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# Combining antisense drugs targeting thymidylate synthase and indoleamine 2,3-dioxygenase in human cancer cells: potentialfor combined therapy

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# Combining antisense drugs targeting thymidylate synthase and indoleamine 2,3-dioxygenase in human cancer cells: *potential for combined therapy*

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## ABSTRACT

Combining therapeutic agents, as a cancer treatment strategy, using antisense targeting **thymidylate synthase (TS)** and **indoleamine 2,3-dioxygenase (IDO)** can potentially reduce tumour cell proliferation, enhance TS-targeting chemosensitivity and enhance tumour immune recognition. However, it must be determined if one reagent modifies the effectiveness of another. The effects of combining **small-interfering RNA (siRNA)** targeting TS and IDO on target mRNA levels and sensitivity to TS-targeting and other drugs were investigated. IDO or TS siRNA alone reduced their mRNA targets specifically and without effects on non-target mRNAs. IDO siRNA combined with TS siRNA did not affect TS siRNA knockdown of TS mRNA. TS siRNA, however, reduced the ability of IDO siRNA to down-regulate IDO mRNA. IDO siRNA did not affect TS siRNA-induced specific drug sensitivity. Targeting multiple mRNAs simultaneously may not have the effect predicted by studies involving single siRNAs. These results are important in determining potential drug combinations in anticancer therapy.



**Key Words:** cancer, antisense, thymidylate synthase, indoleamine 2,3 dioxygenase, siRNA, RNAi, drug resistance, chemotherapy, immune tolerance, combined therapy

## CO-AUTHORSHIP STATEMENT

All work presented in this thesis was fully performed by Kathleen Calonego, with the exception of Figures 3.1.2, 3.1.6 and 3.1.7. Transfection, RNA extraction, reverse transcription and qPCR were performed with the assistance of Mark Niglas (summer student).

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## DEDICATION

***To my Father,***

*I could not have asked for a more loving, supportive, encouraging, and motivating father than you. The impact you made on me in such a short period of time is strong enough to last a lifetime. Your memory will never be lost, and neither will your fight.*
*I will always love you and miss you.*

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| | |
|---|---|
| 1-MT | 1-methyl-tryptophan |
| 5-FU | 5-fluorouracil |
| 5-FUdR | 5- fluorodeoxyuridine |
| Ago2 | Argonaute 2 |
| ANOVA | analysis of variance |
| AS | antisense |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| $CH_2H_4$folate | 5,10-methylene tetrahydrofolate |
| Da | dalton |
| D-MEM | Dulbecco's modified Eagle's medium |
| DHFR | dihydrofolate reductase |
| DHF | dihydrofolate |
| DNA | deoxyribonucleic acid |
| dsRNA | double-stranded RNA |
| dTDP | deoxythymidine diphosphate |
| dTMP | deoxythymidine monophosphate |
| DTT | dithiothreitol |
| dTTP | deoxythymidine triphosphate |
| dUMP | deoxyuridinie monophosphate |
| dUTP | deoxyuridine triphosphate |
| ECL | enhanced chemiluminescence |

| | |
|---|---|
| FBS | fetal bovine serum |
| FdUMP | fluorodeoxyuridine monophosphate |
| FUMP | 5-fluorouridine 5'-monophosphate |
| GCN2 | general control non-derepressible 2 |
| h | hour(s) |
| $H_2$Folate | dihydrofolate |
| $H_4$Folate | tetrahydrofolate |
| HRP | horseradish peroxidase |
| $IC_{50}$ | half maximal inhibitory concentration |
| IDO | indoleamine 2,3-dioxygenase |
| IFN | interferon |
| ISRE | interferon-stimulated response elements |
| kDa | kilodaltons |
| $K_m$ | substrate affinity |
| LNA | locked nucleic acid |
| MAb | monoclonal antibody |
| mg | milligram |
| min | minute(s) |
| miRNA | microRNA |
| mL | milliliter |
| mM | millimolar |
| MMLV | Moloney murine leukemia virus |
| mRNA | messenger RNA |
| MTX | methotrexate |

| | |
|---|---|
| NADPH | dihydronicotinamide adenine dinucleotide phosphate |
| NTC | no template control |
| ODN | oligodeoxynucleotide |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| qPCR | quantitative PCR |
| RA | rheumatoid arthritis |
| RISC | RNA-induced silencing complex |
| RLC | RISC-loading complex |
| $R_n$ | normalized reporter |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| rRNA | ribosomal RNA |
| SDS | sodium dodecyl sulphate |
| SE | standard error |
| siRNA | small interfering RNA |
| TRBP | TAR RNA-binding protein |
| TKI | tyrosine kinase inhibitor |
| $T_{reg}$ | regulatory T cell phenotype |
| TRL | toll-like receptor |
| tRNA | transfer RNA |
| TS | thymidylate synthase |
| µg | microgram |
| µL | microlitre |

| | |
|---|---|
| μM | micromolar |

# CHAPTER 1: INTRODUCTION

## *1.1. The problem: Cancer*

### *1.1.1. Relevance*

In 2008, 73 800 Canadians died of cancer (Canadian Cancer Statistics, 2009). An increase from 159 900 new cases in 2007 to 166 400 new cases in 2008 provides evidence that the number of individuals diagnosed with cancer is rising. An estimated 40% of Canadian women and 45% of Canadian men are expected to develop cancer during their lifetime, with less than half surviving the disease (Canadian Cancer Statistics, 2009). These devastating numbers highlight both the importance and the need to further basic cancer research and to develop enhanced cancer therapies.

### *1.1.2. What is cancer?*

Cancer is not one specific disease; it is a class of diseases consisting of a variety of more specific conditions with some common characteristics. The development of cancer can involve multiple events of genetic changes that can gradually lead to an extremely severe and progressively worsening malignant disease (Hanahan and Weinberg, 2000). Hanahan and Weinberg have suggested that most, if not all, cancers acquire a specific set of characteristics that mediate the malignant phenotype. These characteristics include self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis,

limitless replicative potential, sustained angiogenesis (growth of new blood vessels), and evasion of apoptosis (programmed cell death) (Hanahan and Weinberg, 2000). These cancer "hallmarks", depending on the incidence and severity, correlate with the malignancy of specific tumours and the danger they pose to their human host. Furthermore, they suggest that genomic instability may contribute to these characteristics (Hanahan and Weinberg, 2000). Ultimately, through a combination of these hallmark characteristics, malignant cells can arise and cause devastating effects. The wide diversity of cancer characteristics in individual patients, and in disease sites, suggests diversity in the causes of cancer that is supported by evidence indicating that a multitude of genes, affected by many agents (viruses, chemicals, radiation, dietary constituents, and others) are responsible for acquisition of cancer hallmarks mediating malignancy (Walboomers *et al.*, 1999; de Gruijl, 1999; Frost *et al.*, 2008; Hursting *et al.*, 1990).

