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siRNA Targeting of Thymidylate Synthase, Thymidine Kinase 1 and Thymidine Kinase 2 as an Anticancer Therapy: A Combinatorial RNAi Approach

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Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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siRNA TARGETING OF THYMIDYLATE SYNTHASE, THYMIDINE KINASE 1 AND THYMIDINE KINASE 2 AS A POTENTIAL ANTICANCER THERAPY: A COMBINATORIAL RNAi APPROACH

(Thesis format: Monograph)

by

Christine Di Cresce

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctorate of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Thymidylate synthase (TS) is the only de novo source of thymidylate (dTMP) for DNA synthesis and repair. Drugs targeting TS protein are a mainstay in cancer treatment but off-target effects and toxicity limit their use. Cytosolic thymidine kinase (TK1) and mitochondrial thymidine kinase (TK2) contribute to an alternative dTMP-producing pathway, by salvaging thymidine from the tumour milieu, and may modulate resistance to TS-targeting drugs. We have previously shown that TS antisense molecules (oligodeoxynucleotides, ODNs, and small interfering siRNA, siRNA) sensitize tumour cells, both in vitro and in vivo, to TS targeting drugs. As both TS and TKs contribute to cellular dTMP, we hypothesized that TKs mediate resistance to the capacity of TS siRNA to sensitize tumour cells to TS-targeting drugs. Downregulation of TKs with siRNA enhanced the capacity of TS siRNA to sensitize tumour cells to traditional TS protein-targeting drugs (5FUdR and pemetrexed). Combined downregulation of these enzymes is an attractive strategy to enhance TS-targeted anticancer therapy. TK2 can phosphorylate both thymidine and deoxycytidine to generate dTMP and dCMP, precursors for dTTP and dCTP, respectively. dCTP negatively regulates deoxycytidine kinase (dCK), another enzyme that phosphorylates deoxycytidine as well as the anticancer drug gemcitabine. Antisense knockdown of TK2 could reduce TK2-produced dCMP, thus decreasing dCTP levels and inhibition of dCK, and lead to increased dCK activity, gemcitabine activation, and anticancer effectiveness. Given the substrate promiscuity of TK2, we hypothesized that: (1) TK2 can mediate human tumour cell resistance to gemcitabine, (2) antisense downregulation of TK2 can overcome that resistance, and (3) TK2 siRNA-induced drug sensitization results in mitochondrial damage. siRNA downregulation of TK2 expression sensitized MCF7 and HeLa cells to gemcitabine, but did not sensitize A549 cells (low TK2 expresser). Treatment with TK2 siRNA and gemcitabine: 1) decreased mitochondrial redox status, 2) decreased mitochondrial DNA (mtDNA:nDNA ratio), and 3) decreased mitochondrial activity. This is the first demonstration of a direct role for TK2 in gemcitabine resistance, or any independent role in cancer drug resistance, and further distinguishes TK2 from other dTMP-producing enzymes.
Keywords

small interfering RNA (siRNA), RNA interference (RNAi), combinatorial RNAi, thymidylate synthase (TS), thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), cancer, anticancer drugs, gemcitabine, deoxycytidine kinase (dCK), anticancer therapy, mitochondrial DNA, mitochondrial activity
Co-Authorship Statement

All experimental results presented in this thesis were completed by Christine Di Cresce. Laboratory members contributed invaluable experimental guidance, knowledge and criticisms and are named in the acknowledgements section.
Dedications

To my family!
Rick & Janice (parents), Jordan (brother),
Maria (aunt) and Michel (husband),
along with Mercede, Francesca and Lucio (grandparents)

Without their unwavering support, love and belief in me I never would have been able to pursue and complete my PhD. Mom, Dad, and Mich for keeping me grounded and sane whenever things started to get to crazy. Jordan and Maria for not letting me take myself too seriously.

And to my closest friends!
Lauren, Irina, Juliana, Missa, Rachelle, Sylvia, Dawn, Jill and Laura

Thank you for listening to my rants and providing me with support, comic relief and an escape hatch into the world outside the lab! You are truly some of the most wonderful people I know and I am lucky to have you as my friends.

I would especially like to dedicate this thesis to my grandfather,
Lucio Di Cresce

Gone too soon, but never forgotten or far from my heart my grandfather served as especially inspirational role model for what can be achieved through hard work, perseverance and love. I likely would never have attempted to achieve so much in my life, both personal and academic, without his steadfast insistence that I could “do anything, be anything and go anywhere” I set my mind to.
Acknowledgments

First and foremost, I would like to acknowledge my supervisor, Dr. James Koropatnick for his guidance, support, and open-door policy throughout my PhD years. Even when I thought experiments had failed and my graduate career was circling the drain, he was always able to put a positive spin on things so that “even a bad result done right, is a good result that tells us something”. Dr. Koropatnick has been an excellent role model and supervisor. His laboratory has been my home away from home, and the laboratory members have been my family away from family for the past 6 years. I will very much miss this laboratory when I am done.

I would also like to acknowledge Rene Figueredo and Dr. Peter Ferguson. Both have been invaluable resources of knowledge and support, and I do not think that this project would have been successful without their guidance and methodological expertise. I will miss arguing with them during lab meetings and maxing out my daily “question allowance” just to make Rene laugh (or roll his eyes) at me!

Finally, I have been fortunate enough to work with some amazing and talented people for the last few years. Colin, Saman and Matt - thank you so much for all your comic relief and shenanigans these last few years. You have made coming to work every day a wonderful experience and I will never forget “how NOT to use TRIzol”, “the definition of metro-sexual” and “the North Korean incident”. I also have to say thank you to the three of you for your constructive criticism in lab meetings and helping me with the flow cytometry machine....though Matt definitely was responsible for “breaking” it.
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<th>Definition</th>
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<tbody>
<tr>
<td>2'-F</td>
<td>2’ fluoro</td>
</tr>
<tr>
<td>2'-MOE</td>
<td>2'-O-methoxyethyl</td>
</tr>
<tr>
<td>2'-O-CH2PY(4)</td>
<td>2’-O-methyl4-pyridine</td>
</tr>
<tr>
<td>2'-OMe</td>
<td>2'-O-mthyl</td>
</tr>
<tr>
<td>5FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>5-FUdR</td>
<td>5-fluorodeoxyuridine</td>
</tr>
<tr>
<td>Ago2</td>
<td>Argonaute 2</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>AMEM</td>
<td>alpha modified Eagle’s medium</td>
</tr>
<tr>
<td>ASO</td>
<td>antisense oligodeoxynucleotides</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BRCA</td>
<td>breast cancer gene</td>
</tr>
<tr>
<td>C2</td>
<td>control 2 siRNA</td>
</tr>
<tr>
<td>C3</td>
<td>control 3 siRNA</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>CH₂THF</td>
<td>methylene tetrahydrofolate</td>
</tr>
<tr>
<td>CM</td>
<td>cell membrane</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>dCDP</td>
<td>deoxycytidine diphosphate</td>
</tr>
<tr>
<td>dCK</td>
<td>deoxycytidine kinase</td>
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<td>dCTP</td>
<td>deoxythymidine triphosphate</td>
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<td>dFdC</td>
<td>gemcitabine; 2', 2'-difluoro 2'deoxyctydine</td>
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<td>dFdCTP</td>
<td>gemcitabine triphosphate</td>
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<td>DHFR</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FdUMP</td>
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<td>FdUTP</td>
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<tr>
<td>GARFT</td>
<td>glycinamide ribonucleotide formyl transferase</td>
</tr>
<tr>
<td>hENT1</td>
<td>human equilibriative nucleotide transporter 1</td>
</tr>
<tr>
<td>LF2K, LFA2K</td>
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<td>microRNA</td>
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<tr>
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<td>Moloney murine leukemia virus reverse transcriptase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<td>nuclear DNA</td>
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<td>post-transcriptional gene silencing</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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RISC  RNA induced silencing complex
RNAi  RNA interference
ROS  reactive oxygen species
shRNA  short-hairpin RNA
siRNA  small interfering RNA
SNP  single nucleotide polymorphism
ssRNA  single-stranded RNA
TBS-T  Tris buffered saline with tween
TK1  thymidine kinase 1
TK1 siRNA  thymidine kinase 1 targeting siRNA
TK2  thymidine kinase 2
TK2 siRNA  thymidine kinase 2 targeting siRNA
TLR  toll-like receptor
TRBP  TAR RNA binding protein
TS  thymidylate synthase
TS siRNA  thymidylate synthase targeting siRNA
UTR  untranslated region
Chapter 1

« Introduction »

Each section, and similarly each sub-section, is organized in a reverse pyramid fashion to take the reader from a broad overview of the topic at hand into project-related specifics. The purpose of this section is to establish a basic understanding of RNA interference, cancer, and mechanisms by which deoxythymidine monophosphate (or thymidylate) is produced for DNA synthesis and repair. Subsequently, siRNA in the treatment of human disease is introduced and, specifically, its potential application to the treatment of cancer. In relation to this, small molecule inhibitors of thymidylate production used in anticancer therapy, and mechanisms of resistance to these drugs, are discussed, and antisense is suggested as a way to combat resistance. Finally, a short explanation summarizing and connecting these topics is presented in specific relation to the rationale for the project (the combined use of small interfering RNAs and small molecule drugs to improve upon the inhibition of tumour cell proliferation) prior to stating the hypotheses.

1.1 Small interfering RNA (siRNA)

1.1.1 RNA interference molecules and the RISC complex

In eukaryotic cells, the regulatory mechanism of RNA interference (RNAi) contributes to the control of gene activity and expression. RNAi, a phenomenon first described in the nematode *C. elegans*, is a process by which short double-stranded RNA (dsRNA) molecules down-regulate a specific gene product by complementary, base-guided cleavage of target mRNA [1]. The determination of what constitutes target mRNA is based upon Watson-Crick sequence complementarity with the antisense RNA molecule loaded into the RNA induced silencing complex (RISC): the target mRNA is the sense strand. In RNAi, dsRNA molecules, approximately 19-23 nucleotides in length, are loaded into the RNA-induced silencing complex (RISC) and lead to post-transcriptional
gene silencing (PTGS) by mRNA degradation or translational inhibition of target mRNA transcripts [2, 3]. The optimal siRNA for use in mammalian cells has been determined, by extensive research, to be a 21 nucleotide duplex with 2 nucleotide 3′-overhangs at both ends [4].

There are a number of different RNAi molecules that utilize RISC for control of gene expression, for example: (i) microRNAs (miRNAs), (ii) short-hairpin RNAs (shRNAs), and (iii) small-interfering RNAs (siRNAs) - which may either be synthetically made and introduced or produced endogenously from longer dsRNA molecules in the cell [1, 5, 6]. miRNAs are a form of endogenous RNAi within the cell that regulate the gene expression of multiple target genes through partial- to perfect complementarity sequence matches. shRNAs may be constitutively expressed from a promoter to produce miRNA-like or siRNA-like molecules that result in long-term, more continuous, gene silencing. In contrast, siRNAs are a form of transient RNAi that are generally synthesized to be perfect sequence matches to their target mRNA and, compared to shRNAs, have a decreased risk of toxicity related to the overloading of upstream components of the endogenous miRNA RISC-mediated silencing pathway [1, 2, 5-7]. While all 3 types of RNAi molecules listed utilize RISC for silencing, the process of formation and RISC association is slightly different (Figure 1).

The RISC complex itself is composed of 4 different enzymes: Dicer, TAR-RNA binding protein (TRBP), protein activator of PKR (PACT), and Argonaute 2 (Ago2) [3, 8, 9]. The Dicer enzyme is an RNaseIII endonuclease responsible for processing of pre-miRNA and dsRNA molecules to the appropriate length for incorporation and use in RISC [10-12]. Dicer then interacts with TRBP, which contributes to sensing and loading of dsRNA molecules into a RISC complex with Ago2 present [13-16]. The PACT enzyme is thought to contribute to enhancement of pre-miRNA processing, as well as increased stability and activity of active RISC [14, 17]. Ago2 is the catalytic core of the RISC complex and is responsible for the cleavage and release of the target mRNA [9, 18-24]. In general, perfect or near-perfect complementary matches will result in mRNA cleavage, whereas
partial complementarity will result in the repression of translation (Figure 1). The nucleotides in positions 2-8 of the antisense strand represent the seed region of the molecule and the degree of complementarity between these nucleotides and the target mRNA is most important for determination of the mechanism of silencing (via cleavage, or translational repression and destabilization) and antisense on- and off-target mRNA silencing [3, 25-28].

The antisense molecule of choice for the experiments described in this thesis is siRNA. In particular, the transient nature of siRNA inhibition of mRNA and its decreased toxicity compared to shRNA were ideal for the type of therapeutic application envisioned.
Figure 1: miRNA, shRNA and siRNA processing, RISC association and mRNA silencing. miRNA (A), shRNA (B) and siRNA (C) processing and incorporation into RISC. Perfect or near-perfect sequence matches to mRNA will result in mRNA cleavage, while partial sequence matches result in physical inhibition of mRNA translation followed by degradation within the P-bodies of the cytoplasm. This figure is an adapted version of the image found in [29] and has been used with written authorization and consent from Elsevier Ltd.
1.1.2 Challenges of using siRNA (and RNAi) as therapeutics

siRNA, and other RNAi molecules, are unstable in vivo due to an abundance of endonucleases that promote their degradation, and rapid renal clearance [30]. In the movement towards the use of siRNAs and RNAi molecules as therapeutics, several challenges became apparent and will be discussed in more detail below: (i) delivery of the molecules to the cells, (ii) specificity of the molecules for their intended mRNA target vs. targeting other molecules with similar sequence characteristics, (iii) stability of the siRNA within bodily fluids, and (iv) innate immune system activation as the molecules are recognized as foreign [29]. To meet these challenges, researchers have improved upon the chemical makeup of RNAi molecules and developed nucleic acid delivery vehicles.

1.1.2.1 Chemistry of siRNA molecules

To improve upon the pharmacokinetic and pharmacodynamic properties of siRNAs, and other RNAi molecules, modifications have been made to the base, sugar and/or backbone of the RNA molecules in an attempt to increase specificity and affinity, while decreasing degradation and immune stimulation [31-33]. With respect to backbone substitutions, phosphorothioate modification, where a sulfur atom replaces a phosphate oxygen atom, is widely used to decrease nuclease degradation and prevent renal clearance [34]. Other backbone modifications include: phosphodiester, 2’-O-methyl, and locked nucleic acids that contain an extra bridge between the 2’ oxygen and 4’ carbon of the ribose that locks the sugar into the 3’endo confirmation. Modifications to the ribose sugar include 2’-O-methyl (2’-OMe), 2’fluoro (2’-F), 2’-O-methoxyethyl (2’-MOE) and 2’-O-methyl4-pyridine (2’-O-CH2Py(4)), and 2’-ribose modifications have been shown to increase stability, affinity, and potency [29, 35, 36]. 2’-O-methylation of the ribose, specifically of the RNA in positions 2 and 8 of the antisense strand, has been observed to decrease intravascular degradation and innate immune stimulation as well as reduce off-target mRNA silencing without affecting on-target silencing of perfectly complementary mRNA targets [25, 32, 36-39].
### 1.1.2.2 Innate immune system activation

There are a number of *pattern recognition receptors* that have evolved to recognize *pathogen-associated molecular patterns* and initiate an immune response based on the recognition of structural features of foreign molecules. When exogenous siRNAs and other RNAi molecules are introduced into *in vitro* and *in vivo* systems, activation of the innate immune system and an interferon-mediated activation response can occur as a result of these molecules being recognized as alien, generally in a sequence-independent manner [40]. In particular, toll-like receptors (TLRs) 3, 7, 8, and 9, interferon-induced protein kinase R (PKR), and retinoic acid inducible gene I (RIG-I) are responsible for the activation of the innate immune system in response to RNA- and DNA-based RNAi molecules [40-43]. TLR3 recognizes dsRNA molecules, TLR7 and TLR8 recognize ssRNA, TLR9 recognizes hypomethylated DNA (and RNA) rich in CG dinucleotides, PKR recognizes dsRNA molecules of greater than 33 nucleotides, and RIG-I recognizes uncapped, 5′-tri-phosphorylated dsRNA and dsRNA longer than 100bp [40, 43-49].

siRNAs and RNAi molecules have been experimentally modified to reduce immune stimulation. The list below represents two specific examples of these modifications: Firstly, it has been determined that optimized siRNA are 21-nucleotide duplexes with 2-nucleotide 3′-overhangs that avoid known immunogenic sequences and have reduced capacity to activate TLRs (specifically TLR3 and TLR7), which is based primarily on length. For example, the sequence 5´-GUCCUUCAA-3´ is capable of specifically activating TLR7 [4, 50, 51]. Secondly, the introduction of 2 or 3 residues into an siRNA that are 2′OMe-modified can prevent immune activation as the 2′OMe groups acts as competitive inhibitors of TLR7 activation, thereby inhibiting cytokine production without affecting silencing ability [41, 52]. Other sequence characteristics to optimize siRNAs that do not pertain to avoidance of immune stimulation are discussed below.
1.1.2.3 Delivery vehicles

Naked siRNAs are not stable in vivo or in vitro for long periods of time, may generate an immune response, and suffer from poor cellular uptake [29]. Along with increased susceptibility to enzymatic degradation, intrinsic physicochemical properties of siRNAs pose additional challenges to the successful delivery of siRNAs into cells and therapeutic utilization. Those properties include their small size, hydrophilicity, and net negative charge [53]. Mechanisms to achieve successful delivery of RNAi molecules may be categorized into: (1) physical delivery methods, (2) conjugation to targeting molecules, and (3) carrier-mediated delivery methods [54-56].

Of particular importance to this thesis are carrier-mediated delivery methods, because the transfection reagent (delivery agent) used for experimentation is the cationic-liposome Lipofectamine 2000 (LF2K or LFA2K), which falls into this delivery classification. First demonstrated by Felgner et al. in 1987, lipofection, or the delivery of DNA (or RNA) into the cell via cationic lipids that spontaneous entrap the molecules into liposomes, is an efficient and highly reproducible way to facilitate the entry of RNAi molecules into the cell. This method is associated with less toxicity than other delivery methods, such as viral vectors used to deliver shRNA plasmids [57, 58]. Positively charged cationic lipids work to attract and encompass negatively charged nucleic acid RNAi molecules, resulting in spontaneous formation of liposomes containing RNAi molecules within or on the lipid carrier. These liposomes allow the RNAi molecules to overcome the electrostatic repulsion of the negatively charged cell membrane (CM) and facilitate passage across the CM and/or endocytosis [59, 60]. Since their initial application, these molecules have been further refined and modified to encourage greater cellular uptake, decrease systemic non-specific toxicity, facilitate cell- or tissue-specific targeting, and increase endosomal escape [53, 59, 61, 62]. While the LF2K reagent used here has been shown to be more toxic than other lipofection agents (especially when transfecting DNA plasmids), it was also superior in its ability to specifically delivery siRNAs into cells, allow endosomal escape of the RNAi molecules, can be used in medium containing serum, and
did not form as many large non-functional, and potentially toxic, aggregates [63-65]. As outlined in Materials and Methods, we took both toxicity and other considerations into account [60] and the LF2K toxicity in our experiments was further decreased by reducing the amount of LF2K used without affecting siRNA silencing capacity (Appendix 1, Figure 36).

Large strides have been made towards overcoming the challenge of siRNA delivery in vivo with numerous new and inventive mediators of delivery [56, 66-69]. In 2010, Mark E. Davis and colleagues conducted the first-in-human phase I clinical trial involving the systemic administration of siRNA using a delivery vehicle to patients with solid cancers. They used a cyclodextrin-based, human transferrin protein-targeted nanoparticle delivery system and provided proof of an RNAi-mediated (RISC-mediated) mechanism of gene silencing in humans as a result of action of the administered siRNA [70, 71]. Carrier vehicles and formulation are not the only means of improving RNAi delivery; administration of small molecule drugs can also be augmented by a variety of delivery platforms (liposomes, penetrating-peptides, nanogels, etc.) to increase safety, potency, tissue permeability, and target delivery to the sites of tumours [72-75].

1.1.2.4 Characteristics of a good siRNA sequence

While the seed region, already discussed above, is vitally important to siRNA on-target specificity, other key characteristics have been proposed as important to maximize effectiveness and specificity of siRNAs [76]. These key characteristics are summarized in Table 1, and function to optimize potency and effectiveness while decreasing non-specific side-effects associated with the sequence and concentration [30]. As information increased, algorithms were developed and are updated to aid industry in the creation of optimized, targeting siRNAs [27, 76-78].
Table 1. Characteristics of highly specific and potent siRNAs with reduced off-target effects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Design/Rational</th>
<th>Additional References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC content between 30-52%</td>
<td>Duplexes may have difficulty unwinding (RISC entry) when GC content is greater than 52%. Reduced interaction with mRNA recognition site when GC content is below 30%</td>
<td>[76, 79, 80]</td>
</tr>
<tr>
<td>Lack of siRNA secondary structure formation</td>
<td>Interference with the formation of stable siRNA duplexes during synthesis, RISC incorporation, and target mRNA recognition</td>
<td>[76, 79, 81]</td>
</tr>
<tr>
<td>Asymmetry in siRNA duplex antisense strand selection by RISC</td>
<td>5’-end of the antisense siRNA strand should have lower thermodynamic stability vs. passenger strand. Biases for A residue, and against C or G residues, at position 19 of passenger strand facilitate looser binding at the 5’-end of antisense strand and promotes uptake into RISC.</td>
<td>[78, 82, 83]</td>
</tr>
<tr>
<td>Specific base position biases within the sense strand (or target mRNA)</td>
<td>Bias towards an A at positions 3 and 19, at least 3 “A/U” bases at positions 15-19, and a U at position 10. Bias against a G at position 13 and a G or C at position 19 of the sense strand. These biases may be important for efficient mRNA cleavage, which might involve binding to the target mRNA, cleavage itself, or recycling of the activated RISC. Ago2 has a bias toward cleavage at position 10 when a U is present; though it can still cleave after any nucleotide.</td>
<td>[76, 79]</td>
</tr>
<tr>
<td>Lack of direct immunostimulatory sequences within the siRNA</td>
<td>Several sequences have been identified that will activate TLRs in a sequence-dependent (instead of general independent) manner (e.g. 5’-UGUGU-3’ or 5’-GUCCUUCAA-3’)</td>
<td>[50, 84, 85]</td>
</tr>
<tr>
<td>Avoidance of sequences with known homology to unintended targets - 3’UTR complementarity with unintended targets increases off-target silencing</td>
<td>Sequence-dependent off-target effects should be kept to a minimum via use of bioinformatics searches (e.g. BLASTn or Smith-Waterman dynamic programming sequence alignment algorithm). In particular, avoid sequences that have a completely or nearly complete complementary seed sequence (nucleotides 2–8 of the guide strand). Sequence-independent chemical modification of siRNA RNA bases may help to reduce these effects.</td>
<td>[26, 28, 77, 86, 87]</td>
</tr>
<tr>
<td>Lack of secondary structure at that target site</td>
<td>Secondary structure in the complementary region of the target mRNA could result in decreased RISC mediated cleavage. However, because siRNAs are part of the RISC ribonucleoprotein complex which has putative helicase activity, secondary structure may not be as important for activity. Avoidance of tandem repeats within the RNA so as to prevent the possibility of hairpin-loop formation.</td>
<td>[76, 81, 88]</td>
</tr>
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</table>
1.1.3 siRNA and RNAi in treatment of human diseases

Perhaps the greatest potential of RNAi is as a therapeutic in the treatment of diseases. RNAi molecules would broaden the scope of what can be targeted for therapeutic purposes; targets exist that are considered to be undruggable (i.e., not amenable to attack by small-molecule drugs and antibodies, which are constrained by their need to bind to enzyme active pockets or exposed epitopes) [37]. If RNAi molecules could be used successfully in vivo, the entire spectrum of the proteome could become “druggable” through reduction of RNAs responsible for production of those proteins; the sequence specificity of Watson-Crick base pairing between RNAi and target RNA should, in theory, produce a highly specific means of reducing both RNA and the products encoded by the RNA and/or generated by direct or indirect action of non-coding RNA. siRNA and RNAi molecules are attractive candidates for treatment of cancer, retinal degeneration, viral infections, respiratory disorders, and genetic disorders with well-characterized mutations, such as muscular dystrophy, Down syndrome, and cystic fibrosis [68, 89].

