB2 mRNAs. An siRNA duplex was then designed to simultaneously target human TS and ErbB2 mRNA. Transfection of the multi-targeted siRNA (TS1M17) revealed that both ErbB2 and TS proteins were significantly suppressed in a time and dose-dependent manner in ErbB2-overexpressing human breast cancer SKBR3 cells. The corresponding mRNA levels, as determined by RT-PCR, were also decreased. Protein levels of other ErbB family members, including ErbB1 and ErbB3, remained unchanged with siRNA treatment. An ErbB2-specific siRNA (B2450) inhibited ErbB2, but had no effect on TS expression demonstrating the specificity of the multi-targeted siRNA against both TS and ErbB2. Mismatched (TS1-Mismatch) and control (GL2) siRNAs had no inhibitory effects on expression of the two target proteins. Suppression of activated ErbB2, as determined by expression of phosphorylated ErbB2 protein, was observed with transfection of TS1M17 siRNA. In addition, the expression of downstream signaling proteins, such as

phosphorylated mitogen activated protein kinase (p-MAPK), $p27^{Kip1}$, $p21^{Cip1}$, cyclin D1, and survivin were significantly changed. In contrast, control siRNAs did not exert any inhibitory effects on downstream signaling. Taken together, these findings suggest that TS1M17 siRNA inhibits signaling of the ErbB2 pathway. The effect of TS1M17 siRNA on cytotoxicity was analyzed by WST-1 assay. Upon transfection into SKBR3 cells, the TS1M17 siRNA significantly suppressed cell proliferation with an IC_{50} value of 0.65 nM, which is 154-fold more potent than ErbB2- and TS-specific siRNAs. This study suggests that targeting expression of ErbB2 and TS, two key proteins involved in distinct and critical pathways for cancer growth and proliferation, with a single siRNA molecule may provide a novel approach for cancer chemotherapy.

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Introduction

Cancer is the second leading cause of death in the United States. The American Cancer Society recently reported 1,400,000 new cases of cancer and 570,000 cancer-related deaths in 2006. With recent improvements in public awareness, screening and detection, the rate of cancer deaths has been trending down since the 1990's. Another major contribution to reducing cancer-related deaths has been the development of new cytotoxic and biologic agents (1). Clinical progress in chemotherapy has been fueled by our enhanced understanding of the molecular and genetic basis for cancer and cancer biology. Significant efforts have focused on characterizing key signaling networks that regulate cellular activities involved in cellular survival, invasion/metastasis, and angiogenesis. Many of these networks are found to be radically altered or upregulated in neoplastic cells versus normal host tissues. By the early 1990's, research developing effective agents that specifically target defective signaling pathways in cancer cells led to the era of "targeted therapy" in oncology (2). These new targets include growth factor receptor pathways, modulators of the cell-cycle, proteins involved in apoptosis, and signaling molecules responsible for angiogenesis.

Despite the recent successes in cancer drug development, there remain a number of serious limitations. First, cancer is a heterogeneous disease. Second, despite their specificity, targeted therapies still show toxic side effects that limit their potential clinical use. Finally, the well-studied phenomenon of acute and chronic development of resistance persists. Therefore, understanding the molecular features of human tumors,

improving the overall safety profile, and elucidating the mechanisms of cellular drug resistance continue to be key aspects of cancer research. Such efforts are essential as we continue the effort to improve cancer-related morbidity and mortality.

Thymidylate Synthase as a Chemotherapeutic Target

Thymidylate synthase (TS) is a folate-dependent enzyme that catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (Figure 1). Once synthesized, dTMP is metabolized intracellularly into deoxythymidine triphosphate (dTTP), which is a key nucleotide for DNA replication and repair (3). Although these DNA precursors can be formed through the salvage pathway, a mechanism catalyzed by thymidine kinase, the reaction catalyzed by TS provides the only intracellular *de novo* source of dTMP. Inhibition of this reaction results in immediate blockade of cellular proliferation and growth. Given the central role that TS plays in cellular proliferation, TS has been an attractive target for cancer chemotherapy (4).

In 1957, the synthesis of 5-fluorouracil (5-FU) was first reported by Heidelberger et al. based on the finding that rat hepatoma cells utilized uracil more efficiently than normal tissue cells (5). Research at that time led to the development of novel fluoropyrimidine compounds which targeted essential biosynthetic processes involved in the growth and proliferation of cancer cells (6). 5-FU exerts its anticancer effects through various mechanisms, including inhibition of thymidylate synthase (TS), incorporation of 5-FU metabolites into DNA, and incorporation of 5-FU metabolites into RNA. While the

precise molecular mechanism remains unclear, there is now a large body of evidence supporting the view that inhibition of TS is a necessary prerequisite for cancer cell death. For the last 50 years, the fluoropyrimidines, and in particular 5-fluorouracil (5-FU), have become the backbone of treatment for a wide range of human malignancies, including colorectal, gastrointestinal, breast, and head and neck cancer (7).

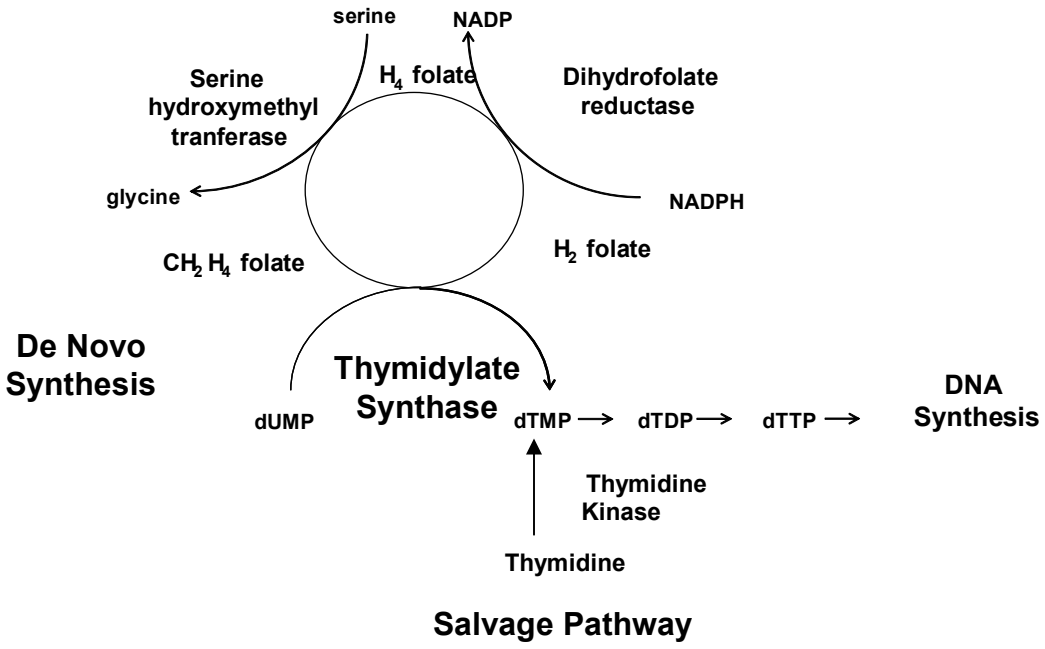

Figure 1. Enzyme reaction catalyzed by TS.

There are two major classes of drugs targeting TS: fluoropyrimidines, which include 5-FU and the oral prodrug capecitabine, and the antifolate TS inhibitors, raltitrexed, OSI-7904L, and pemetrexed. Both drug classes play a pivotal role in the treatment of a wide range of human solid cancers. In particular, these agents have shown their greatest clinical activity in patients with colorectal cancer (8). However, as with all chemotherapeutic regimens, mechanisms of innate and induced drug resistance continue

to limit their clinical use. Several studies have documented a strong correlation between increased TS enzyme levels and resistance to fluoropyrimidines. Innate resistance to fluoropyrimidines has also been observed in patients with breast and colon cancer whose tumors express high basal levels of TS. These patients have subsequently been shown to be less responsive to 5-FU therapy (9-11).

The molecular pathways involved in the development of acquired drug resistance are complex and their understanding provides a foundation to improve current chemotherapeutic regimens. There are now known to be different intracellular mechanisms involved in the development of resistance in chronic and acute drug exposure. In both settings it has been noted that exposure to 5-FU results in an increase in TS enzyme level (12,13). It has been hypothesized that the induction of TS protein is a mechanism for acquired resistance to fluoropyrimidines (12,14). The cellular events that can be altered to induce protein are found at all levels of cellular regulation from gene amplification to post-translational modifications. Studies have shown that cancer cells under continuous exposure to 5-FU treatment have increased level of protein secondary to amplification of the TS gene and increased transcription (15,16). Given the processes of gene amplification, transcription and post-transcription require a cascade of intracellular pathways, it is logical that these mechanisms leading to increased protein expression would require more time and only be seen in the setting of long-term drug exposure. Exposure to fluoropyrimidines in *in vitro* and clinical model systems, results in a rapid induction of TS enzyme level (17,18). The prompt increase in TS enzyme level must rely on cellular processes that do not require time-consuming intracellular signaling.

The acute induction of TS must therefore be modulated by immediate changes in mRNA translation and/or protein modifications. A complete understanding of the regulatory elements involved in the acute induction of TS after exposure to chemotherapy would provide a rational basis to study therapeutic strategies for patients with 5-FU resistant tumors and prevent further development of resistance.

Studies noting the importance of translational regulation in drug-mediated TS protein induction were conducted by Chu et al., who showed that short term treatment of human colon cancer cells with 5-FU resulted in acute increases in both TS enzyme activity and protein expression that were not associated with a corresponding change in TS mRNA levels (19). Given the regulation of TS expression after exposure to TS-inhibitors seemed to be partly regulated by translational control, the regulation of TS mRNA was further elucidated. Subsequent work showed that, in addition to its catalytic activity, TS is an RNA binding protein that negatively regulates translation of TS mRNA (20-22). The first site of binding is located within a region containing the translational start site, and the second site is contained within nucleotides 434 to 634 in the protein-coding region of TS mRNA. RNA binding studies revealed that TS binds maximally to its own TS mRNA when it is in a ligand-free state, resulting in complete translational repression of TS mRNA. Further studies demonstrated that when TS protein is bound to its physiological substrate, an antifolate substrate, or by the 5-FU metabolite, FdUMP, TS is unable to bind to its own mRNA and suppress its own mRNA translation, thereby leading to increased synthesis of TS protein. The current working model for translational autoregulatory control of TS mRNA is presented in figure 2. The binding of TS to its own mRNA is an

efficient mechanism for regulating intracellular levels of TS. This process allows the cell to respond rapidly to cytotoxic exposure and ensure the desired level of TS protein for cell growth and proliferation. This mechanism provides a rational explanation for the acute induction of TS protein seen in cancer cells upon drug exposure and a possible etiology for the rapid development of drug resistance (16,23).

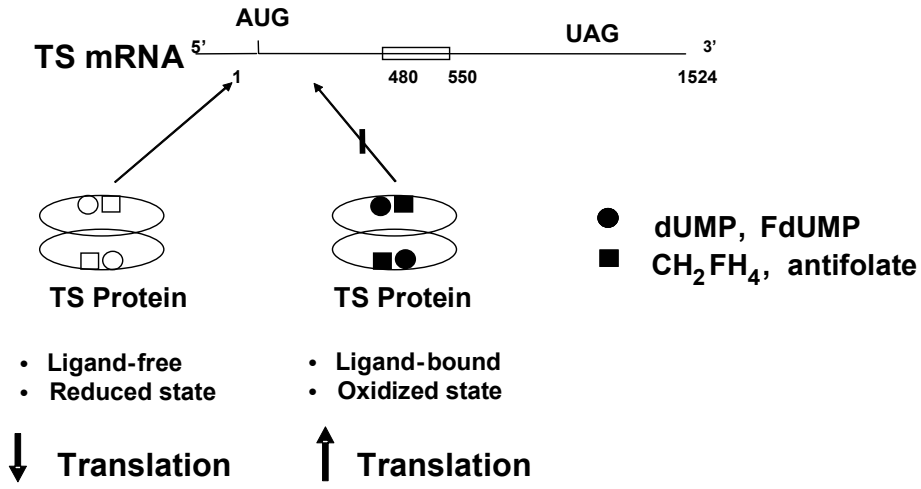

Figure 2. Model of TS translational autoregulation.