### *1.1.3. Cancer therapy: treatment options*

Diversity in both cancer causes and acquired malignant characteristics has resulted in a wide variety of cancer treatments. Treatments of choice for cancer types and individual patients is based on the location and severity of the tumour, and the characteristics of the patient (their general state of health, age, and individual characteristics mediating sensitivity to specific treatments) (DeVita Jr *et al.*, 2008). Treatment options include surgical removal of the cancer

(particularly solid tumours at surgically accessible sites) (Guarneri and Conte, 2004), radiation therapy (for tumours sensitive to ionizing radiation, at sites where radiation exposure to normal tissues is minimal and/or normal tissues are relatively radiation resistant) (Welsh *et al.*, 2002), cytotoxic chemotherapy (to preferentially kill tumour cells on the basis of their accelerated rate of proliferation rather than tumour-specific differences in molecular targets) (NIH, 2009) or targeted therapy (to specifically kill tumour cells by targeting genes/proteins they express uniquely or preferentially) (DeVita Jr *et al.*, 2008; NIH, 2009).

Traditional chemotherapy uses cytotoxic drugs to destroy dividing cancer cells (DeVita Jr *et al.*, 2008). Chemotherapeutic agents may exhibit their effect through inhibiting necessary enzymes for cell division, including thymidylate synthase (TS), an enzyme responsible for *de novo* production of intracellular thymidylate for DNA synthesis and repair. TS can be inhibited by a variety of drugs including fluoropyrimidines (such as 5-fluorouracil [5-FU] and 5-fluorodeoxyuridine [5-FUdR]) and antifolates (such as raltitrexed and pemetrexed) (Danenberg *et al.*, 1999; Heidelberger *et al.*, 1957; Scagliotti *et al.*, 2008; Chu *et al.*, 2003; Calvert *et al.*, 1986). Other classes of chemotherapeutic agents can act through targeting multiple intracellular sites, such as the antifolate methotrexate (MTX), or by preferentially killing proliferating tumour cells, for example, by using platinum-based small molecules such as cisplatin (Kim *et al.*, 1993; Hussain *et al.*, 1997; Jacobs *et al.*, 1992).

Advances in both technology and our understanding of the genetic nature of cancer have translated into advances in cancer therapy, particularly in the development of targeted therapy (DeVita Jr *et al.*, 2008). Targeted therapy, using drugs that block cancer cell growth and reduce their viability by interacting relatively specifically with molecules uniquely or preferentially expressed by cancer cells (and which mediate their malignant characteristics and/or survival), is an increasingly prevalent strategy in cancer therapy. Targeted drugs include monoclonal antibodies (MAbs), tyrosine kinase inhibitors (TKIs), and antisense technology. Validation of targeted therapy success can be found from applying the MAb Herceptin, a commonly used targeted drug in breast cancer (DeVita Jr *et al.*, 2008). MAb Herceptin targets the HER2 transmembrane tyrosine kinase receptor (part of the epidermal growth factor receptor, EGFR, family), which is over-expressed in breast cancer and a poor prognostic marker (Baselga *et al.*, 1996; Daniele and Sapino, 2009; McNeil, 1998, 2000). Antisense approaches represent a second targeted therapy of increasing interest in the medical setting because they provide both increased specificity with fewer non-specific toxicities (DeVita Jr *et al.*, 2008). Antisense drugs are short RNA or DNA molecules that can bind to complementary RNA sequences (Fire *et al.*, 1998). Once antisense molecules are bound to their target mRNA transcripts, they can cause target degradation or translational inhibition (Fire *et al.*, 1998). Through targeting mRNA encoding oncogenic proteins, production of the protein is inhibited. Currently there are a variety of antisense drugs undergoing clinical trials for cancer therapy, including Genasense, that targets Bcl-2 (which can be over-

expressed in a number of cancers and may prevent chemotherapy-induced cell death) (Cotter *et al.*, 1999) and OGX-427, that targets heat shock protein 27 (Hsp27) (which can inhibit stress-induced cell death and is associated with chemotherapeutic drug resistance) (Garrido *et al.*, 1997; Oesterreich *et al.*, 1993).

### *1.1.4. Cancer therapy: obstacles*

One principle obstacle in effective cancer therapy, particularly with traditional chemotherapy, is tumour drug resistance (DeVita Jr *et al.*, 2008). Heterogeneous populations of cancer cells can contain cells that are constitutively resistant to some treatments, and those cells are selected for survival and tumour regrowth during treatment (Heppner *et al.*, 1978). On the other hand, cancer cells originally arising as relatively treatment-sensitive can acquire transient or permanent drug resistance through a variety of mechanisms, including over-expression of a chemotherapeutic target (Goldie, 2001). It is possible to target the molecules that mediate resistance and protect cells from chemotherapeutic agents through combining traditional chemotherapy with innovative targeted therapy (DeVita Jr *et al.*, 2008). An example of acquired treatment resistance is through over-expression of a common therapeutic target, such as TS (Longley *et al.*, 2003; Pestalozzi *et al.*, 1997; Lenz *et al.*, 1996; Johnston *et al.*, 1994; Marsh, 2005). TS-related drug resistance can be overcome by reducing TS mRNA levels (Ferguson *et al.*, 2001). This has been achieved *in vitro* through the use of TS mRNA-targeted therapy and will be discussed in

further detail throughout this thesis (Ferguson *et al.*, 2001; Ferguson *et al.*, 1999).

Other obstacles to effective cancer therapy are the inability of the immune system to detect tumour cells and the varying ability of tumour cells to evade immunodetection and/or immune cell-mediated induction of tumour cell death. If normal cells undergo transformation into cancer cells, they are typically targeted by immune surveillance, where they can be eliminated by the immune system (Figure 1.1.4.) (Prendergast, 2008; Zou, 2005). Immune surveillance can exert selective pressure for tumour cells that are immune resistant (Prendergast, 2008). Tumours can also acquire the ability to inactivate this immune response (Zou, 2005; Prendergast, 2008). These cells can then escape the immune system and go on to form a malignant tumour (Prendergast, 2008). One potential therapeutic approach to overcoming this obstacle is through down-regulation of specific molecules in tumours mediating immune escape through suppression of immune cells, such as indoleamine 2,3-dioxygenase (IDO) (Mellor and Munn, 2004; Fallarino *et al.*, 2002; Grohmann *et al.*, 2003). Previous studies have investigated the benefits of using IDO mRNA-targeted therapy in cancer, demonstrating great potential as anti-cancer therapy (Zheng *et al.*, 2006; Yen *et al.*, 2009).

Overcoming these obstacles by combining targeting therapies is an attractive strategy in cancer treatment. Understanding the biochemical and physiological consequences of combining therapies (specifically, antisense therapies) is an essential prerequisite and a focus of this thesis.