Along with the challenges mentioned above, there are other barriers to the successful use of siRNA, and RNAi molecules, in vivo that include: biological barriers (e.g., tissue penetrance and intracellular delivery and trafficking), toxicities of RNAi (e.g., reaction to delivery vehicle, RISC saturation), tissue specificity (e.g., healthy versus diseased tissue), pharmacokinetics and pharmacodynamics, and potential resistance to RNAi (e.g., inherent resistance based on genetic variations between different ethnic groups in allele frequency and/or SNPs, as well as altered RNAi processing machinery) [37, 90-92]. Despite these issues, there are a number of RNAi molecules currently in clinical trials, summarized in Table 2. While no RISC-mediated RNAi molecules have currently been approved for use in the clinic, a few of the more well-established oligodeoxynucleotides (ODNs), a form of RNAi that utilizes RNase H for mRNA cleavage, have advanced into clinical trials and some have also been approved for treatment of human disease [54]. These approved ODNs are Mipomersen (approved in 2013, which targets apolipoprotein
B-100 and is used to treat familial hypercholesterolemia) and Vitravene (which targets CMV IE2 (cytomegalovirus immediate early protein 2) protein and is used to treat retinitis caused by CMV in HIV-infected patients; approved in 1998 and since withdrawn due to lack of use) [54, 93-97].
Table 2. siRNAs in human clinical trials.
This table was compiled using information contained at clinicaltrials.gov and [29, 61, 98, 99]

<table>
<thead>
<tr>
<th>siRNA Drug Name</th>
<th>Disease</th>
<th>Disease Category</th>
<th>Target</th>
<th>Phase</th>
<th>Company</th>
</tr>
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<tbody>
<tr>
<td>Bevasirnaib</td>
<td>Age-related macular degeneration (AMD) and macular edema (ME)</td>
<td>Ophthalmology</td>
<td>VEGF</td>
<td>III (AMD), II (ME)</td>
<td>Opko Health</td>
</tr>
<tr>
<td>PF-4523655 (PF-665)</td>
<td>(diabetic) AMD and ME</td>
<td>Ophthalmology</td>
<td>RTP801 (DDIT4)</td>
<td>II (AMD &amp; ME)</td>
<td>Quark</td>
</tr>
<tr>
<td>Sirna-027 (AGN211745)</td>
<td>AMD</td>
<td>Ophthalmology</td>
<td>VEGF-R1</td>
<td>I/II</td>
<td>Allergan</td>
</tr>
<tr>
<td>QPI-1007</td>
<td>Chronic optic nerve atrophy (NAION)</td>
<td>Ophthalmology</td>
<td>proNGF, caspase2</td>
<td>I</td>
<td>Quark</td>
</tr>
<tr>
<td>TD1010</td>
<td>Pachyonychia congenita</td>
<td>Genetic disorder</td>
<td>Keratin K6a</td>
<td>Ib</td>
<td>International Pachyonychia Congenita Project</td>
</tr>
<tr>
<td>Proteosome siRNA (iPsiRNA)</td>
<td>Metastatic lymphoma and melanoma</td>
<td>Oncology</td>
<td>Immuno-proteosome B-subunits</td>
<td>I</td>
<td>Duke University</td>
</tr>
<tr>
<td>CALAA-01</td>
<td>Solid tumours</td>
<td>Oncology</td>
<td>M2 subunit of ribonucleotide reductase</td>
<td>I</td>
<td>Pharmaceutica l Inc.</td>
</tr>
<tr>
<td>Atu027</td>
<td>Solid tumours (gastrointestinal, lung)</td>
<td>Oncology</td>
<td>PKN3</td>
<td>I</td>
<td>Silence Therapeutics AG</td>
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<tr>
<td>ALN-VSP02</td>
<td>Solid tumours (liver)</td>
<td>Oncology</td>
<td>KSP and VEGF</td>
<td>I</td>
<td>Alnylam</td>
</tr>
<tr>
<td>I5NP (QPI-1002)</td>
<td>Delayed graft function and acute kidney inflammation</td>
<td>Inflammation</td>
<td>p53</td>
<td>II</td>
<td>Quark</td>
</tr>
<tr>
<td>PRO-040201</td>
<td>Hypercholesterolemia</td>
<td>Metabolic disease</td>
<td>ApoB</td>
<td>I</td>
<td>Pharmaceutica ls Corp.</td>
</tr>
<tr>
<td>ApoB SNALP</td>
<td>Hypercholesterolemia</td>
<td>Metabolic disease</td>
<td>ApoB</td>
<td>I</td>
<td>Tekmira</td>
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<td>ALN-RSV01</td>
<td>Respiratory Syncytial Virus (RSV)</td>
<td>Viral infection</td>
<td>RSV nucleocapsids</td>
<td>II</td>
<td>Alnylam/Cubist</td>
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<td>siG12D LODER</td>
<td>Solid tumours (pancreatic adenocarcinoma)</td>
<td>Oncology</td>
<td>KRAS</td>
<td>I</td>
<td>Silenseed Ltd.</td>
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<td>ALN-TTR02</td>
<td>Transthyrein (TTR)-mediated amyloidosis</td>
<td>Genetic disorder</td>
<td>TTR</td>
<td>II</td>
<td>Alnylam/Genzyme</td>
</tr>
<tr>
<td>SLY040012</td>
<td>Intraocular pressure</td>
<td>Ophthalmology</td>
<td>ADRB2</td>
<td>II</td>
<td>Sylentis</td>
</tr>
</tbody>
</table>
1.1.4 Combinatorial RNAi

Several properties of RNAi activity must be considered when using RNAi molecules in combination. For example, RNAi substrates (such as miRNAs, shRNAs and siRNAs) can compete with each other for RISC incorporation and processing [100]. In addition, the RNAi pathway is saturable, containing rate-limiting components such as Exportin-5 [101] and TRBP [15, 102] and Ago2 [103]. Exportin-5 is particularly important in regard to miRNAs and shRNAs, which require it for nuclear export [100, 101]. Oversaturation of Exportin-5 in the RNAi pathway with excessive miRNA/shRNAs resulted in mortality in mice due to severe liver toxicity [104]. TRBP is important for all double-stranded RNAi substrates as it acts as a sensor for selection and incorporation of the antisense guide strand into RISC [15, 100, 105]. Oversaturation of the RNAi pathway results in modulation of endogenous miRNA pathways important in cell growth, differentiation, and death [104]. This highlights the importance of ensuring that the RNAi pathway is not oversaturated in in vitro and in vivo studies and that shRNAs inherently carry an augmented risk of saturation over siRNAs (which bypass the necessity of Exportin-5 and other upstream RNAi processing enzymes).

Combining multiple siRNAs targeting different RNAs in the same cell is relatively novel. Previous studies have not focused on combining multiple siRNAs, but various combinations of two of the following with or without concurrent drug treatment have been reported: ODN, siRNA, miRNA and shRNA [106-108]. The concept of an antisense siRNA cocktail to downregulate multiple targets has been more actively pursued for treatment of virally-induced human diseases than for treatment of cancer [89, 106, 109]. In a very rare example, Chen et al. experimented with using 7 different siRNAs to simultaneously target multiple viral genomic components as a potential treatment for Hepatitis B [110].

Competitive inhibition upon combining several distinct siRNAs can result in a loss of silencing activity. The capacity of a given siRNA to interfere with the ability of another siRNA to downregulate its target mRNA is referred to as competition potency [111].
Factors contributing to a given siRNA’s competition potency, whether it itself is a targeting siRNA or non-targeting siRNA (i.e., where there are no known sequences with sufficient complementarity in cells in which they are applied to mediate silencing), have not been solidly determined. Work in the field has focused on the chemical makeup of a given siRNA that may contribute to silencing efficiency, silencing potency, competition potency and, more recently, modifying the concentration of a given siRNA when using it in combination with other siRNA(s) [111-114].

Combining multiple siRNAs targeting different mRNAs into one treatment is an especially intriguing idea for anticancer therapy, where use of drug cocktails in chemotherapy regimens is routinely applied in order to have an effect on several cancer-related proteins at the same time. While one would generally attempt to mix multiple different siRNA duplexes together to achieve this, Tiemann et al. took an approach significantly ahead of its time (given the field) and created a prediction algorithm for dual-targeting siRNAs in which both strands of the siRNA duplex stand a 50:50 chance of being incorporated into RISC and where each is completely complementary to different mRNA transcripts; dual targeting siRNA were subsequently created, tested, and found to mediate target mRNA downregulation [115].

1.2 Cancer: A general introduction to cancer and cancer treatments

1.2.1 What is the problem and how big is it?

The Canadian Cancer Society estimated that in 2013 there would be 187,600 new cases of cancer diagnosed, that the general probability of developing cancer would be 2 in 5 Canadians, and that 75,500 deaths from cancer will occur during this same year. Consequently, cancer is the leading cause of death in Canada accounting for approximately 30% of all deaths [116, 117]. There are risk factors associated with the potential development of cancer, some of which are modifiable, including: age (increased incidence in people over 50 years of age), genetics (e.g., a family history of cancer/inherit cancer risk, BRCA gene mutations, and sporadic mutations), poor diet,
lack of physical activity, obesity, exposure to radiation (including sunlight), alcohol, infection with certain bacteria and viruses (e.g., Hepatitis C virus and human papillomavirus), smoking (including frequent exposure to second-hand smoke), and exposure to other environmental risks (e.g., asbestos, pesticides, and geographic location, both within and between countries) [116, 117]. There is an urgent need for better treatment options as cancer represents a significant global crisis and is a substantial burden to the Canadian population and healthcare system. Canada is a developed country with a cancer incidence greater than the global average, though incidences in poor and developing countries are quickly increasing [118-120].

1.2.2 The origins of cancer: Cancer as a disease of uncontrolled cellular proliferation

Cancer is not one disease, but rather a term that can be applied to a diverse group of diseases sharing the common characteristic of abnormal, uncontrolled, cell division. These uncontrolled cells eventually form tumours (a mass of these cells) within a particular organ and which may eventually progress to form malignant tumours (tumours that invade or spread throughout the original organ and to nearby organs or other distant sites within the body)[121]. The underlying causes of transformation from “healthy cell” to “cancerous cell” are immense in number, and it is uncommon for any two cancer types to share exactly the same underlying deviations that permit cancer development, survival of the tumour, and increased morbidity and mortality in cancer patients.

Nevertheless, some broad generalizations can be attributed to cancer cells and these were eloquently summarized by Hanahan and Weinberg in their 2000 and 2011 “Hallmarks of Cancer” reviews. The hallmarks of cancer (including emerging hallmarks) have been described as:

1) Sustained proliferative signalling
2) Deregulated cellular energetics
3) Resistance to cell death
4) Genome instability and mutation
5) Induction of angiogenesis
The overarching theory is that cancer begins with mutations within the DNA of the cell’s genome (activation of oncogenes and/or inhibition of tumour suppressor genes). These mutations accumulate at such a rate that they go unrepaired (or occur in a DNA repair enzyme leading to decreased capacity to repair mutations occurring at relatively low rates) and confer a mutant phenotype onto the cell which provides it with the ability to replicate and reproduce chaotically, often in the face of signaling to the contrary. Through the hallmarks described above, cancer cells gain a proliferative advantage over the surrounding non-cancerous cells within their environment, domination of their surroundings (i.e., acting autonomously and without regard for the viability and function of normal surrounding cells), and capacity to accumulate, over time, progressively more malignant characteristics.

1.2.3 Cancer as a metabolic disease: The Warburg and Crabtree hypotheses

From the 1920s through the 1950s, Otto Warburg advanced and revolutionized the field of tumour cell metabolism. He demonstrated that tumour cells, compared to normal cells, prefer to metabolize glucose by glycolysis rather than respiration even in the presence of sufficient oxygen [122]. He then went on to postulate that tumorigenesis occurs through cellular defects in mitochondrial respiration that cause the cell to adopt glycolysis as a means to survive; that cancer is initiated by respiratory impairment and increased glycolytic rate occurs as a result [122-124]; this became known as the Warburg Effect and Warburg Hypothesis.

Around the same time as Warburg’s initial discoveries in the 1920’s, Herbert Crabtree demonstrated and argued an alternative explanation for Warburg’s observations. Crabtree demonstrated that, in both normal and cancerous cells, increased glycolysis
inhibited respiration and suggested that this explained the deceased respiration noted by Warburg in tumour cells but, that actual defects in respiration were not the cause of the increased glycolysis [125, 126]; this became known as the Crabtree Effect and Crabtree Hypothesis. It is important to note that the Crabtree Effect does not actually explain the difference in magnitude between glycolysis in cancer cells as compared to normal cells and for this reason Warburg’s hypothesis became predominant in the cancer field even though a direct casual relationship has not been definitively elucidated.

We are now aware that the relationship between cancer and metabolism, specifically with respect to glycolysis, is a complex one and below are some points that highlight this diverse phenomenon and opportunities which may also be exploited for cancer therapy [127]:

I. *altered metabolism is selected for by the tumour microenvironment*: Tumours experience hypoxic conditions when they outgrow their local blood supply and decreased reliance upon respiration and increased reliance upon glycolysis is advantageous - changes in hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF) occur [128, 129]

II. *the activation of certain oncogenes can drive changes in cell metabolism*: For example, oncogenic K-ras promotes metabolic changes in glucose metabolism and elevated glucose uptake consistent with a K-ras-mediated increase of glycolysis in tumours [130, 131]

III. *lactate produced and secreted by cancer cells from increased glycolysis may serve to support cancer cell survival, invasion, metastasis and immune evasion*: For example, in *in vitro* experiments, lactic acid suppressed the activity of human cytotoxic T lymphocytes (proliferation by up to 95% and killing by up to 50%) and activity was restored when cells were allowed to recover in lactic acid free medium [132, 133]
IV. *that altered metabolism may serve to support macromolecule biosynthetic pathways:* Proliferating cells require not only ATP but, also amino acids, nucleotides and fatty acids to support the increased demand on macromolecular synthesis for cellular replication. A function of increased glycolysis in rapidly proliferating cells may be to provide high levels of glycolytic intermediates capable of supporting biosynthetic pathways [134, 135]. Glycolysis is a major source of carbons (5 to 9 carbons out of a required 10) for nucleotide biosynthesis [134].

It is important to note, that often, the Warburg hypothesis is interpreted to mean that glycolysis occurs instead of respiration; that damage to respiration is thought to occur instead of deregulation of glycolysis. However, many tumour have been shown to demonstrate the Warburg effect (glucose fermentation) while still performing mitochondrial respiration (glucose oxidation) [136]. Part of the confusion stems from Warburg’s initial misinterpretation of his own early data and which was later clarified - relative to their consumption of glucose. Indeed, the respiration of cancer cells is low but, it is not low in comparison to the respiration of normal cells; in fact the high glucose consumption rate is used for tumour visualization via positron emission tomography (PET) [135, 136]. This means that respiration and mitochondria can still play important, functional, roles in cancer cells even when glycolysis is increased.

1.2.3.1 *Relevance of mitochondria and mitochondrial DNA (mtDNA) in cancer*

Recall that mitochondria are sub-cellular organelles producing ATP through oxidative phosphorylation (OXPHOS) and playing an important role in the control of cellular apoptosis. Deregulation of mitochondria does not explain the Warburg effect, however, there is strong evidence that defective mitochondria do accumulate in tumours [137]. Oncogenes and tumour suppressor genes can influence mitochondrial mass (which, in healthy cells, is proportion to cell size and energy demands) by having downstream effects on mitochondrial biogenesis (formation) and mitophagy (intracellular mitochondrial turnover or degradation); this make sense as mitochondrial biogenesis
involves replication of the mitochondrial genome which is a coordinated effort between mitochondrial and nuclear genome encoded proteins [135, 138]. For example, members of the Bcl-2 superfamily, regulators of cell death, have a role in both the regulation of apoptosis and in mitochondrial fission and fusion dynamics (important in responding to energy demands during cellular stress); indicating a interrelated fluidity to mitochondrial cell death and energy functions and highlighting Bcl-2 family members as potential anticancer targets [137, 139].

The mitochondrial genome is 16,569 bp large and encodes 13 distinct proteins which are primarily involved in the synthesis of the electron transport chain (ETC) for the OXPHOS production of ATP. Each mitochondrion typically contains 2 to 10 copies of mitochondrial DNA (mtDNA) and each cell has, on average, 1000 mitochondria resulting in thousands of mtDNA genomes/copies per cell [140]. Mutations within the mitochondrial genome have been reported in cancers of the pancreas, breast, prostate, bladder, thyroid and colon (see Chatterjee et al. [141] for a review of cancer sites and associated mutations in mtDNA). The mitochondria contribute to the formation of reactive oxygen species (ROS) within the cell via leakage of electrons from the ETC. An increase in ROS, that cannot be balanced by antioxidants, can result in oxidative stress, (implicated in a variety of pathologies including aging and cancer) that can lead to the oxidation of proteins, lipids, and nucleic acids such as nuclear DNA (nDNA) and mtDNA; thus, mitochondria serve not only as source of ROS but also as a target of ROS [142-144]. For example, mitochondrial-produced ROS can contribute to mtDNA and nDNA mutations, which can then lead to further cellular deregulation and increased ROS production in a circuitous cycle involving damage to mtDNA and ETC components [142, 145].

mtDNA alterations and copy number changes may also have a diagnostic value in human cancers. qPCR can be used to evaluate quantitative variations in mtDNA content. Many cancer types have shown either increased mtDNA content (acute lymphoblastic leukemia, colorectal, head and neck, ovarian, etc.) or a decrease in mtDNA content
(breast, non-small cell lung cancer, hepatocellular carcinoma, etc.) and chemotherapy resistant phenotypes have been associated with both increased and decreased mtDNA in cancers of the head and neck and colon [146-150]. Studies of mtDNA content in human tumours have shown that there is some degree of stability in the range of mtDNA copy number (within 2-fold) for a particular tumour type or primary site when compared to the content in normal surrounding tissue [151, 152]. As numerous studies have made an association between mtDNA copy number and cancer risk and/or disease progression, mtDNA copy number variations are being explored as a novel biomarker for human cancers [146, 153-157]. mtDNA can be easily detected from bodily fluids (e.g. whole blood, saliva, urine) and as such, monitoring mtDNA copy quantitative changes is a potentially non-invasive method for screening pre-malignant lesions and high-risk individuals along with monitoring or charting cancer progression, identifying disease recurrence and predicting prognosis.

1.2.3 The treatment of cancer

Current anticancer treatments can be broken down into three general categories; surgery, chemotherapies, and radiation [118, 158]. Typically, cancer treatment will involve a combination of these categories and/or multiple agents within a given category so as to do battle with cancer on multiple fronts; for example, combined drug cocktails such as R-CHOP (rituximab with cyclophosphamide, doxorubicin, vincristine and prednisone) in the treatment of lymphoma [159, 160]. The determination of what treatment(s) a patient receives depends upon the location, size, and severity of the disease, as well as characteristics of the individual (age, state of health, treatment preference and, when known, specific characteristics of the tumour that can modulate sensitivity to treatment i.e. personalized medicine) [118]. Further discussion of cancer treatments will focus exclusively on thesis relevant chemotherapies.
1.2.3.1 Drugs used in cancer chemotherapy

Chemotherapeutics used during experimentation in this thesis include 5FUdR, pemetrexed, cisplatin and gemcitabine. As both 5FUdR and pemetrexed are thymidylate synthase (TS) inhibitors, they will be discussed in the next section under inhibitors of TS. Directly below will be a brief discussion regarding cisplatin and gemcitabine. Cisplatin is used in chapter 3 as a non-TS-targeting drug for the purposes of an experimental control. Gemcitabine is used in experiments described throughout that chapter.

1.2.3.1.1 Cisplatin

Cisplatin (or cis-Diamminedichloroplatinum(II), CDDP) is a platinum-based anticancer drug that exerts its cytotoxicity via the cross-linking of DNA to form platinum (Pt)-DNA adducts which accumulate and ultimately cause apoptosis [161, 162]. While highly effective against multiple cancers (tesicular, overian, bladder, colon, etc.), until resistance develops, cisplatin is also associated with severe nephro- and neuro- and ototoxicity which, may be partly reversible but are also therapeutically limiting [163]. Resistance to cisplatin may be intrinsic or acquired after a patient exposure. Resistance mechanisms include alterations in pharmacokinetics (i.e. decrease drug accumulation, increased drug efflux, increased detoxification), increased DNA repair (i.e. over expression of nucleotide excision repair enzymes) and increased tolerance to cisplatin-induced damage (i.e. reduced apoptotic response) [164, 165].

1.2.3.1.2 Gemcitabine

Gemcitabine (2’, 2’-difluoro 2’deoxyctydine, dFdC) is a deoxycytidine analogue that is used to treat a wide variety of tumour types (lung, breast, ovarian, bladder and pancreas) [166]. Gemcitabine is a pro-drug that is activated intracellularly via phosphorylation by deoxycytidine kinase (dCK) to gemcitabine monophosphate and subsequently processed into di- and tri- phosphorylated forms which are active metabolites in mediating an anti-tumour response [166, 167]. Ridonucleotide
reductase, the enzyme responsible for the production of nucleotides for DNA synthesis and repair, is inhibited by gemcitabine diphosphate and contributes to gemcitabine cytotoxicity [168, 169]. Tri-phosphorylated gemcitabine (dFdCTP) exerts cytotoxicity by a mechanism known as *masked chain termination* (MCT) which inhibits DNA synthesis and eventually results in apoptosis [166, 170]. Briefly, in MCT, dFdCTP is recognized as a “normal” nucleotide and incorporated into DNA. However, once incorporated it prevents further DNA chain elongation and inhibits DNA synthesis as only one additional nucleotide can be added by DNA polymerases to the growing DNA chain (this is the “masking” part). Polymerase exonuclease activity and DNA repair mechanisms are ineffective at detecting, removing, and replacing dFdCTP and apoptosis results from lack of DNA synthesis. Resistance can result due to increased drug efflux, decreased intracellular uptake, genetic polymorphisms in patients and decreased pro-drug conversion into active metabolites [171-173].

### 1.3 Enzymes that synthesize deoxythymidine monophosphate (dTMP)

#### 1.3.1 *De novo* synthesis of dTMP by thymidylate synthase

Thymidylate synthase (TS) is the enzyme responsible for *de novo* synthesis of deoxythymidine monophosphate (dTMP or thymidylate) via the reductive methylation of deoxyuridine monophosphate to dTMP using methylene tetrahydrofolate (CH$_2$THF), a folate co-factor, as the methyl donor [174]; this has previously been referred to as the thymidylate synthesis cycle ([Figure 2](#)) [175]. The enzyme is highly conserved and functions as a homodimer comprised of 35kDa subunits[174].

The TS enzyme is an essential enzyme for the production of dTMP used in DNA synthesis/replication ([Figure 2](#)). It plays an important role in cellular proliferation and has been an invaluable drug target in the treatment of cancer since the late 1950’s with the introduction of 5-fluorouracil (an antimetabolite, nucleoside analogue to be discussed more below) [176, 177].
Figure 2: Thymidylate synthesis cycle and dTTP incorporation into DNA

TS catalyzes the reductive methylation of dTMP from dUMP using CH$_2$THF as the methyl donor; dihydrofolate is generated as a biproduct in a 1:1 ratio with dTMP. Using NADPH, DHFR regenerates THF. THF is then converted back into CH$_2$THF by SHT to be used again by TS to generate dTMP. Once dTMP is generated, it is then become di- and tri-phosphorylated into dTTP which is incorporated into DNA during synthesis and repair.

SHT, serine hydroxyl transferase; THF, tetrahydrofolate; DHF, dihydrofolate; dTDP, deoxythymidine diphosphate; dTTP, deoxythymidine triphosphate
1.3.1.1 Regulation of TS protein expression

TS expression and activity is cell cycle-dependent, being highest during S phase and decreasing by G2 [178-181]. It has been observed that rapidly proliferating cells in general have increased levels of TS compared to those cells in G0, and that human tumour cells have variably higher levels of TS than normal, non-tumour proliferating cells [180, 182]. TS enzyme levels are tightly regulated at the level of translation. The TS protein can bind to its own mRNA to inhibit translational processing of the mRNA - termed translational repression and specifically autoregulation [183, 184]. Exogenously-administered allosteric inhibitors of TS (including anticancer drugs that target TS) bind to the protein and suppress it's capacity to generate thymidylate but, in doing so, can also suppress the capacity of TS to repress translation of its own mRNA: the resulting translational derepression induces a transient two-fold to four-fold increase in TS enzyme levels, shown both in vitro and in vivo, including in human patients [185-189]. The transient TS increase counters the intended therapeutic effect of TS-targeting drugs and is a significant clinical problem. Hence, TS has a dual function: it is both an enzyme and a regulatory feedback molecule, capable of modulating its own level by mediating translation of its own mRNA. In addition to binding its own mRNA, TS has also been shown to bind to, and inhibit the translation of, the mRNAs of both c-myc and p53 [190-193]. These data suggest a role for TS in the translational regulation of other proteins in addition to its self-regulatory function [194].