Given the current understanding of TS translational regulation as outlined above, alternative strategies have been developed to target TS expression. One approach would include developing small molecules that inhibit TS enzyme activity but do not affect binding activity to TS mRNA, thereby circumventing translational induction of TS protein. Another approach would be to develop molecules that directly target sequences on TS mRNA, resulting in translational inhibition and/or RNA degradation. Such an

approach would include the use of small molecules, antisense oligoribonucleotides (ORNs), ribozyme-based molecules or short interfering RNAs (siRNAs) that directly target TS mRNA. Studies by Schmitz et al. showed that treatment of human colon cancer cells with 30-nt 2'-O-methyl antisense ORNs, targeting the 5'-upstream *cis*-acting element of TS mRNA, specifically inhibited TS expression in a time and dose-dependent manner (24). They found that antisense ORNs did not affect TS mRNA or the half-life of TS protein, suggesting that the mechanism of action of antisense ORNs was by translational arrest. Most importantly, the therapeutic potential of antisense ORNs was also studied. Preliminary cytotoxicity experiments showed that the 30-nt antisense ORN inhibited proliferation of human colon cancer RKO cells with an IC_{50} value of 200 nM. However, upon further testing, it was noted that control ORNs, including sense and mismatched sequences, also achieved IC_{50} values in the submicromolar range. Despite their effects on cell toxicity, these controls did not affect TS protein expression. Therefore, the cytotoxic effects of the 30-nt antisense ORN could not be fully attributed to TS protein inhibition. These nonspecific effects limited their potential use as therapeutic agents and led to additional research developing other potential molecules, such as TS-specific siRNAs.

RNA Interference

RNA interference (RNAi) is a recently discovered, gene-silencing process in which sequence-specific, double-stranded RNA (dsRNA) promotes the degradation of cellular mRNA encoded by targeted genes. This evolutionarily conserved process of gene silencing is now known to exist in a host of eukaryotic organisms. Although its biological

functions are still being elucidated, it is thought to be a regulatory adaptation of cells to mount specific attacks against invading nucleic-acid sequences such as those seen in viral infections as well as a mechanism of genomic protection from transposon insertion and translocation (25,26).

The discovery of RNAi was made when Fire et al. observed that gene silencing could be triggered in the nematode *Caenorhabditis elegans* by injection or feeding of dsRNA (27). In this system, silencing is systemic and spreads throughout the entire organism and in addition can be transmitted to their progeny. Their studies also showed that dsRNA was ten-fold more potent than either sense or antisense single strands alone as silencing triggers. The molecular process of RNAi is a simple mechanism in which long dsRNA is processed into siRNA of 21-25 nucleotides (nts) in length by a intracellular dsRNA-specific endonuclease known as Dicer and is depicted in figure 3. These siRNAs are then incorporated into a nuclease complex known as RNA-induced silencing complex (RISC). Finally, the RISC-siRNA complex becomes activated and is guided to its homologous target mRNA by Watson-Crick base pairing for cleavage and degradation by cellular nucleases (25).

At the time of its initial discovery, this potent mechanism of post-transcriptional gene silencing was limited to basic science research as an ideal functional approach to study specific genetic pathways in animal models such as *C. elegans* and *Drosophila*. Using RNAi in mammalian cells was initially of restricted use secondary to nonspecific responses of cells to long dsRNAs. This was until Elbashir and colleagues showed that

introduction of synthetic 21-nucleotide siRNA duplexes into mammalian cell lines could effectively suppress expression of endogenous genes without initiating nonspecific gene silencing pathways (28). Presently, RNAi has played a fundamental role in changing biological research and has unveiled a novel tool for human genomic study and further understanding of genetic pathways (29).

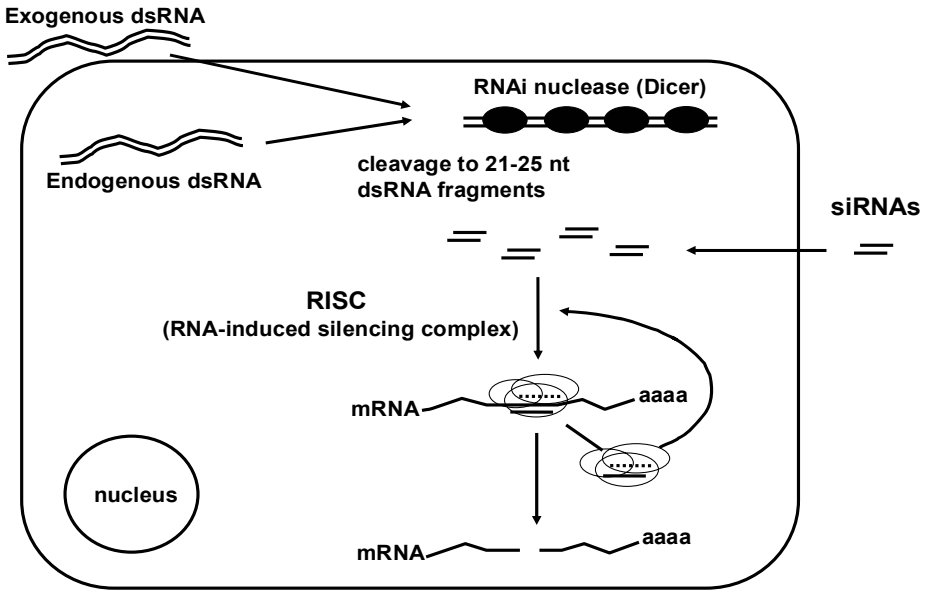

Figure 3. Model of RNA interference.

Harnessing RNAi has also extended into the realm of clinical medicine secondary to its enormous therapeutic potential. In an era where molecular etiologies of diseases are constantly being discovered, developing the use of siRNAs as inhibitors of disease-causing genes is an obvious application. Studies have already begun to explore the use of RNAi as a therapy for diseases such as hepatitis C and B, human immunodeficiency virus (HIV) infection, and age-related macular degeneration (AMD) (30-33). However, there

are several obstacles that must be overcome before siRNAs move from the laboratory bench to the clinic. An obvious limitation of using RNAi is its rapid susceptibility to nuclease degradation. Cellular uptake is also an issue, given siRNAs are negatively charged molecules that require a cationic transport system to efficiently be taken into cells. Another concern with cellular introduction of siRNAs is the potential nonspecific effects. The design of efficient siRNAs is an empirical process, given that the precise molecular mechanisms of RNAi are not fully understood. Different siRNAs show different levels of inhibition, and in practice, only some are effective in gene silencing (34). Many studies have shown that siRNAs with base-pair mismatches at specific locations on the 21-nucleotide duplexes can still bind to targeted mRNA and efficiently initiate target degradation. In fact, it has been shown that guanine (G): uracil (U) base pair mismatches, also known as "wobble mutations" are viable in RNA:RNA binding, and in particular siRNA:mRNA binding (34,35). In addition, studies have shown that base pairing between adenine (A) and cytosine (C) are also well tolerated in siRNA binding (36). Furthermore, studies have demonstrated that siRNAs with as few as 7 complimentary nts to the mRNA target can induce gene silencing. Given this flexibility, introduction of siRNAs can target unintended genes that share minimal homology to the desired targeted sequence. Of note, possible nonspecific siRNA effects may also arise from unintended induction of natural cellular defense mechanisms. Although it was initially thought that the small sized siRNA would bypass the immune system, recent studies have documented induction of interferon-α (IFN-α) and interleukin-6 (IL-6) in mouse serum when lipid-encapsulated siRNAs containing the sequences 5'-UGUGU-3' or 5'-GUCCUUCAA-3' were introduced systemically (37). Nucleoside modification and

proper sequence selection are two strategies currently being used to avoid the immunostimulatory effects of siRNAs.

Despite its current limitations, much is being learned about harnessing RNAi for clinical therapy. In fact, RNAi has already been introduced to the clinic for the treatment of AMD. Two siRNA molecules, Sirna-027 and Cand5, are already in phase I clinical trials. These siRNAs respectively target vascular endothelial growth factor receptor-1 (VEGFR-1) and vascular endothelial growth factor (VEGF) mRNA. The VEGF-signaling pathway, of which VEGF and VEGFR are two critical proteins, is a key mediator of angiogenesis, an important process that mediates the development of wet AMD. Interim data have shown a trend towards improvement of vision. If these siRNAs are found to be safe and effective, they have the potential to be approved by the FDA by 2008 as the first therapeutic siRNAs.

The use of siRNAs would also be a tremendous tool in cancer therapy and expectations for the development of new target-specific drugs remains high. Genetic mutations and overexpression of tumor-specific oncofusion proteins have been implicated in an increasing number of cancers including leukemia, colon and breast carcinomas, and melanomas. The specificity of RNAi makes it possible to silence these disease-causing mutant alleles or oncogenes without affecting the wild-type alleles, thereby bypassing the negative side affects often seen with traditional cytotoxic chemotherapy. Using RNAi to target genes such as *bcr-abl* and *Ras*, make it a valuable and novel therapeutic option for a broad range of human cancers (31-33).

Thymidylate Synthase-Targeted siRNA

Following the successful use of ORNs to inhibit TS expression by Schmitz and colleagues, further work was conducted to identify additional molecular approaches to target TS mRNA that would inhibit translation (24). The discovery of siRNA technology provided a direct mechanism for the degradation of TS mRNA and provided a novel approach to prevent TS protein expression as well as negative autoregulation of TS mRNA by its own protein end-product. Studies proceeded to elucidate the chemotherapeutic potential of siRNA in human cancer. By inhibiting protein expression at the mRNA level, it would be possible to prevent the development of drug resistance to TS-targeted compounds.

Schmitz et al. investigated whether the use of TS-targeted siRNA duplexes *in vitro* could inhibit the expression of TS protein and prevent the previously studied mechanism of drug-induced resistance (38). An siRNA targeting a region on human TS mRNA corresponding to nucleotides 1058 to 1077 was designed and transfected into the human colon cancer RKO cell line. They showed that treatment of cells with TS-targeted siRNA (TS7) potently inhibited the expression of both TS mRNA and protein. These studies showed that, compared to ORNs, TS7 siRNA had a longer duration of intracellular effects, a greater affinity to TS mRNA as seen by RNA analysis, and a 500- fold increase in biological activity. Significant potential therapeutic applications were also suggested from these *in vitro* pre-clinical studies. Schmitz et al. showed that treatment with TS7 siRNA completely prevented TS protein induction following exposure to TS inhibitors, decreasing the development of cellular drug resistance. In addition, TS7 restored

chemosensitivity to cells overexpressing TS protein and resistant to various TS inhibitors. These studies showed that siRNAs effectively and specifically induced the process of RNAi in human colon cancer cells. More importantly, these studies identified a potent therapeutic application of siRNAs as single agents and in combination with TS inhibitors to overcome the development of cellular drug resistance. This work provided new insights towards the development of siRNAs as novel therapeutic molecules directed at key cellular pathways involved in cancer growth, proliferation, and survival.

Multi-Targeted Cancer Drug Development

Chemotherapeutic drugs have traditionally been developed to target one specific pathway. Combination chemotherapy has been a mainstay of cancer treatment since the recognition of its beneficial effect for childhood acute lymphoblastic leukemia (ALL) in the 1970's (2). This approach continues to be the cornerstone of therapeutic regimens for virtually all cancers, including breast and colorectal tumors. The benefit of combination therapy is due to the lower clinical efficacy seen with the use of one drug in the treatment of these cancers as well as the prevention of drug-resistance. The novel approach of developing molecules that target two or more key pathways in cancer cell growth has become more prominent given its beneficial implications in chemotherapy. First, it provides increased efficacy against tumor cells. Second, it improves and possibly prevents the development of cellular drug resistance to one chemotherapeutic agent. Finally, such an approach may simplify patient regimens by targeting multiple pathways with a single molecule.

Novel multi-targeted inhibitors are currently being designed to specifically target key signaling pathways in cancer cell proliferation and metastasis (39). Several of these new agents are under clinical investigation (40). A few examples of this class of anti-cancer drugs are the tyrosine-kinase inhibitors (TKIs) imatinib, ZD6474, and lapatinib (Table 1). Imatinib is a well-established small molecule found to not only target *abl*, a constitutively active kinase in chronic myelogenous leukemia (CML), but also the kinases associated with *c-kit* and platelet derived growth factor (PDGF)-α receptors. This agent is currently approved for use by the FDA for CML and gastrointestinal stromal tumors (GIST). ZD6474 is a potent inhibitor of VEGFR-2 that also has activity against epidermal growth factor receptor (EGFR) and *ret* (41). Phase I clinical trials showed good therapeutic potential in patients with refractory non small cell lung carcinoma (NSCLC) leading to phase II trials as first line therapy in NSCLC. Lapatinib, a TKI that targets both EGFR and ErbB2, is currently under phase III trials for advanced and refractory breast cancer. Recent studies have shown that in combination with capecitabine, lapatinib decreases progression of disease without an increase in serious side effects (42,43). Inhibition of the EGFR and ErbB2 signaling pathways have resulted in the induction of apoptosis in *in vitro* models of colon carcinoma (44,45).