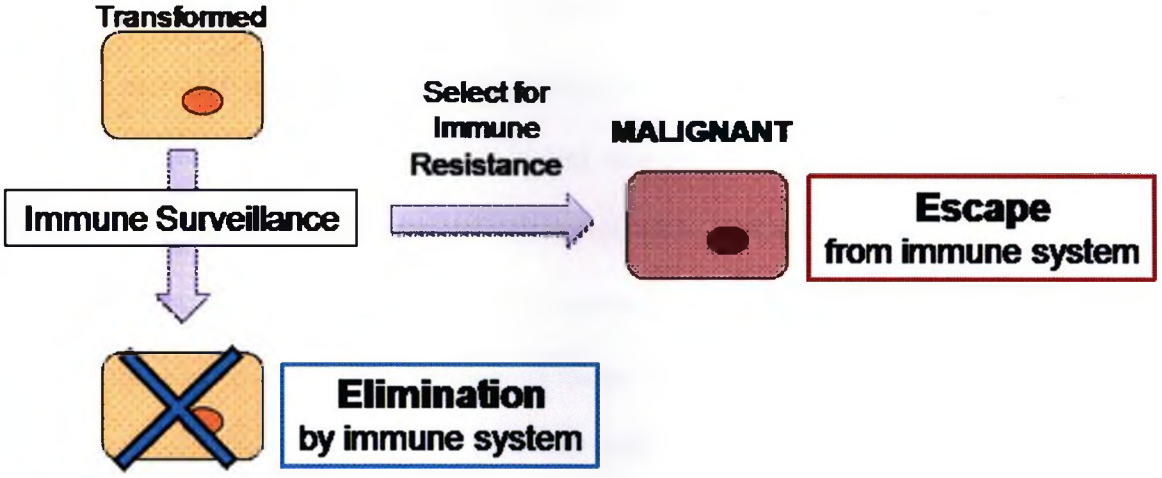


**Figure 1.1.4. The role of immune surveillance in detection of malignant cells.** When normal cells undergo transformation into cancer cells, they typically are detected by immune surveillance, where they can be eliminated by the immune system. This immune surveillance can generate selective pressure for tumours that are immune resistant. These cells can then escape the immune system and go on to form a malignant tumour. Modified from Prendergast, 2008.

### *1.1.5. Cancer therapy: combination therapy*

Combined cancer therapy is one of the main strategies for overcoming obstacles faced in cancer therapy (DeVita Jr *et al.*, 2008). Combining multiple treatment types, such as different combinations of surgery, radiation, and chemotherapeutic drugs, is common (DeVita Jr *et al.*, 2008). Combined cancer therapy can also apply to combining multiple therapies of the same type, such as multiple chemotherapies. The combined use of two or more chemotherapeutic drugs, with different modes of action or similar modes of action but with non-overlapping undesirable side effects, is common in cancer treatment (DeVita Jr *et al.*, 2008; Fisher *et al.*, 1993; Van Cutsem *et al.*, 2009). Combining 5-FU and cisplatin is standard therapy for patients with head and neck cancers and gastric cancers (Kim *et al.*, 1993; Hussain *et al.*, 1997; Jacobs *et al.*, 1992). Standard treatment of childhood leukemia, Hodgkins's lymphoma and diffuse B-cell lymphomas involves combined chemotherapy programs and have effectively provided curative therapy (DeVita Jr *et al.*, 2008).

It is possible to combine traditional chemotherapeutics with newer targeted therapeutics to enhance the efficacy of cancer therapy. A combination of both traditional chemotherapy and targeted therapy is used in the treatment of breast cancer and is suggested to be used as an initial treatment in metastatic colon cancer (Van Cutsem *et al.*, 2009). Combining the classic chemotherapeutic agent paclitaxel with siRNA targeting EphA2 (an over-expressed tyrosine kinase receptor in ovarian cancer) in a nude mouse model reduced ovarian tumour size

by 90%, compared to a reduction of only 50% when antisense approaches were used alone (Landen *et al.*, 2005). These examples emphasize the importance of combining multiple treatments, particularly combined chemotherapy with antisense therapy, to enhance the management of cancer. A combination of anticancer treatments that do not negatively interfere with each other is ideal. Improvement in anticancer therapy through combination therapy does not require synergy; it relies on additive effects of combining multiple treatments.

This thesis focused on combining multiple treatments targeting the same, and multiple pathways, to enhance cancer therapy. More specifically, it focused on the use of combining two specific antisense molecules simultaneously and the effect this combination therapy has on traditional chemotherapeutic sensitivity.

## *1.2. RNA Interference*

### *1.2.1. History*

RNA interference (RNAi) is a form of antisense (AS) technology where double-stranded RNA (dsRNA) can be used to silence the expression of a gene in a sequence-specific manner (Fire *et al.*, 1998). Small interfering RNA (siRNA) molecules can bind to complementary target mRNA sequences via Watson-Crick base pairing leading to mRNA degradation or translation inhibition (Fire *et al.*, 1998; Zeng *et al.*, 2003; van den Berg *et al.*, 2008). This process was first observed in 1990 (Napoli *et al.*, 1990), but the exact mechanism of RNAi (described in *Section 1.2.23*) was not entirely understood until over a decade

later. Fire and Mello characterized RNAi through a series of experiments studying gene expression in nematodes (*Caenorhabditis elegans)* during the 1990's (Fire *et al.*, 1998). It was then that the term RNAi was first coined and described as the use of double-stranded RNA to specifically break down mRNA, block translation and ultimately prevent subsequent protein production (Fire *et al.*, 1998). For their discovery, both Fire and Mello jointly received the Nobel Prize in Physiology and Medicine in 2006 (Mello, 2007; Fire, 2007). The escalating number of publications, over 33 000 since the discovery of RNAi in 1998, demonstrates the increasing interest in this exciting field that is applicable in both the basic research and the clinical setting.

### *1.2.2. Types of RNAi*

The RNAi mechanism can be exploited in the medical and research setting by introducing exogenous forms of RNAi, such as longer dsRNA or short siRNA, or by other methods of antisense mRNA modulation, such as antisense oligodeoxynucleotides (ODNs). ODNs are chemically synthesized single-stranded DNA molecules that bind to complementary mRNA sequences. ODNs can inhibit protein synthesis and also cause mRNA degradation in the nucleus via RNase H, a ubiquitously expressed endonuclease that recognizes DNA/RNA duplexes (Stein and Cheng, 1993; Grunweller *et al.*, 2003). Through both the RNAi and ODN pathways, target mRNA is ultimately blocked from the translational machinery or degraded (Zamaratski *et al.*, 2001; Fire *et al.*, 1998). ODNs can be

modified to enhance their cellular uptake, stability as well and efficiency, as discussed in *Section 1.2.4*. ODNs were the first antisense agents used in clinical practice, however, evidence suggests that other forms of antisense molecules may be more specific and more potent (DeVita Jr *et al.*, 2008).

Naturally occurring RNA interference is an innate mechanism in cells and plays a very important role in innate immune responses against viral infections (Berkhout and Haasnoot, 2006; Schott *et al.*, 2005; Wilkins *et al.*, 2005). Viruses may also contain an RNA genome or produce RNA replicative intermediates. RNAi is a method to degrade double-stranded viral RNA found within an infected cell (Obbard *et al.*, 2009). dsRNA produced by viruses can be recognized and cleaved by Dicer (an RNase III-like endonuclease) (Bernstein *et al.*, 2001) to produce a viral form of siRNAs, termed viRNAs (Ding and Voinnet, 2007). The target RNA in this form of RNAi is typically the viral RNA, resulting in an antiviral response (Obbard *et al.*, 2009; Tolia and Joshua-Tor, 2007).