1.3.1.2 TS and cancer

As an important enzyme in DNA synthesis and replication, TS has had a lengthy, intimate, and intertwined relationship with cancer research and cancer treatment. Elevated TS levels in malignancy, at a plethora of body sites, have been well established since the 1950s [176, 182, 195-197]. Elevated TS levels in multiple human tumour types have been correlated with cancer progression, poor prognosis, and poor response to
treatment [187, 188, 198-201]. Specific inhibitors of TS and the development of resistance to treatment are discussed below.

1.3.1.3 TS enzyme inhibitors

The following sections focus on TS inhibitors used in experiments described in this thesis (5FU, 5FUdR, and pemetrexed).

1.3.1.3.1 dUMP analogues

TS is an important enzyme with respect to cancer and drugs targeting TS-protein have been a mainstay in cancer chemotherapy since the synthesis of fluorinated pyrimidines [177, 195]. Fluorinated pyrimidines including 5-fluorouracil (5FU) and 5-fluorodeoxyuridine (5FUdR) belong to the nucleoside analogue class of drugs and inhibit TS by mimicking dUMP and irreversibly binding the enzyme. 5FU is a uracil analogue with a fluorine atom introduced in place of hydrogen at the carbon-5 position, used in the treatment of cancers of the breast, and colon. Once inside the cell, the 5FU prodrug is converted to metabolites (5FUdR) and active metabolites (fluorodeoxyuridine-monophosphate and -triphosphate, FdUMP and FdUTP). FdUMP binds to and inhibits the capacity of TS to produce dTMP, resulting in dTMP and dTTP pool depletion. Thymine-less death results from the subsequent disruption of DNA synthesis and repair as dUTP is inappropriately incorporated into DNA, resulting in cell death [202-205].

1.3.1.3.2 Antifolates

A second class of TS inhibitors are folate analogues, such as pemetrexed, which target TS by blocking its use of CH$_2$THF as a co-factor in the production of dTMP [201, 206]. Pemetrexed is, in fact, a multi-targeting antifolate analogue that inhibits dihydrodofolate reductase (DHFR) and glycinamide ribonucleotide formyl transferase (GARFT) in addition to inhibiting TS as its primary pharmacological target [207]. Thymine-less death is similarly thought to be an important contributing factor to the
mechanism of pemetrexed-induced cell death, along with a general depletion of purines associated with DHFR and GARFT inhibition [208].

1.3.1.4 Resistance to TS-targeting drugs

TS-targeting drug-induced increases in TS levels within tumour cells is a mechanism of resistance to TS-targeting chemotherapies [187, 188]. TS levels can increase in tumour cells in the presence of TS inhibitors due to translational derepression and/or enzyme stabilization [195, 209, 210]. In addition, TS increases are mediated by TS gene amplification and patients with increased levels of TS enzyme have been shown to have a poor prognosis [197, 198, 205, 211-214]. Increased levels of TS protein in tumour cells provide "sacrificial targets" for anti-TS chemotherapeutic drugs, increased capacity to withstand exposure to those drugs, and decreased likelihood of suffering the consequences of preferential tumour-associated, chemotherapy-induced toxicity (i.e., apoptosis, necrosis, growth arrest). For the above reasons, it is imperative that new anti-cancer therapies be developed to specifically overcome TS overproduction (and associated chemotherapy resistance) in human tumour cells. An attractive strategy to do so includes the use of antisense molecules targeting TS, either as single agents or in combination with small molecule drugs that target TS protein. The Koropatnick laboratory has demonstrated this to be a valuable approach to improve the effectiveness of TS-targeting drugs, both in vitro and in vivo animal models [215-219].

Along with increased TS, the thymidine kinase salvage pathways (discussed below) that contribute to alternative dTMP production are also contributors to resistance to TS-targeted drugs [220, 221].

1.3.2 Salvage synthesis of dTMP by thymidine kinases

1.3.2.1 Thymidine kinases

There are two thymidine salvage enzymes in the cell that produce dTMP by ATP-dependent phosphorylation of deoxythymidine: cytosolic thymidine kinase 1 (TK1) and
mitochondrial thymidine kinase 2 (TK2). TK1 and TK2 are different proteins that both phosphorylate endogenous and exogenous (imported by nucleoside transporters) thymidine to produce dTMP necessary for DNA synthesis and repair. They have different patterns of expression in tissues and intracellular localization (to be discussed in more detail below) and are encoded by different genes located on different chromosomes (TK1 on chromosome 17 and TK2 on chromosome 16) [222]. Both TK1 and TK2 activity and expression are negatively regulated via feedback inhibition of their downstream phosphorylation products - mainly by high levels of dTTP (and dCTP, for TK2 as well) within the cell[222, 223].

1.3.2.1.1 Thymidine kinase 1 (TK1)

TK1 is a cytosolic, cell cycle-dependent enzyme that functions primarily as a homodimer of two 25 kDa subunits[224]. It has an expression pattern similar to that of TS and activity and expression are high in proliferating cells, including malignant cancer cells, and absent in quiescent cells [225-227]. TK1 protein is regulated at the transcriptional level by E2F; at the translational level, potentially by its own mRNA; and at the post-translational level (for example, rapid mitotic degradation via the anaphase-promoting complex) [224, 225, 228, 229]. TK1 has a more constrained preference for thymidine as a phosphorylation substrate than does TK2 (discussed below)[230].

1.3.2.1.2 Thymidine kinase 2 (TK2)

Historically, TK2 is the least studied nucleoside kinase and information on the enzyme, especially compared to TK1, is lacking. Human TK2 is a 29-35 kDa, constitutively expressed, mitochondrial nucleoside kinase encoded by a nuclear gene with a half-life of around 33 h [231, 232]. In normal proliferating cells, TK2 activity is low (an estimated 1-5% of total TK activity), but in quiescent, non-proliferating cells TK2 accounts for 100% of the TK activity [233, 234]. TK2 expression in different tissues has previously been shown to have a positive correlation with number of mitochondria [222]. Unlike TK1, TK2 displays substrate promiscuity in that it will phosphorylate thymidine, cytosine and
adenosine to fulfill the requirements of mitochondrial DNA replication and genome maintenance [235]. On the other hand, TK2 does not phosphorylate deoxyguanosine: that event is mediated, in mitochondria, by deoxyguanosine kinase [222, 230].

1.3.2.2 TK1 and cancer

Immunostaining for TK1 has confirmed its increased expression in a number of different cancers (those arising in breast, liver, lung, bladder, thyroid, and white blood cells) and increased serological TK1 levels have been correlated with increased malignancy and poor prognosis [236-243]. Upregulation of TK1 has been demonstrated both in vitro and in vivo in response to treatment with anticancer drugs and DNA damaging agents such as 5FU (a TS inhibitor) and doxorubicin [244-246], prompting speculation that TK1 could contribute to resistance to anticancer therapies. The relative ease of measurement of TK1 and the presence of elevated TK1 level in multiple human tumours has prompted assessment of TK1 in serum as a proliferation marker and screening tool to monitor cancer in the clinic with respect to prognosis, treatment, and detection of recurrence [236, 242, 246-250].

In healthy replicating cells, de novo thymidylate synthesis pathways are thought to be capable of providing the necessary amounts of dTMP required for DNA synthesis. However, in cancerous and other rapidly dividing cells, the role and contribution of salvage enzymes may be greater [222]. It is possible that TK1 (and/or TK2), through their activity as mediators of salvage pathway production of dTMP, mediate resistance to small molecule drugs and antisense molecules capable of reducing TS activity and TS level, respectively. Unlike TS, there are currently no pharmacological agents targeting TK for the purposes of anti-cancer therapy. Thus, an antisense approach was taken in experiments described in this thesis, to reduce TS, TK1, and TK2, alone and in combination with each other, to determine the capacity of combined reduction of TS and TK to sensitize human tumour cells to current TS-targeting anticancer drugs. Given the greater contribution of de novo TS synthesis to dTMP production in cycling cells, we
expect the that reduction of TK1 or TK2 alone will be insufficient to sensitize to TS-targeted chemotherapy.

1.3.2.3 TK2 in human disease

Mutations in, and dysregulation of, the TK2 gene are associated with a heterogeneous group of autosomal recessive disorders known as mitochondrial DNA depletions syndromes (MDS), specifically the myopathic forms (progressive muscle weakness) and encephalomyopathic form (stroke-like episodes, migraine headaches, seizure) [251-254]. Mutations in genes that function in mitochondrial DNA (mtDNA) replication and/or mitochondrial dNTP synthesis result in impaired mtDNA and genome maintenance [253, 255, 256]. A reduction in mitochondrial DNA (mtDNA) content leads to impaired energy production in the affected tissues and organs, and the leading hypothesis is that this is due to insufficient synthesis of mitochondrial electron transport chain components [255, 257, 258]. There is some evidence to show that the tissue specificity and severity of MDS resulting from TK2 dysregulation may be affected by the ability of TK1 and nucleotide transporter proteins to compensate for reductions in dTTP in mitochondrial dNTP pools [259, 260]. MDS can affect either a specific organ or combination of organs, which most often include muscle, liver, kidney and brain. TK2 associated MDS patients are predominantly pediatric and death occurs within a few years of diagnosis, resulting from rapidly progressing muscle weakness and respiratory failure [251, 254, 261-263].

1.4 Project rationale

Antisense targeting of TS in combination with TS-targeting small molecule drugs resulted in potentiation of inhibition of tumour cell proliferation and cell death. It is possible that the TK enzymes mediate resistance to TS-targeting small molecule drugs and/or -antisense. There are currently no pharmacological inhibitors of human TKs. Thus, a combinatorial RNAi approach (in which siRNAs against TS, TK1 and TK2 are combined in single treatments) was taken to investigate if one or both of the TK
enzymes may contribute to decreased effectiveness of TS targeting drugs (5FUdR and pemetrexed) and/or antisense siRNA molecules.

As TK2 displays substrate promiscuity and contributes to levels of both dTMP and dCMP, it is possible that TK2 mediates resistance to the anticancer drug gemcitabine via feedback inhibition of the gemcitabine-activating enzyme dCK; this would be the downstream production of deoxycytidine triphosphate molecules by way of TK2-produced dCMP. As small molecules inhibitors of TK2 are not commercially available (or available in sufficient quantities for experimentation at the start of this project), siRNAs targeting TK2 were used in combination with gemcitabine to address this question. Given that TK2 is a mitochondrial enzyme important for the synthesis and maintenance of mtDNA and that gemcitabine can negatively affect mitochondrial DNA polymerase y, changes in mtDNA content and activity where also monitored after exposure to combined TK2 siRNA and gemcitabine treatment to elucidate what impact, if any, there was on mitochondria.

1.5 Hypotheses

1. TKs mediate resistance to TS-targeting small molecule drugs

2. TKs mediate resistance to the capacity of TS siRNA to sensitize tumour cells to TS-targeting anticancer drugs.

3. TK2 can mediate human tumour cell resistance to gemcitabine, and antisense downregulation of TK2 can overcome that resistance.

4. TK2 siRNA-induced drug sensitization causes increased mitochondrial damage.
Chapter 2

« Materials and Methods »

Details of the materials used and methods employed are taken from my already published paper in the Journal of Pharmacology and Experimental Therapeutics [264], with expanded information.

2.1. Human tumour cell lines

Human cervical carcinoma (HeLa), breast epithelial adenocarcinoma (MCF7), lung epithelial carcinoma (A549), mesothelioma (lung derived, Meso H28) and colorectal adenocarcinoma (HT-29) cell lines were purchased from the American Type Culture Collection (Manassas, VA and Rockville, MD, USA). HeLa and MCF7 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Wisent Inc., St-Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco, Burlington, Ontario, Canada). A549 and HT-29 cell lines were cultured in Alpha MEM (AMEM) (Wisent Inc.) supplemented with 10% FBS (Gibco). Meso H28 cells were cultured in RPMI (Wisent Inc.) supplemented with 10% FBS (Gibco). All cell lines were maintained in a humidified incubator at 37°C with 5% CO₂ in air. Cultured cells were kept under 75% confluency at all times.

2.2. siRNAs

All siRNAs (ON-TARGET plus or siGENOME) were obtained from Dharmacon RNAi Technologies (Lafayette, CO, USA) as annealed and desalted duplexes. ON-TARGET plus siRNAs contain a chemical modification that enhances siRNA antisense strand entry into the RNA-induced silencing complex (RISC) for certain sequences (identified by Dharmacon’s siRNA design algorithm) where the modification is likely to enhance specific activity without increasing off-target binding [27, 77, 78, 87, 265]. ON-TARGET plus or siGENOME reagents were used as recommended by Dharmacon for each
targeted mRNA sequence (Table 3). All siRNAs were resuspended in siRNA buffer (supplied by Dharmacon) to generate 5 μM or 10 μM stock solutions. Throughout experiments, total siRNA concentration was held constant at 10 nM. C2 siRNA was used as a supplement when the combined concentration of targeting siRNAs was less than 10 nM (Appendix 1). Results sections will indicate specific siRNA concentrations used.
Table 3: siRNA sequences.
Control siRNAs (C2, C3) contain 4 or more mismatches with all known human RNAs. Cy3-labeled TS siRNA #4 has the Cy3 fluorophore attached to the 5’ end on the antisense strand to avoid interference with the important seed region at the 3’ end.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>siRNA short-form</th>
<th>Targeted RNA</th>
<th>Target mRNA Sequence</th>
<th>mRNA transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON-TARGETplus Non-targeting siRNA Control 2; C2</td>
<td>C2</td>
<td>No Target</td>
<td>5’-UGGUUUACA-UGUUGUGUGA-3’</td>
<td></td>
</tr>
<tr>
<td>ON-TARGETplus Non-targeting siRNA Control 3; C3</td>
<td>C3</td>
<td>No Target</td>
<td>5’-UGGUUUACA-UGUUUUCUGA-3’</td>
<td></td>
</tr>
<tr>
<td>TYMS siGENOME siRNA TS siRNA #3</td>
<td>CH 3. TS siRNA (1)</td>
<td>TS mRNA</td>
<td>5’-ACAGAGAUA-UGGAAUCAGA-3’</td>
<td>576-594 Coding region</td>
</tr>
<tr>
<td>TYMS siGENOME siRNA TS siRNA #4</td>
<td>CH 3. TS siRNA (2), CH 4. TS siRNA #4, or TS siRNA</td>
<td>TS mRNA</td>
<td>5’-GGACUUGGG-CCCAGUUUAU-3’</td>
<td>526-544 Coding region</td>
</tr>
<tr>
<td>Cy3 labelled TS #4 siRNA Cy3-TS siRNA</td>
<td>Cy3-TS siRNA</td>
<td>TS mRNA</td>
<td>5’-GGACUUGGG-CCCAGUUUAU-3’</td>
<td>526-544 Coding region</td>
</tr>
<tr>
<td>Human TK1 ON-TARGETplus siRNA TK1 siRNA #11</td>
<td>CH3. TK1 siRNA (1), or TK1 siRNA CH4. TK1 siRNA</td>
<td>TK1 mRNA</td>
<td>5’-GCACAGAGU-UGAUGAGACG-3’</td>
<td>308-326 Coding region</td>
</tr>
<tr>
<td>Human TK1 ON-TARGETplus siRNA TK1 siRNA #12</td>
<td>CH3. TK1 siRNA (2)</td>
<td>TK1 mRNA</td>
<td>5’-CAAAGACAC-UCGCUACAGC-3’</td>
<td>578-596 Coding region</td>
</tr>
<tr>
<td>Human TK2 ON-TARGETplus siRNA TK2 siRNA #9</td>
<td>CH 3. TK2 siRNA (1), or TK2 siRNA CH 4. TK2 siRNA #9</td>
<td>TK2 mRNA</td>
<td>5’-AAAUCGGGA-UCAAUUAUUA-3’</td>
<td>1101-1119 Coding region</td>
</tr>
<tr>
<td>Human TK2 ON-TARGETplus siRNA TK2 siRNA #11</td>
<td>CH 3. TK2 siRNA (2), CH 4. TK2 siRNA #11</td>
<td>TK2 mRNA</td>
<td>5’-UCACAGCGC-AAGAUACAUU-3’</td>
<td>759-777 Coding region</td>
</tr>
</tbody>
</table>
2.3. Cytotoxic drugs

5FUdR was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Pemetrexed (Alimta, manufactured by Eli Lilly and Co., Toronto, Ontario, Canada), cisplatin (Platinol, manufactured by Bristol-Myers Squibb, Montreal, Quebec, Canada) and gemcitabine (Gemzar, Eli Lilly and Co., Toronto, ON, Canada) were obtained from the pharmacy at London Regional Cancer Program (London, Ontario, Canada).

2.4. siRNA transfection

2.4.1. General transfection methodology

Twenty-four hours prior to siRNA transfection, cells were plated in NUNC 25 cm$^2$ tissue culture flasks (T-25, Nunclon™ Δ surface)(VWR International, Ontario, Canada) in 2mL of medium supplemented with 10% FBS. The next day (approximately 24 h later), cells were transfected with 10 nM siRNA using Lipofectamine 2000 (LF2K or LFA2K, Invitrogen, Carlsbad, CA). Transfections were performed according to a modification of the manufacturer’s protocol that included a 50% reduction of the amount of LF2K used, which allowed for the same mRNA down regulation obtained when following the standard protocol, while decreasing off-target toxicity effects associated with LF2K (see Appendix 1 for supporting information). Briefly, equal volumes of LF2K mixture (120 μL serum-free medium and 5 μL LFA2K) were combined with siRNA mixture (total siRNA concentration of 10 nM when added to cells in serum-free media volume of 125 μL). Mixture was incubated for 20 mins to allow loading of liposomes with siRNA. Experimental conditions included untreated cells (NTC, only medium) and LF2K-treated cells (LF2K only without siRNA) as controls. After 20 mins, 250 μl of the combined mixture were added to each T25-flask. Four hours after transfection, one of the following was done to the flasks depending on the nature of the experiment.
2.4.1.1. Experiments lasting only 24h post-transfection with siRNA (not applicable to time course results)

In these experiments, cells were initially plated at 1.5x10^5 cells/flask (HeLa) or 2.0x10^5 cells/flask (MCF7). Four hours post-transfection, 4 mL of medium containing 10% FBS were added to each flask. Cells were harvested for analysis 20 h later.

2.4.1.2. Experiments lasting longer than 24 h post-transfection with siRNA (including time-course and drug-treatment experiments)

In these experiments, cells were initially plated at 3.5x10^5 cells/flask regardless of cell line used. Four hours post-transfection, cells were re-plated at specific densities (Table 4), in triplicate flasks for the siRNA treatment conditions and the drug treated conditions. When cells were intended for analysis by immunoblotting or flow cytometry, 6 x T25 flasks were re-plated for each drug-treated condition to allow 2 x T25 flasks to be pooled at random to ensure enough cells were available for analysis. Unless otherwise indicated, flasks were then incubated until the time of sample collection.

When re-plating was required for the experiment and there was more than one flask per treatment group at time of transfection, the cells were pooled prior to re-plating. The pooling of cells from multiple flasks per specific treatment group and their re-plating at a lower cell density was done to carry cells for the length of the experiment without over-growth of controls and to maintain more consistent transfection conditions.
### Table 4: Cell numbers and media volumes upon re-plating

<table>
<thead>
<tr>
<th>Analysis Method</th>
<th>Cell Number Used for Re-plating (cells/flask or well)</th>
<th>Volume of Media for Re-plating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Counting - Coulter Counter</td>
<td>$5.0 \times 10^4$</td>
<td>$3 \text{ mL}$</td>
</tr>
</tbody>
</table>
| Immunoblots (end points are post transfection and re-plating) | 48 h end point = $1.5 \times 10^5$  
72 h end point = $1.0 \times 10^5$  
96 h end point = $5.0 \times 10^4$ | 3 mL  
(4 mL when experiment did not involve gemcitabine) |
| qPCR - mRNA or DNA (end points are post transfection and re-plating) | 24 h end point = $2.0 \times 10^5$  
48 h end point = $1.5 \times 10^5$  
72 h end point = $1.0 \times 10^5$  
96 h end point = $5.0 \times 10^4$ | 3 mL  
(4 mL when experiment did not involve gemcitabine) |
| alamarBlue assay | $1.0 \times 10^3$ | 100 μL |
| Flow Cytometry | $5.0 \times 10^4$ | 3 mL |
2.4.2. siRNA transfection with Subsequent Cytotoxic Drug Treatment

When drugs were used, the cells were treated 4 h after re-plating as follows:

i) The drug-untreated group received 1 mL of medium (when using T25 flasks or 6-well plates; 100 μL when using 96-well plates)

ii) The drug-treated group received 1 mL of medium (when using T25 flasks or 6-well plates; 100 μL when using 96-well plates) containing drug at the required concentration to yield indicated final concentration used in experiment

Cells were incubated for the time required for each experiment and then collected for analysis.

2.5. Measures of cellular proliferation

2.5.1. Cell counting

The effects of siRNA treatment on cellular proliferation, alone or in combination with anticancer drugs, were assessed. Cells were exposed to various treatments, grown for 4 days, washed with PBS, trypsinized, and counted on a Beckman Coulter Z1 Particle Counter (Beckman, Mississauga, Ontario, Canada). Results were analyzed by calculating the fold change in cell number after 4 days of growth (relative to the starting number of plated cells) as:

\[
\text{Fold Change} = \left[ \frac{(\text{number of cells, day 4}) - (\text{number of cells, day 0})}{(\text{number of cells, day 0})} \right]
\]

Differences in proliferation induced by treatment were expressed as “% Fold Change” and were calculated using “Fold change” in cell number in treated cells (“treatment fold change”) and the fold change in cell number under appropriate matched control conditions (“control fold change”):

\[
\% \text{ Fold Change} = \left[ \frac{\text{treatment fold change}}{\text{control fold change}} \right] \times 100
\]
2.5.2. alamarBlue assay

After transfection, cells were re-plated into 96-well plates. Each 96-well plate contained drug-untreated conditions as controls for the gemcitabine concentration(s) in that plate. Plates also contained wells with only medium supplemented with 10% FBS (no cells present, 200 μL) for assay background control.

Ninety-six hours after re-plating, 100 μL of medium was removed from each well and replaced with 100 μL of a 1:10 dilution of alamarBlue reagent (Invitrogen, Carlsbad, CA) in medium containing 10% FBS (final dilution of 1:20). Plates were placed back in the incubator and the red-ox reaction, indicated by color change, was allowed to occur for 4.5 - 6 h. Fluorescence at 595nm was detected using a Wallac Victor²™ 1420 multilabel counter (PerkinElmer, Woodbridge, ON, Canada). An average background absorbance value, from medium-only wells, was subtracted from all experimental wells. Data are expressed as a percentage of C2 siRNA without drug.

2.6. Measurement of TS, TK1 and TK2 mRNA levels

2.6.1. RNA isolation and reverse transcription

Cells were lysed for mRNA analysis using TRIzol Reagent (Invitrogen, CA, USA) and total cellular RNA was isolated according to the manufacturer’s protocol. RNA was quantified using UV-spectrometry.

Purified RNA (1 μg) was used to synthesize cDNA by reverse transcription mediated by Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Invitrogen) and random primers according to the protocol provided by the manufacturer. Samples were incubated at 25°C for 10 mins (annealing of primers), 37°C for 1 h (synthesis of cDNA by MMLV-RT), and 95°C for 5 mins (inactivation of MMLV-RT and separation of cDNA from RNA) using an Eppendorf Mastercycler Gradient thermal cycler (Eppendorf, Westbury, NY).
2.6.2. Quantitative PCR (qPCR)

qPCR was used to determine the relative levels of TS, TK1, TK2 mRNA compared to either 18S rRNA or GAPDH mRNA levels (housekeeping genes/internal standard) using a TaqMan Gene Expression Assay kit (Applied Biosystems, USA) and specific, to gene of interest (GOI), primers and probes sets (Table 5). Standard curves were prepared using the cDNA of the sample with the highest expected level of target mRNA. Standard curves were composed of serial dilutions of cDNA and a no template control of RNase-free water. The 1X standard curve initial sample contained 2.5 times the amount of cDNA of experimental sample; experimental samples contained 1 μL of cDNA in RNase-free water. A general primer and probe master mix contained: 2X TaqMan® Universal PCR Master Mix (PE Applied Biosystems, Streetsville, ON), and either i) GAPDH or r18S Pre-Developed TaqMan® assay (Applied Biosystems), or ii) 100 μM of GOI Forward Primer, 100 μM GOI Reverse Primer, and 100 μM GOI fluorescently labeled probe.

qPCR amplification was performed using either the ABI Prism® 7900HT Detection System (Applied Biosystems), for Chapter 3, or the Viia 7™ Real-time PCR System (Applied Biosystems, Life Technologies Holdings, Singapore), for Chapter 4, using the default standard instrument run properties: 50°C for 2 mins and then 95°C for 10 mins (incubation steps), followed by 40 cycles of 95°C for 10 seconds (denature), and 60°C for 1 min (annealing of primers and DNA polymerization).