TS has also been an important focus for multi-targeted treatment given its importance as a chemotherapeutic target. Development of the antifolate analog pemetrexed is a prime example of a clinically active agent approved by the FDA for the treatment of NSCLC and mesothelioma that targets three integral enzymes involved in the synthesis of nucleic acids (46). This drug is known as a multi-targeted antifolate (MTA), as it inhibits TS,

dihydrofolate reductase (DHFR), and GAR transformylase (GARTF), a key enzyme involved in *de novo* purine biosynthesis (Figure 1).

Table 1. Examples of Multi-Targeted TKIs.

	Molecular Target	*Tumor Type*	*Phase/Therapy*
Imatinib	*abl* *c-kit* PDGFR-α	CML GIST	Approved
ZD6474	VEGFR-2 EGFR	NSCLC	Phase II/ with Docetaxel
Lapatinib	EGFR ErbB2/Her2	Breast cancer	Phase III/ with Capecitabine

Multi-Targeted siRNA

The novel approach to multi-targeted drug design is not necessarily limited to kinase inhibitors and antimetabolites. Few studies have successfully implemented the use of siRNAs to simultaneously inhibit expression of two proteins. Conceptually, it would be possible to transfect several siRNAs into cells targeting a variety of different mRNAs. In practice, this has been shown to result in intracellular competition between the siRNAs, suggesting that some component of the RISC complex is limiting (47,48). It is conceivable that the transfection of multiple siRNA sequences may also increase the possibility for a natural immune response. Therefore, the novel technique of using a single siRNA molecule to target multiple pathways involved in cancer progression is an innovative approach to cancer drug development.

Another group of critical proteins involved in cancer growth and proliferation are the ErbB family. These proteins play a pivotal role in the proliferation of many human

cancers. Within this family, EGFR and ErbB2 are of particular interest in the study of tumor biology given their overexpression in a broad range of solid epithelial tumors including colon and breast (49).

ErbB Family

The ErbB family is among the best studied growth factor receptor systems since their implication in cancer development in the 1980's, when the avian erythroblastosis tumor virus was found to encode a mutated form of the human epidermal growth factor receptor (EGFR) (49). The ErbB family is a group of receptor tyrosine kinases (RTKs) whose members are membrane spanning proteins expressed in cells of mesenchymal and ectodermal origin and normally involved in mediation of human organogenesis and cell-cell interaction during adulthood (50). It consists of four members: EGFR (HER1/ErbB1), ErbB2 (HER2/*neu*), ErbB3 (HER3), and ErbB4 (HER4). All members of the ErbB protein family are composed of an extracellular ligand-binding domain, a transmembrane region, and an intracellular tyrosine kinase domain. The kinase activity of the ErbB family is activated through ligand-induced receptor homo- and heterodimerization which triggers autophosphorylation of tyrosine residues in the cytoplasmic tail. This in turn can result in a complex and diverse network of protein activation involved in the regulation of cell proliferation, differentiation, and survival. Two signaling pathways downstream of most active ErbB dimers are the mitogen-activated protein kinase (MAPK) pathway, and the PI(3)K-activated Akt pathway which are both essential pathways in cancer growth (Figure 4) (51).

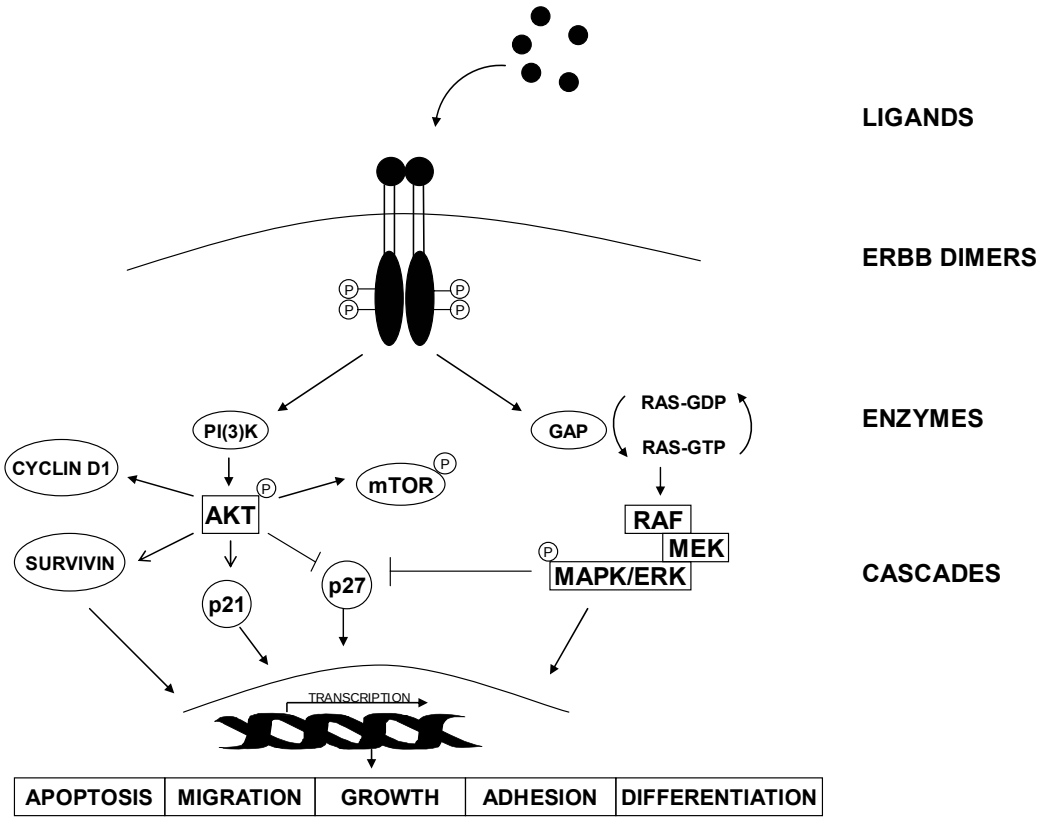

Fig 4. ErbB signaling network.

ErbB2 is a ligand-free receptor and is the preferred dimerization partner for all the other ErbB members, including EGFR (52). ErbB2-containing heterodimers have prolonged and enhanced downstream signaling when compared to homodimers of the same receptors. Moreover, ErbB2 serves as a critical component of breast carcinoma cell invasion when coupled to other ErbB receptors (53). Clinically, the pivotal role of ErbB2 in breast has been well-established as 15-25% of these tumors are known to overexpress the ErbB2 receptor (54). In addition, tumors that have varying alterations in ErbB receptors generally demonstrate more aggressive disease and poorer clinical outcome (54, 55).

The role of ErbB2 in breast cancer has been discussed above. Although rarely overexpressed in colorectal cancer, studies have shown constitutive expression of both ErbB2 and EGFR in colon cancer cell lines (56). Evidence suggests that hyperactivation of ErbB2 receptors at normal expression levels in colon cancer cells is due to elevated levels of growth factor ligands, such as TGF-α and heregulin, produced by the tumors themselves (57). This finding indicates that despite normal levels of ErbB2 receptor, ErbB2 and its associated dimerization partners (EGFR, ErbB3, ErbB4) could provide the signaling basis for tumor growth in cancers that do not overexpress ErbB2, such as colorectal, lung, and a majority of breast cancers (58). There is now strong evidence to show that the cooperation between these receptors is integral to the oncogenic transformation of several human cancers as opposed to the discrete function of the individual receptors.

The treatment of various solid tumors with molecules targeting ErbB receptors has gained recent validity in the clinic (49,59,60). There are currently two major classes of anti-ErbB therapeutic agents. The first includes monoclonal antibodies binding to the extracellular domain of the ErbB receptors. Within this group are trastuzumab, an ErbB2-targeted monoclonal antibody and cetuximab and panitumumab, two EGFR-targeted antibodies. Trastuzumab is presently approved for the treatment of ErbB2-overexpressing breast cancer, while cetuximab and panitumumab are approved for advanced/metastatic colorectal cancer (61-63). The second class of anti-ErbB agents are small-molecule tyrosine-kinase inhibitors (TKIs). These molecules compete with ATP in the ATP binding pocket of the tyrosine-kinase domain of the receptor and have taken center stage

in modern molecular cancer treatment (64,65). Erlotinib and gefitinib are molecules that target EGFR and these agents are approved for the treatment of NSCLC. Lapatinib is a dual inhibitor that targets both EGFR and ErbB2, and the expectation is this novel molecule will soon be approved by the FDA for the treatment of advanced breast cancer. Although these agents show significant promise, the development of cellular drug resistance remains a major issue that limits their clinical efficacy (66).

Statement of Purpose

For this research project, I hypothesize that siRNAs can be designed and developed to inhibit the expression of multiple key proteins involved in cancer cell growth and proliferation. Given the central role of TS in cellular proliferation, I set out to design a TS-targeted siRNA that targets TS as well as other critical proteins implicated in the growth of human cancers. Both TS and EGFR are important targets of current chemotherapeutic agents used for colorectal cancer, and as such, sequence analysis was performed in search of an overlapping sequence between TS and EGFR mRNAs. However, no homologous sequences could be identified. With the understanding that ErbB family members function primarily as heterodimers and that targeting other ErbB receptors may lead to similar downstream effects on colon cancer cell proliferation, the search was extended to other ErbB family members. A comparison between TS and ErbB2 mRNAs revealed a 19-nt sequence with 80% base-pair homology. ErbB2 mRNA encodes an important growth factor receptor that is critical for the growth and proliferation of breast cancers as well as other solid tumors. In addition, TS is an important target for current breast cancer therapy. Taking advantage of the innate flexibility of designing siRNAs, an siRNA that simultaneously targets human ErbB2 and TS mRNA was developed.

The main goal of my project is to investigate the therapeutic application of multi-targeted siRNAs. To achieve this goal, my research project has 3 main aims, and they are as follows:

Aim 1: Characterize the molecular mechanisms by which multi-targeted siRNAs control and suppress the expression of ErbB2 and TS protein using human breast cancer cells as my *in vitro* model system

Aim 2: Optimize the *in vitro* effects of multi-targeted siRNAs

Aim 3: Investigate the *in vitro* cytotoxic effects of multi-targeted siRNAs

Materials and Methods

siRNA Synthesis. siRNA duplexes were designed to simultaneously target sequences on human TS mRNA corresponding to nucleotides 2-20 (5'-CCACUUGGCCUGCCUCCGU-3') and human ErbB2 mRNA corresponding to nts 903-921 (5'-CUGCCUGGCCUGCCUCCAC-3'). A series of siRNAs with base pair mismatches to these targets were additionally designed. The selected sequences were screened in a BLAST search against all known human genes to verify that no other genes were targeted. siRNA duplexes were obtained from Dharmacon Research (Lafayette, CO). In addition, an siRNA duplex previously studied by Schmitz et al. (38) targeting a non-homologous region of TS corresponding to nts 1058-1077, an siRNA duplex targeting ErbB2 corresponding to nts 2450-2469, and a control siRNA duplex GL2, were obtained from Dharmacon. All siRNA's contained two symmetric 3' overhangs of two 2'-deoxythymydine nts.

Cell Culture. Human breast cancer cell lines SKBR3 and MCF-7 were obtained from the American Type Culture Collection (Manassas, VA). All cells were maintained in 75-cm^2 tissue flasks (BD Bioscience; San Jose, CA) in a 100% humidified incubator at 37°C. Cells were grown continuously in growth medium containing RPMI-1640 (Invitrogen; Carlsbad, CA) with 10% dialyzed fetal bovine serum (Invitrogen). Baseline growth experiments were performed to determine the doubling time of the human breast cancer cell lines. SKBR3 and MCF-7 cells were found to have doubling times of 40 and 57 hrs, respectively.

siRNA Transfection. SKBR3 cells were plated in 25-cm^2 flasks in 3 ml of RPMI-1640 at a density of 5 x 10^5 cells per flask. siRNA duplexes were complexed with the cationic lipid Lipofectamine (Invitrogen) in OPTI-MEM medium as described by the manufacturer's protocol. Briefly, 2 µl of Lipofectamine were diluted in 148 µl of OPTI-MEM medium and allowed to sit at room temperature for 5 min. Diluted Lipofectamine was added to diluted siRNA of desired concentration and incubated for 20 min at room temperature. Total aliquots of 300 µl were added to the 25-cm^2 flasks under sterile conditions. At 48 hrs post-transfection, SKBR3 cells were deprived of growth serum by incubating in non-supplemented medium for 24 hours. This change in medium conditions was sufficient to induce protein phosphorylation. Cells were washed twice with ice-cold PBS and either trypsinized or detached with a rubber policeman.