MicroRNA (miRNA) is another form of endogenous RNAi, where the cell produces its own natural 18-25 base pair RNA molecules complementary to the cell's mRNA (Lagos-Quintana *et al.*, 2001; Carthew and Sontheimer, 2009). These miRNAs bind complementary mRNA transcripts within the cytoplasm of a cell and can reduce mRNA levels through degradation, or prevent protein translation in a similar fashion as siRNA via RISC complex activation (as described in *Section 1.2.3*) (Chendrimada *et al.*, 2005; Gregory *et al.*, 2005; Carthew and Sontheimer, 2009). miRNA alters mRNA stability and function (Liu, 2008). This allows the cell to regulate gene expression post-

transcriptionally at critical times throughout development (Lau *et al.*, 2001; Carthew and Sontheimer, 2009).

Throughout this thesis, the focus will be on siRNA specifically. dsRNA, a form of RNAi, has progressively replaced ODNs due to the greater efficiency of RNAi over the RNase H pathway (Fire *et al.*, 1998). Direct use of siRNA has also recently become the main strategy used in therapeutic applications, primarily due to the increased stability of double-stranded RNA and efficiency paired with decreased off-target effects also made possible through chemical modifications (Bertrand *et al.*, 2002; Castanotto and Rossi, 2009). The potency of siRNA is evident in the $IC_{50}$ (approximately 0.06 nM) compared to other forms of antisense ($IC_{50}$ 0.4-220 nM) (Grunweller *et al.*, 2003). Doses as low as 0.05 nM siRNA can effectively reduce target mRNA levels *in vitro*, with 0.1 nM siRNA reaching a maximum reduction of 90% (Grunweller *et al.*, 2003). There are, however, conflicting publications on this topic. Previous studies have shown that siRNA and modified ODNs may actually have similar efficiencies when used in the same concentrations (Vickers *et al.*, 2003). It has been suggested that this discrepancy between the two approaches may be due to the differences in the most efficient target sites of the technologies (Jason *et al.*, 2004). Although ODNs have been studied for decades (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978), siRNA may be superior. Both technologies exploit pathways endogenous to cells, however, siRNAs are naturally present in the cell and ODNs are not.

Based on the increasing knowledge in this area and the trend towards a greater focus on siRNA, siRNA will be the specific technology used throughout the course of this thesis.

### *1.2.3. Mechanism of RNAi action*

RNAi is an RNA-dependent gene silencing process at the mRNA level (Fire *et al.*, 1998). Starting from long dsRNA, RNAi is initiated in eukaryotes when Dicer (an RNase III-like endonuclease) cleaves the dsRNA (Bernstein *et al.*, 2001). The products of this cleavage are double-stranded siRNA molecules that are approximately 21 base pairs in length and contain dinucleotide 3' overhangs (McManus and Sharp, 2002; Elbashir *et al.*, 2001; Wall and Shi, 2003). Within the cytoplasm of the cell, double-stranded siRNA can associate with several proteins to form the RNA-induced silencing complex (RISC complex) (Figure 1.2.2.). This loading of siRNA into the RISC complex is thought to be facilitated by the RISC-loading complex (RLC), composed of Dicer and the TAR RNA-binding protein (TRBP) in mammalian cells (Step 1; Figure 1.2.2) (Tomari and Zamore, 2005; Haase *et al.*, 2005; Kurreck, 2009). Another component that associates with the RISC complex is the endonuclease Argonaute 2 (Ago2) (Tahbaz *et al.*, 2004).

Once loaded into the RISC complex, the sense strand (complementary to the antisense strand) is unwound by Dicer (Schwarz *et al.*, 2003) and cleaved between nucleotides 9 and 10 (Matranga *et al.*, 2005) by Ago2 and removed from