In Chapter 3 results, TS was multiplexed with the r18S housekeeping gene; r18S is used as the internal control throughout the chapter. In Chapter 4, TK2 was multiplexed with the GAPDH housekeeping gene; GAPDH is used as the internal control throughout the chapter. Multiplexing allows for assaying of multiple genes in a single well by using gene-specific probes with different, non-overlapping emission spectra, fluorescent tags. TS, TK1 or TK2 mRNA levels were normalized to the internal standard (r18S or GAPDH) and expressed as a percentage of the experimental control condition (C2 siRNA, unless otherwise indicated).
Table 5: Primers and probes for mRNA detection and quantification by qPCR.
FAM, NED, and VIC are laser-activated fluorescent probes. MGB/NFQ refers to the specific quencher present in the probe.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>TaqMan Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>5’-GGCCTCGG</td>
<td>5’-GATGTGCAGCAATCATGTACGT-3’</td>
<td>6FAM-AACATCGCCAGCTACGCGCTGC-MGB/NFQ</td>
</tr>
<tr>
<td>TK1</td>
<td>5’-TTCTCTACCTCTGGTGATGGTTTC-3’</td>
<td>5’-TGCCACCATCTTGTTGAA-3’</td>
<td>NED-ACAGGAACAAAGCAGCATC-MGB/NFQ</td>
</tr>
<tr>
<td>TK2</td>
<td>5’-CTGGCGAAGGCAGAACACTT-3’</td>
<td>5’-TTCTCAAGAGCAGACCCACATG-3’</td>
<td>6FAM-CTACCAGGAATCTTG-MGB/NFQ</td>
</tr>
<tr>
<td>r18S</td>
<td>As supplied</td>
<td>As supplied</td>
<td>VIC-labelled - as supplied</td>
</tr>
<tr>
<td>GAPDH</td>
<td>As supplied</td>
<td>As supplied</td>
<td>VIC-labelled - as supplied</td>
</tr>
</tbody>
</table>
2.7. Measurement of TS, TK1, TK2 and dCK protein levels

2.7.1. Isolation and quantification of total protein

Total cell protein lysates were obtained at indicated times as follows. Flasks or wells were placed on ice, cells were washed twice with 4°C -cold PBS, scraped and re-suspended into cell lysis buffer (10 mM Tris at pH 7.6, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), and sonicated at 4°C using a Vibra Cell™ ultrasonic processor (Sonics & Materials Inc., Danbury, CT) to disrupt membranes.

Protein concentration was estimated using a Bio-Rad Protein Assay kit (Bio-Rad, QC, Canada) following the protocol provided by the manufacturer. The required amount of total protein (Table 6) was then combined with water and loading buffer (supplemented with 2′-β-mercaptoethanol) and heated to 95°C for 5 mins (denaturing).

2.7.2. Gel electrophoresis and immunoblotting

Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare-Amersham Biosciences, UK). See Table 6 for pertinent antibody information, total protein loaded amounts and percentages of acrylamide in gels. Membranes were blocked for 1 hour (5% milk in TBS-T: TBS plus Tween 20 [0.2%]) and then washed with fresh TBS-T. Bands were detected as described in methods sections below.
<table>
<thead>
<tr>
<th>Protein of Interest</th>
<th>Protein Size (and band size if different)</th>
<th>Total Protein Loaded</th>
<th>% of Acrylamide in Gel</th>
<th>Antibody Used &amp; Dilution</th>
<th>Chapter used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidylate Synthase (TS)</td>
<td>~35 kDa</td>
<td>25 µg</td>
<td>12 %</td>
<td>Anti-TS antibody (Taiho Pharmaceuticals, Hanno-City, Japan) 1:800</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Thymidine Kinase 1 (TK1)</td>
<td>~25 kDa</td>
<td>40 µg</td>
<td>12 %</td>
<td>Anti-TK1 antibody (34003, QED Bioscience Inc., San Diego, CA, USA) 1:400</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Thymidine Kinase 2 (TK2)</td>
<td>~35 kDa</td>
<td>40 µg</td>
<td>12 %</td>
<td>Anti-TK2 antibody (SAB1300098, Sigma-Aldrich, Saint Louis, MO, USA) 1:150</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Thymidine Kinase 2 (TK2)</td>
<td>(~35 kDa and at times 31 kDa) more reliable antibody</td>
<td>35 µg</td>
<td>15 %</td>
<td>Anti-TK2 antibody (HPA041162, Sigma-Aldrich) 1:12000</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Deoxycytidine Kinase (dCK)</td>
<td>~31 kDa (28 kDa)</td>
<td>35 µg</td>
<td>15 %</td>
<td>Anti-dCK antibody (ab83046, Abcam, 1:400 in 5% BSA)</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Actin</td>
<td>~42 kDa</td>
<td>Depends on above loaded amounts - internal standard</td>
<td>12-15 %</td>
<td>Anti- actin antibody (α-actin, Sigma-Aldrich) 1:1000</td>
<td>Chapter 3 and 4</td>
</tr>
</tbody>
</table>
2.7.2.1. Immunoblotting in Chapter 3

2.7.2.1.1. TS and Actin immunoblots

Membranes were probed with rabbit polyclonal anti-human TS primary antibody (1:800 in TBS-T with 1% skim milk, 1 h, 20°C), followed by rabbit polyclonal anti-human Actin primary antibody (1:1000 in TBS-T with 1% skim milk, 1 h, 20°C), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (GE Healthcare-Amersham Biosciences, UK, 1:3,000 in TBS-T with 1% skim milk, 1 h, 20°C) in preparation for TS and Actin band detection. Washing with TBS-T occurred after each probe.

2.7.2.1.2. TK1, TK2 and Actin immunoblots

Membranes were probed with rabbit polyclonal anti-human TK2 primary antibody (1:150 in TBS-T with 1% skim milk, 16 h, 4°C) followed by horseradish peroxidase-conjugated α-rabbit IgG secondary antibody (1:3000 in TBS-T with 1% skim milk, 1 h, 20°C) in preparation for TK2 band detection. Washing with TBS-T occurred after each probe. Bands were detected and quantified as described in methods section 2.7.3.

*Immunoblot band detection and quantification.* Membranes were then stripped using Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA, 15 minutes, 20°C), washed with TBS-T, blocked in TBS-T with 5% skim milk and cut into two pieces (≥ 37 kDa and ≤ 37 kDa) using the Kaleidoscope Precision Plus Protein Standards Ladder (BioRad, Carlsbad, CA, USA) as a guide.

Membranes containing proteins of a 37kD size or smaller were probed with mouse polyclonal anti-human TK1 primary antibody (1:400 in TBS-T with 1% skim milk, 1 h, 20°C) followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (GE Healthcare-Amersham Biosciences, UK 1:3,000 in TBS-T with 1% skim milk, 1 h, 20°C) in preparation for TK1 band detection. Washing with TBS-T occurred after each probe.
Membranes containing proteins of a 37 kDa size or greater were probed with rabbit polyclonal anti-human Actin primary antibody (1:1000 in TBS-T with 1% skim milk, 1 h, 20°C) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:3,000 in TBS-T with 1% skim milk, 1 h, 20°C) in preparation for Actin band detection. Washing with TBS-T occurred after each probe.

2.7.2.2. Immunoblotting in Chapter 4

2.7.2.2.1. TK2 and Actin immunoblots

After blocking, membranes were cut into two pieces (>37 kD and <37 kD) using the Kaleidoscope Precision Plus Protein Standards Ladder as a guide.

Membranes containing proteins of a 37kD size or smaller were probed with rabbit polyclonal anti-human TK2 primary antibody (1:12,000 in TBS-T with 1% skim milk, 1h, 20°C) followed by horseradish peroxidase-conjugated α-rabbit IgG secondary antibody (1:8000 in TBS-T with 1% skim milk, 1 h, 20°C) in preparation for TK2 band detection. Washing with TBS-T occurred after each probe.

Membranes containing proteins of a 37 kDa size or greater were probed with rabbit polyclonal anti-human Actin primary antibody (1:1000 in TBS-T with 1% skim milk, 1 h, 20°C) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:8,000 in TBS-T with 1% skim milk, 1 h, 20°C) in preparation for Actin band detection. Washing with TBS-T occurred after each probe.

2.7.2.2.2. dCK and Actin immunoblots

After blocking, membranes were cut into two pieces (>37 kD and <37 kD) using the Kaleidoscope Precision Plus Protein Standards Ladder as a guide.

Membranes containing proteins of a 37kD size or smaller were probed with rabbit anti-human dCK primary antibody (1:400 in TBS-T with 5% BSA, 36 h, 4°C) followed by horseradish peroxidase-conjugated α-rabbit IgG secondary antibody (1:10,000 in TBS-T
with 1% skim milk, 1 h, 20°C) in preparation for dCK band detection. Washing with TBS-T occurred after each probe.

Membranes containing proteins of a 37 kDa size or greater were probed with rabbit polyclonal anti-human Actin primary antibody (1:1000 in TBS-T with 1% skim milk, 1 h, 20°C) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:8,000 in TBS-T with 1% skim milk, 1 h, 20°C) in preparation for Actin band detection. Washing with TBS-T occurred after each probe.

2.7.3. Immunoblot band detection and quantification

Horseradish peroxidase activity associated with protein bands was detected using Enhanced Chemiluminescence Plus (ECL Plus; GE Healthcare-Amersham Biosciences, UK - Chapter 3) or Pierce® ECL 2 Western Blotting Substrate (Thermo Scientific, Rockford, IL - Chapter 4) and a STORM 860 Molecular Imager (phosphoimager/fluoroimager) (Amersham Biotech-Molecular Dynamics Inc, CA, USA). Band intensity was quantified using ImageQuant 5.1 software (Amersham Biotech-Molecular Dynamics Inc., Sunnyvale, CA, USA). TS, TK1, TK2, and dCK results are expressed normalized to the Actin internal standard and then as a percentage of an experimental control condition (C2 siRNA unless otherwise indicated).

2.8. Measurement of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA)

The methods for this section, targets chosen, and primer and probe sequences used were based on published literature [266-270]. The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) allows determination of the effect of experimental treatments on mtDNA. It also serves as an indirect representation of the effect of treatment on mitochondrial health, function and biogenesis and allows determination of whether treatments preferentially affect mitochondrial DNA as compared to nuclear DNA.
2.8.1. DNA isolation

Cells were washed twice with cold PBS, trypsinized, collected in medium containing 10% FBS, centrifuged at 218 rcf for 10 mins, washed with cold PBS, and centrifuged again. Total DNA was isolated from cell pellet using a phenol-ethanol extraction protocol with Digestion Buffer (100 mM NaCl + 50 mM Tris HCl pH 8.0 + 50 mM EDTA) supplemented with Proteinase K (200 μg/mL) and an additional ethanol precipitation step to further purify the DNA. DNA was quantified using UV-spectrometry.

2.8.2. Quantitative PCR (qPCR) for mtDNA:nDNA ratio

mtDNA and nDNA reactions were not multiplexed. Reactions were amplified using TaqMan-based primer-probe sets specific to a mtDNA or nDNA target GOI (Table 7). Standard curves were prepared using DNA from the sample with the highest expected amount of target. The curve was composed of 1:10 serial dilutions of DNA and a no template control of RNase free water. The 1X standard curve initial sample contained 4000 ng of DNA. Each experimental sample contained 300 ng of DNA. A general primer and probe master mix contained: 2X TaqMan® Universal PCR Master Mix (PE Applied Biosystems), 100 μM of GOI Forward Primer, 100 μM GOI Reverse Primer, and 100 μM GOI fluorescently labeled probe.

qPCR amplification was performed using the ViiA 7™ Real-time PCR System (Applied Biosystems) and the default standard instrument run properties: 50 °C for 2 mins and then 95°C for 10 mins (incubation steps), followed by 40 cycles of 95°C for 10 seconds (denature), and 60 °C for 1 min (annealing of primers and DNA polymerization). mtDNA levels were normalized to r18S and then expressed as a percentage of the experimental control condition (C2 siRNA).
### Table 7: Primers and probes used for qPCR to produce mtDNA:nDNA ratio

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>TaqMan Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADHD1 (mtDNA)</td>
<td>5’-CACCCAAGA</td>
<td>5’-TGGCCATGG</td>
<td>6FAM-5’-TTACCGTCT</td>
</tr>
<tr>
<td></td>
<td>ACAGGTTTTGT-3’</td>
<td>GTATGTAA-3’</td>
<td>GCCATCT-3’-MGBNFQ</td>
</tr>
<tr>
<td>r18S (nDNA)</td>
<td>5’-TAGAGGGAC</td>
<td>5’-CGCTGAGCC</td>
<td>VIC-5’-AGCAATAGG</td>
</tr>
<tr>
<td></td>
<td>AAGTGGCGTTC-3’</td>
<td>AGTCAGTG-3’</td>
<td>TCTGTGATG-3’-MGBNFQ</td>
</tr>
</tbody>
</table>
2.9 Flow cytometry

Cells were washed with 4°C cold PBS, trypsinized, re-suspended in 4°C cold medium containing 10% FBS, pelleted by centrifugation, washed and re-suspended in 4°C cold PBS, and pelleted again by centrifugation at 218 rcf for 10 mins.

2.9.1 Determination transfection efficiency using Cy3-labelled siRNA

In keeping with our experimental protocol of when additional medium or re-plating occurs, 4 h after transfection cells were re-suspended in 400 uL of PBS and run using the FLH-2 channel of a BD FACSCalibre flow cytometer (Becton Dickson Immunocytometry Systems, San Jose, CA) without fixation. The number of events collected per sample run was set to 10,000.

2.9.2 Determination of mitochondrial activity using MitoTracker® Red CMXRos

Cells were processed as per manufacturer’s protocol. Briefly, cell pellets were re-suspended in a 200nM final concentration of MitoTracker® Red CMXRos (Invitrogen, Eugene, OR) in 37°C PBS and incubated in the dark at 37°C for 20 minutes. Cells were then washed with PBS and pelleted twice by centrifugation at 218 rcf for 10 mins. Cell pellets were resuspended in 400 uL of PBS and run using the FLH-2 channel of a BD FACSCalibre flow cytometer (Becton Dickson Immunocytometry Systems) without fixation. A sample containing the same cell lines as experimental samples, but without exposure to MitoTracker® was also run as a non-stained control. The number of events collected per sample run was set to 12,000.

2.9.3 Analysis of flow cytometry results

Analysis of flow cytometry results was carried out using FlowJo V10 software (Tree Star Inc., Ashland, OR). Forward scatter and side scatter of control, non-labeled cells were used to placed a “live cell” gate upon the experimental samples. A histogram for each sample was then generated for FLH-2 detection events (representing either Cy3-labelled
siRNA or MitoTracker®). Mean fluorescent intensity (MFI) was calculated. MFI refers to the average fluorescence intensity of each event and is a representation of abundance in expression, activity, etc, depending on the fluorescent probe. Results are expressed as a percentage of control treatment MFI.

2.10. Statistical analysis of results

In most experiments, three independent replicate experiments were performed (n=3 in each experiment). For each experiment, the mean was calculated. The average of 3 means ± SEM was calculated (i.e. a “mean of means” ± standard error of the “mean of means”) and is presented in the results sections. Thus, the “mean of means” reflects an n of 9 and includes both intra- and inter-experimental error. Unless otherwise indicated in figure legends, the “mean of means” analysis applies in all cases.

ANOVA was used to determine significant differences among normally distributed means. Student’s t tests were used to determine significant differences between normally distributed means. The level of significance for all statistical analyses was chosen a priori to be p < 0.05.
Chapter 3

« Results Part 1: Combining siRNAs targeting TS, TK1 or TK2 sensitizes human tumour cells to 5F UdR and pemetrexed »

The majority of results presented in this chapter were previously published in 2011 in The Journal of Pharmacology and Experimental Therapeutics [264] and have been reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics (all rights reserved) (see Appendix 2). The text and figures have been modified to conform to the monograph style. Unpublished data are discussed and pertain to Figures 15-19.

Currently, there are no small molecule drugs commercially available to target human TK1 or TK2. Thus, antisense molecules were used to decrease the activity of these enzymes. TK1 siRNA and TK2 siRNA were used alone and in combination with TS siRNA and/or TS-targeting drugs to assess whether TK plays a role in mediating resistance to TS-targeting interventions. Cell lines were treated with low siRNA concentrations that maximized knockdown of target mRNAs, minimized non-specific effects, and yielded consistent target downregulation in replicate experiments. Unless otherwise stated, TS, TK1, and TK2 targeting siRNAs were each used at 5 nM. Total siRNA concentrations were held constant at 10 nM by adding non-targeting C2 siRNA to siRNAs targeting TS, TK1, or TK2 where necessary, a maneuver that did not alter the effectiveness of siRNAs (Appendix 1).

3.1. Abstract

Thymidylate synthase (TS) is the only de novo source of thymidylate (dTMP) for DNA synthesis and repair. Drugs targeting TS protein are a mainstay in cancer treatment, but off-target effects and toxicity limit their use. Cytosolic thymidine kinase (TK1) and mitochondrial thymidine kinase (TK2) contribute to an alternative dTMP-producing pathway, by salvaging thymidine from the tumour milieu, and may modulate resistance
to TS-targeting drugs. Combined downregulation of these enzymes is an attractive strategy to enhance cancer therapy. We have previously shown that antisense targeting TS enhanced tumour cell sensitivity to TS-targeting drugs in vitro and in vivo. As both TS and TKs contribute to increased cellular dTMP, we hypothesized that TKs mediate resistance to the capacity of TS siRNA to sensitize tumour cells to TS-targeting anticancer drugs. We assessed the effects of targeting TK1 or TK2 with siRNA alone and in combination with siRNA targeting TS and/or TS-protein targeting drugs on tumour cell proliferation. Downregulation of TK with siRNA enhanced the capacity of TS siRNA to sensitize tumour cells to traditional TS protein-targeting drugs (5FUdR and pemetrexed). The sensitization was greater than that observed in response to any siRNA used alone, and was specific to drugs targeting TS. Upregulation of TK1 in response to combined 5FUdR and TS siRNA suggests that TK knockdown may be therapeutically useful in combination with these agents. TKs may be useful targets for cancer therapy when combined with molecules targeting TS mRNA and TS protein.

3.2. siRNAs downregulate respective target mRNAs

3.2.1. TS and TK siRNAs decrease target mRNAs in HeLa and MCF7 cells

Two different siRNAs targeting TS [TS siRNA(1) and TS siRNA(2)] were evaluated. TS siRNA(1) and TS siRNA(2) each downregulated TS mRNA by 70-85% in HeLa (Figure 3A) and MCF7 (Figure 4A) cells. The capacity of either TS siRNA to downregulate TS was unaffected by simultaneously targeting TK1 or TK2 using one of two different TK siRNAs (in combination with TS siRNA), in both HeLa (Figure 3A) and MCF7 (Figure 4A) cells.

Similarly, two different siRNAs targeting TK1 [TK1 siRNA(1) and TK1 siRNA(2)] downregulated TK1 mRNA by 60-80% in HeLa (Figure 3B) and MCF7 (Figure 4B) cells. In MCF7 (but not HeLa) cells, TK1 siRNA(1) downregulated TK1 more effectively than TK1 siRNA(2). The capacity of either of the two TK1 siRNAs to downregulate TK1 was unaffected by simultaneous administration of TS siRNA in HeLa cells (Figure 3B) and MCF7 (Figure 4B) cells.
TK2 mRNA was also downregulated by siRNAs, although to a more variable and lesser degree than TS and TK1. Two different siRNAs [TK2 siRNA(1) and TK2 siRNA(2)] downregulated TK2 by more than 80% in HeLa cells (Figure 3C) and 45-50% in MCF7 cells (Figure 4C), and combined treatment with TS siRNAs did not alter their effectiveness. TK2 siRNA(1) was a more effective siRNA than TK2 siRNA(2) in both HeLa (Figure 3C) and MCF7 cells (Figure 4C).

These experiments tested 2 different siRNAs for each target mRNA (TS, TK1, and TK2) in HeLa and MCF7 cell lines. Results were obtained 24 h post-transfection. TS siRNA(2), TK1 siRNA(1), and TK2 siRNA(1) were the most effective among the 6 siRNAs tested and were selected for use in subsequent experiments. They are referred to as TS siRNA, TK1 siRNA and TK2 siRNA, without distinguishing (1) or (2) for the remaining figures and results in this chapter.
Figure 3: siRNAs targeting TS, TK1, and TK2 reduce target mRNA in HeLa cells at 24 h post-transfection.

HeLa cells were treated with various combinations of 5 nM TS, TK1, and/or TK2 siRNA or control siRNAs (C2, C3; 10 nM total siRNA for each treatment). TS mRNA levels are shown in panel A, TK1 mRNA levels are shown in panel B, and TK2 mRNA levels are shown in panel C at 24 h post transfection. Two different siRNAs were used against target mRNA. Changes in target mRNA levels were calculated as a percent of mRNA levels in cells transfected with control (C2) siRNA. *different from cells treated with control (C2) siRNA (p<0.05, Student’s t test). * different from cells treated with siRNA targeting a different sequence in the same mRNA (p <0.05, ANOVA).
Figure 4: siRNAs targeting TS, TK1, and TK2 reduce target mRNA in MCF7 cells at 24 h post-transfection.

MCF7 cells were treated with various combinations of 5 nM TS, TK1, and/or TK2 siRNA or control siRNAs (C2, C3; 10 nM total siRNA for each treatment). TS mRNA levels are shown in panel A, TK1 mRNA levels are shown in panel B, and TK2 mRNA levels are shown in panel C at 24 h post-transfection. Two different siRNAs were used against target mRNA. Changes in target mRNA levels were calculated as a percent of mRNA levels in cells transfected with control (C2) siRNA. * different from cells treated with control (C2) siRNA (p<0.05, Student’s t test). ‡ different from cells treated with siRNA targeting a different sequence in the same mRNA (p<0.05, ANOVA).
3.2. Effects of targeting TS and/or TK1 or TK2 with siRNA in combination with the cytotoxic drug 5FUdR

3.2.1. Combined treatment with TS and TK2 siRNA sensitized HeLa cells to 5FUdR better than TS siRNA alone

To assess the outcome of combined reduction of both TS and TKs to enhance the activity of a TS-targeting drug, the effect of siRNA downregulation on HeLa cell proliferation was measured using: (i) TS or TK siRNAs alone; (ii) in combination, and (iii) followed by a period of treatment with or without 5FUdR. Results were obtained 96 h post-transfection and treatment with drug.

As single agents, siRNAs targeting TS, TK1, and TK2 had no effect on HeLa cell proliferation compared to treatment with control, non-targeting siRNA (Figure 5A). As expected, treatment with 5FUdR (IC_{50}) without knockdown of TS, TK1, or TK2 inhibited proliferation by approximately 50%. Neither TK1 siRNA nor TK2 siRNA enhanced the anti-proliferative effect of 5FUdR (Figure 5A). siRNA knockdown of TS prior to 5FUdR treatment enhanced the anti-proliferative effect of 5FUdR by approximately by two-fold (compared to C2 siRNA, Figure 5A) as reported previously [215, 216, 219, 271]. However, in the context of 5FUdR treatment, when cells were treated with both TS siRNA and TK2 siRNA, proliferation was further reduced by approximately 20% compared to treatment with TS siRNA alone (Figure 5B). This is an overall decrease of approximately 60% versus 50% when each was compared to C2 siRNA with drug (Figure 5A). The capacity of TK knockdown to increase TS siRNA-mediated enhancement of the anti-proliferative effect of 5FUdR was specific to TK2 (Figure 5). siRNA knockdown of TK1 had no effect on TS siRNA enhancement of 5FUdR activity.
Figure 5: Simultaneous treatment with TS and TK2 siRNAs sensitizes HeLa cells to 5FUdR better than treatment with TS siRNA alone. 