For cell proliferation assays, SKBR3 and MCF-7 cells were plated in 96-well tissue culture plates (BD Bioscience) in 100 µl of RPMI-1640 at a density of 4 x 10^4 and 2.5 x 10^4 cells/well, respectively. For cytotoxicity experiments, the cationic lipid Oligofectamine (Invitrogen) was used. Oligofectamine (0.4 µl, 0.6 µl) was added to OPTI-MEM medium for a total volume of 15 µl and allowed to sit for 5 min at room temperature. This dilution was combined with 35 µl of diluted siRNA and incubated for 15 min before addition to individual wells.

Western Immunoblot Analysis. SKBR3 cells were plated, transfected, and harvested as described above. Cell pellets were resuspended in RIPA cell lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Igepal (Sigma-Aldrich Co., Milwaukee, WI), 0.5%

deoxycholic acid and 1% SDS] containing freshly added phenylmethylsulfonyl fluoride and protease inhibitor mixture (Sigma; St. Louis, MO). Phosphorylated proteins were extracted by resuspension of cell pellets in PhosphoSafe Extraction Buffer (Novagen; Darmstadt, Germany) along with the protease inhibitor mixture. Suspensions were incubated on ice for 20 min and then centrifuged at 14,000 rpm for 10 min at 4°C. Protein concentrations of the supernatant were determined using the DC protein assay (Bio-Rad; Hercules, CA). Equivalent amounts of protein (10-70 μg) from each cell lysate were resolved on SDS-PAGE (7.5-12.5% acrylamide) and electroblotted onto a nitrocellulose membrane (0.45 μm filter; Bio-Rad). Membranes were incubated in blocking solution (1 x PBS or 1 x TBS, 0.1% Tween-20, and 5% nonfat dry milk powder) for 1 hour at room temperature. Membranes were incubated with primary antibodies overnight at 4°C in 5% TBST, 5% PBST or 5% bovine serum albumin. The following antibody dilutions were used: TS, 1:2,000 (Zymed Lab.; 4H4B1); EGFR, 1:500 (BD Trans. Labs; 555996); ErbB-2, 1:2,500 (Oncogene; Ab-3); phospho-ErbB2, 1:1,000 (Neomarkers; Ab-18); ErbB3, 1:500 (Neomarkers; Ab-2); phospho-MAPK, 1:500 (Santa Cruz Biotechnology; sc-7383); α-tubulin, 1:2,500 (Santa Cruz; sc-8035); cyclin D1, 1:500 (Santa Cruz; sc-20044); p21, 1:500 (Cell Signaling); p27, 1:500 (Calbiochem; Ab-2) and survivin 1:1,000 (Cell Signaling; 2802). The polyclonal MAPK protein (sc-94) antibody was purchased from Santa Cruz (Santa Cruz, CA) and used at a dilution of 1:1,000. The anti-topoisomerase I monoclonal antibody was a gift from Dr. Yung-chi Cheng, Yale University, New Haven, CT and used at a dilution of 1:2000. After five 10 min washes, membranes were incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibody (IgG goat antimouse/IgG goat antirabbit; Bio-Rad) for 1 hr at room

temperature. This was followed by five additional 10 min washes. Membranes were then processed by the enhanced chemiluminescence method (SuperSignal West Pico substrate; Pierce, Rockford, IL), and protein bands were visualized by autoradiography. Quantitation of signal intensities was performed using densitrometry on a Hewlett-Packard ScanJet 5370C (Palo Alto, CA) with NIH image 1.62 software.

Reverse Transcriptase PCR. SKBR3 cells were plated and transfected in 25-cm^2 flasks as described. Total RNA was purified 72 hours after transfection using the RNeasy Mini Kit (Qiagen; Valencia, CA) as described by the manufacturer's protocol. RNA concentration was then determined and verified by running 1% agarose/formaldehyde gels. cDNA was synthesized from 1 μg of total RNA using the ThermoScript RT-PCR System (Invitrogen) and amplified by PCR using *Taq* DNA polymerase (Qiagen). The primers used for PCR amplification are as follows:

ErbB2 (484 bp):	(F) 5'-GAGATCTTGAAAGGAGGGGTCT-3'
	(R) 5'-CGTCCGTAGAAAGGTAGTTGTAGG-3'
EGFR (550 bp):	(F) 5'-AGAGGAGAACTGCCAGAAACTG-3'
	(R) 5'-AGGAGGAGTATGTGTGAAGGAGTC-3'
ErbB3 (499 bp):	(F) 5'-GTGGATGGCCCTTGAGAGTA-3'
	(R) 5'-ACGTGGCCGATTAAGTGTTC-3' (67)
TS (440 bp):	(F) 5'-GGGCAGATCCAACACATCCT-3'
	(R) 5'-CAAGCGCACATGATGATT-3'

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 440 bp) specific PCR products using (F) 5'-TCATCTCTGCCCCCTCTGCT-3' and (R) 5'-CGACGCCTGCTTCACCACCT-3' primers from the same samples were amplified and served as internal controls, where F and R are the forward and reverse primers, respectively. Each amplification cycle consisted of 30 seconds at 95°C for denaturation, 30 seconds at 55°C for primer annealing, and 1 minute at 72°C for extension. After amplification, DNA products were analyzed by 1% agarose gel electrophoresis.

Cell Growth Assay. Growth inhibition and cell toxicity was evaluated by WST-1 assay (Roche Applied Science; Indianapolis, IN). In this assay, quantification of cell proliferation and cell viability is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. In brief, SKBR3 and MCF-7 cells were seeded in 96-well tissue culture plates, as described above. At 24 hours after seeding, cells were treated with siRNA:Oligofectamine complexes. A volume of 15 µl of WST-1, equaling 10% of the total well volume, was added to each well of SKBR3 and MCF-7 plates after 7 and 6 days of siRNA exposure, respectively. Reactions were incubated at 37°C for 1 hr. Absorbance at 630 nm was recorded using a 96-well plate reader. All assays were performed in duplicate on each plate.

Results

Inhibition of ErbB2 and TS protein expression by a multi-targetd siRNA in SKBR3 cells. Previous studies by Schmitz et al. have shown that thymidylate synthase (TS)-targeted siRNAs effectively and specifically induced the process of RNAi in human colon cancer cells (38). siRNAs were able to inhibit TS expression in the low nanomolar range, suggesting the potent therapeutic potential of siRNAs. I subsequently investigated the ability of a single siRNA duplex to simultaneously inhibit expression of TS and another key cellular protein involved in cancer cell growth and proliferation, namely the ErbB2 growth factor receptor. Sequencing analysis identified a 19-nt sequence on TS mRNA (nt 2 -20) that had 15-nt homology to nt 903-921 on ErbB2 mRNA (Figure 5A). Of note, the ErbB2 mRNA encodes an important growth factor receptor that is critical for the growth of breast cancer as well as other solid tumors. An siRNA duplex, TS1, was designed to be complementary to this region on human TS mRNA. Preliminary studies using the human colon cancer RKO cell line showed that transfection with the TS1 siRNA markedly reduced expression of both ErbB2 and TS protein (data not shown). However, the decrease in ErbB2 protein expression was not as significant as that seen with an siRNA molecule designed to specifically target ErbB2, B2450 (data not shown).

To extend these studies to a biological model that would be more responsive to a multi-targeted siRNA that inhibited both TS and ErbB2, further work was conducted in a human breast cancer cell line known to overexpress ErbB2, SKBR3 cells. In an attempt to improve potency of TS1 in downregulating expression of ErbB2, siRNAs were designed that contained corrected mismatches to ErbB2 mRNA. All positions in this

study are reported from the 5'-end of the antisense strand of the siRNA. As seen in figure 5A, TS1M15 was designed to contain a guanine (G) at the 15th position instead of an adenine (A). TS1M17 was designed to contain a cytosine (C) on the 17th position instead of a uracil (U). TS1M17M18 had two corrected mismatches on the 17th and 18th positions, which in addition to changing a U for a C, changed a G residue to an A at the 18th position. The effect of these mismatched siRNAs on protein expression was investigated by Western blot analysis. Upon transfection into SKBR3 cells at a concentration of 10 nM, both TS1M17 and TSM17M18 significantly inhibited ErbB2 and TS protein expression (Figure 5B). The siRNA TS1M15 lost its inhibitory effects on both ErbB2 and TS protein expression. The expression of two control proteins, α-tubulin and topoisomerase-I, was unaffected by siRNA treatment. In addition, treatment with a control GL2 siRNA, at a concentration of 10 nM, had no effect on levels of ErbB2, TS, or control proteins, suggesting that transfection of the ErbB2/TS siRNAs had specific effects on protein expression. Additional experiments suggested that the TS1M17 siRNA showed greater inhibitory activity on expression of both ErbB2 and TS proteins than TS1 or TS1M17M18 (data not shown). Given this observation, I proceeded to focus on elucidating the biological effect and therapeutic potential on TS1M17 siRNA.

A time course experiment was then performed to determine the duration of effect of TS1M17 siRNA treatment on levels of ErbB2 and TS protein. As seen in figure 6, TS1M17 effectively decreased levels of both ErbB2 and TS protein by 48 hours of

A

TS1 siRNA:

TS mRNA 5'-CCACUUGGCCUGCCUCCGU -3'

siRNA duplex dTdT- GGUGAACCGGACGGAGGCA
CCACUUGGCCUGCCUCCGU-dTdT
19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

ErbB2 mRNA 5'- CUGCCUGGCCUGCCUCCAC -3'

B

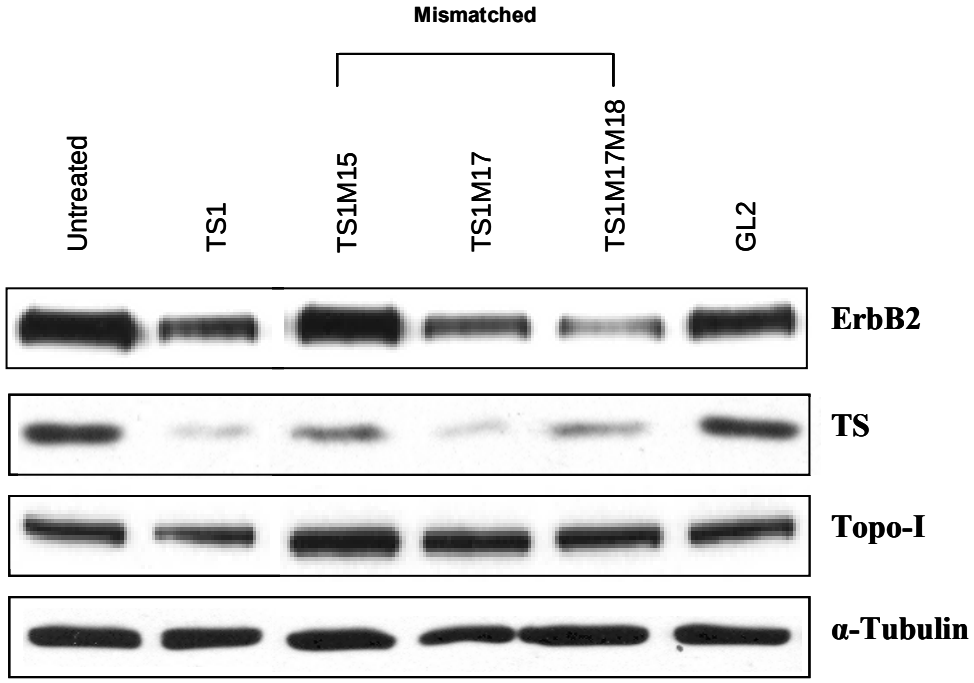

Fig. 5. Effect of multi-targeted small interfering RNAs (siRNAs) on ErbB2 and TS protein expression in SKBR3 cells. *(A)* Schematic of TS1 siRNA duplex with targeted region on TS and ErbB2 mRNA. Solid lines denote non-homologous base-pairs to the antisense strand of TS1 siRNA; *(B)* Western blot analysis of SKBR3 cells after treatment with TS1, mismatched (TS1M15, TS1M17, TS1M17M18) and control (GL2) siRNAs. Cells were transfected with Lipofectamine alone (untreated) or with 10 nM of TS1, TS1M15, TS1M17, TS1M17M18 and GL2 siRNAs for 72 hrs. Cell extracts were processed for Western blot analysis as described in "Materials and Methods".

siRNA exposure. The expression of both proteins rapidly decreased to <10% and <5%, respectively, following a 24-hr incubation period (Figure 6). By 96-hrs, ErbB2 and TS expression had increased and returned to base-line levels by 120-hrs (data not shown), demonstrating that maximal protein inhibition occurred at 72-hrs of siRNA exposure in SKBR3 cells. The expression of α-tubulin remained unaffected over this same time course. Given this data, all subsequent siRNA transfection experiments were performed at 72-hrs.