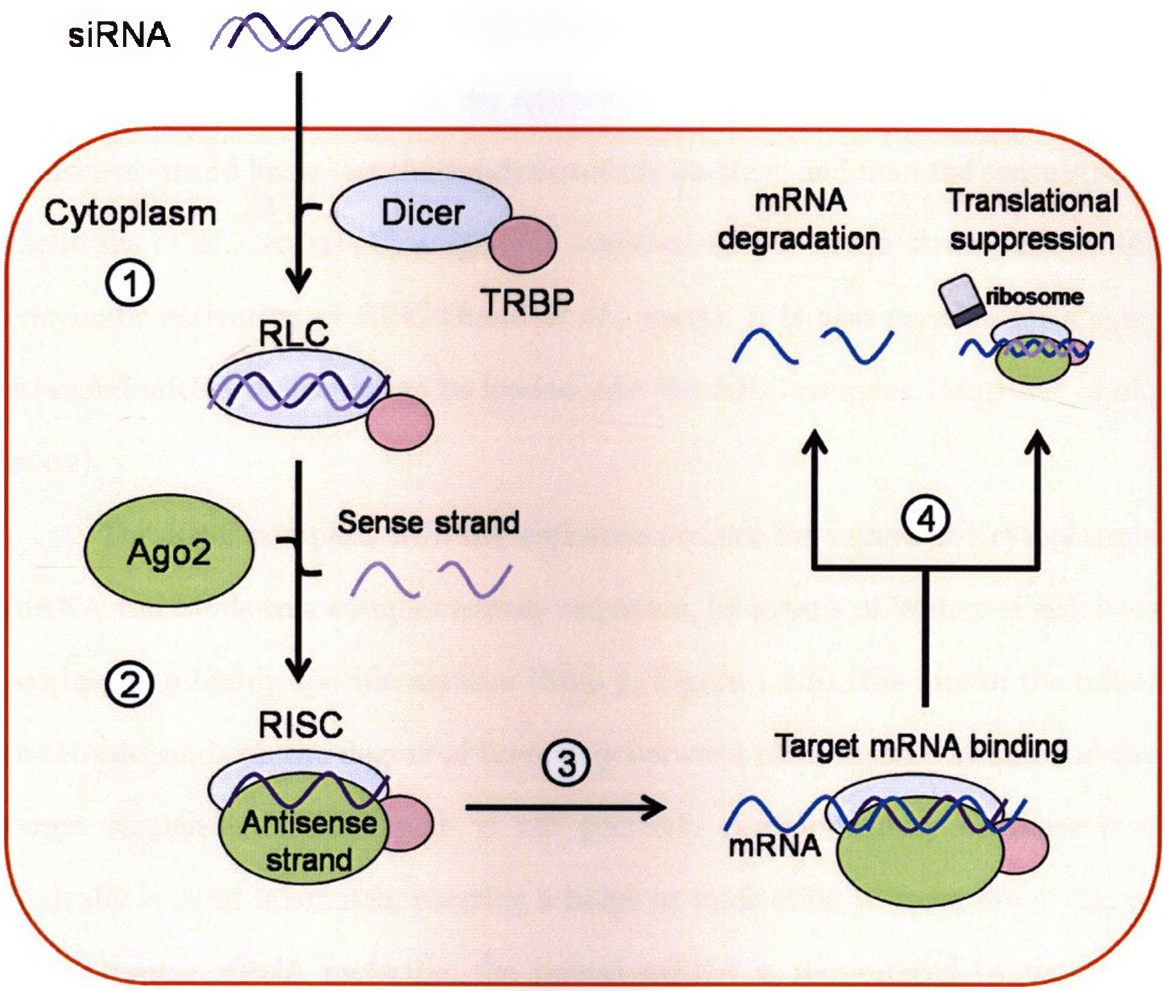


**Figure 1.2.2. The mechanism of siRNA action.** Double stranded siRNA molecules associate with Dicer and TAR RNA-binding protein (TRBP) to for the RISC-loading complex (RLC). Argonaute 2 (Ago2) is recruited to the RLC, the sense strand is removed and the activated RNA-induced silencing complex (RISC) is formed. The RISC complex with the antisense strand then scans through the cytoplasmic mRNA and binds to a complementary sequence. The target mRNA can either be degraded or RISC can block the ribosomal machinery and cause translational suppression. Modified from Rohl and Kurreck, 2006 and Kurreck, 2009.

the complex, leaving the antisense strand loaded in RISC (Step 2; Figure 1.2.2) (Meister *et al.*, 2004; Rand *et al.*, 2005; Hammond *et al.*, 2001). The RISC complex is able to distinguish the antisense strand from the sense strand. The antisense strand has a less thermodynamically stable 5' end than the sense strand (Schwarz *et al.*, 2003). Cleavage and removal of the sense strand allows for enzymatic activation of RISC (Rand *et al.*, 2005). It is also possible for single-stranded siRNA molecules to be loaded into the RISC complex (Martinez *et al.*, 2002).

The RISC complex, with the antisense strand, then scans the cytoplasmic mRNA and binds to a complementary sequence, by means of Watson-Crick base pairing, in a highly specific manner (Step 3; Figure 1.2.2). The fate of the target mRNA depends on the degree of homology between the antisense strand and the target sequence. If the match is not perfectly complementary or there is a centrally located mismatch, creating a bulge at nucleotide 10 from the 5' end of the antisense siRNA molecule, the bound mRNA is sequestered by the RISC complex thus blocking the mRNA transcript from accessing the ribosomal translational machinery (Step 4; Figure 1.2.2) (van den Berg *et al.*, 2008; Zeng *et al.*, 2003). If the match is perfectly complementary, the target mRNA is cleaved (Step 4; Figure 1.2.2) (Zeng *et al.*, 2003). Ago2 is also responsible for cleavage of the target mRNA, specifically at the site adjacent to 10 nucleotides from the 5' end of the antisense siRNA strand (Liu *et al.*, 2004; Elbashir *et al.*, 2001; Hammond *et al.*, 2001). Ago2 aggregates in cytoplasmic processing bodies (p-bodies) (Sen and Blau, 2005), where most mRNA degradation occurs, including

RNAi induced degradation through siRNA as well as naturally occurring RNA degradation (Cougot *et al.*, 2004; Sheth and Parker, 2003). The cleaved mRNA, lacking the necessary components required for stabilization, including the 5'-cap and 3'-poly(A) tail, is degraded by RNases. Ultimately, through either pathway (blockage or degradation), mRNA is no longer available for protein translation.

### *1.2.4. siRNA chemical modifications*

siRNAs can be modified to increase their stability and prevent nucleolytic degradation, particularly for *in vivo* use (Kurreck, 2009). On the antisense strand, the 5' end must be phosphorylated in order for the antisense molecule to activate the RNAi pathway (Martinez *et al.*, 2002). Phosphorothioate DNA is a frequently used modification for ODNs and can also be used for siRNA (Figure 1.2.4.) (Kurreck, 2009). This is the substitution of an unbound oxygen atom for a sulfur atom. Substitution of the hydroxyl group in the 2'-position of the ribose moiety with a 2'-*O*-methyl or a 2'-fluoro moiety are also common modifications and can enhance siRNA stability and increase potency (Figure 1.2.4.) (Layzer *et al.*, 2004; Allerson *et al.*, 2005). Another modification that enhances stability as well as target affinity is the locked nucleic acid (LNA) (Figure 1.2.4.) (Grunweller and Hartmann, 2007; Kauppinen *et al.*, 2006). LNAs contain an extra methylene bridge that helps to fix antisense structure and enhance antisense potency, compared to oligonucleotides (Grunweller *et al.*, 2003). It is possible to combine