(A) HeLa cells were treated with control siRNA, TS siRNA, TK1 siRNA, and TK2 siRNA, in various combinations, followed by 5FUdR (IC\textsubscript{50} black bars) or no drug (white bars) for 96 h and proliferation was measured by counting cells (as described in Methods Section 2.4. siRNA and 2.5.1. Cell counting). (B) Proliferation of cells treated with 5FUdR plus TS siRNA, with or without TK2 siRNA. Proliferation of cells untreated with drug or siRNAs, and cells treated with various siRNAs alone, were calculated as a percentage of the fold increase in number of cells treated with control (C2) siRNA only. Proliferation of cells treated with 5FUdR was calculated as a percent of respective siRNA-treated cells fold-change without 5FUdR. NTC and LF2K data show that transfection conditions had minimal effects on proliferation. *different from cells treated with control (C2) siRNA and 5FUdR (p<0.05, ANOVA). \textsuperscript{a}different from cells treated with TS siRNA(1) plus C2 siRNA and 5FUdR (p<0.05, ANOVA and/or Student’s t test). For all conditions in which 5FUdR is present, there is a statistically significant reduction in proliferation (p<0.05, ANOVA).
3.2.2. siRNA downregulation of TS, TK1 and TK2 persisted up to 96 h post-transfection with, and without, 5FUdR treatment

TS siRNA, TK1 siRNA, and TK2 siRNA were applied to HeLa cells in four different treatment protocols: (1) as single agents, (2) TS siRNA combined with TK1 siRNA, (3) TS siRNA combined with TK2 siRNA, and (4) followed by treatment with 5FUdR. All siRNAs downregulated their target mRNA, and protein, regardless of regimen, up to 96 h post-transfection (Figure 6 for TS levels, Figure 7 for TK1 levels, and Figure 8 for TK2 levels). The degree of mRNA downregulation at 96 h was less than that at 24 h post-transfection (Figure 3). At 96 hours after treatment with TS siRNA alone, TS mRNA levels were 28% of control (Figure 6A) compared to 7% of control at 24 h (Figure 3A). At 96 h after TK1 siRNA treatment alone, TK1 mRNA was 52% of control (Figure 7A) compared to 10% of control at 24 hours (Figure 3B). At 96 h after TK2 siRNA treatment alone, TK2 mRNA was 75% of control (Figure 8A) compared to 16% of control at 24 h (Figure 3C). Similarly, time-dependent attenuation of target mRNA was evident under conditions where TS siRNA was combined with TK1 siRNA or TK2 siRNA, with or without 5FUdR (i.e., the degree of mRNA downregulation was greater at 24 h than at 96 h).

All siRNAs decreased target protein levels at 96 h post-transfection. TS siRNA alone, or in combination with TK1 siRNA or TK2 siRNA, decreased TS protein by 70% (Figure 6B). A similar level of TS mRNA and protein downregulation was observed in the presence of 5FUdR (Figure 6A & C). 5FUdR did not affect the capacity of TS siRNA to downregulate TS mRNA and protein. TK1 siRNA alone, or in combination with TS siRNA, reduced TK1 protein by 57% (Figure 7B) and the capacity of TK1 siRNA to downregulate TK1 mRNA and protein was unaffected by 5FUdR (Figure 7A & C). TK2 siRNA alone, or in combination with TS siRNA, reduced TK2 protein by 30% (Figure 8B). 5FUdR did not affect the ability of TK2 siRNA to downregulate TK2 mRNA and protein (Figure 8A & C) compared to conditions without 5FUdR.
Figure 6: TS siRNA reduces both TS mRNA and protein in HeLa cells, with or without simultaneous administration of TK siRNAs and/or 5FUdR.

HeLa cells were treated with control and/or targeting siRNAs in various combinations, followed by media alone or media plus 5FUdR, and specific mRNA and protein levels were measured 96 h later (as described in Methods and Methods Sections Section 2.6. Measurement of TS, TK1 and TK2 mRNA levels and

2.7.2.1. Immunoblotting in Chapter 3). (A) Relative TS mRNA (expressed as a percent of C2 siRNA without 5FUdR). (B & C) Relative TS protein (expressed as a percent of C2 siRNA without 5FUdR) without (B) and with (C) 5FUdR. Representative immunoblots (one treatment condition per lane) are shown (Ld, molecular weight ladder; lane 1, untreated control; lane 2, LFA2K; lane 3, C2; lane 4, C3; lane 5, TS + C2; lane 6, TK1 + C2; lane 7, TK2 + C2; lane 8, TS +TK1; lane 9, TS +TK2). Double TS protein bands (C) are due to the presence of both unbound and 5FUdR-bound TS. *, Different from cells treated with control (C2) siRNA (p≤0.05, ANOVA and/or Student’s t test). a*, Different from cells treated identically but without 5FUdR (p≤0.05, Student’s t test).
Figure 7: TK1 siRNA reduces both TK1 mRNA and protein in HeLa cells, with or without simultaneous administration of TS siRNAs and/or 5FUdR. HeLa cells were treated as described in the legend to Figure 6. (A) Relative TK1 mRNA (expressed as a percent of C2 siRNA without 5FUdR). (B & C) Relative TK1 protein (expressed as a percent of C2 siRNA without 5FUdR) without (B) and with (C) 5FUdR. Representative immunoblots (one treatment condition per lane) are shown (Ld, molecular weight ladder; lane 1, untreated control; lane 2, LFA2K; lane 3, C2; lane 4, C3; lane 5, TS + C2; lane 6, TK1 + C2; lane 7, TK2 + C2; lane 8, TS + TK1; lane 9, TS + TK2). *Different from cells treated with control (C2) siRNA (p<0.05, ANOVA and/or Student’s t test). a, Different from cells treated identically but without 5FUdR (p<0.05, Student’s t test).
Figure 8: TK2 siRNA reduces both TK2 mRNA and protein in HeLa cells, with or without simultaneous administration of TS siRNAs and/or 5FUdR.

HeLa cells were treated as described in the legend to Figure 6. (A) Relative TK2 mRNA (percent of that in cells treated with C2 siRNA). (B & C) Relative TK2 protein (percent of that in cells treated with C2 siRNA) without (B) and with (C) 5FUdR. Representative immunoblots (one treatment condition per lane) are shown (Ld, molecular weight ladder; lane 1, untreated control; lane 2, LFA2K; lane 3, C2; lane 4, C3; lane 5, TS + C2; lane 6, TK1 + C2; lane 7, TK2 + C2; lane 8, TS + TK1; lane 9, TS + TK2). Bars represent means ± SE (panel B: n=6 independent experiments, panel C: n= 3 independent experiments, triplicates per treatment group).

*Different from cells treated with control (C2) siRNA (p<0.05, ANOVA and/or Student’s t test).
3.2.3. TK1 protein increased as a result of combined TS siRNA and 5FUdR treatment

In the context of treatment with 5FUdR, cells treated with TS siRNA had a twofold increase in TK1 protein levels (Figure 7C) compared to the level seen after treatment with TS siRNA without 5FUdR (Figure 7B). Similar results are observed for combined treatment with TS and TK2 siRNA. The increase was not due to increased TK1 mRNA (Figure 7A) and was abolished when TK1 siRNA was combined with TS siRNA prior to 5FUdR treatment (Figure 7C). A similar increase in TK2 protein levels was not seen when using TS siRNA, alone or in combination with TK1, and/or 5FUdR (Figure 8B & C).

3.3. Effects of targeting TS and/or TK1 or TK2 with siRNA in combination with pemetrexed

3.3.1. Combined treatment with TS and TK1 siRNAs sensitized HeLa cells to the cytotoxic drug pemetrexed better than TS siRNA alone

HeLa cells were treated with siRNAs and pemetrexed (at the IC_{50} concentration) and the effects on proliferation were evaluated 96 h later. TS siRNA alone, or combined with TK1 or TK2 siRNAs, had no effect on cellular proliferation, without pemetrexed present, when compared to treatment with C2 siRNA (Figure 9A). Compared to C2 siRNA, treatment with TS siRNA increased sensitivity to pemetrexed by more than 30% (Figure 9A). Combined treatment with TK1 siRNA and TS siRNA enhanced pemetrexed-induced inhibition of proliferation by 25% more than that of pemetrexed plus TS siRNA alone (Figure 9 A & B). Overall, TS siRNA plus TK1 siRNA enhanced the antiproliferative effect of pemetrexed by approximately two-fold compared to C2 siRNA with pemetrexed (Figure 9A). The capacity of TK knockdown to increase TS siRNA-mediated enhancement of the antiproliferative effect of pemetrexed was specific to TK1. siRNA knockdown of TK2 had no effect on TS siRNA enhancement of pemetrexed activity (Figure 9 A & B).
Figure 9: Simultaneous treatment with TS and TK1 siRNAs sensitizes HeLa cells to pemetrexed treatment better than TS siRNA alone.

(A) HeLa cells were treated with control siRNA, TS siRNA, TK1 siRNA, and TK2 siRNA, in various combinations, followed by pemetrexed (IC_{50}) (black bars) or no drug (white bars) for 96 h. Proliferation was measured as described in Methods Section 2.5.1. Cell counting. (B) Proliferation of cells treated with pemetrexed plus TS siRNA, with or without TK1 siRNA. Proliferation results in panels A and B are expressed in the same format as those of Figure 5.

* Different from cells treated with control (C2) siRNA and pemetrexed (p<0.05, ANOVA). a, Different from cells treated with TS siRNA plus C2 siRNA and pemetrexed (p<0.05, ANOVA and/or Student’s t test). For all conditions in which pemetrexed is present, there is a statistically significant reduction in proliferation (p<0.05, ANOVA).
3.3.2. siRNA downregulation of TS, TK1, or TK2 persists up to 96 hours post-transfection, with and without pemetrexed treatment

As seen after combined treatment with siRNAs targeting TS or TS plus TK1 or TK2, with or without subsequent 5FUDR (Figure 6, Figure 7, Figure 8), siRNA-mediated downregulation of mRNA and protein targets was maintained up to 96 h post-transfection of HeLa cells in the absence and presence of pemetrexed (TS, Figure 10; TK1, Figure 11; TK2, Figure 12). Control data, presented throughout the figures in this chapter, were generated from similar experiments, shown in each case because they were performed simultaneously with, and controlled for, potential variation in inter-experiment culture conditions and cell characteristics. Data shown in Figure 8 B and Figure 12 B are identical and are shown in both figures to facilitate comparison with novel data.

The addition of pemetrexed to TS siRNA increased TK2 protein to a level 70% higher than that observed after treatment with TS siRNA alone (Figure 12 B and C). This effect was negated by the presence of TK2 siRNA. There was no concomitant increase in TK1 protein (Figure 11 C).
Figure 10: TS siRNA reduces both TS mRNA and protein in HeLa cells, with or without simultaneous administration of TK1 or TK2 siRNA and/or pemetrexed.

HeLa cells were treated with control and/or targeting siRNAs in various combinations, followed by media alone or media plus pemetrexed, and specific mRNA and protein levels were measured 96 h later as described in Materials and Methods. (A) Relative TS mRNA (percent of that in cells treated with C2 siRNA). (B,C) Relative TS protein (percent of that in cells treated with C2 siRNA) without (B) and with (C) pemetrexed. Representative immunoblots (one treatment condition per lane) are shown (Ld, molecular weight ladder; lane 1, untreated control; lane 2, LFA2K; lane 3, C2; lane 4, C3; lane 5, TS + C2; lane 6, TK1 + C2; lane 7, TK2 + C2; lane 8, TS + TK1; lane 9, TS + TK2). *Different from cells treated with control (C2) siRNA (p<0.05, ANOVA and/or Student’s t test). †Different from cells treated identically but without 5FUdR (p<0.05, Student’s t test).
Figure 11: TK1 siRNA reduces both TK1 mRNA and protein in HeLa cells, with or without simultaneous administration of TS siRNA and/or pemetrexed.

HeLa cells were treated as described in the legend to Figure 7. (A) Relative TK1 mRNA (percent of that in cells treated with C2 siRNA). (B,C) Relative TK1 protein (percent of that in cells treated with C2 siRNA) without (B) and with (C) pemetrexed. Representative immunoblots (one treatment condition per lane) are shown (Ld, molecular weight ladder; lane 1, untreated control; lane 2, LFA2K; lane 3, C2; lane 4, C3; lane 5, TS + C2; lane 6, TK1 + C2; lane 7, TK2 + C2; lane 8, TS + TK1; lane 9, TS + TK2). *Different from cells treated with control (C2) siRNA (p<0.05, ANOVA and/or Student’s t test). †Different from cells treated identically but without pemetrexed (p<0.05, Student’s t test).
Figure 12: TK2 siRNA reduces both TK2 mRNA and protein in HeLa cells, with or without simultaneous administration of TS siRNA and/or pemetrexed.

HeLa cells were treated as described in the legend to Figure 7. (A) Relative TK2 mRNA (percent of that in cells treated with C2 siRNA). (B,C) Relative TK2 protein (percent of that in cells treated with C2 siRNA) without (B) and with (C) pemetrexed. Representative immunoblots (one treatment condition per lane) are shown (Ld, molecular weight ladder; lane 1, untreated control; lane 2, LFA2K; lane 3, C2; lane 4, C3; lane 5, TS + C2; lane 6, TK1 + C2; lane 7, TK2 + C2; lane 8, TS + TK1; lane 9, TS + TK2). Bars represent means ± SE (panel B: n=6 independent experiments, triplicates per treatment group). *Different from cells treated with control (C2) siRNA (p<0.05, ANOVA and/or Student’s t test). 

*Different from cells treated identically but without 5FUdR (p<0.05, Student’s t test).
3.4. The effect of TS siRNA and/or TK1 or TK2 siRNAs in combination with a non-TS-targeting drug

3.4.1. siRNA-mediated reduction of TS and TKs does not enhance the cytotoxicity of cisplatin

To investigate if the enhancement of sensitivity to drugs when using TS siRNA and a TK siRNA was specific to TS-targeting drugs, the previous methodology was used in combination with the non-TS-targeting drug cisplatin. TS and TK siRNAs, alone or in combination, had no effect on cisplatin-mediated inhibition of proliferation (Figure 13). Therefore, the enhancement of drug-mediated antiproliferative effects by siRNA knockdown of TS and TK in HeLa cells was specific to 5F UdR and pemetrexed.

3.5. siRNAs do not effect proliferation

To assess whether the siRNAs had an effect on cellular proliferation, the no-drug-treatment conditions from Figure 5, Figure 9, and Figure 13 were compiled and statistical analysis was performed. Compared to the control siRNA, TS siRNA, TK1 siRNA, and TK2 siRNA used as single agents, or in various combinations, do not cause a reduction in HeLa cell proliferation (Figure 14). Compared to untreated cells, or cells treated with LF2K but without siRNA, the transfection of siRNAs, in general, into HeLa cells does cause a non-specific reduction in cellular proliferation (Figure 14). The reduction in proliferation is non-specific, as the decrease in proliferation is similar among both targeting and control, non-targeting siRNA sequences.
HeLa cell sensitivity to cisplatin is not affected by TS and/or TK siRNAs.

HeLa cells were treated with control siRNA, TS siRNA, TK1 siRNA, and TK2 siRNA, in various combinations followed by cisplatin (IC$_{50}$) (hatched bars) or no drug (white bars) for 96 h and proliferation was measured as described in Methods Sections 2.4. siRNA and 2.5.1. Cell counting. Proliferation of cells untreated with drug or siRNAs, and cells treated with various siRNAs alone, was calculated as a percent of cells treated with control (C2) siRNA only. Proliferation of cells treated with cisplatin was calculated as a percent of identically-treated cells without cisplatin. NTC and LF2K data are included to show that transfection conditions had minimal effects on proliferation. For all conditions in which cisplatin is present, there is a statistically significant reduction in proliferation ($p<0.05$, ANOVA).
Figure 14: Targeting siRNAs do not cause a reduction in HeLa proliferation. HeLa cells were treated with control siRNA, TS siRNA, TK1 siRNA, and TK2 siRNA, in various combinations for 96 h and proliferation was measured as described in Methods Sections 2.4. siRNA and 2.5.1. Cell counting. Proliferation of cells untreated with drug or siRNAs, and of cells treated with various siRNAs alone, was calculated as a percent of cells treated with control (C2) siRNA. Bars represent means ± S.E.M. of n=9 independent experiments, with triplicates per treatment group for each experiment. *Different from NTC cells and LF2K treated cells (p<0.05, ANOVA).
3.6. The effect of combined TS, TK1, and TK2 siRNAs used with TS-targeting drugs: Using 3 different targeting siRNA sequences simultaneously

3.6.1. Combined treatment with TS, TK1, and TK2 siRNAs does not cause further sensitization to 5FUdR compared to cells treated with TS and TK2 siRNA

To assess the effect of targeting all 3 dTMP-producing enzymes on inhibition of cellular proliferation by 5FUdR, HeLa cells were treated with TS siRNA [3 nM], TK1 siRNA [3 nM] and TK2 siRNA [4 nM], or various combinations thereof, using C2 siRNA to top up siRNA concentrations to a total of 10 nM in all cases. Proliferation was measured by counting cells after 96 h. TS siRNA sensitized HeLa cells to the antiproliferative effects of 5FUdR. Combined treatment with both TS and TK2 siRNAs further sensitized HeLa cells to 5FUdR beyond that seen with TS siRNA (Figure 15 and as previously shown in Figure 5). The addition of TK1 siRNA to this combination did not cause further sensitization to 5FUdR (Figure 15). In fact, the addition of TK1 siRNA to the combination of TS and TK2 siRNAs negated the further sensitization to 5FUdR that was previously seen when compared to TS siRNA.

3.6.2. Combined treatment with TS, TK1, and TK2 siRNAs does not cause further sensitization to pemetrexed compared to cells treated with TS and TK1 siRNA

HeLa cells were treated with the same siRNA concentrations used above (TS siRNA [3 nM], TK1 siRNA [3 nM] and TK2 siRNA [4 nM]) and a similar experiment was carried out using pemetrexed as the TS-targeting drug. At 96 h post-transfection, TS siRNA resulted in an increased reduction in HeLa cell proliferation when cells also treated with pemetrexed (Figure 16). While treatment with TS and TK1 siRNAs resulted in increased sensitivity to pemetrexed, the addition of TK2 siRNA did not cause further sensitization to pemetrexed beyond that seen with TS siRNA (Figure 16 and as previously seen in Figure 9). Similar to results seen above with 5FUdR, the addition of TK2 siRNA to the combination of TS and TK1 siRNAs negated the further sensitization to pemetrexed that
was previously seen when compared to sensitization induced by treatment with TS siRNA alone.

### 3.6.3. Total siRNA concentration used affects sensitization to TS-targeting drugs.

Prior to the siRNA triple combination containing TS siRNA (3 nM), TK1 siRNA (3 nM), and TK2 siRNA (4 nM), triple combination experiments at a higher concentration of each targeting siRNA (4 nM) were attempted (i.e., the total siRNA concentration was increased to 15 nM). After transfection, cells were replated and exposed to drug-free medium, medium containing 5FUdR, or medium containing pemetrexed. For both 5FUdR (Figure 17) and pemetrexed (Figure 18), increasing the total siRNA concentration to 15 nM negated the prior sensitization effects seen with either drug. At this total concentration of siRNA, HeLa cells did not experience a further sensitization to the anti-proliferative effects of 5FUdR when TS siRNA (5 nM + 10 nM of C2 siRNA) was used or when TS and TK2 siRNAs (5 nM of each + 5 nM of C2 siRNA) were used as previously seen in Figure 5, Figure 15 (using 10 nM siRNA), and in prior publications (using TS-targeting ODNs or siRNAs) from this laboratory [215, 219, 271]. Similarly, cells did not experience a further increase in sensitization to pemetrexed when TS and TK1 siRNAs were used or when only TS siRNA was used as previously seen in Figure 9, Figure 16, prior publications [215, 219, 271].

To assess if this phenomenon was specific to HeLa cells, similar experiments were carried out using Meso H28 cells, 5FUdR and either a 10 nM or 15 nM total concentration of siRNA (Figure 19). At the 10 nM total concentration of siRNA, in Meso H28 cells TS siRNA enhanced the inhibition of proliferation by FUdR by 35%. There was a further reduction in proliferation to 48% compared to C2 siRNA when TS siRNA was combined with TK2 siRNA, but not with TK1 siRNA (Figure 19 A). At the 10 nM total concentration of siRNA, Meso H28 cells show a 90% reduction in proliferation in all treatment conditions involving 5FUdR and TS siRNA (Figure 19 A). The effect of siRNAs and of 5FUdR in combination with siRNAs is decreased in the Meso H28 cell line when a total of 15 nM of siRNA is used compared to the 10 nM results. At the 15 nM total
concentration, TS siRNA alone did not cause a reduction in Meso H28 proliferation and combined effects with 5FUdR are decreased (Figure 19 B). This indicates that the decreased magnitude of the antiproliferative response which was based on an overall increase in the total concentration of siRNA used is not specific to HeLa cells.
Figure 15: Combined treatment with TS siRNA, TK1 siRNA and TK2 siRNA does not cause further sensitization to 5FUdR compared to cells treated with TS and TK2 siRNAs.

HeLa cells were treated with TS siRNA [3 nM], TK1 siRNA [3 nM] and TK2 siRNA [4 nM], or various combination thereof using C2 siRNA to top-up to a 10 nM total concentration of siRNA when necessary. Cell were then treated with either medium alone or medium containing 5FUdR and cellular proliferation was then measured 96 h later as outlined in Methods Sections 2.4.  

siRNA and 2.5.1. Cell counting. *different from cells treated with control (C2) siRNA and 5FUdR (p<0.05, ANOVA).  "different from cells treated with TS siRNA plus C2 siRNA and 5FUdR (p<0.05, ANOVA and/or Student’s t test). For all conditions in which 5FUdR is present, there is a statistically significant reduction in proliferation (p<0.05, ANOVA).
Figure 16: Combined treatment with TS siRNA, TK1 siRNA and TK2 siRNA does not cause further sensitization to pemetrexed compared to cells treated with TS and TK1 siRNAs.

HeLa cells were treated with TS siRNA [3 nM], TK1 siRNA [3 nM] and TK2 siRNA [4 nM], or various combination thereof using C2 siRNA to top-up to a 10 nM total concentration of siRNA when necessary. Cells were then treated with either medium alone or medium containing pemetrexed and cellular proliferation was measured 96 h later as outlined in Methods Sections 2.4. siRNA and 2.5.1. Cell counting. *different from cells treated with control (C2) siRNA and pemetrexed (p<0.05, ANOVA). a different from cells treated with TS siRNA plus C2 siRNA and pemetrexed (p<0.05, ANOVA and/or Student’s t test). For all conditions in which pemetrexed is present, there is a statistically significant reduction in proliferation (p<0.05, ANOVA).
At 15 nM total siRNA, siRNAs targeting TK2 siRNA and/or TS siRNA did not sensitize HeLa cells to 5FUdR.

HeLa cells were treated with 5 nM each of TS siRNA, TK1 siRNA and TK2 siRNA, or various combination thereof using C2 siRNA to top-up to a 15 nM total concentration of siRNA when necessary. Cell were then treated with either medium alone or medium containing 5FUdR and cellular proliferation was measured 96 h later as outlined in Methods Sections 2.4. siRNA and 2.5.1. Cell counting. For all conditions in which 5FUdR is present, there is a statistically significant reduction in proliferation (p<0.05, ANOVA).

Figure 17: At 15 nM total siRNA, siRNAs targeting TK2 siRNA and/or TS siRNA did not sensitize HeLa cells to 5FUdR.
Figure 18: At 15 nM total siRNA, targeting TS and TK1 siRNAs did not sensitize to pemetrexed.

HeLa cells were treated with 5 nM each of TS siRNA, TK1 siRNA and TK2 siRNA, or various combination thereof using C2 siRNA to top-up to a 15 nM total concentration of siRNA when necessary. Cell were then treated with either medium alone or medium containing pemetrexed and cellular proliferation was measured 96 h later as outlined in Methods Sections 2.4. siRNA and 2.5.1. Cell counting. For all conditions in which pemetrexed is present, there is a statistically significant reduction in proliferation (p<0.05, ANOVA).
Meso H28 Cell Line

Figure 19: In Meso H28 cells, total siRNA concentration effects sensitivity to TS siRNA, TS siRNA plus TK2 siRNA and siRNAs in combination with 5FUdR.