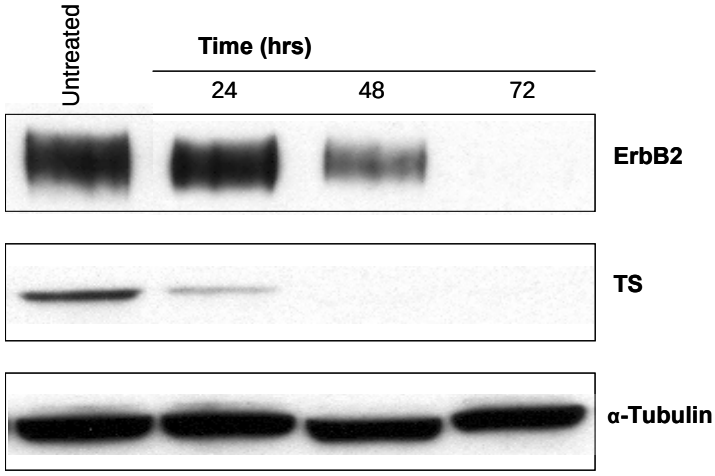

Fig. 6. Duration of effect of TS1M17 siRNA on expression of ErbB2 and TS protein in SKBR3 cells. SKBR3 cells were transfected with 10 nM of TS1M17 siRNA and harvested prior to transfection (untreated) and at the following times: 24, 48 and 72 hrs. Cell extracts were processed for Western Blot analysis as described in "Materials and Methods".

A dose-dependent experiment was subsequently performed using varying concentrations of the siRNA in order to identify the range of concentration in which TS1M17 siRNA was able to coordinately inhibit the expression of ErbB2 and TS protein in SKBR3 cells,. As seen in figure 7, TSM17 siRNA inhibited the expression of ErbB2 and TS protein in a dose-dependent manner with 50% inhibition observed at 0.1 nM. Maximal inhibition of

ErbB2 (>90%) and TS (>95%) was observed at 72 hrs of 10 nM TSM17 siRNA transfection. Of note, inhibition of ErbB2 and TS protein expression was not enhanced when SKBR3 cells were treated with higher concentrations of TS1M17 siRNA, up to 100 nM (data not shown). This difference in protein inhibition suggests that the increased levels of ErbB2 protein in SKBR3 cells does not allow for complete inhibition of ErbB2 by the TS1M17 siRNA. Expression of control proteins, topoisomerase-I and α-tubulin, remained unaffected with siRNA treatment. As an important control, treatment with 10 nM of the control GL2 siRNA also had no effects on levels of ErbB2, TS, or control proteins.

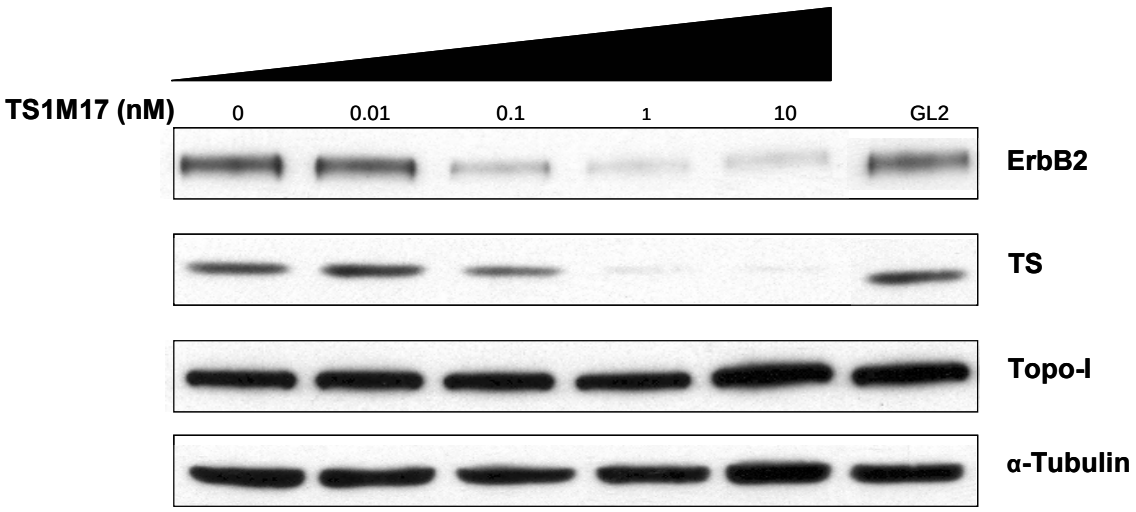

Fig. 7. Dose-dependent effect of TS1M17 siRNA on ErbB2 and TS protein in SKBR3 cells. SKBR3 cells were transfected with Lipofectamine alone (0 nM) and varying concentrations (0.01, 0.1, 1, 10 nM) of TSM17 siRNA for 72 hrs. Cell protein extracts were processed for Western Blot analysis as described in "Materials and Methods".

Specific inhibition of ErbB2 and TS expression by TS1M17 siRNA in SKBR3 cells. As described above, our results documented the successful implementation of simultaneously inhibiting two proteins with one siRNA in an *in vitro* model. These

results suggested a potential therapeutic approach to targeting multiple critical proteins involved in tumor cell growth. However, previous studies have shown that siRNAs can target unintended genes that share minimal homology to the desired targeted sequence (34). I therefore set out to confirm target specificity of the multi-targeted siRNA, TS1M17, in SKBR3 cells. To demonstrate that the decrease in ErbB2 and TS were secondary to direct RNAi of each target, ErbB2- and TS-specific siRNAs were designed. The ErbB2-specific siRNA, B2450, targets a protein coding region (nt 2450-2458) on ErbB2 mRNA. The TS-specific siRNA TS7 was previously studied and shown to specifically target the 3'-UTR immediately downstream of the translational stop site (nt 1058-1077) of TS mRNA (38). Sequences were screened by BLAST search to verify that no other genes were targeted. Western blot analysis showed that SKBR3 cells transfected with TS1M17 had significant effects on ErbB2 and TS protein (Figure 8A). Based on this analysis, B2450 and TS7 siRNAs only inhibited their intended protein targets, ErbB2 and TS, respectively (Figure 8A). In addition, transfection with B2450 siRNA demonstrated limited silencing ability compared to TS1M17, reducing ErbB2 by only 50% of control. The mismatched control siRNA, TS1 Mismatch (TS1-MM), and GL2 control siRNA had absolutely no effect on expression of either protein at 10 nM concentrations. In addition, the expression of control housekeeping proteins, topoisomerase-I and α-tubulin, remained unchanged in response to treatments with any of the siRNAs.

There are 4 members of ErbB family of receptors, and they share variable sequence homology. Therefore, experiments to determine effects of TS1M17 siRNA on expression

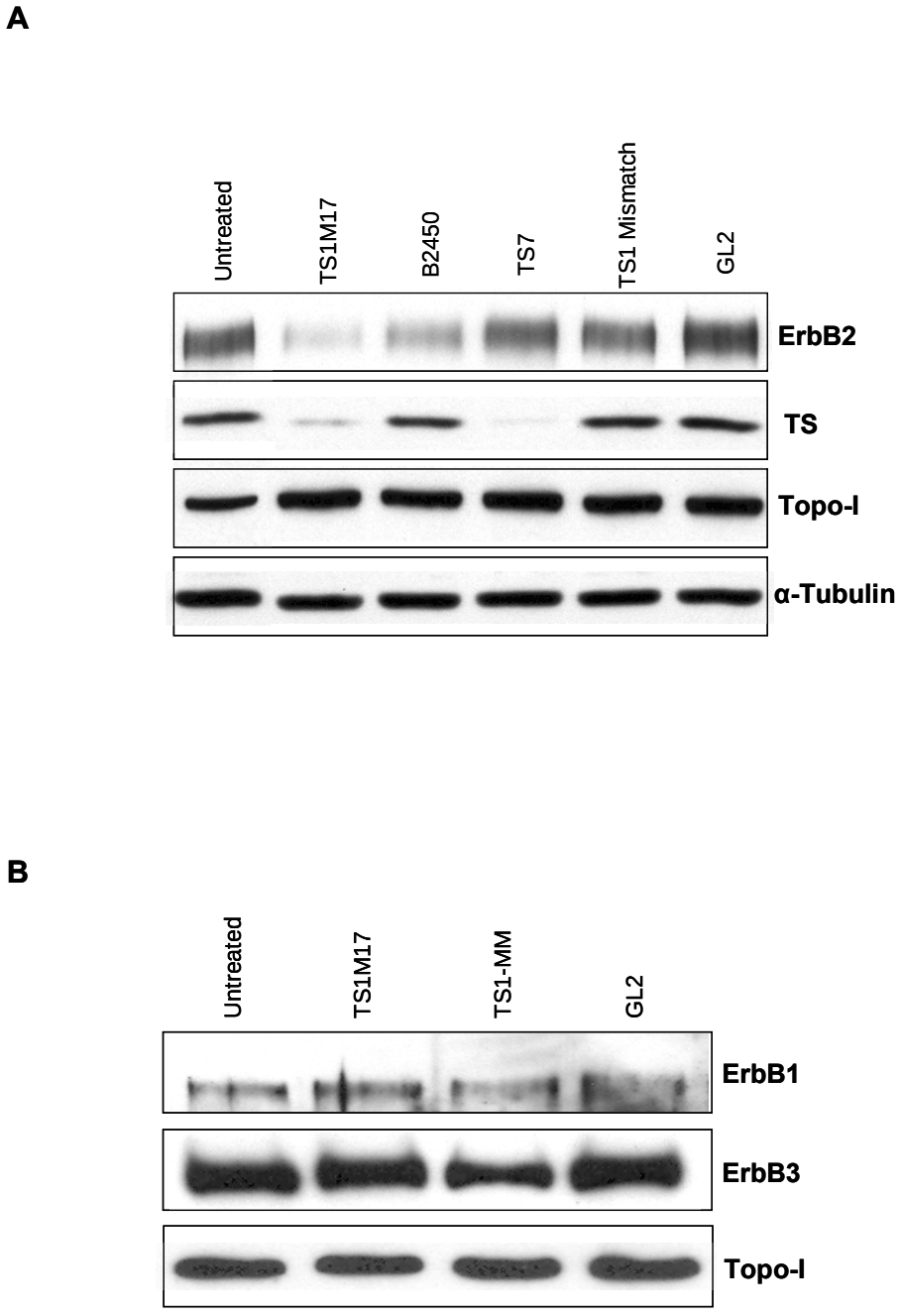

Fig. 8. Specificity of effect of TS1M17 siRNA on ErbB2 and TS protein expression in SKBR3 cells. *(A)* Western blot analysis of SKBR3 cells after treatment with 10 nM of TS1M17, ErbB2-specific (B2450), TS-specific (TS7) and control (TS1 Mismatch and GL2) siRNAs; *(B)* Effect of TS1M17 on expression of ErbB1 and ErbB3 in SKBR3 cells. Cells were transfected with Lipofectamine alone (untreated) or with 10 nM of TS1M17, TS1 Mismatch (TS1-MM), and GL2 siRNAs for 72 hrs. Cell extracts were processed for Western blot analysis as described in "Materials and Methods".

of other ErbB family members in SKBR3 cells were done. Previous work (68) has shown that these cells express ErbB1 and ErbB3 but not ErbB4. Given this finding, Western blot analysis confirmed that SKBR3 cells indeed expressed ErbB1 and ErbB3 protein levels. Our studies revealed that levels of both ErbB1 and ErbB3 protein remained unaffected after transfection with 10 nM of TS1M17 siRNA (Figure 8B). In addition, control, TS1 Mismatch and GL2, siRNAs had absolutely no affect on expression of either receptor protein. As an important control, the levels of topoisomerase-I protein remained unchanged under all conditions.