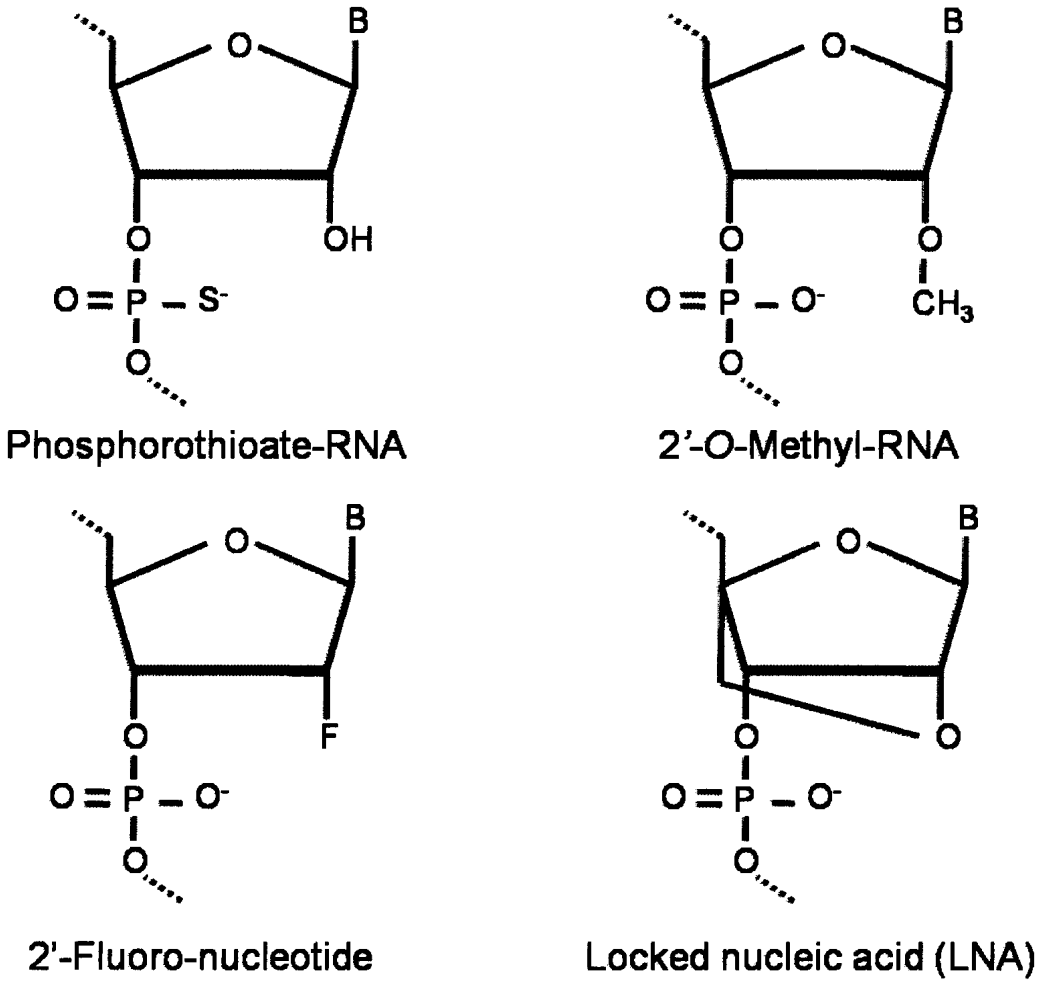


**Figure 1.2.4. siRNA chemical modifications.** siRNAs can be modified to enhance their stability *in vivo*. These modifications include phosphorothioate-RNA, 2'-*o*-Methyl-RNA, 2'-Fluoro-nucleotides and locked nucleic acids (LNAs). Modified from Kurreck, 2009.

these modifications and further enhance stability and activity (Kierzek *et al.*, 2005; Allerson *et al.*, 2005).

### *1.2.5. Potential interferon response*

Introducing dsRNA (greater than 30 base pairs in length) into mammalian cells has the potential to trigger a non-specific interferon (IFN) response that may have adverse side-effects (Sledz *et al.*, 2003). IFN-γ is a cytokine that can induce expression of immunomodulatory molecules, such as IDO (Takikawa *et al.*, 1988). Since the discovery of chemically synthesized siRNA molecules 18-25 base pairs in length, research has indicated that this non-specific IFN response can potentially be avoided, allowing for use of RNAi in mammalian systems (Kurreck, 2009). Of importance, one study observed that treating mammalian cells with a non-targeted siRNA resulted in IFN-γ induction and gene silencing (Kleinman *et al.*, 2008). They observed that toll-like receptor 3 (TLR3), which normally recognizes dsRNAs (Alexopoulou *et al.*, 2001), can also recognize 21-nucleotide siRNAs and that siRNAs can induce IFN-γ protein (Kleinman *et al.*, 2008). Other studies support the hypothesis that TLR3 plays a critical role in the IFN-γ-related dsRNA response (Negishi *et al.*, 2008). There is also evidence to suggest that TLR-mediated immune responses vary depending on the siRNA sequence and their structural motif (Kurreck, 2009; Judge *et al.*, 2005). This suggests that the therapeutic use of certain siRNAs can potentially induce

immune effects and that such siRNAs can be avoided or selected for (Kleinman *et al.*, 2008).

### *1.2.6. RNAi as a targeted drug*

In the laboratory, synthetic RNAi can be used as a tool to explore and understand the function of particular genes within the cellular environment by reducing the levels of specific mRNAs and by observing the change in cell characteristics. This technique has been used to study a wide variety of phenomena, such as the role of IDO in dendritic cells (DCs) and peripheral immune tolerance (Belladonna *et al.*, 2009). In the clinical setting, synthetic RNAi has the potential to be used as targeted anticancer therapy to control over-expressed genes, including TS and IDO (Yen *et al.*, 2009; Zheng *et al.*, 2006; Pandyra *et al.*, 2007; Ferguson *et al.*, 1999; Ferguson *et al.*, 2001). Since the mechanism of siRNA action relies on Watson-Crick base pairing of DNA and RNA molecules and therefore a high degree of specificity, it is expected that using RNAi in a therapeutic setting can help diminish off-target effects often seen with small molecule therapies. RNAi can also provide a reduction in non-specific toxicity and an improvement in anticancer activity compared to traditional chemotherapeutics (Tafech *et al.*, 2006).

In the clinical setting, RNAi is referred to as a promising new approach in developing therapies (Castanotto and Rossi, 2009). The first study to investigate the use of siRNA *in vivo* in mammals was reported in 2002 when McCaffrey and

colleagues applied siRNA methods to target the hepatitis C virus in mice (McCaffrey *et al.*, 2002). As of early 2009, there are several RNAi therapies in clinical trials, some of which have already been approved by the American Food and Drug Administration (FDA) (Table 1.2.6.).

### *1.2.7. Combining multiple siRNAs*

Combining drugs in anticancer therapy is commonly used and is very successful (Fisher *et al.*, 1993; Van Cutsem *et al.*, 2009). A previously discussed, this applies to traditional chemotherapies; however, this may also be applicable to combining antisense agents. Previous studies have reported that combining antisense agents targeting different molecules can result in independent effects at the molecule level and greater than additive effects at the physiological level (Normanno *et al.*, 1996; Skorski *et al.*, 1995; Skorski *et al.*, 1996). Despite these successes, Pandyra *et al.* (2007) observed that combining antisense *in vitro* can produce antagonistic responses at the molecular and functional levels. It was determined that Bcl-2 siRNA antagonized the capacity of TS siRNA to reduce TS mRNA and protein levels and to sensitize tumour cells to traditional TS-targeting chemotherapeutics (Pandyra *et al.*, 2007). Therefore, it is important to recognize that siRNAs may not behave the same in combinations as they do as single agents.

With regards to RISC saturation and preference for particular siRNAs, there is currently little known. However, previous literature has provided

| RNAi Technology | Name | Company | Target mRNA | Disease | Phase |
|---|---|---|---|---|---|
| **siRNA** | Sirna-027 | Merck-Sirna Therapeutics | VEGFR-1 | Age-Related Macular Degeneration | Phase II |
| **siRNA-transfected dendritic cells** | N/A | N/A | LMP2, LMP7 and MECL1 | Metastatic Melanoma | Phase I |
| **Antisense oligonucleotide** | OGX-427 | OncoGenex Technologies | HSP27 | Neoplasms | Phase I |
| **Antisense oligonucleotide** | LErafAON-ETU | Neopharm | c-Raf | Neoplasms | Phase I |
| **siRNA** | Genasense® G3139 | Genta | Bcl-2 | 15 different neoplasms | Phase I-III |

**Table 1.2.6. Antisense drugs currently used in clinical trials.** Information obtained from the National Institute of Health Clinical Trials website (www.clinicaltrials.gov).

evidence that two components of the RISC complex, Dicer and TRBP, can become saturated and that there may be strand biases for loading of competing siRNAs in to RISC (Andersson *et al.*, 2005; Bennasser *et al.*, 2006).

Clearly, it is essential to investigate the molecular and functional effects of combining antisense agents prior to therapeutic intervention. Therefore, this study will investigate the effects of combining siRNA targeted towards thymidylate synthase and indoleamine 2,3-dioxygenase for a potential cancer therapeutic approach.