Meso H28 cells were treated with various concentrations of targeting siRNAs as previously described such that the total concentration of siRNA was held constant at either 10 nM, for A), or 15 nM for B) using C2 siRNA to top-up when necessary. Cell were then treated with either medium alone or medium containing 5FUdR and cellular proliferation was measured 96 h later as outlined in Methods Sections 2.4. siRNA and 2.5.1. Cell counting. For all conditions in which 5FUdR is present, there is a statistically significant reduction in proliferation ($p<0.05$, ANOVA). *different from C2 siRNA with 5FUdR ($p<0.05$, ANOVA). #, different from C2 siRNA with 5FUdR ($p<0.05$, ANOVA). Different from TS siRNA without 5FUdR ($p<0.05$, ANOVA).
Chapter 4
« Results Part 2: siRNA targeting of mitochondrial thymidine kinase 2 (TK2) to sensitize cancer cells to gemcitabine »

The results of this Chapter are currently being submitted for publication.

The data presented here seeks to address if mitochondrial TK2 contributes to resistance, or decreased effectiveness of, gemcitabine chemotherapy. There exists a relationship between dCK, TK2 and gemcitabine (described in sections 1.3.2.1 Thymidine kinases, 1.4 Project rationale, and in the abstract below), such that TK2 may contribute to levels of dCTP that negatively regulate dCK; both under normal conditions and when using gemcitabine.

4.1. Abstract

Mitochondrial thymidine kinase 2 (TK2) preferentially phosphorylates thymidine to generate thymidine monophosphate (dTMP). TK2 also phosphorylates deoxycytidine to generate dCMP, a precursor for dCTP -- an example of TK2 substrate promiscuity. dCTP negatively regulates deoxycytidine kinase (dCK), the enzyme that primarily phosphorylates deoxycytidine, but also phosphorylates the anticancer drug gemcitabine. Gemcitabine activation requires phosphorylation. Thus, there is a therapeutic advantage to high dCK in tumour cells treated with gemcitabine, as it activates the drug. Antisense knockdown of TK2 could reduce TK2-produced dCMP, thus decreasing dCTP levels and its inhibition of dCK. This would subsequently lead to increased dCK activity, gemcitabine activation, and anticancer effectiveness.

Importantly, gemcitabine is a very poor target for direct phosphorylation by TK2. Given the substrate promiscuity of TK2, we hypothesized that: (1) TK2 can mediate human tumour cell resistance to gemcitabine, and (2) antisense downregulation of TK2 can overcome that resistance. Downregulation of TK2 using siRNA sensitized MCF7 and HeLa cells (high and moderate TK2 expressers, respectively) to gemcitabine, but did not
sensitize A549 cells (low TK2 expresser). Combined treatment with TK2 siRNA and gemcitabine increased dCK enzyme levels. We also explored the hypothesis that TK2 siRNA-induced drug sensitization is mediated by mitochondrial damage. Consistent with this hypothesis, we observed that treatment of TK2 expressing human tumour cells with TK2 siRNA and gemcitabine, compared to the control siRNA and gemcitabine: 1) altered mitochondrial redox status, 2) decreased mitochondrial DNA (mtDNA:nDNA ratio), and 3) decreased mitochondrial activity. This is the first demonstration of a direct role for TK2 in gemcitabine resistance, or any independent role in cancer drug resistance, and further distinguishes TK2 from other dTMP-producing enzymes [cytosolic TK1 and thymidylate synthase (TS)]. siRNA knockdown of TK1 and/or TS in combination with TK2 siRNA and gemcitabine did not cause further sensitization. This phenomenon is specific to targeting of TK2.

4.2. TK2 expression in human tumour cells

Immunoblot analysis indicates that MCF7, HeLa and A549 human tumour cell lines differ with respect to basal TK2 protein expression levels (Figure 20). They will be designated as TK2\textsuperscript{HIGH} (MCF7), TK2\textsuperscript{MEDIUM} (HeLa) and TK2\textsuperscript{LOW} (A549) cells. A549 cells have 25% of the amount of TK2 seen in HeLa levels and 7% of the amount in MCF7.
Figure 20: Basal TK2 expression in MCF7, HeLa and A549 cells.

Basal TK2 expression levels in MCF7(TK2<sup>HIGH</sup>), HeLa(TK2<sup>MEDIUM</sup>) and A549(TK2<sup>LOW</sup>) cell lines. Immunoblots show representative triplicate independent samples (Ld, molecular weight ladder).
4.3. TK2 siRNA knockdown

4.3.1. TK2 siRNA decreased TK2 protein and mRNA

MCF7 (TK2\textsuperscript{HIGH}) cells were transfected with either 10 nM of control siRNA (C2 or C3) or 10 nM of TK2-targeting siRNA (TK2#9 or TK2#11) and replated 4 h post-transfect as indicated in materials and methods section 2.4.1.2. Experiments lasting longer than 24 h post-transfection with siRNA (including time-course and drug-treatment experiments) and Table 4. Cell lysates and mRNA were then collected at 24, 48, 72, and 96 h post-transfection and immunoblotting and qPCR were performed.

In MCF7 cells, TK2-targeting siRNAs (TK2#9 and TK2#11) decreased TK2 protein by 20-25% compared to control siRNAs (C2 and C3) at 72 and 96 h post-transfection (Figure 21A). Similar to MCF7 cells, TK2 protein down regulation could be seen in HeLa cells 96 h post-transfection with TK2 siRNAs; a 35-45% reduction in TK2 protein levels (Figure 22). Results for TK2 mRNA levels taken from concurrent experimental MCF7 samples show that TK2 mRNA was reduced by 60-75% as compared to control treated cells at 24-96 h post-transfection (Figure 21B).

TK2\textsuperscript{LOW}, A549 cells line served as a control for some experiments as TK2 downregulation by siRNA was modest. A549 cells have 25% of the amount of TK2 seen in HeLa levels and 7% of the amount in MCF7 (Figure 20).
Figure 21: TK2 protein and mRNA levels 24-96 h after TK2 siRNA transfection in MCF7 cells.

MCF7 (TK2\textsuperscript{HIGH}) cells were transfected with 10 nM and replated 4 h post-transfect as indicated in methods section. Cell lysates were collected at 48, 72, and 96 h post-transfection and concurrent mRNA samples were collected at 24, 48, 72, and 96 h post-transfection. 

A) Relative TK2 protein levels in MCF7 cells, 48-96 h post-transfection with 10 nM TK2 siRNA. 

B) TK2 mRNA in MCF7 cells 24-96 h post-transfection with 10 nM siRNA. For both graphs, results are shown as a percent of the amount in cells transfected with control, non-targeting C2 siRNA on that day. 

*different from cells treated with C2 or C3 siRNA (\(p < 0.05\) Student’s t test and/or ANOVA). 

\#different from cells treated with C2 siRNA (\(p < 0.05\) Student’s t test and/or ANOVA).
Figure 22: TK2 expression in HeLa cells 96 h post-transfection with 10 nM of siRNA. HeLa cells, 96 h post-transfection with 10 nM TK2 siRNA. In both cases, relative TK2 protein is shown as a percent of the amount in cells transfected with control, non-targeting C2 siRNA. Results are shown as a percent of the amount in cells transfected with control, non-targeting C2 siRNA. *different from cells treated with C2 or C3 siRNA (p < 0.05, Student's t test or ANOVA). Immunoblots show representative triplicate independent samples (Ld, molecular weight ladder).
4.3.2. Differences in transfection efficiency do not account for variable responses to TK2 siRNA.

To help ensure that any future biological responses, or lack thereof, to TK2 antisense siRNA downregulation were not attributable to variability in siRNA transfection efficiency, experiments looking at transfection efficiency of MCF7, HeLa, and A549 cells were carried out. The HT-29 cell line was used as a negative control as the cell line does not transfect well using LF2K transfection reagent. Cells were transfected with either 10 nM Cy3-TS siRNA, or TS siRNA #4 (which has the same sequence as Cy3-TS siRNA but lacks the Cy3 fluorophore on the 5’-end). Untreated cells were used as experimental controls. Flow cytometry for Cy3 fluorescence was performed at 4 h post-transfection; the timing was in keeping with experimental protocol used where cells are replated 4 h after transfection (Materials and Methods Section 2.4.1.2. Experiments lasting longer than 24 h post-transfection with siRNA (including time-course and drug-treatment experiments)).

MCF7, HeLa, and A549 cells had similar siRNA transfection efficiencies (Figure 23) indicating that differences among the cell lines with respect to the consequences of transfection with TK2 siRNA are not attributable to differential transfectability. By comparison, under the same conditions as the MCF7, HeLa and A549 cell lines, the HT-29 cell line showed a much lower increase in Cy3 mean fluorescence intensity (MFI) compared to its control when transfected with Cy3 siRNA (Figure 23).
Figure 23: siRNA transfection efficiencies of MCF7, HeLa, and A549 cell lines. 

A) Cells were untransfected (white bars), transfected with unlabeled TS siRNA (grey bars), or transfected with Cy3-labeled TS siRNA (black bars). Mean fluorescence intensity (MFI) was determined by flow cytometry 4 h post-transfection with 10 nM siRNA using LF2K. Data are shown as the percent increase in MFI compared to untransfected cells. *different from untransfected cells or labeled TS siRNA cells ($p < 0.05$, ANOVA).

B) Representative histograms of the results from A) for each cell line showing untreated cells (red), TS siRNA treated cells (blue) and Cy3-TS siRNA treated cells (orange). HT-29 cell line is a hard-to-transfect cell line that was used as a control.
4.4. Antisense knockdown of TK2 sensitizes TK2<sub>MEDIUM</sub> (HeLa) and TK2<sub>HIGH</sub> (MCF7) cells, but not TK2<sub>LOW</sub> A549 cells, to gemcitabine

The effect of antisense-mediated TK2 knockdown on human tumour cell proliferation and sensitivity to gemcitabine was assessed in response to two different control (C2 or C3) and two different TK2-targeting siRNAs (#9 and #11, complementary to different portions of the TK2 mRNA sequences, Table 3). TK2#9 siRNA (compared to C2 non-targeting siRNA), or TK2#11 siRNA (compared to C3 non-targeting siRNA), were used to reduce TK2 in all 3 cell lines and the effect on sensitivity to growth inhibition by gemcitabine was assessed.

TK2 siRNA sensitized TK2<sub>HIGH</sub> MCF7 cells and TK2<sub>MEDIUM</sub> HeLa cells to gemcitabine (multiple drug concentrations from IC<sub>20</sub> to IC<sub>80</sub>) (Figure 24 and Figure 25). TK2 knockdown in TK2<sub>HIGH</sub> MCF7 cells by either TK2 siRNA enhanced gemcitabine-mediated reduction in cell proliferation by 30-50% (Figure 24 A-B) and TK2<sub>MEDIUM</sub> HeLa cells by 15-50% (Figure 25 A-B). TK2#11 siRNA treatment of HeLa cells sensitized only to mid-range concentrations of gemcitabine (4 nM and 6 nM). TK2 siRNA treatment did not sensitize TK2<sub>LOW</sub> (A549) cells to any tested gemcitabine concentration (Figure 26). Experiments were carried out concurrently for these cell lines, meaning they were exposed to the exact same transfection mixture.

To isolate siRNA-induced sensitization to gemcitabine from off-target siRNA effects, treatment with siRNAs alone (without gemcitabine) were deemed to have 100% survival, in accord with our observation that there was no detectable reduction in proliferation induced by control siRNAs alone (C2 or C3) or TK2-targeting siRNAs alone (TK2#9 or TK2#11)(Figure 27).
Figure 24: siRNAs targeting TK2 sensitize MCF7 (TK2^{HIGH}) human tumour cells to gemcitabine. MCF7 cells were transfected with 10 nM of A) C2 or TK2#{9}, or B) C3 or TK2#{11} siRNAs and treated with gemcitabine as described in Materials and Methods. Proliferation was measured by cell counting at 96 h post-transfection. Data is expressed as a percent of the number of cells after treatment with siRNA alone (without drug). * different from cells treated with control, non-targeting siRNA (C2 or C3) at the same gemcitabine concentration (p < 0.05, Student’s t test).
Figure 25: siRNAs targeting TK2 sensitize HeLa (TK2\textsuperscript{MEDIUM}) human tumour cells to gemcitabine.
HeLa cells were transfected with 10 nM of A) C2 or TK2\#9, or B) C3 or TK2\#11 siRNAs and treated with gemcitabine as described in Materials and Methods. Proliferation was measured by cell counting at 96 h post-transfection. Data is expressed as a percent of the number of cells after treatment with siRNA alone (without drug). *different from cells treated with control, non-targeting siRNA (C2 or C3) at the same gemcitabine concentration (\( p < 0.05 \), Student’s t test).
Figure 26: siRNAs targeting TK2 did not sensitize A549 (TK2\textsuperscript{LOW}) human tumour cells to gemcitabine. A549 cells were transfected with 10 nM of A) C2 or TK2#9, or B) C3 or TK2#11 siRNAs and treated with gemcitabine as described in Materials and Methods. Proliferation was measured by cell counting at 96 h post-transfection. Data is expressed as a percent of the number of cells after treatment with siRNA alone (without drug). There are no significant differences to report.
Figure 27: TK2 siRNAs alone do not affect human tumour cell proliferation. Data for cell lines are the results of the 0 nM gemcitabine condition presented in Figure 24-26. A) MCF7, B) HeLa, and C) A549 cells were transfected with control or TK2-targeting siRNAs. Proliferation was measured by counting cells as described in Materials and Methods. Proliferation of cells treated with control, non-targeting C2 siRNA was normalized to 100% and values for cells transfected with TK2#9 siRNA are expressed as a percent of values in cells transfected with C2 siRNA. Proliferation of cells treated with control, non-targeting C3 siRNA was normalized to 100% and values for TK2#11 siRNA are expressed as a percent of values in cells transfected with C3 siRNA. There are no significant differences to report.
4.5. Combined treatment with TK2 siRNA and gemcitabine reduces TK2 and increases dCK

4.5.1. Analysis of TK2 and dCK protein levels

Cell lysates (and mRNA) for TK2\textsuperscript{HIGH}(MCF7) and TK2\textsuperscript{MEDIUM}(HeLa) cells were collected 96 h after transfection with siRNA and treatment with gemcitabine (4 nM). Immunoblot analysis of cell lysates assessed TK2 and dCK enzyme levels under the same conditions. In both cell lines, with or without gemcitabine, TK2 protein levels are decreased by TK2 siRNA (Figure 28 A & C).

Combined treatment with TK2 siRNA and gemcitabine reduced TK2 protein levels (as seen without gemcitabine present) and increased dCK levels in both MCF7 and HeLa cells (Figure 28 B & D). When HeLa cells were also exposed to gemcitabine, treatment with TK2 siRNA #9 resulted in a 25% decrease in TK2 protein and a 60% increase in dCK protein compared to cells treated with C2 siRNA (Figure 28 C-D); similar results are seen with TK2 siRNA #11 is compared to C3 siRNA.

In TK2\textsuperscript{HIGH}(MCF7) cells, a similar increase in dCK accompanied TK2 siRNA downregulation of TK2 (Figure 28 A-B). When MCF7 cells were also exposed to gemcitabine, treatment with TK2 siRNA #9 resulted in a 30% decrease in TK2 protein and a 45% increase in dCK protein levels compared to C2 siRNA treated cells (Figure 28 A-B). When treated with TK2 siRNA #11, after exposure to gemcitabine, there was a 30% decrease in TK2 protein levels and a 70% increase in dCK protein levels compared to C3 siRNA (Figure 28 A-B).

4.5.2. Analysis of TK2 and dCK mRNA levels

At 96 h post-transfection with siRNA and treatment with gemcitabine, mRNA samples were collected for both MCF7 and HeLa cells. qPCR was performed to assess TK2 siRNA downregulation of TK2 mRNA in the presence and absence of gemcitabine (4 nM). In both cell lines, with or without gemcitabine, TK2 mRNA levels are decreased by TK2
siRNAs (Figure 29) at 96 h post-transfection. In the MCF7 cells, treatment with either TK2#9 or TK2#11 siRNA resulted in a 55% decrease in TK2 mRNA levels compared to control siRNAs (C2 or C3) (Figure 29 A). In the HeLa cell line, there was a 30-45% reduction in TK2 mRNA levels, as compared to control siRNAs, after treatment with TK2-targeting siRNAs (Figure 29 B).
Figure 28: TK2 knockdown and gemcitabine treatment increased dCK in MCF7 and HeLa cells.

MCF7 (A-B) and HeLa (C-D) cells were transfected with C2, C3, TK2#9 or TK2#11 siRNAs, treated with gemcitabine, and relative TK2 and dCK protein levels measured 96 h post-transfection as described in Materials and Methods. Bars represent means ± S.E.M for n = 8 – 9 samples representing 3 independent experiments. * different from cells transfected with control, non-targeting siRNA (p < 0.05, Student’s t test). # different from cells treated identical siRNA but without gemcitabine (p < 0.05, Student’s t test). See Figure 29 for siRNA-mediated knockdown of TK2 mRNA in the same cells for which these data are shown.
Figure 29: TK2 mRNA levels in MCF7 and HeLa cells after treatment with TK2 siRNAs and 4 nM gemcitabine.

MCF7 (A) and HeLa (B) cells were transfected with siRNA, treated with gemcitabine and relative TK2 mRNA levels measured 96 h post-transfection as described in Materials and Methods. TK2 and dCK protein was measured in the same experiment and that data is presented in Figure 28. Data is expressed as a percent of non-targeting control C2 siRNA without drug. *different from cells transfected with C2 or C3 siRNA (p < 0.05, ANOVA).
4.6. Sensitization effects are specific to TK2: siRNAs targeting other dTMP-producing enzymes do not sensitize to gemcitabine

TK2 is only one of three enzymes that mediating dTMP synthesis: thymidylate synthase (TS) is responsible for *de novo* dTMP production and TK1 is an alternative salvage enzyme to TK2 [174, 272]. Consequently, further experiments were performed to assess if sensitization to gemcitabine was specific to TK2 enzyme or to dTMP-producing enzymes in general. Sensitization of TK2\textsuperscript{MEDIUM} (HeLa) cells to gemcitabine (0-7.5 nM) in the context of siRNA knockdown of TS and TK1, in addition to knockdown of TK2, was assessed.

Only TK2 knockdown, and not TS or TK1 knockdown, sensitized HeLa cells to gemcitabine (*Figure 30 A-D*); of 4 tested gemcitabine concentrations, at only one (6 nM) did TK1 reduction sensitize to a detectable degree, which was minimal and to a lesser degree than reduction of TK2 (*Figure 30 B*). When siRNA treatments were combined to: i) reduce both TS and TK2, ii) reduce both TK1 and TK2, or (iii) reduce TS, TK1, and TK2 simultaneously in a triple-targeting approach, the combinations did not increase sensitization to gemcitabine to a greater extent that observed by TK2 knockdown alone, at all tested gemcitabine concentrations (3-7.5 nM). Thus, sensitization to gemcitabine appears to be specific to knockdown of TK2.
Figure 30: siRNA targeting of TK2, but not TS or TK1, contributes to sensitization to gemcitabine.

HeLa cells were transfected with siRNAs targeting TS, TK1 and TK2 in various combinations, treated with gemcitabine at either A) 3 nM, B) 4 nM, C) 6 nM, or D) 7.5 nM and the effect on proliferation measured at 96 h post-transfection as described in Materials and Methods. Bars indicate means ± SEM (n = 3-6 independent experiments) as a percent of proliferation of cells treated with control, non-targeting C2 siRNA without gemcitabine. *different from cells transfected with C2 siRNA but otherwise treated identically (p < 0.05, ANOVA).
4.7. Treatment with TK2 siRNA and gemcitabine affects mitochondria

4.7.1. Combined treatment with TK2 siRNA and gemcitabine decreases mitochondrial DNA content

AlamarBlue (resazurin) is an indicator of redox activity and dependent on mitochondrial function including electron transport and oxidation during cellular respiration [273]. As TK2 is a mitochondrial enzyme, sensitization to impairment of mitochondrial function as a consequence of TK2 knockdown in the context of gemcitabine treatment was subsequently assessed.

Total DNA was collected from MCF7 and A549 cells 96 h after treatment with TK2 siRNA and gemcitabine (treated at the IC50, as determined in cells treated with TK2 siRNAs) and mtDNA:nDNA ratios assessed as described in methods section 2.8. Measurement of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). TK2 siRNA-induced sensitization to gemcitabine in TK2 expressing MCF7 cells (Figure 24) was accompanied by reduction in the mtDNA:nDNA ratio (Figure 31 A). There was no reduction in that ratio in identically-treated TK2 LOW A549 cells (Figure 31 B), consistent with the lack of gemcitabine sensitization induced by TK2 siRNA in those cells (Figure 26).
Figure 31: TK2 siRNA and gemcitabine decrease mitochondrial DNA in TK2\textsuperscript{HIGH} (MCF7) cells but not in TK2\textsuperscript{LOW} (A549) cells.

MCF7 and A549 cells were transfected with siRNA, treated with gemcitabine (the IC\textsubscript{50} as determined in each cell line after TK2 siRNA transfection) and the mtDNA:nDNA ratio determined 96 h later as described in Materials and Methods. Data are expressed as a percent of cells treated with C2 siRNA without gemcitabine. *different from cells transfected with C2 or C3 control siRNAs and otherwise treated identically (p < 0.05, ANOVA).
4.7.2. Combined treatment with TK2 siRNA and gemcitabine decreases mitochondrial activity.

Relative mtDNA content (the mtDNA:nDNA ratio) is an indirect indicator of mtDNA function and mitochondrial biogenesis and activity [140]. MitoTracker CMX ROS staining depends on intact, functioning, mitochondrial membrane and the degree of staining is correlated with intact mitochondrial membrane potential and mitochondrial activity. We assessed mitochondrial functioning more directly using a MitoTracker probe and flow cytometry (see material and methods section 2.9.2. *Determination of mitochondrial activity using MitoTracker® Red CMXRos*).

TK2 knockdown in TK2\textsuperscript{HIGH} (MCF7) cells decreased mitochondrial activity at both tested concentrations of gemcitabine (3 and 5 nM) (Figure 32 A). TK2 siRNA treatment of TK2\textsuperscript{MEDIUM} (HeLa) cells decreased mitochondrial activity at only the highest concentration (7 nM) of gemcitabine (Figure 32 B). TK2 siRNA treatment of TK2\textsuperscript{LOW} (A549) cell line did not affect mitochondrial activity in combination with gemcitabine (Figure 32 C). In TK2 expressing cell lines, combine treatment with TK2 siRNA and gemcitabine decreases mitochondrial membrane potential and activity. Treatment with TK2 siRNA, as a single agent, did not result in changes in MitoTracker staining compared to non-targeting control siRNA (C2) in these cell lines (Figure 32).
Figure 32: TK2 siRNA and gemcitabine decrease mitochondrial activity in TK2 expressing MCF7 and HeLa cells, but not in TK2\textsubscript{LOW} A549 cells.

MCF7 (A), HeLa (B) and A549 (C) cells were transfected with siRNAs and treated with gemcitabine (2 different concentrations) for 96 h, and mitochondrial activity measured as described in Materials and Methods. Data are expressed as a percent of the mean in cells transfected with C2 siRNA and exposed to the same gemcitabine concentration. *different from cells transfected with C2 siRNA and treated with the same concentration of gemcitabine ($p < 0.05$, Student’s t test).
Chapter 5

« Discussion »

Research data will be discussed with a focus on implications for the field and future directions. The chapter has been broken down into subheadings of major points for discussion. Discussion points previously published have been updated and expanded.

5.1. Resistance to TS-targeting drugs and antisense mediated by TK1 or TK2

5.1.1. TS-targeting drugs in combination with siRNAs targeting TS and TK1 or TK2

Both thymidylate synthase and thymidine kinases mediate production of dTMP for DNA synthesis and repair. The activity of both pathways increases intracellular dTMP and can potentially contribute to tumour cell resistance to drugs targeting TS. I hypothesized that, when TS is inhibited by anti-TS drugs and/or TS siRNA, TKs are important in mediating resistance to those agents. Consequently (and in addition), I hypothesized that siRNA targeting TK alone or in combination with TS siRNA would further enhance the effectiveness of TS-targeting drugs.