The proposed mechanism of RNAi is post-transcriptional cleavage of target mRNA (25). To gain insight into the potential molecular mechanism by which TS1M17 siRNA was exerting its effects on ErbB2 and TS protein expression, levels of ErbB2 and TS mRNA were verified. Preliminary Northern blot analysis showed decreased expression of ErbB2 mRNA with TS1M17 siRNA transfection (data not shown). To confirm these results, I used RT-PCR analysis to show effects of TS1M17 siRNA on ErbB2 and TS mRNA levels. As seen in figure 9, transfection with TS1M17 significantly decreased expression of ErbB2 and TS mRNAs to barely detectable levels in SKBR3 cells (<5% of control). As two important controls, the TS1 Mismatch and GL2 siRNAs had no effect on levels of either mRNA. As another important control, GAPDH mRNA expression remained unchanged with siRNA treatment. Although these results are not exactly quantitative, the absence of ErbB2 and TS PCR products confirms that TS1M17 siRNA inhibits protein expression through degradation of target mRNA and not through translational inhibition. These experiments, taken together, lend further support that TS1M17 siRNA successfully

and specifically targets both ErbB2 and TS protein in SKBR3 cells through simultaneous RNAi.

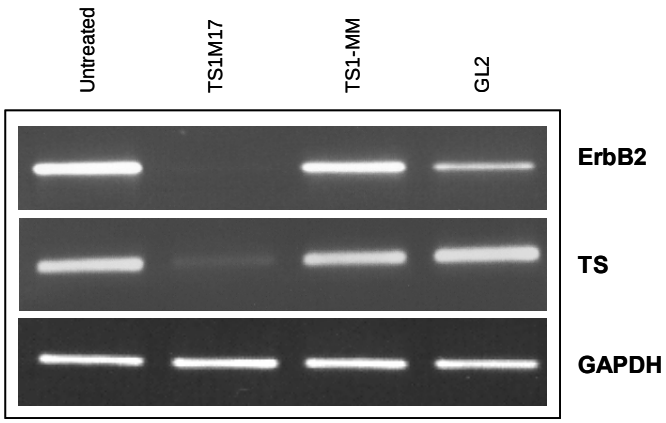

Fig. 9. Effect of TS1M17 siRNA on ErbB2 and TS mRNA in SKBR3 cells. SKBR3 cells transfected with Lipofectamine alone (untreated) or 10 nM of TS1M17 and control (TS1 Mismatch and GL2) siRNAs. Cells were transfected for 72 hrs, and total RNA from cells was isolated, reverse-transcribed, and then amplified using gene-specific primers as described in "Materials and Methods". DNA products were resolved on 1% agarose gels and visualized. GAPDH was used as an internal control.

TS1M17 siRNA inhibits ErbB2 phosphorylation and downstream signaling molecules in SKBR3 cells. The effects of ErbB2 activation on cytoplasmic signal transduction proteins in the Ras/MAP kinase pathway have been well defined (Figure 4). Previous studies have shown that inhibition of ErbB2 by monoclonal antibodies or receptor tyrosine kinase inhibitors (TKIs) have subsequent effects on phosphorylation of ErbB2 as well as downstream phosphorylation of MAPK (69,70). In addition, Ras/MAPK and the PI(3)K/Akt signaling have been implicated in the regulation of cell cycle modulators $p27^{Kip1}$, $p21^{Cip1}$, cyclin D1 and the apoptosis inhibitor, survivin (71,72). In contrast to TKIs, which inhibit the tyrosine kinase domain of the growth factor

receptors, siRNAs exert their effects at the mRNA level. Although previous experiments showed that TS1M17 siRNA effectively inhibited the basal expression of ErbB2, its effects on phosphorylated ErbB2 were unknown. The effects of TS1M17 siRNA on tyrosine phosphorylation of ErbB2 in SKBR3 cells were studied. As seen in figure 10, TS1M17, at a concentration of 10 nM, was capable of inhibiting expression of both the unactivated and activated forms of ErbB2 to <5% compared to baseline levels at 72 hrs after transfection.

I next examined whether a decrease in ErbB2 phosphorylation levels secondary to TS1M17 siRNA transfection, resulted in downstream signaling effects on the ErbB2 signaling pathway. Western blot analysis revealed that MAPK expression was similar for all transfection conditions (Figure 10). However, activated MAPK, as measured by phospho-MAPK levels, was reduced by 44% in cells treated with 10 nM of TS1M17 siRNA for 72 hours. The expression of other key downstream proteins was then analyzed. I observed that the same concentration of 10 nM TS1M17 siRNA decreased expression of cyclin D1, p21, and survivin to 13, 30, and 15%, respectively, compared to baseline protein levels and control transfections with 10 nM of TS1 Mismatch (TS1-MM) and GL2. In addition, transfection with TS1M17 siRNA upregulated the expression of p27 to 7.3-fold higher than baseline levels (Figure 10). The level of topoisomerase-I remained unchanged with each of the conditions, providing an important negative control. These results suggest that treatment with TS1M17 siRNA results in inhibition of ErbB2 downstream signaling.

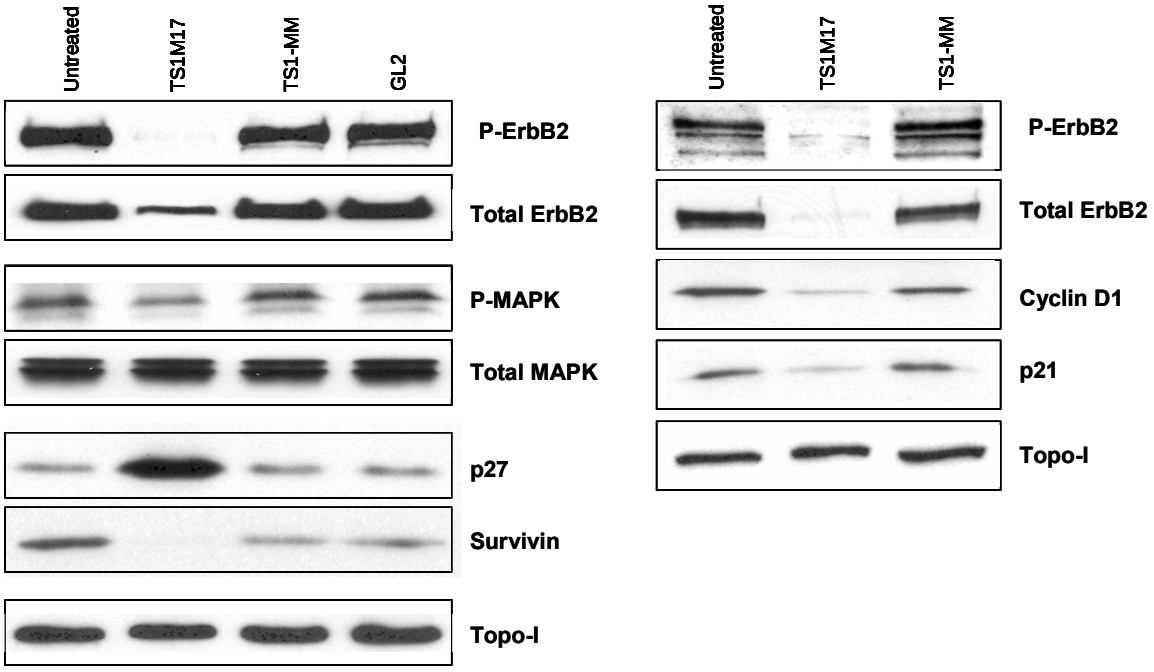

Fig. 10. Effect of TS1M17 siRNA on ErbB2 receptor phosphorylation and signaling molecules in SKBR3 cells. SKBR3 cells were transfected for 72 hrs with Lipofectamine alone (untreated) or 10 nM TS1M17 and control (TS1 Mismatch and GL2) siRNAs. Cells were then harvested for Western Blot analysis as described in "Material and Methods". Phosphoprotein levels were examined by using anti-pErbB2 (p-Tyr^{1248}) antibody and anti-pMAPK (p-Tyr^{204}) antibody.

TS1M17 inhibits cellular proliferation in SKBR3 and MCF7 cells. To determine the effect of TS1M17 siRNA on the growth and proliferation of SKBR3 cells, a series of cell proliferation experiments was performed. SKBR3 cells were treated with a concentration range of 0.01 to 100 nM siRNA for a total of 7 days. Cell growth was marginally suppressed by TS7 siRNA, a TS-specific siRNA previously shown to have no effect on ErbB2 protein expression, and cell growth was inhibited by only 30%, using a concentration of 100 nM (Figure 11A). B2450, the ErbB2-specific siRNA, had a greater effect than TS7, resulting in 51% inhibition of cell proliferation at a concentration of 100 nM. The most significant effect was seen after treatment with the multi-targeted siRNA,

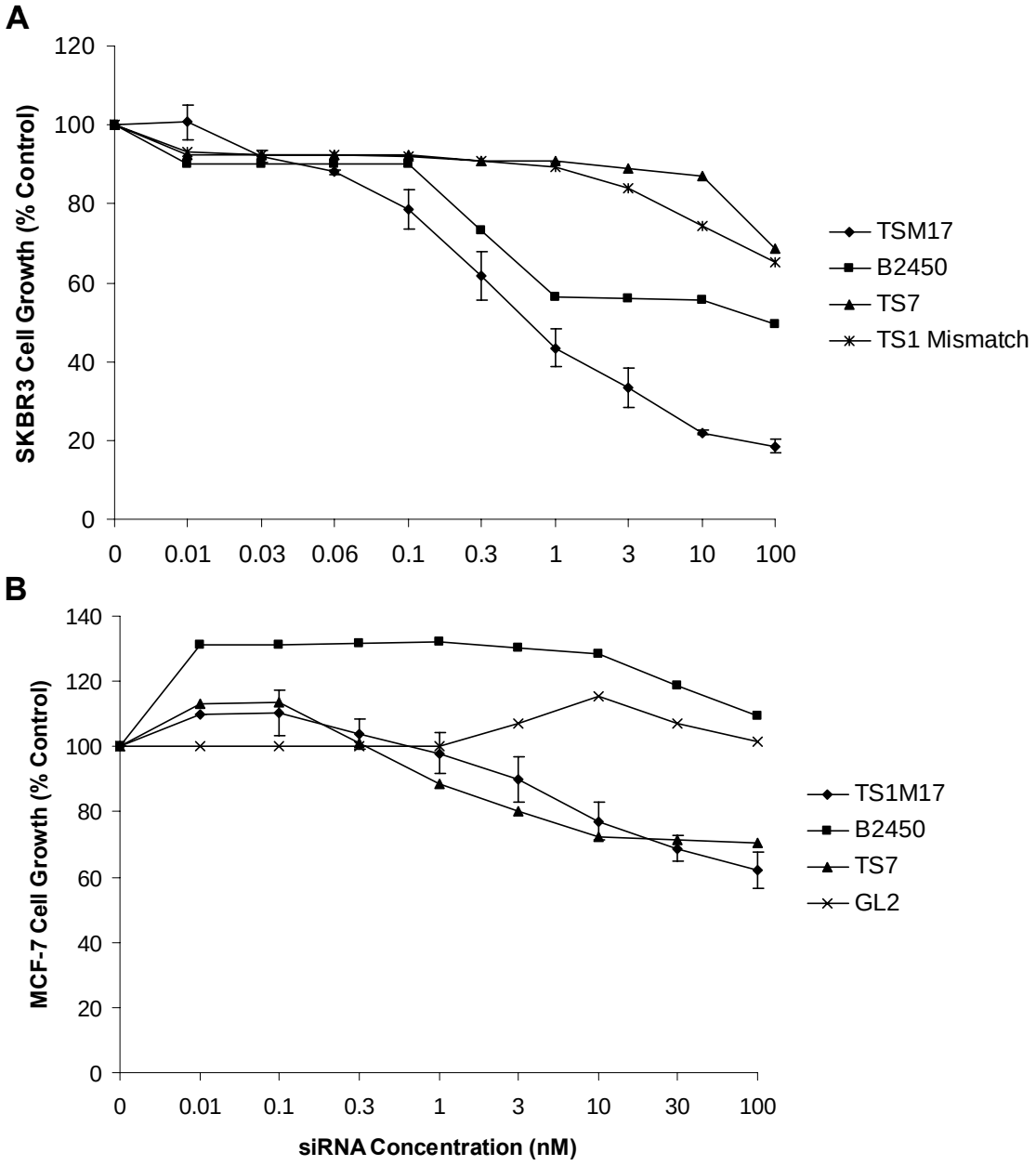

Fig. 11. Effect of ErbB2 and TS siRNAs on cellular proliferation in SKBR3 and MCF-7 cells. Cells were transfected with various concentrations of TS1M17, ErbB2-specific (B2450), TS-specific (TS7), and control (TS1-Mismatch and GL2) siRNAs. Cell proliferation was determined by the WST-1 assay as described in "Materials and Methods". *(A)* SKBR3 cells were treated for 7 days; *(B)* MCF-7 cells were treated for 6 days. Values represent the mean ± SD from 4 to 7 experiments.