## *1.3. Thymidylate Synthase*

### *1.3.1. Properties and biochemical function of thymidylate synthase*

Thymidylate synthase (TS) is the enzyme responsible for the *de novo* production of thymidylate, a precursor required for both DNA synthesis and repair (Danenberg, 1977; Danenberg *et al.*, 1999; Johnson, 1994). In an irreversible and rate-limiting step, TS catalyzes the transfer of a methyl group from 5,10-methylene tetrahydrofolate ($CH_2H_4$folate) to the 5-position of deoxyuridine monophosphate (dUMP) (Figure 1.3.1). This reductive methylation results in the production of thymidylate, also referred to as 2'-deoxythymidine-5'-monophosphate (dTMP), and dihydrofolate ($H_2$folate) (Friedkin and Kornberg, 1957; Carreras and Santi, 1995). In order for thymidylate to be incorporated into DNA, it must subsequently be tri-phosphorylated to 2'-deoxythymidine-5'-triphosphate (dTTP) (Brandt and Chu, 1997).

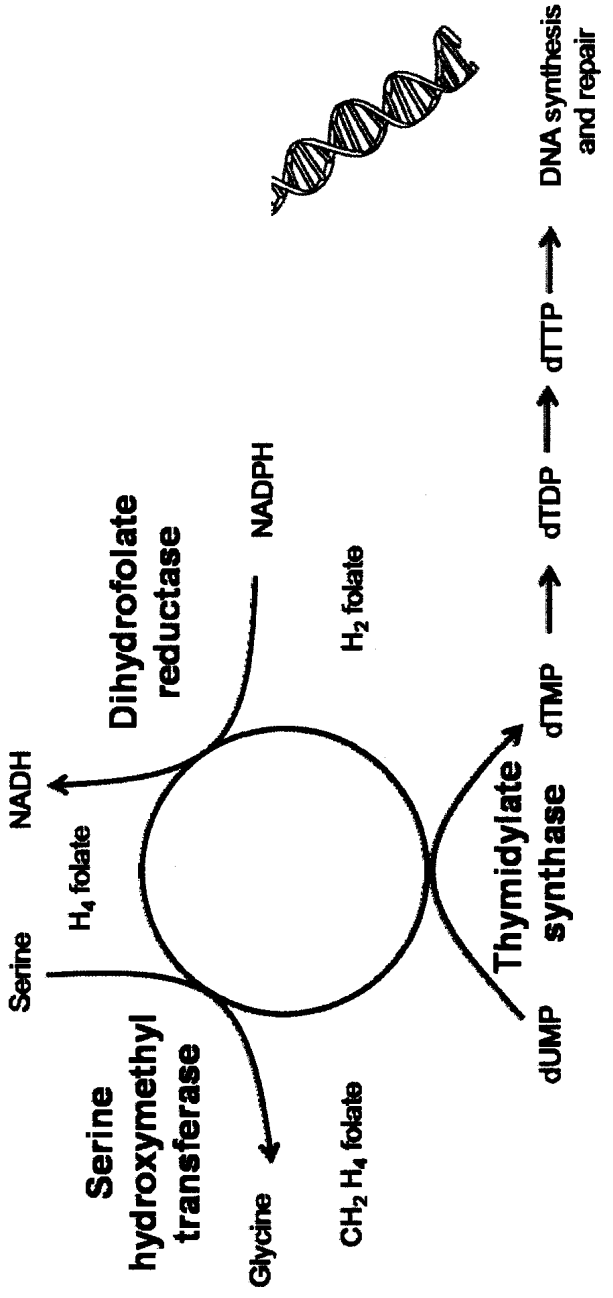


**Figure 1.3.1. The thymidylate synthesis cycle.** TS catalyzes the transfer of a methyl group from $CH_2H_4$folate to dUMP. This produces thymidylate (dTMP) and $H_2$folate. dTMP must subsequently become phosphorylated to dTDP, and then to dTTP, in order to be incorporated into DNA. Dihydrofolate reductase requires NADPH to convert $H_2$folate to $H_4$folate, which is then converted to $CH_2H_4$folate by serine hydroxymethyl transferase. $CH_2H_4$folate can be used by TS. Modified from Tai *et al.*, 2004.

The human TS gene is located on chromosome 18 at location 18p11.32 and spans 15 kb, and consists of 7 exons (Hori *et al.*, 1990). The TS protein consists of 313 amino acids (36 kDa) that homodimerizes and is localized in both the cytoplasm and nuclear region (primarily in the nucleoli) (Kucera and Paulus, 1986; Samsonoff *et al.*, 1997; Johnston *et al.*, 1991; Brown *et al.*, 1965). The half-life of rodent TS mRNA, particularly in mouse fibroblasts, is 9.6 hours in resting cells and 7.3 hours in growing cells (Jenh *et al.*, 1985). The half-life of the human TS enzyme in a colon cancer cell line is 7.3 ± 0.3 hours (Kitchens *et al.*, 1999).

### *1.3.2. Physiological function of thymidylate synthase*

Thymidylate synthase is the sole intracellular *de novo* source of thymidylate, and therefore has a critical role in DNA synthesis and repair. Both proliferating normal cells and cancer cells rely on TS for DNA synthesis (Berg *et al.*, 2002). TS levels are positively correlated with cell proliferation (Maley and Maley, 1960). TS mRNA expression levels are normally up-regulated 2- to 4-fold at the $G_1$/S boundary of the cell cycle due to the requirement of thymidylate during the synthesis phase of the cell cycle (Navalgund *et al.*, 1980). This up-regulation is likely mediated by the E2F transcription factor (Banerjee *et al.*, 1998; Banerjee *et al.*, 2000). At this boundary, TS enzymatic activity is increased up to 10- to 20-fold (Navalgund *et al.*, 1980). This discrepancy between mRNA expression and TS activity provides evidence of post-transcriptional regulation of TS protein levels (Johnson, 1994). The TS protein is able to bind to TS mRNA,

thus inhibiting the translational machinery and preventing TS protein production (Chu *et al.*, 1991). There is also evidence that TS protein binds p53 mRNA and controls p53 expression, as well as mRNA of other cell cycle-dependent proteins, including *c-myc* mRNA (Ju *et al.*, 1999; Chu *et al.*, 1995).

### *1.3.3. Thymidylate synthase in cancer*

High levels of TS are a characteristic of tumour cells. TS mRNA and protein levels correlate with poorer prognosis for patients with a variety of cancers, including breast, gastric and colorectal (Longley *et al.*, 2003; Pestalozzi *et al.*, 1997; Lenz *et al.*, 1996; Johnston *et al.*, 1994). Over-expression of TS is also associated with resistance to traditional chemotherapeutics that target TS on the protein level (Marsh, 2005), and will be expanded on in *Section 1.3.4.*

Due to the fact that TS is the sole source of *de novo* thymidylate and is required for cell proliferation and cell survival, targeting TS in cancer therapy has been a useful strategy. Down-regulation of TS through both traditional and non-traditional chemotherapeutics targeting TS should be very beneficial in anticancer therapy. It is expected that cancer cells would be sensitive to TS-targeting treatment due to the high proliferative capacity of cancer cells. In fact, TS has been an important target for chemotherapy for over 5 decades (Bollag, 1957; Haggmark, 1962).