To test these hypotheses, I treated HeLa and MCF7 cells with siRNAs targeting TS alone, TK1 or TK2 alone, or TS siRNA in combination with siRNA targeting TK1 or TK2. To validate this approach, it was important to first establish that simultaneous administration of siRNAs targeting different mRNAs, at the siRNA concentrations employed in these experiments, did not affect the capacity of each siRNA to lower the level of its target mRNA. Previous reports (by our group and others) have suggested that combining different siRNAs can have reciprocal and non-reciprocal inhibitory effects on each other’s activity due to competition for entry into RISC complexes, and/or direct base-pairing with each other because of short regions of complementarity [77, 100, 219]. Each of the siRNAs used in this study downregulated their target mRNA, alone or
in combination. Targeting TS mRNA had no effect on TK1 or TK2 mRNA levels and vice versa (Figure 3 and Figure 4). All siRNAs were more potent in reducing target mRNA levels in HeLa than in MCF7 cells, and TS siRNAs were generally more effective than siRNAs targeting TK1 or TK2 (Figure 3 and Figure 4). The capacity of 2 different siRNAs to downregulate each mRNA target were compared, and only the most potent was used for subsequent experiments in HeLa cells (described in Chapter 3).

TS siRNA increased 5FUdR-mediated inhibition of HeLa cell growth by approximately 50% (Figure 5A) as previously reported by our group [195, 215-217, 219, 271]. When TK2 siRNA was combined with TS siRNA, it increased sensitivity to 5FUdR by approximately 25% more than treatment with TS siRNA alone (Figure 5 A-B). The phenomenon was enzyme-specific: TK1 siRNA combined with TS siRNA did not add to the increase in drug sensitivity induced by TS knockdown. These data strongly suggest that at least one TK enzyme (TK2) mediates sensitivity to 5FUdR. However, that participation appears to be indirect as siRNA-mediated knockdown of either TK2 or TK1 alone had no effect, under these experimental conditions, on 5FUdR sensitivity (Figure 5B). This is consistent with a model in which TS-mediated synthesis of thymidylate is the predominant cellular source (which is the case under normal conditions [222]), but where alternative TK-mediated production can partially compensate when de novo thymidylate synthesis is impaired by combined treatment with TS siRNA (to decrease TS mRNA) and 5FUdR (to decrease TS enzyme activity).

Although our data provide evidence of a role only for TK2 with respect to 5FUdR sensitivity, involvement of TK1 under different conditions cannot be excluded. TK1 activity and expression is generally higher in proliferating cells compared to TK2 [222, 274] and the degree of TK1 knockdown achievable using this technology may not be sufficient to enhance the effects of combined TS siRNA and 5FUdR. We have previously reported that a threshold exists in antisense knockdown of TS to enhance sensitivity to TS-targeting drugs [215]. I suggest that a similar threshold could exist for TK1 and/or TK2, and that threshold may differ between the two. If so, a smaller decrease in TK2,
compared to TK1, might be all that is required to induce a significant effect to 5FUdR under our experimental conditions. The converse may be true for pemetrexed. Finally, although we assessed the role of both TK1 and TK2 in combination with 5FUdR treatment at the IC$_{50}$ concentration, the effect of TK1 downregulation might be evident only in combination with higher 5FUdR concentrations that reduce TS activity and cell viability more profoundly. These possibilities are appropriate for future investigation.

Issues such as those discussed above might be responsible for reports that targeting TK1 alone had no effect on sensitivity to TS-targeting drugs [220, 245]. Those studies may also have been limited by the use of 5FU (a less specific TS inhibitor than 5FUdR [202]), their sole focus on TK1 (TK2 had not previously been assessed as a potential cancer therapeutic target), and the use of dipyridamole for global inhibition of nucleoside transporters rather than specific inhibition of thymidylate production. Early clinical trials aimed at disruption of the TK-mediated salvage pathway were likely limited by the available technology, method of administration, and bioavailability of dipyridamole [220, 221, 275]. Regardless, these early results still suggested that thymidine salvage was an important factor in response to TS-targeting drugs and folate inhibitors.

TK siRNA-mediated enhancement of the increase in drug sensitivity induced by TS siRNA was not restricted to 5FUdR. Combined treatment with TK1 siRNA and TS siRNA enhanced sensitivity to pemetrexed (a multi-targeted antifolate with a different mode of action than 5FUdR) by approximately 30% more than the enhancement induced by TS siRNA alone (Figure 9 A-B). Similar to TK effects on 5FUdR sensitivity, the phenomenon was enzyme-specific. In this case, however, enhancement was caused by TK1 siRNA and not TK2 siRNA (Figure 9 A). Therefore, although our data show that TK plays a role in sensitivity to two different TS-targeting drugs, sensitivity to 5FUdR and pemetrexed in response to knockdown of each of the two TK enzymes is different, for reasons that are not yet clear. Although both pemetrexed and 5FUdR are TS inhibitors, their interactions with TS and other cellular targets are quite different. Pemetrexed, unlike 5FUdR, interacts with multiple folate-dependent enzymes required for production of DNA and
RNA intermediates, including GARFT and DHFR [276]. Differential roles for TK enzymes in connection with inhibition of individual or multiple folate-requiring enzymes have not yet been explored. In particular, TK1 is localized primarily in cytoplasm and TK2 in mitochondria [222], and the contribution of mitochondrial thymidine and folate metabolism to drug sensitivity is unknown. Our data suggest non-overlapping functions of TK1 and TK2, perhaps based on cellular location, that require additional investigation and this will be discussed further in the paragraphs that follow.

An unexpected finding was that treatment with TS siRNA in combination with 5FUdR increased TK1 protein (Figure 7C), and TS siRNA combined with pemetrexed increased TK2 protein (Figure 12C). The increases were not apparently due to increased gene transcription since they were evident in the absence of increased TK1 or TK2 mRNA (Figure 7A and Figure 12A). Whatever the mechanism, these increases in response to TS inhibition support the hypothesis that TKs could mediate enhanced cell survival (and, conversely, knockdown of TKs may reduce that survival in tumour cells) when TS activity is reduced. In support of this, induction of TK1 in response to TS inhibition by 5-FU (the prodrug of 5-FUdR) has been reported and invoked as a mechanism for increased uptake of 3′-deoxy-3′-[¹⁸F]fluorothymidine (the so-called “flare response”) [245, 277]. That report is consistent with our observation of increased TK1 after treatment with TS siRNA and 5FUdR. Our observation of a specific increase in TK2 protein in response to pemetrexed (indeed, to any TS-targeting drug) is novel and currently under investigation in our laboratory.

It is possible that the observed increases in the TK1 or TK2 protein levels are a compensatory mechanism to allow the cell to cope with reduced dTMP levels when the level of both TS and one of the two TK enzymes are reduced. It has previously been reported that there is cross-talk between nuclear and mitochondrial dNTP pools [278, 279]. It is therefore possible that dTMP produced by the action of TK1 (nuclear encoded gene; cytosolic protein) could compensate for lack of TK2 (nuclear encoded gene; mitochondrial protein), and vice versa. However, the enzyme-specific increase in TK1
and TK2 in response to 5FUdR and pemetrexed, respectively, does not explain our observation that siRNA-mediated knockdown of TK2 (but not TK1) contributes to 5FUdR sensitivity and knockdown of TK1 (but not TK2) contributes to enhanced pemetrexed sensitivity. A partial explanation is suggested by the fact that TK1 is required for phosphorylation of 5FUdR (a prodrug) to produce the active FdUMP metabolite that inhibits TS [202, 280]. This dual role for TK1 – both in generating thymidylate and in activating 5FUdR – could explain the observation that knockdown of TK1 does not sensitize cells to combined TS antisense and 5FUdR treatment (i.e., TK1 reduction could simultaneously reduce thymidylate and sensitize to 5FUdR, but also reduce the amount of active 5FUdR available to act on tumour cells). Regardless of this, however, the different sensitization induced by combined knockdown of TS and TK1 (increased sensitivity to pemetrexed) compared to combined knockdown of TS and TK2 (increased sensitivity to 5FUdR) remains unexplained. Experiments to further explore the differential involvement of TK1 and TK2 in sensitivity to different classes of TS-targeting drugs (where those drugs are applied at concentrations other than the IC\textsubscript{50}, and where TK1 and TK2 are over-expressed) are appropriate future steps.

siRNA-mediated down-regulation of TS, alone or in combination with siRNA knockdown of TK1 or TK2, had no effect on sensitivity to cisplatin (Figure 13). This agrees with previous reports that antisense against TS increased tumour cell sensitivity specifically to TS-targeting drugs, but not to chemotherapeutics that do not target TS [219, 271]. It also supports the hypothesis that modulation of TK activity affects sensitivity to anti-TS drugs. This is further supported by the results of Figure 30 (see Chapter 4), where results of experiments using TS siRNA in combination with gemcitabine are presented.

Overall, these data support the hypothesis that TK enzymes are capable of reducing cellular sensitivity to TS-targeting drugs when the drugs are administered in combination with antisense molecules (siRNA) against TS. I demonstrate, for the first time, that a combinatorial RNAi approach (TS siRNA plus TK siRNA) enhanced human tumour cell sensitivity to two different TS-targeting drugs, and did so more effectively
than by reduction of TS using TS siRNA alone. In addition, these data revealed a novel
distinction between TK1 and TK2 in their roles in sensitivity to TS-targeting drugs with
different modes of action (TK2 and the nucleoside inhibitor 5FUdR, TK1 and the
antifolate drug pemetrexed). TK1 and TK2 are potential therapeutic targets to enhance
tumour sensitivity to TS-targeting drugs with there being TK enzyme-specificity for
certain classes of drug (antimetabolite or antifolate).

5.1.2. TS-targeting drugs in combination with siRNAs targeting TS, TK1 and TK2

To further assess the role of the TK enzymes in mediating resistance to TS-targeting
drugs and antisense, experiments were carried out in HeLa cells in which all 3 dTMP
producing enzymes (TS, TK1 and TK2) were knocked down with siRNA and treated with
either 5FUdR or pemetrexed. In the context of treatment with 5FUdR, the addition of
TK1 siRNA to the combination of TS siRNA and TK2 siRNA did not yield further
sensitization beyond the level induced by TS siRNA alone. Specifically, treatment with
the triple combination of targeting siRNA molecules did not improve HeLa cell sensitivity
beyond that of TS siRNA (striped bars, Figure 15). A similar result was observed when
the triple combination of siRNAs was used in combination with pemetrexed as the TS-
targeting small molecule drug. The addition of TK2 siRNA to the combination of TS siRNA
and TK1 siRNA did not result in further sensitization to the anti-proliferative effects of
pemetrexed beyond that induced by TS siRNA alone (hatched bars, Figure 16).

These results were surprising, as prior data showed that the level of both TK enzymes
increased in response to TS downregulation, suggesting that both TK1 and TK2 are
involved in compensating for the lack of de novo production of thymidylate through the
action of TS. Specifically: (i) TK1 protein levels increased after combined treatment with
TS siRNA and TS plus TK2 siRNA in the context of 5FUdR (Figure 7), and (ii) TK2 protein
levels increased after combined treatment with TK1 and TS siRNA in the context of
pemetrexed (Figure 12). It is possible that downregulation of all 3 dTMP-producing
enzymes by transient siRNA transfection was not sufficient to assess the effect of
inhibition of both de novo and salvage pathway production of dTMP in the context of
TS-targeting drugs. However, the likelihood of this is small as antisense knockdown of TK2 in combination with antisense knockdown of TS and/or TK1 sensitized tumour cells to all tested concentrations of gemcitabine, including the triple combination siRNAs targeting TS, TK1, and TK2 (Figure 30).

Published reports by others, however, suggest that these data may be consistent with each other. For example, Villarroya et al. [281] showed that human equilibrative nucleoside transporter 1 (hENT1) was more important than TK1 in maintaining dNTP pools when there is decreased TS and TK2 function. In their experiments with quiescent TK2-deficient fibroblast cells with an undepleted mtDNA phenotype, TK1 mRNA levels were increased without a concomitant increase in TK1 activity (in itself a somewhat surprising result, as dNTP pools were unchanged compared to control cells). The TK2− quiescent fibroblast cells were used as a model system to investigate TK enzyme function as de novo synthesis of dTMP should be minimal in non-cycling cells. They showed that, as expected for quiescent non-cycling cells, TS mRNA was decreased compared to TK2− fibroblasts cells that were actively cycling. While measurement of TS enzyme and/or activity (rather than simply reporting TS mRNA levels) would have been more informative about the potential for TS-mediated de novo production of thymidylate to compensate for the lack of TK2 in these cells, Villarroya’s data show that TK1 activity was not the essential factor responsible for the observed lack of mtDNA depletion phenotype. Instead, hENT1 appeared to be more important, because mtDNA depletion was induced in these cells when hENT1 was downregulated. Essentially, in quiescent TK2-deficient fibroblasts with decreased levels of TK2 protein and TS (due to quiescence), hENT1 is more important for maintenance of dNTP pools than TK1.

I observed that when both TS and TK2 are targeted with siRNA during treatment with 5UdR there is an increase in TK1 protein levels, but not mRNA (Figure 7). However, TK1 activity, in addition to TK1 protein and mRNA levels, was not measured. If the combination of TS siRNA and TK2 siRNA with 5FUdR results in cytostatic, as opposed to cytotoxic effects, then the situation observed by Villaroya et al. would suggest that
concomitant targeting of TK1 with siRNA might have no further effect on the sensitization of the HeLa cells to 5FUdR. If, in this situation, the state of the HeLa cells is similar to that of the quiescent TK2-deficient fibroblasts, and TK1 mRNA and protein levels do not reflect fluctuations in TK1 activity, then TK1 would not be expected to compensate for reductions in of TK2 and TS in order to maintain HeLa cell survival. Future investigation into the combined treatment of TS-targeting drugs and TS siRNA and TK2 siRNA should address: (i) TK1 activity in the HeLa cells, (ii) the underlying mechanism for decreased HeLa (or other cell line) cell number (i.e., cell death or cellular arrest), and (iii) the contribution of hENT1, as this protein is present on both mitochondrial and cell membranes to import nucleosides into cellular compartments (cytosol or mitochondria) and has been implicated in compensating for decreased nucleotide and nucleoside levels in reports by Villarroya et al. and others [260, 281, 282].

hENT1 is one of the transporters responsible for the import of thymidine, and other nucleosides, into cells (there are multiple hENTs as well as concentrative nucleoside transporters) [283]. Indeed, all nucleoside transporters have been implicated in having an effect on the efficacy of anticancer and antiviral nucleoside drugs [284]. Preliminary experiments to assess the effect of increased extracellular thymidine on combined siRNA and drug treatment were conducted (Figure 33). Increased extracellular thymidine was expected to be imported into cells and mitochondria by nucleoside transporters, including hENT1. Results revealed that treatment of HeLa cells with thymidine (10 μM) could protect against the inhibition of proliferation induced by 5FUdR (administered at the IC50 in these cells) and by the inhibition of proliferation induced by administration of combined TS siRNA and 5FUdR. When TK2 siRNA and TS siRNA were used under the same conditions, there was still rescue of the cells by added thymidine, but not to the same degree as in cells treated with only with TS siRNA and 5FUdR. Specifically, in the context of 5FUdR treatment, TS siRNA-treated cells were rescued so that proliferation in the presence of 5FUdR was increased to 90% of control cells untreated with 5FUdR, while TS siRNA- and TK2 siRNA-treated cells were rescued
to 80% of control (Figure 33). The triple combination of all three siRNAs (targeting TS, TK1, and TK2) did not reduce the capacity of added thymidine to protect cells from 5FUdR toxicity beyond the reduction in protection from 5FUdR toxicity mediated by TK2 siRNA in combination with TS siRNA (Figure 33). Further experimentation was not pursued. This data strongly suggest that TK1 and TK2 enzymes have independent, non-overlapping functions in normal cellular metabolism, and with respect to resistance to different classes of TS-targeting small molecule drugs. Those differences have not been previously hypothesized or reported to our knowledge.
Figure 33: siRNA targeting of dTMP enzymes in combination with 5FUdR and medium supplemented with 10 μM thymidine.

Experimental design and analysis was the same as described in the legend to Figure 15. HeLa cells were treated with TS siRNA [3 nM], TK1 siRNA [3 nM] and TK2 siRNA [4 nM], or various combination thereof using C2 siRNA to bring each siRNA treatment to a 10 nM total concentration of siRNA wherever required. For those conditions in which 10 μM thymidine is present, HeLa cells were grown in medium containing thymidine for a minimum of 72 h prior to transfection and throughout the duration of the experiment. Results are expressed as a percentage of the C2 siRNA with medium condition fold change. Bars represent mean ± S.D. for n = 6 samples from 2 independent experiments. *different from cells treated identically, but with non-targeting control (C2) siRNA (p≤0.05). *a different from cells treated identically, but with TS siRNA alone (p≤0.05).
5.2. The effect of siRNA concentration on the magnitude of biological response: the pitfalls of a combinatorial RNAi approach?

An unexpected finding while investigating siRNA-mediated reduction in cellular TS, TK1 and TK2 was that the magnitude of biological response with respect to siRNAs in combination with 5F UdR or pemetrexed could be increased or decreased depending upon the total concentration of siRNA used. The results of Figure 15, Figure 16, and Figure 19 A (where the total siRNA concentration was 10 nM) as compared to Figure 17, Figure 18 and Figure 19 B (where the total siRNA concentration was 15 nM) showed that transfection of higher amounts of the targeting siRNAs (TS and/or TK1 and/or TK2) dampened or negated sensitization to TS-targeting drugs observed after transfection of lesser amounts of siRNA. Differences in response seem to be dependent upon the total amount of siRNA administered and were not specific to the siRNA target.

The use of a 15 nM total siRNA concentration, in general, resulted in a lesser response to combined treatment with 5F UdR or pemetrexed than compared to the 10 nM total siRNA concentration. In fact, less total targeting siRNA combined with 5F UdR or pemetrexed appeared to mediate a larger decrease in HeLa and Meso H28 cellular proliferation than higher total concentrations of the same combination of siRNAs (TS and/or TK1 and/or TK2). Specifically, when HeLa cells were treated with 5F UdR or pemetrexed at the IC$_{50}$ of those drugs, and 10 nM total siRNA concentration was used, there was a 50-60% increase in capacity of 5F UdR to inhibit proliferation compared to no sensitization induced by targeting siRNAs when a 15 nM total siRNA concentration was used. A similar result was obtained after treatment of Meso H28 cells (Figure 19), which showed a 30-45% reduction in proliferation in response to treatment with the TS siRNA and combinations of TS and TK siRNAs alone (no drug) at a concentration of 10 nM, and a 0-15% reduction in proliferation when 15 nM of siRNA was used.

Prior to carrying out the above experiments with variable total concentrations of combined siRNAs, it was known that when two or more siRNA (or other antisense)
molecules are simultaneously introduced into a system, the capacity of one or both of the siRNA to silence genes can be compromised [100, 112, 219, 285, 286]. Based on sequence, the siRNAs chosen for our studies do not bind to each, ruling out this trivial mechanism as a means of preventing target downregulation. I showed, that at a 10 nM concentration, when two targeting siRNAs were combined, the ability of the TS siRNAs, TK1 siRNAs and TK2 siRNAs to downregulate target mRNA and protein was the same as when used as a single agent (results presented in Chapter 3, for example Figure 3, Figure 6, Figure 11, and Figure 12). Also, in the case of TK2 siRNA and gemcitabine, the triple combination of siRNA did not prevent TK2 siRNA from sensitizing human tumour cells to gemcitabine toxicity (Figure 30).

In general, competition between RNAi molecules can become apparent when using either a high concentration of antisense molecules and/or multiple antisense molecules within the same cell. It was surprising that the 5 nM difference between the 10 nM and 15 nM concentrations resulted in such a profound abrogation of sensitization. This was particularly true because: (1) previous studies published by our lab [219] used siRNAs in combination with each other at concentrations as high as 50 nM and still showed a biological phenomenon, and (2) the results from Figure 38 in Appendix 1 indicated that using a targeting siRNA at lower concentrations (5 nM - 0.01 nM) and adding control, non-targeting siRNA to maintain constant concentrations between and among experiments did not impede mRNA silencing.

Specifically, and taking TS siRNA transfection followed by 5FUdR treatment in HeLa cells as an example, there was a 75% vs 55% decrease in proliferation induced by 5FUdR when cells were transfected beforehand with a total concentration of 10 nM siRNA vs 15 nM concentration of siRNA, respectively (Figure 15 vs. Figure 17). Furthermore, in the human mesothelioma-derived Meso H28 cells, there was a 90% vs 45% decrease in proliferation induced by 5FUdR when cells were transfected beforehand with a total concentration of 10 nM siRNA vs 15 nM concentration of siRNA, respectively (Figure 19 A vs. B) when compared to drug untreated cells. These data suggest a more widespread,
and not target-specific, effect on cells that cannot be attributed solely to the presence of multiple different targeting siRNAs applied simultaneously to cells. Further to this point, these results are seen in the above figures both when TS siRNA is the only targeting siRNA present and when targeting siRNAs are used in combination.

I hypothesize that these results are off-target effects caused by non-sequence-specific events related to competition for RISC. Differences in the magnitude of biological response produced by the siRNA concentrations indicate that these results are likely due to competition between the siRNA(s), and endogenous miRNAs, for entry into RISC complexes. I speculate that this is the case because it is the increased siRNA concentration that produces the lesser biological response. As these experiments use siRNA as the antisense molecule of choice, we can assume that competition between our siRNAs and endogenous miRNAs, if this is the case, is at the level of RISC complex proteins (Ago-2, TRBP, Dicer) and not for upstream components of the miRNA pathway like Dicer (for pre-processing) or Exportin-5 (for export from of miRNAs from the nucleus,) as the siRNAs do not require the same upstream processing by these components that shRNAs and miRNAs do [15, 100, 101, 103, 104, 287-289].

With respect to dose in our initial experiments, the use of a 10 nM total siRNA concentration was investigated as a strategy to reduce siRNA treatments to the lowest concentrations possible, while maintaining maximum capacity to reduce mRNA target levels. Previously, the lowest total concentration of siRNA used by our group was 25 nM [219]. Jackson et al. [26] reported dose response effects associated with transfection of MAPK14 siRNA at 5 different doses from 0.16 nM - 100 nM into HeLa cells. They showed that the relative magnitude of sequence non-specific off-target effects did not decrease with decreasing siRNA concentration. Data presented in this thesis shows that on-target effects can also be perturbed by overall siRNA dosage and greater on-target effectiveness was achieved at a lower siRNA dose compared to a higher siRNA dose. These data could also be interpreted to show that the relative magnitude of off-target effects does decrease with decreasing siRNA concentration (i.e., when using 10 nM
siRNA vs. 15 nM siRNA and as measured by the decrease in biological phenomenon - the sensitization to drugs).

Khan et al. [286] and others [290] have shown that when even one exogenous antisense molecule is transfected into a cell system, there are global changes in the microRNA network within those transfected cells that lead to the upregulation of genes known to be targeted by miRNAs. Of particular interest, Khan’s computational analysis models, with a focus on HeLa cell transfections, determined that there was upregulation of a number of specific oncogenes as a result of transfection with exogenous siRNAs. In addition, HeLa cells were particularly sensitive to perturbations in their microRNA regulatory pathways. Because exogenous, transfected siRNAs and endogenous miRNAs compete for a limited pool of RISC proteins, factors such as siRNA potency (including the relative differences between exogenous siRNAs and between exogenous siRNAs and endogenous miRNAs) and RISC preferences for antisense strand selection may be exacerbated in experiments which include the introduction of multiple, exogenous, targeting siRNAs. It is not currently known if cells can respond to an increased load of siRNAs (i.e., an increased demand for RISC) by upregulation of RISC proteins to deal with the augmented demand and if this would restore normal patterns of miRNA regulation of gene expression within the cell. This information could be revealing. If this was the case, it could indicate that early toxicities and/or off-target effects associated with the initial competition for RISC would eventually lessen with time.