TS1M17. Transfection resulted in 78% inhibition with 10 nM of TS1M17 siRNA and an IC_{50}.value of 0.65 nM. Higher concentrations of TS1M17 siRNA (up to 100 nM) resulted in only slightly higher growth inhibition (82%). This result correlated with our initial observation that concentrations greater than 10 nM did not result in improved silencing effect on ErbB2 or TS protein expression. This finding also suggested that the increase in growth-inhibitory effects of TS1M17 siRNA, although mostly secondary to ErbB2 protein inhibition, could possibly be accounted for by the additional inhibition of TS protein expression. As an important control, TS1 Mismatch had minimal effect on cell growth.

Given that SKBR3 cells overexpress ErbB2, cell growth assays were also performed using a human breast cancer cell line, MCF-7, which expresses lower baseline levels of ErbB2 (68,73). For these experiments, MCF-7 cells were treated with varying concentrations (0.01-100 nM) of siRNA for a total of 6 days. The doubling time for the cell line was noted to be 17 hrs shorter than SKBR3 cells and therefore required a shorter incubation period (data not shown). As seen in figure 11B, TS7 and TS1M17 siRNAs inhibited cell proliferation to a similar extent. These findings suggest that the observed effect on cell growth in MCF-7 cells is mainly secondary to TS inhibition. The IC_{50} value of TS1M17 siRNA in MCF-7 cells was not calculated given maximum growth inhibition was 48%. Although statistically not significant ($p \leq 0.1$), at 100 nM concentration, TS1M17 had a slightly greater effect on suppression of cellular proliferation than the TS-specific siRNA TS7, 48 ± 5.6% and 39 ± 4%, respectively. These findings suggest the possibility of enhanced effects secondary to ErbB2 protein inhibition at higher doses. Transfection with B2450 and GL2 had absolutely no effect on MCF-7 cell proliferation.

Discussion

In this research project, I investigated the therapeutic potential of a novel multi-targeted siRNA in human breast cancer cells. Schmitz and colleagues had previously identified an siRNA that specifically and potently inhibited expression of TS by targeting TS mRNA (38). This and other studies revealed the therapeutic potential of utilizing RNAi as an approach to targeted cancer drug development. As previously mentioned, there is a large investigational effort to develop drugs that target multiple cellular pathways. Previous work transfecting cells with two siRNAs simultaneously, showed that once introduced into the cell, siRNAs compete with each other, suggesting that there is a limiting aspect to the RNAi machinery (47,48). Given this work, as well as the successes seen with multi-targeted drug therapies, an siRNA duplex that targeted TS mRNA as well as a cellular mRNA that encodes a different cellular protein involved in cancer cell growth and proliferation, such as a member of the ErbB family, was designed and developed. Through gene analysis, a 19-nt sequence on human TS mRNA was found that was 80% homologous to human ErbB2 mRNA (Figure 5A). In analyzing this multi-targeted siRNA, TS1, all five mismatches to ErbB2 mRNA were noted to be G:U or A:C base-pairs (Figure 5A). Previous RNAi work had shown that both of these mismatches are viable base-pairs in siRNA binding, providing support that TS1 siRNA could successfully and simultaneously target human ErbB2 and TS mRNAs (35,36). Our studies showed that, despite the mismatched base-pairs, there was significant suppression of both ErbB2 and TS protein (Figure 5B). As seen in figure 5B, altering one of five mismatches on the same siRNA resulted in a significant difference in ErbB2 and TS protein expression in SKBR3 cells. By correcting the 15^{th} nucleotide mismatch, TS1M15

siRNA lost its effects on ErbB2 and TS protein expression. In contrast, correcting the 17th base-pair mismatch showed greater inhibitory activity on both ErbB2 and TS protein expression than TS1 siRNA. Taken together, these experiments support previous findings reported by Tuschl et al. that the design of effective siRNAs is an empirical process given that base-pair mismatches at specific locations in the siRNA duplex can enhance or decrease the effect of the siRNA (34). In addition, given the enhanced effect of TS1M17 siRNA, I proceeded to characterize in greater detail the biological activity of this siRNA.

Studies revealed that transfection of TS1M17 siRNA significantly inhibited expression of ErbB2 and TS protein for up to 72 hrs (Figure 6). Previous studies by Schmitz et al. had shown that a TS-specific siRNA exhibited a longer inhibitory effect in human colon cancer RKO cells (38). These studies showed that inhibition of TS was maximal at 48 hr and lasted up to 5 days. The discrepancy between duration of protein repression could be explained, at least in part, by the different model system used in each study. The half-life of siRNAs in SKBR3 cells may be significantly shorter than the half-life of siRNAs in RKO cells. In addition, the overexpression of ErbB2 protein in SKBR3 cells may also have direct impact on the effective duration of RNAi of ErbB2 mRNA. As seen in figure 7, TS1M17 siRNA also inhibited ErbB2 and TS protein expression in a dose-dependent manner. Maximal inhibition of ErbB2 and TS protein, 90% and 95%, respectively, occurred at a concentration of 10 nM. Increasing the concentration of TS1M17 siRNA to 100 nM did not further enhance the inhibitory effects on protein expression in SKBR3 cells (data not shown). This finding is noteworthy for two reasons. First, despite higher concentrations of TS1M17 siRNA, ErbB2 protein levels were not completely suppressed.

This finding can be explained by the increased levels of ErbB2 protein expressed in SKBR3 cells. Second, treatment with 100 nM concentrations of TS1M17 siRNA did not significantly inhibit more cells than 10 nM concentrations (78 and 82%) in SKBR3 cells (Figure 11). This correlation strongly supports our underlying hypothesis that the growth inhibitory effects of TS1M17 siRNA are secondary to inhibition of protein expression caused by RNAi and not secondary to non-specific effects of introducing exogenous siRNA to the cells.

As discussed above, the use of a single siRNA molecule to inhibit two proteins was successfully implemented in SKBR3 cells. A concern with the use of siRNAs is non-specific targeting, particularly in light of previous findings that mismatched siRNAs can induce inhibition of non-homologous targets (34). To address this concern, a series of experiments that would enhance our understanding of the specificity and mechanism of ErbB2 and TS protein inhibition by TS1M17 siRNA were performed. To prove specificity of target, experiments were designed to show that treatment with TS1M17 siRNA did not affect other cellular proteins, particularly other members of the ErbB family, which have a high level of sequence homology to ErbB2. As seen in figure 8B, TS1M17 siRNA did not affect ErbB1 or ErbB3 protein levels. Western blot analysis shows that TS1M17 siRNA treated SKBR3 cells express the same levels of protein as untreated and treated controls (TS Mismatch and GL2 siRNAs). In addition, other cellular proteins remained unaffected. Levels of α-tubulin and topoisomerase-I remained unchanged in response to treatments with siRNAs, further reinforcing the specificity of TS1M17 siRNA. To provide insights into the possible mechanism of action of TS1M17

siRNA, levels of ErbB2 and TS mRNA in SKBR3 cells were verified. Inhibition of protein expression can be accomplished by post-trancriptional changes to cellular RNA, translational repression or by post-translational modifications. For the latter two mechanisms, levels of cellular mRNA would remain intact. To confirm preliminary results observed by Northern blot analyses, I subsequently performed an RT-PCR analysis. As seen in figure 9, levels of both ErbB2 and TS mRNA decreased significantly after treatment with TS1M17 siRNA. Given that the proposed mechanism of gene silencing through RNA interference is cleavage of target mRNA, this finding provides strong evidence that the expression of ErbB2 and TS protein are both inhibited by TS1M17 siRNA through the process of RNA interference. It should be noted that further studies looking at quantitative expression of mRNA by Northern blot analysis will show comparative data between untreated and treated cells and will be conducted in the future. However, taken together, these data provide evidence that TS1M17 siRNA, successfully and specifically induces RNAi of two different proteins in SKBR3 cells.

An extensive series of studies have shown that activation of ErbB2 and other members of the ErbB family of RTKs results in cell signaling with activation of downstream effector pathways. These signaling cascades are responsible for the cellular processes that promote cancer cell growth and proliferation, as seen in figure 4. Although the signaling networks that are activated subsequent to receptor dimerization and intracellular kinase phosphorylation are complex, two important and distinct pathways involved in ErbB family signal transduction have been well-defined in the proliferation of tumor cells. These are the MAPK/Ras and PI(3)K-induced Akt pathways. It has been shown that

inhibition of ErbB2, with agents such as monoclonal antibodies and TKIs, affects cellular proliferation through inhibition of these two mentioned pathways. Reports show that this inhibition may generate cell cycle arrest and/or induce apoptosis in *in vitro* breast and colon cancer cells (44). Therefore, the termination of these signaling pathways is an important component to successful anticancer therapy (74). Unlike monoclonal antibodies and TKIs which inhibit protein signaling by preventing receptor phosphorylation, siRNAs exert their effects directly at the mRNA level. Prior to the present research, no studies had analyzed the effects of ErbB2-specific siRNA on the level of phosphorylated ErbB2 receptor protein. I therefore set out to confirm that TS1M17 siRNA affects the signaling cascades that are implicated in the effective development of anti-ErbB2 drugs. To perform this task, levels of phosphorylated ErbB-2 protein, which is the initiating event that leads to downstream signaling, were analyzed. As seen in figure 10, both unphosphorylated and phosphorylated forms of the ErbB2 receptor in SKBR3 cells were markedly decreased. In fact, after treatment with TS1M17 siRNA, levels of active ErbB2 protein were decreased to <5% of baseline value in SKBR3 cells after 72 hrs.

Given the finding that TS1M17 siRNA effectively inhibited expression of phosphorylated ErbB2 protein, I analyzed other downstream effects of the ErbB2 signaling pathway. One concern in this study was the limited duration of effect of TS1M17 siRNA on both active and inactive ErbB2 protein in SKBR3 cells. I verified that 72 hr inhibition of phosphorylated ErbB2 protein was sufficient to initiate downstream signaling effects on the MAPK/Ras pathway. As seen in figure 10, despite the limited time course, a

concentration of 10 nM of TS1M17 siRNA was significant enough to inhibit levels of phosphorylated MAPK by 44%. These findings suggest that the MAPK pathway was affected by TS1M17 siRNA treatment. Given this observation, other downstream effector proteins were analyzed by Western blot. Cell cycle modulators, such as $p21^{Cip}$, $p27^{Kip}$ and cyclin D1 have been linked to ErbB2 activation (74). Studies have shown that increased expression of ErbB2 receptor in breast cancer cells results in increased turnover of p27 as well as increased levels of cyclin D1 and p21. These downstream effects have also been implicated as a mechanism for cancer growth and proliferation. We observed that treatment of SKBR3 cells with TS1M17 siRNA resulted in 7.3-fold higher levels of p27 protein than baseline and control cells (Figure 10). In addition, levels of cyclin D1 and p21, which have both been shown to decrease after treatment with ErbB2 inhibitors, were inhibited by 87% and 70%, respectively. Sato et al. has linked the expression of the inhibitor of apoptosis protein (IAP), survivin, with ErbB2 expression in breast cancer cells (72). Survivin levels also decreased by 85% in cells treated with 10 nM of TSM17 siRNA. The inhibition of these proteins could be attributed to three mechanisms. These are direct effects of TS1M17 siRNA on: (1) MAPK/Ras signaling, (2) up-regulation of p27, and (3) other signaling pathways that were not analyzed, such as the PI(3)K/Akt pathway (Figure 4). Studies have shown that the expression of survivin in breast cancer cells is clearly regulated by the PI(3)K/Akt pathway. Further studies will focus on the effects of TS1M17 siRNA on this specific signaling cascade. Given that control siRNAs (TS1 Mismatch and GL2) did not induce change in baseline levels of any downstream protein in SKBR3 cells, I concluded that the process of RNAi itself did not cause the noted changes. The effect of RNAi on downstream signaling was previously shown by

Reagan-Shaw and colleagues (75). In these studies, an siRNA targeting the mRNA encoding the PI(3)K protein was designed and shown to decrease cell cycle proteins and induce apoptosis in breast cancer cells. Accumulation of p27 and inhibition of cyclin D1 was also seen after transfection. These studies suggest that siRNAs can inhibit downstream signaling at the initiating event of cytoplasmic receptor phosphorylation. Although not directly implicated in these pathways, it is conceivable that TS inhibition may also have played a role in our experimental results. Future studies will investigate the effect of ErbB2- and TS-specific siRNAs on downstream signaling molecules. However, past studies along with the data presented herein suggest that inhibition of ErbB2 phosphorylation by TS1M17 siRNA led to the downstream signaling changes.

Our study also set out to investigate the potential benefit of targeting two separate and distinct pathways involved in cancer growth and proliferation over inhibiting either target alone. The development of targeted cancer drugs has evolved over recent years. Initially, the excitement over designing molecules that specifically targeted one protein involved in oncogenesis, led to the development of an array of antibodies and TKIs. Although these molecules provide enhanced specificity in terms of targeting, they have not shown especially significant clinical activity as single therapeutic agents. For example, drugs targeting ErbB1 such as the monoclonal antibody, cetuximab, and the small molecule TKI, gefitinib, have shown limited response as monotherapy in patients with ErbB1 positive colon cancers. In recent studies, they have generated only a 10% response rate if given without another chemotherapeutic drug (63,76,77). One reason for the limited efficacy of these molecular targeted therapies is that there is tremendous redundancy in

growth factor signaling pathways in cancer cells that allows for their continued growth and proliferation. These targets are ultimately affected by a variety of pathways involved in signal transduction that lead to neoplastic disease. One example of the intricate networking of signaling pathways is seen in the ErbB family. Therefore, blocking only one of these pathways still allows the cell to act through other cellular signaling pathways and escape death. Inhibition of multiple targets would potentially prevent this phenomenon and induce higher rates of response in cancers such as colorectal, breast, and lung (49,78). Clinical trials have begun testing drugs that simultaneously inhibit multiple pathways in cancer progression, such as the TKIs ZD6474 and imatinib (Table 1). Our study also provides data to support targeting of two proteins involved in cancer cell growth and proliferation, which may provide enhanced cellular toxicity than targeting either protein alone. As seen in figure 11, TS1M17 siRNA inhibits the expression of both ErbB2 and TS proteins, and thus has greater growth inhibitory effects than SKBR3 cells treated with ErbB2-specific (B2450) or TS-specific (TS7) siRNAs. It was shown that both ErbB2- and TS-specific siRNAs inhibit proliferation of SKBR3 cells by 51 and 30%, respectively. In contrast, incubation with TS1M17 siRNA resulted in nearly 80% growth inhibition. These findings suggest significant suppression on SKBR3 cell proliferation with an IC_{50} value of 0.65 nM, which is 154-fold more potent than the cytotoxic effects of either ErbB2- or TS-specific siRNAs. Although enhanced cytotoxic effects were seen with TS1M17 siRNA treatment, protein analysis also showed that TS1M17 siRNA had greater inhibitory effects on ErbB2 protein expression than the ErbB-2 specific siRNA, B2450 (Figure 8A). This finding may suggest that the enhanced therapeutic effect may be secondary to enhanced inhibitory effects on ErbB2 protein

expression rather than the additional inhibitory effects on TS protein expression. Further work will analyze ErbB2-specific siRNAs that have improved ErbB2 protein inhibitory effects and determine their subsequent effect on cellular proliferation. However, these preliminary findings still provide validity to the potential use of multi-targeted siRNAs as single agents in the treatment of human malignancies. Given these findings, future studies will also focus on developing combination therapies and analyzing possible enhanced growth inihibition when multi-targeted siRNAs are used in conjunction with other ErbB2 and TS inhibitor compounds. With these future studies, we will analyze the potential therapeutic application of TS1M17 siRNA in combination therapy for cancer treatment.

The use of a multi-targeted siRNA in combination with other chemotherapeutic agents would broaden the therapeutic potential of TS1M17 siRNA. One of the major obstacles of cancer chemotherapy is the development of cellular drug resistance. Previous studies have shown that exposure to TS-inhibiting compounds results in acute induction of TS expression (13). This process has been implicated in the development of cellular drug resistance to TS inhibitors. The autoregulation of TS mRNA by its own protein end product mediates this process (Figure 2). Schmitz et al. showed that TS-specific siRNAs prevented the induction of TS protein in the human colon cancer RKO cell line following incubation with TS-inhibitors (38). In addition, they showed that RKO cells overexpressing TS enzyme, and therefore chronically resistant to TS inhibiting compounds, regained sensitivity to these drugs upon introduction of the TS-specific siRNA. Thus, siRNAs may provide a novel approach to circumvent both acquired and innate resistance. In this study, the combination of inhibition on ErbB2 and TS

expression with a single siRNA molecule may provide enhanced cytotoxicity in ErbB2 overexpressing cells (Figure 11). Further studies need to be conducted by combining TS1M17 siRNA with TS inhibitor compounds, such as 5-FU and raltitrexed, to determine whether inhibition of two key cellular signaling pathways can lead to enhanced chemosensitivity to tradiational cytotoxic chemotherapy.

The development of targeted drugs has led to a large selection of agents targeting specific intracellular pathways. Each of these key cellular signaling pathways is generally altered or upregulated in a limited variety of neoplastic cells, narrowing the therapeutic range of these target-specific drugs to a few types of cancers. For example, the use of trastuzumab is mainly restricted to treatment of ErbB2-positive human breast cancers. This is in contrast to the wide therapeutic range of traditional cytotoxic drugs such as 5-FU and paclitaxel which have been successfully used in the therapeutic regimens of colorectal, breast, and lung cancers. Development of novel multi-targeted drugs has also mostly been limited to agents that inhibit multiple pathways altered in specific tumor types, such as EGFR, ErbB2 and *bcr-abl*. Therefore, developing a multi-targeted drug that incorporates both targeted and cytotoxic effects could be beneficial for several important reasons. First, such an approach would broaden the clinical range of use to multiple tumor types. Second, this strategy could possibly reduce the side-effect profiles of cytotoxic agents. Current studies are being conducted that combine multi-targeted agents that have both target-specific and cytotoxic components (79). As discussed, TS is an important target for traditional chemotherapy agents and its inhibition, with drugs such as 5-FU, has been successful in the treatment of colorectal, breast, lung, and GI cancers (4). In this study,

the inhibition of a selective target (ErbB2) and an enzyme involved in multiple critical pathways of human cells (TS) in one molecule was accomplished. In order to test the range of efficacy of the multi-targeted siRNA, cytotoxic experiments were conducted in a non-ErbB2 receptor expressing human cancer cell line. Previous studies have shown that MCF-7, a human breast cancer cell, expresses extremely low base-line levels of ErbB2 protein. In fact, some have characterized MCF-7 cells as non-ErbB2 expressing (68,73). Previous studies from our lab also showed that ErbB2 protein levels were at times undetectable at base-line in MCF-7 cells (data not shown). The effect of TS1M17 siRNA on the growth of MCF-7 cells was determined, with the knowledge that these cells are not dependent on ErbB2 protein for proliferation. As seen in figure 11, the observed effect of TS1M17 siRNA was equal to that of a TS-specific (TS7) siRNA. This finding suggests that the effect on cell growth was mostly secondary to TS protein inhibition. Targeting two separate pathways with TS1M17 siRNA did not seem to produce an enhanced effect than targeting one protein alone, but the low-expression of ErbB2 in MCF-7 cells might explain this finding. Given that this cell line does not express any ErbB2 receptor, there would be no target ErbB2 mRNA for TS1M17 siRNA to inhibit and the siRNA would behave as a TS-specific siRNA, as seen in our results. This is supported by the finding that the ErbB2-specific siRNA had absolutely no effect on MCF-7 cellular proliferation. Previous drug studies also showed that the ErbB2 monoclonal antibody, trastuzumab, had absolutely no effect on MCF-7 cellular proliferation (data not shown). These studies suggest that in order to see an enhanced effect of TS1M17 siRNA when compared to drugs inhibiting either target alone, a cell system that expresses a minimum amount of both proteins may be needed. Further studies with other cell lines will need to be

conducted to evaluate the range of cancers that this type of molecule effects, particularly when compared to more selective agents.

Despite the promising effects of TS1M17 siRNA, studies remain to be done in order to elucidate the precise mechanisms of cell inhibition of this multi-targeted siRNA. This study shows that TS1M17 siRNA potently inhibits SKBR3 cell proliferation (Figure 11). There are two possible mechanisms of inhibiting cell proliferation. These are through cytotoxic (apoptosis/necrosis) or cytostatic (cell-cycle arrest) effects of the siRNA on cell growth. As seen in figure 10, TS1M17 siRNA had downstream signaling effects on the cell-cycle modulators p27 and cyclin D1. These results have been previously reported with inhibition of ErbB2 and indicate treatment with TS1M17 siRNA resulted in cell cycle arrest (71). However, studies also showed increased inhibition on survivin expression after treatment with TS1M17 siRNA, which would suggest induction of apoptosis. Some have implicated higher concentrations of drug induce apoptosis, while inhibition of ErbB2 phosphorylation at low doses induces G_0-G_1 phase cycle arrest (44). Although these data are applicable to our study, the effect of TS inhibition also plays a role in cytotoxicity. Previous studies have demonstrated that TS regulates p53 expression by binding directly to a sequence in the coding region of p53 mRNA and represses translation (80). Further evidence has shown that inhibition of TS mRNA results in a rapid rise in p53 protein levels (24). The p53 tumor suppressor protein plays an essential role in preserving the integrity of the genome and for maintaining cell-cycle progression. Its expression has been associated with cell-cycle arrest, which allows the cell time to repair DNA damage. Levels of p53 have been shown to acutely increase in malignant

cells after exposure to chemotherapeutic agents that exert their effects through DNA damage. Studies have also shown that the synthesis of p53 is regulated by direct binding of TS protein to p53 mRNA (80). Since p53 expression seems to be partly controlled by the cellular levels of TS protein, siRNAs targeting TS mRNA may inhibit expression of TS protein allowing restoration of p53 levels in cells, and thereby inducing cell-cycle arrest. It would also facilitate the apoptotic response seen at high dose concentrations of ErbB2 inhibitors. Therefore, future studies using flow cytometry as well as analyzing markers of apoptosis, may elucidate the precise mechanism of decreased cell proliferation after treatment with TS1M17 siRNA. In addition, analyzing cellular levels of p53 in TSM17 siRNA treated cells would verify secondary effects of TS protein inhibition.

The data presented herein provides encouraging results as to the potential therapeutic benefit of multi-targeted siRNAs. To continue the development of multi-targeted siRNAs as an anticancer agent, the cytotoxic effects of TS1M17 siRNA must be replicated in an *in vivo* model system. Current work is being conducted in harnessing siRNAs for systemic use. Attempts to advance the use of siRNA as therapeutic agents are limited by several different factors. The first limitation is, because of their negative charge and nuclease susceptibility, siRNAs are difficult to deliver and transport into tumor cells. As such, siRNAs must be encapsulated in cationic molecules in order to have successful uptake into cells. Coupling of siRNAs with a cationic lipid molecule has been difficult in *in vivo* models secondary to toxicity profiles of the cationic molecules. Several strategies have been developed to overcome this limitation. One such approach by Urban-Klein et al. was to complex siRNA with polyethylenimine (PEI) (81). They observed a 40%

reduction in target mRNA levels with tumor growth inhibition following systemic application in mice. Another approach is the use of nanoparticles to encapsulate siRNA duplexes and permit systemic transport to tumor cells while evading nuclease degradation. Schiffelers et al. showed tumor-selective uptake, siRNA sequence inhibition, and tumor growth inhibition with intravenous administration of ligand-directed nanoparticles containing VEGFR2-specific siRNA into tumor bearing mice (82). Given the potential of nanotechnology for cancer therapy, studies from our own lab are currently analyzing the delivery of an optimized TS-targeted siRNA to colon cancer cell tumors in nude mice with nanoparticles. The second limitation relates to the potential non-specific effects of non-homologous targeting. These effects could prove to be problematic in a clinical setting. Recently, Grimm et al. showed that transfection of mice carrying small-hairpin RNAs suffered from hepatoxicity. These findings were felt to be secondary to competition between endogenous siRNAs and foreign siRNAs for cellular transport (83). Therefore, the *in vivo* and clinical systemic use of siRNAs must wait until further research is done in elucidating and harnessing the mechanisms of RNAi.

In the present report, I have developed a novel approach of using a single siRNA molecule to target multiple pathways involved in cancer proliferation. The use of RNAi to inhibit two proteins is an innovative approach to cancer drug development. This thesis has shown that a multi-targeted siRNA directed at both ErbB2 and TS mRNA specifically and potently inhibited the expression of ErbB2 and TS protein in human breast cancer SKBR3 cells via the process of RNAi. This approach showed that inhibition of both ErbB2 and TS protein may result in enhanced cytotoxic effect than inhibition of

either protein alone. In addition, downstream signaling pathways were shown to be effected by treatment of low concentrations of multi-targeted siRNA. The use of multi-targeted drugs has become a valuable tool in cancer treatment secondary to its therapeutic benefits. These results, taken together, highlight the therapeutic promise of multi-targeted siRNAs as a strategy to be used as monotherapy or in combination with other chemotherapeutic agents against a broad range of human cancers.

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