Antisense molecules are being actively pursued as therapeutics [35, 68, 71, 291-293]. Consequently, when using RISC-mediated antisense technology where target downregulation is carried out by RISC cleavage, studies to observe the overall effect on miRNA pathways will be important for understanding and predicting on-target effects (associated with a specific sequence) and off-target effects (which may also be sequence-specific and the result of sequence similarity among RNAs, or associated with pertubations in endogenous miRNAs not related to sequence) [294]. Depending on the objective of the study, measurement of differences in RISC-associated RNAi molecules
(by qPCR) before and after treatment with RNAi substrates from an exogenous source may be a method to optimize dosage or concentration, such that a balance is achieved between exogenous antisense (and on-target effects) and endogenous miRNAs (to reduce non-specific off-target effects) [295]. For example, to assess the extent to which exogenous siRNAs compete with endogenous miRNAs for RISC association, one might transfect in tagged siRNAs (for example, biotin), isolate RISC (or more specifically Ago-2) associated with tagged siRNAs by column immunoprecipitation (in keeping with the above example, streptavidin) from RISC associated with endogenous miRNA substrates, and quantify the amount of siRNAs and miRNAs associated with the complex (again, by qPCR) [295-299]. It would be important to use a panel of multiple targeting siRNAs and control non-targeting siRNAs as it is possible that the residency time in RISC is different for these two types, and may also be sequence-dependent. A difference in the amount and type (sequence) of miRNAs associated with RISC would be taken as evidence that transfection of exogenous siRNAs perturbs the regulatory control of gene expression carried out by endogenous miRNAs. Transfecting non-targeting siRNAs in addition to targeting siRNAs will help distinguish between that possibility, and the possibility that changes in miRNA metabolism occur because of the knockdown of target mRNAs by siRNA and not competition for RISC. One might also use antisense oligonucleotides (ASOs) targeting the same mRNAs targeted with siRNA to determine whether that perturbs miRNA association with RISC in a fashion similar to pertubations induced by siRNAs. This will help distinguish between the two possibilities, as ASOs exert their antisense activity via an RNase H-dependent, RISC-independent pathway.

5.3. siRNA Knockdown of TK Sensitizes Cancer Cells to Gemcitabine

5.3.1. The relationship between TK2, dCK and gemcitabine

TK2 siRNA knockdown induced a relatively modest reduction in TK2 protein. Regardless, the capacity of that reduction to enhance the antiproliferative effect of gemcitabine was relatively large (up to 50%) in certain TK2-expressing cell lines (MCF7, Figure 24; HeLa, Figure 25).
A relationship among dCK, gemcitabine and TK2 that incorporates cellular responses to antisense knockdown of TK2 is proposed (Figure 34). Importantly, gemcitabine is poorly phosphorylated by TK2. dCK is required to activate gemcitabine and increased dCK levels mediate gemcitabine anti-tumour action. In fact, decreased dCK levels in vitro mediate gemcitabine resistance [172, 300, 301]. In cells that express TK2 to a certain degree, reliance upon TK2 to contribute to the production of dCMP/dCTP may be greater than in cells with lower TK2 levels (for example, MCF7 and HeLa cells compared to A549 cells). When these cells are targeted with both TK2 siRNA and gemcitabine, dCK levels increase, potentially to compensate for decreased dCTP. However, the increased dCK serves only to further activate gemcitabine. Examination of the interplay between TK2, dCK and gemcitabine shows, for the first time, increased dCK levels in human tumour cells in response to combined targeting with TK2 siRNA and gemcitabine treatment (Figure 28). Importantly, the increase did not occur in response to either TK2 siRNA or gemcitabine used as single agents.
Figure 34: Proposed relationship between TK2, dCK and gemcitabine.

dCK phosphorylates both deoxycytidine and the anticancer drug gemcitabine. dCK is negatively regulated by the feedback inhibition of dCTP. TK2 phosphorylates both deoxythymidine and deoxycytidine. Thus, TK2 contributes to levels of dCTP that negatively regulate dCK. Inhibition of dCK, by TK2-produced dCTP, decreases the amount of gemcitabine that can be activated by dCK when used as an anticancer agent. siRNA-mediated reduction in TK2, in combination with gemcitabine, results in increased dCK enzyme levels and a greater response to the antiproliferative effects of gemcitabine. Thus, TK2 contributes to gemcitabine resistance.
5.3.2. Effects on mitochondria after combined treatment with TK2 siRNA and gemcitabine

The implications of decreased TK2 in the context of combined treatment with gemcitabine were also explored with a focus on mitochondrial toxicity. Combined TK2 siRNA and gemcitabine appears to reduce mtDNA in preference to nDNA (Figure 31). The concurrent decrease in mtDNA:nDNA ratio and sensitization to gemcitabine induced by TK2 siRNA in TK2\textsuperscript{HIGH} (MCF7) cells, but not A549 cells with low TK2, implies that TK2 is critically involved in tumour cell mitochondrial dysfunction and cellular toxicity in response to combined treatment with TK2 siRNA and gemcitabine in these cells.

The decrease in mtDNA:nDNA ratio in the TK2\textsuperscript{HIGH} (MCF7) cell line implicated increased mitochondrial toxicity as a potential mediator of the sensitization phenomenon seen when gemcitabine was combined with TK2 siRNA. Flow cytometry using MitoTracker CMX Ros confirmed a decrease in mitochondrial activity in TK2-expressing MCF7 and HeLa cells, but not in TK2\textsuperscript{LOW} A549 cells (Figure 32). This is in keeping with the fact that mtDNA encodes genes specific to electron transport chain (ETC) enzymes, and that the magnitude of mtDNA and Mitotracker reduction in gemcitabine and in TK2 siRNA-treated cells are not disproportionate with respect to each other.

Fowler et al. reported that gemcitabine can inhibit the activity of human mitochondrial DNA polymerase γ [302]. This, in combination with a reduction in TK2, could contribute to a preferential decrease in mtDNA. The profile of gemcitabine toxicity has been shown to be similar to that of some anti-viral agents, and induces events similar to the pathologies seen in a heterogeneous group of disorders known as mtDNA depletion disorders. Interestingly, mutations and deletions in the TK2 gene have been found to cause mtDNA depletion disorders [251, 252, 303, 304]. We have shown that combined treatment with TK2 siRNA and gemcitabine results in decreased mtDNA and mitochondrial membrane potential and activity. These data implicate increased mitochondrial toxicity as a critical event in gemcitabine sensitization. Whether mitochondrial toxicity is the result of sensitization, or contributes to sensitization,
remains to be determined. However, preliminary experiments using MCF7 cells and a 72 h end-point did not show similar mitochondrial effects, as measured by mtDNA:nDNA ratio and MitoTracker (*data not shown*), suggesting that mitochondrial toxicity is likely a consequence of treatment that could contribute to decreased cellular proliferation or increased cell death in treated cells, but only during later time points.

There is an alternative explanation to these results based upon relative mtDNA content between the MCF and A549 cell lines. When we compare the relative mtDNA:nDNA ratio of untreated A549 cells and MCF7 cells, we see that MCF7 cells have a mtDNA:nDNA ratio that is approximately 85% lower than that of A549 cells. As such, it is possible that the MCF7 cells are inherently primed to experience increased toxicity in response to treatments that affect mtDNA and mitochondrial functioning. Thus, this effect would not be specific to TK2 targeting, although it would be specific to mitochondrial targeting, and gemcitabine could be combined with any other treatment which negatively affects mitochondrial functioning to improve upon anticancer therapy. These include, for example, rotenone (an ETC inhibitor) or atractyloaside (an inhibitor of the ADP/ATP exchanger) or other mitochondrionally-targeted anticancer drugs (mitocans) already approved for use or under investigation [305]. Given the implied role of the mitochondria in contributing to tumour cell dysfunction and ROS generation (discussed in the Introduction, section 1.2.3.1 *Relevance of mitochondria and mitochondrial DNA (mtDNA) in cancer*) it is no surprise that targeting mitochondria as an anticancer therapy or to improve upon current anticancer therapies is currently being explored. Mitocans, as mentioned above, are defined as [anti-cancer] drugs whose action is achieved by way of mitochondrial destabilization [306, 307]. In fact, Neuzil *et al.* [306] reviewed a number of drugs that act as mitocans and classified them into 8 separate classes depending upon the mechanism by which they affect the mitochondria. One of these classes includes “drugs targeting mtDNA”, which can occur via inhibition of DNA polymerase y and would imply that gemcitabine itself should be considered to have mitocan-like effects secondary to its action upon nDNA [302].
Figure 35: mtDNA:nDNA ratio in untreated A549 and MCF7 cells.
mtDNA and nDNA in MCF7 and A549 cells were measured as described Materials and Methods. Data are expressed as a percent of A549 cell’s mtDNA:nDNA ratio. *different from A549 cells (p < 0.05, Student’s t test).
Others have previously suggested that TK2 plays a role in the effectiveness of deoxycytidinenucleoside analogues such as gemcitabine, and that the mitochondria could play a role in both toxicity and antitumour activity [171, 308-310]. Prior to this study, there has been no report of the consequences of antisense-mediated TK2 reduction on gemcitabine effectiveness and mitochondrial function. Indeed, the work described here is the first report of a tangible connection between theory and observation. These data identify TK2 as a potentially valuable target for anticancer therapy in combination with gemcitabine. Although siRNAs are difficult to administer effectively in vivo due to stability and pharmacokinetic challenges [30, 53, 311], experimental TK2-inhibiting small molecules including those reported by others [312, 313] may be appropriate candidate TK2-targeting agents for use in combination with gemcitabine. Targeting of TK2 is likely to be of therapeutic value, but only in combination with gemcitabine or related cytidine-nucleoside-analogue drugs as siRNA-mediated knockdown of TK2 alone did not reduce tumour cell proliferation, nor (in previous studies published by us) did TK2 knockdown in combination with pemetrexed, cisplatin, or 5FUdR reduce proliferation or sensitize to chemotherapy [264].

5.4 Summary

Downregulation of TK with siRNA enhanced the capacity of TS siRNA to sensitize tumour cells to traditional TS protein-targeting drugs (5FUdR and pemetrexed). Combined antisense targeting of TS and TK1/TK2 was more effective than either siRNA used alone to sensitize tumour cells to the effects of TS-targeting chemotherapeutic drugs.

Upregulation of TK1 in response to combined 5FUdR and TS siRNA, and TK2 in response to pemetrexed and TS siRNA, suggests new and diverging properties of TK1 and TK2.

Enhanced sensitization to 5FUdR and pemetrexed by targeting both TS and TK with siRNAs suggests that the TK salvage pathways are potential targets for anticancer therapies when combined with molecules targeting TS mRNA and TS protein.

Downregulation of TK2 using siRNA sensitized cancer cells to gemcitabine by as much as 50% compared to cells treated with control siRNA. Combined treatment with TK2 siRNA
...and gemcitabine also resulted in increased dCK enzyme levels. It was observed that treatment of TK2-expressing human tumour cells with TK2 siRNA and gemcitabine, compared to the control siRNA and gemcitabine: (i) decreased mitochondrial redox status (as indicated by alamarBlue), (ii) decreased mitochondrial DNA (as measured by mtDNA:nDNA ratio), and (iii) decreased mitochondrial activity (as measured by flow cytometry with MitoTracker CMX ROS). This is the first demonstration of a direct role for TK2 in gemcitabine resistance, or any independent role in cancer drug resistance, and further distinguishes TK2 from other dTMP-producing enzymes [cytosolic TK1 and thymidylate synthase (TS)]. siRNA knockdown of TK1 and/or TS in combination with TK2 siRNA and gemcitabine did not cause further sensitization. This phenomenon is specific to targeting of TK2.

Overall, the TKs are implicated in reducing the effectiveness of TS-targeting drugs and may be useful targets for cancer therapy when combined with TS-targeting antisense molecules, drugs targeting TS protein or, specifically in the case context of TK2, gemcitabine.
References


Appendix 1

The information in this appendix pertains to initial studies done to determine experimental conditions, including the amount of LF2K used to transfect, siRNA concentrations, and whether topping up with control siRNA (so as to keep siRNA concentrations constant between single agent and combined treatments) would effect the capacity of the -targeting siRNA to downregulate its target mRNA. The assumption was made that, as administered siRNA concentrations increased, the potential for undesirable non-specific toxicity and/or off-target effects (related to the siRNA sequence or to competition with miRNAs for RISC) would increase as the concentration of administered siRNA increased. Therefore, I assessed the capacity for targeting siRNA using substantially lower concentrations (picomolar) than those used in most in vitro cell culture studies (nanomolar) to effectively reduce target mRNA levels. In addition, reduction in the amount of liposomal transfection reagents to similarly reduce toxicity and/or off-target effects was also assessed.

A1.1 Determining the Amount of Lipofectamine 2000 (LF2K) to Use for Transfections

siRNA transfection reagents can, themselves induce off-target effects (non-specific mRNA downregulation) [60]. I sought to determine if it would be possible to decrease the total amount of LF2K used per flask without decreasing desired on-target effects (siRNA downregulation of target mRNA).

HeLa cells were transfected with either C2 siRNA (5 nM) or TS siRNA #4 (5 nM) using different amounts of LF2K per flask (similar to what is described in Materials and Methods, section 2.4.1.1). The different amounts used relate to: (i) the minimum recommended amount for a T25 flask (10 μL) as suggested by the manufacturer, (ii) half of the minimum recommended amount (5 μL), (iii) a quarter of the recommended
amount (2.5 µL), and (iv) one tenth the minimum recommended amount (1 µL). Cells were collected for mRNA analysis by qPCR 24 h post-transfection.

The results were used to identify half of the minimum recommended amount (5 µL per flask) as the amount of LF2K to use for our transfections. This amount was chosen as it allowed for TS mRNA downregulation to the same extent as the manufacturer’s recommended amount, but decreased off-target toxicity associated with using LF2K (Figure 36). At lower amounts of LF2K used (2.5 µL and 1 µL) the off-target effects of LF2K were further decreased, but on-target TS mRNA downregulation suffered.
Figure 36 Appendix: siRNA transfection of HeLa cells using different amounts of LF2K transfection reagent.

HeLa cells were transfected with 5 nM total siRNA using varying amounts of LF2K transfection reagent. qPCR was used to examine off-target TS mRNA downregulation associated with use of a specific amount of LF2K and to compare on-target TS mRNA downregulation as well. Data are expressed as a percentage of the LF2K (5 uL) condition. Bars represent means ± S.E.M. for n = 6 samples from 2 independent experiments. * different from LF2K (5 uL) condition identically treated cells (p < 0.05).
A1.2 Determining the Parameters of siRNA Concentrations Used for Experiments

Prior to the experiments presented in this thesis, the lowest concentration of siRNA used for experimentation in this laboratory was 25 nM for siRNAs used as single agents and 50 nM for combinations of 2 siRNAs [219]. Others in the field had published work using lower siRNA concentrations (0.1 nM - 20 nM) and so I knew that using a lower concentration of siRNA was a realistic possibility [112, 314]. In order to plan experiments where multiple siRNAs could be administered simultaneously without increasing total siRNA concentrations to levels with potential for undesired toxicity and/or off-target effects, minimization of administered siRNA concentrations was desirable. In addition, I wished to control for the overall amount of siRNA that cells were exposed to during experiments, such that all conditions were equal: consequently I assessed the effect of addition of control non-targeting siRNA (C2 siRNA) to targeting siRNAs to bring total siRNA concentrations to 10 nM to the capacity of targeting siRNA to decrease target mRNA.

HeLa cells were transfected with TS siRNA #3 at concentrations ranging from (0.01 - 10 nM) with or without C2 siRNA top-up to 10 nM total siRNA. Cells were collected for mRNA analysis by qPCR 24 h post-transfection. Results show that TS mRNA was downregulated by TS siRNA #3 at all concentrations tested both without C2 siRNA top-up (Figure 37) and with top-up (Figure 38); though siRNA concentration dependent effects on mRNA downregulation become apparent at concentrations less than 0.5 nM. Throughout the results presented in this thesis, siRNA concentrations of 10, 5, 4, and 3 nM siRNA were consistently used. Results here show: (i) that there should be no difference in the different siRNA-concentration’s ability to downregulate target mRNA levels, and (ii) that topping up to a 10 nM total siRNA concentration, using non-targeting control siRNA (C2), does not interfere with the targeting siRNA’s ability to downregulate target mRNA (Figure 37 and Figure 38).
Figure 37 Appendix: TS siRNA knockdown of TS mRNA using siRNA at concentrations ranging from 0.01 to 10 nM.

HeLa cells were transfected with either C2 siRNA [10 nM], C3 siRNA [10 nM] or TS siRNA # 3 [0.01 - 10 nM] and TS mRNA downregulation was assessed at 24 h post-transfection by qPCR. Data are shown as a percentage of the C2 siRNA condition and bars represent means ± S.E.M. for n = 6 samples from 2 independent experiments. * different from cells transfected with non-targeting control siRNA (C2 or C3) (p < 0.05 by ANOVA). # different from cells transfected with 0.5-10 nM TS siRNA #3 (p < 0.05 by ANOVA). a different from cells transfected with 0.1 and 0.05 nM TS siRNA #3 (p < 0.05 by ANOVA).
Figure 38 Apendix: TS siRNA knockdown of TS mRNA using TS siRNA at concentrations ranging from 0.01 to 10 nM and topping up with C2 siRNA to a 10 nM total concentration.

HeLa cells were transfected with either C2 siRNA [10 nM], C3 siRNA [10 nM] or a range of TS siRNA #3 concentrations [0.01-10 nM] using C2 siRNA to top up each condition to 10nM siRNA. TS mRNA downregulation was assessed at 24 h post-transfection by qPCR. Data are shown as a percentage of the C2 siRNA condition and bars represent means ± S.E.M. for n = 3 independent experiments. * different from cells transfected with non-targeting control siRNA (C2 or C3) (p < 0.05 by ANOVA). # different from cells transfected with 0.1-10 nM TS siRNA #3 (p < 0.05 by ANOVA). a different from cells transfected with 0.05 nM TS siRNA #3 (p < 0.05 by ANOVA).
Appendix 2

A2.1 Permission from Journal of Pharmacology and Experimental Therapeutics

February 11, 2014
Christine Di Cresce

Dear Christine Di Cresce:

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C. Di Cresce, R. Fimaroedo, P. J. Fernuson, M. D. Vincent, and J. Koropatkine, Combining Small Interfering RNAs Targeting Thymidylate Synthase and Thymidine Kinase 1 or 2 Sensitizes Human Tumor Cells to 5-Fluorodeoxyuridine and Pemetrexed, J Pharmacol Exp Ther September 2011 338:952-963

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   Manuscript in Preparation

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   European Journal of Cancer 2010; 8(Supplements 7), 168
   Abstracts: 22nd EORTC-NCI-AACR International Symposium: Molecular Targets and Cancer Therapeutics--Nov 16–19, 2010; Berlin, GERMANY

4. Abstract C147: siRNA Targeting TS and TK as an Anticancer Therapy
   C Di Cresce, R Figueredo and J Koropatnick
   Molecular Cancer Therapeutics (Meeting Supplement), C147, December 10, 2009
   Abstracts: AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics--Nov 15–19, 2009; Boston, MA

PATENTS

1. TITLE: Chemo- and Radiation Sensitization of Cancer by Indoleamine-2,3, dioxygenase (IDO) Inhibitors
   AUTHORS: Saman Maleki Vareki, Mateusz Rytelewski, Peter John Ferguson, Christine Di Cresce, Mark David Vincent, Colin Way, Donald James Koropatnick
   US Patent Office # : 753-128pr
   Full patent filed 31-May-2013

RESEARCH PRESENTATIONS

   Platform Presentation - 1st place winner
March 2014

2. Department of Oncology Research and Education Day, London, Ont
   Poster
   June 2013

   Poster
   March 2013

4. EORTC-NCI-AACR Symposium: Molecular Targets and Cancer Therapeutics, Dublin, Ireland
   Poster – *International Conference*
   Nov 6-9, 2012

5. Department of Oncology Research and Education Day
   **Platform presentation** – *1st place winner*
   June 2012

   Poster
   March 2012

7. AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics, San Francisco, CA
   Poster – *International Conference*
   Nov 12-16, 2011

8. Department of Oncology Research and Education Day, London, Ont
   Poster - *1st place winner*
   June 2011

9. Lawson Research Day, LHSC-Victoria Hospital, London, Ont
   Poster
   March 2011

10. EORTC-NCI-AACR Symposium: Molecular Targets and Cancer Therapeutics, Berlin, Germany
    Poster – *International Conference*
    Nov 16-19, 2010
11. Department of Oncology Research and Education Day, London, Ont
   **Poster**
   June 2010

12. Lawson Research Day, LHSC-Victoria Hospital, London, Ont
   **Poster**
   March 2010

13. AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics, Boston, MA
   **Poster – International Conference**
   Nov 15-19, 2009

14. Department of Oncology Research and Education Day, London, Ont
   **Poster**
   June 2009

15. Lawson Research Day, LHSC-Victoria Hospital, London, Ont
   **Poster**
   March 2009

**SCHOLARSHIPS and AWARDS**

PhD Scholarship Award ($18,000)
*Translational Breast Cancer Research Unit*
Sept 2013

Microbiology and Immunology Graduate Travel Award ($1,000)
*Dept. Microbiology and Immunology, University of Western Ontario*
Nov 2012

Ontario Graduate Scholarship ($15,000)
*Government of Ontario*
Sept 2012-Aug 2013

1st Place - Oral Presentation ($100)
*Oncology Research and Education Day*
June 2012

CIHR Strategic Training Program – Cancer Research and Technology Transfer ($10,000)
*CIHR Doctorate Training STIHR*
Sept 2012-2013
Institute Community Support (ICS) Travel Award ($923)
Canadian Institute of Health Research (CIHR)
Nov 2011

Poster Award Winner ($100)
Oncology Research and Education Day
June 2011

Graduate Student Travel Award ($1,000)
Division of Medical/Experimental Oncology – University of Western Ontario
2011

Microbiology and Immunology Graduate Travel Award ($1,000)
Dept. Microbiology and Immunology, University of Western Ontario
Nov 2010

CIHR Strategic Training Program – Cancer Research and Technology Transfer ($26,000/yr)
CIHR Doctorate Training STIHR
Sept 2010-2012

Western Graduate Research Award (~ $7,600/yr)
University of Western Ontario
Sept 2009-present

Schulich Graduate Scholarship ($7,200)
University of Western Ontario
Sept 2008-2009

COMMITTEES, VOLUNTEER EXPERIENCE AND EXTRA CURRICULAR

Schulich Graduate Affairs Committee, University of Western Ontario, London, Ontario
Graduate Student Representative to Graduate Affairs Committee (2012)
Sept 2012–present

Vice Dean, Basic Medical Sciences Selection Committee, University of Western Ontario, London, Ontario
Graduate Student Representative - Schulich School of Medicine and Dentistry
Aug 2012–Jan 2013

Space Assessment Committee, University of Western Ontario, London, Ontario
Graduate Student Representative - Schulich School of Medicine and Dentistry
Feb 2012-present
Schulich Graduate School Student Council, University of Western Ontario, London, Ontario
Microbiology & Immunology Representative, Council Chair (2012/13)
Sept 2011–present

Speaker Selection Committee, CIHR-STP-Cancer Research and Technology Transfer, London, Ont
Participant/Volunteer
Sept 2011

Program Advisory Committee, CIHR-STP-Cancer Research and Technology Transfer, London, Ont
Student Representative on Program Advisory Committee
Sept 2011-2012

Virtual Researcher On Call (VROC): An Educational Initiative of Partners in Research - VROC Science Careers Weekly E10: Translational Cancer Research
Participant
Shot By: Kevin Cougler in London, ON
http://www.youtube.com/watch?v=qw2UjFIM1ac
July 2011

Pamela Greenway-Komlemier Translation Breast Cancer Research Unit
London, Ontario
Outreach Activities Volunteer
Sept 2009-2012

Lawson Association of Fellows and Students (LAFS)-South-End, Victoria Hospital, London, Ont
Secretary (2009), Events Coordinator (2010 – present)
April 2009-present

Terry Fox Run, London, On
Participant
Sept 2009 & 2011

CIBC Run for the Cure, London, Ont
Participant (2008-2013) and Juggernauts Team Captain (2009-2011)
Oct 2008-2013

Let’s Talk Science, University of Western Ontario, London, Ont
Volunteer (2008-2012) and Department Rep. (Sept 2009 - 2012)
Sept 2008-2012
Microbiology and Immunology Social Committee, University of Western Ontario, London, Ont
**Member (2008-2011) and Co-Chair (Sept 2009-2010)**
Sept 2008-2011

Varsity Swim Team, University of Western Ontario, London, Ont
**Competitive Swimmer (Canadian National and CIS Level)**
Sept 2003-2007

Richmond Hill Aquatic Club, Richmond Hill, Ont
**Competitive Swimmer (Provincial and Canadian National Level)**
Sept 1996-2003

**SPECIAL TRAINING and CERTIFICATIONS**

Advanced Mouse Training Certification – Injections and Anaesthesia
**University of Western Ontario Animal Care and Veterinary Services**
Sept 2010-present

Certified Scuba Diver- Open Water
**SDI and PADI Certifications**
Feb 2010-present

WHMIS (Workplace Hazardous Materials Information System) Certified
**University of Western Ontario and Victoria Hospital**
Sept 2008- present

Radiation Handling and Safety Training
**University of Western Ontario** – certified in North America
Sept 2008- present