### *1.3.4. Thymidylate synthase as a target in cancer therapy: chemotherapeutic drugs*

Several inhibitors of TS enzyme activity are well established as clinical chemotherapy agents (DeVita Jr *et al.*, 2008). The first major TS-protein-targeting drug to be used clinically was 5-fluorouracil (5-FU). This compound, first synthesized and patented in 1957, belongs to the class of drugs known as fluoropyrimidines (Figure 1.3.2) (Heidelberger *et al.*, 1957). This category also includes 5-fluorodeoxyuridine (5-FUdR). To become a TS inhibitor, both 5-FU and 5-FUdR must become activated through intracellular metabolism to form 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), the active form of both drugs (Chu *et al.*, 2003). FdUMP can then compete with dUMP binding to TS, forming a ternary complex with TS and $CH_2H_4$folate to irreversibly block the catalytic site. FdUMP can also become phosphorylated to form 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP). This can be incorporated into DNA and ultimately decrease DNA synthesis, cause mutations, and lead to futile repair (blocks DNA synthesis) (Chu *et al.*, 2003). 5-FU can also be metabolized to 5-fluorouridine 5'-monophosphate (FUMP), which is then incorporated into RNA (Noordhuis *et al.*, 2004). 5-FU has been successfully used to treat a variety of solid tumours including breast, head and neck, ovarian, gastric and colon cancers (DeVita Jr *et al.*, 2008).

Another class of TS protein-targeting drugs are the antifolates (Figure 3.1.2) (Rose *et al.*, 2002). These drugs, first developed in the late 1980s, compete

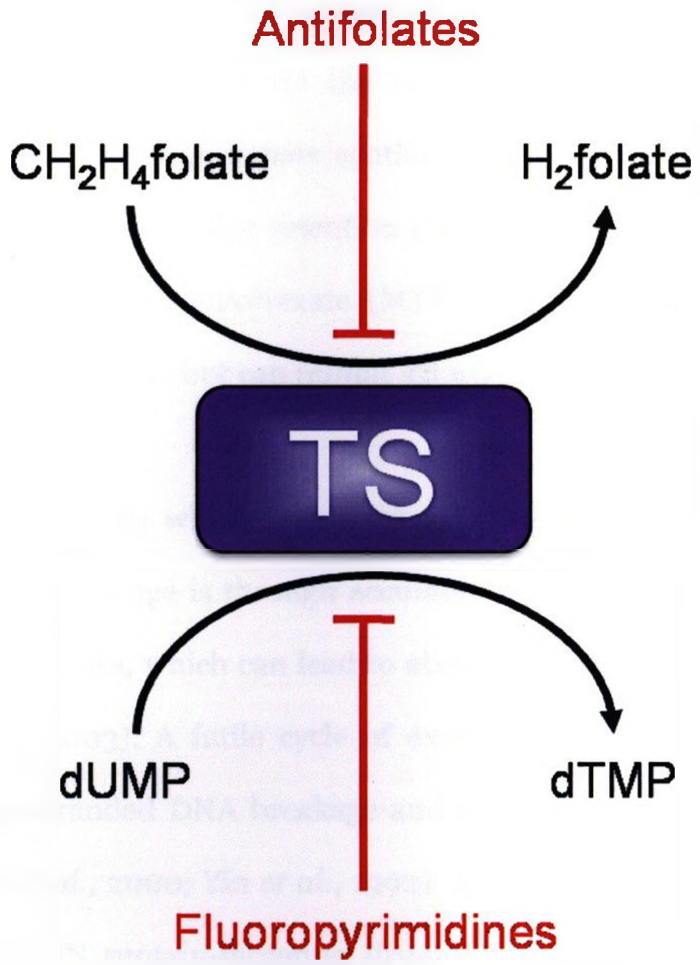


**Figure 1.3.2. Current chemotherapies target thymidylate synthase protein.** Fluoropyrimidines compete with dUMP binding to TS and prevent the conversion of dUMP to dTMP. Antifolates also target TS by competing with $CH_2H_4$folate binding to TS. Modified from a figure from Dr. James Koropatnick.

with $CH_2H_4$folate by binding to TS (Calvert *et al.*, 1986). One example of this class is the quinazoline-based drug raltitrexed (ZD1694, Tomudex®), which is used as first-line therapy to treat colon and rectal cancers (Cocconi *et al.*, 1998). Once raltitrexed enters the cell via the reduced folate carrier, it undergoes polyglutamation by folypolyglutamate synthase, enhancing its enzyme-binding-affinity as well as its intracellular retention (Jackman and Calvert, 1995). Other folate analogues, such as methotrexate (MTX), can inhibit other intracellular enzymes that require folate, but can inhibit TS when used at high concentrations (Berg *et al.*, 2002).

The mechanism by which fluoropyrimidines and antifolates can lead to cytoxicity and DNA damage is through accumulation of dUTP combined with a reduction of dTTP levels, which can lead to aberrant incorporation of dUTP into DNA (Chu *et al.*, 2003). A futile cycle of excision-repair pathways eventually results in double-stranded DNA breakage and subsequent apoptosis (Matsui *et al.*, 1996; Peters *et al.*, 2000; Yin *et al.*, 1992). Although this is a p53-dependent process for some TS protein-targeting drugs (Peters *et al.*, 2000), 5-FUdR-induced cell death occurs in p53-depleted cells (Munoz-Pinedo *et al.*, 2001), suggesting that cell death may also occur via p53-independent pathways (Berg *et al.*, 2002).

TS-targeting drug resistance is a major obstacle in cancer therapy and can be mediated through a variety of mechanisms in both normal and tumour cells. Tumours can be constitutively resistant or can acquire transient or permanent drug resistance through a variety of mechanisms. Mechanisms of TS-targeting

drug resistance include reduced drug uptake (Patino-Garcia *et al.*, 2009; Brigle *et al.*, 1995), TS gene amplification (Wang *et al.*, 2004), or increased drug efflux (Assaraf, 2007; Berg *et al.*, 2002). Also, when human cancer cell lines are treated *in vitro* with TS protein-targeting drugs, TS protein levels rise 10- to 40- fold, while TS mRNA levels remain unchanged (Welsh *et al.*, 2000; Keyomarsi *et al.*, 1993). It is thought that the presence of TS-targeting drugs may relieve binding of TS protein to TS mRNA, preventing the negative autoregulatory role of TS protein and allowing more TS protein to be produced (Chu *et al.*, 1993; Chu *et al.*, 1990). The inhibition of translation repression has been termed "translational derepression" (Chu *et al.*, 1994). This can eventually lead to TS-targeting drug resistance in tumours. It is important that these obstacles are overcome to further cancer therapy.

There has been a growing emphasis, both in research and clinically, on combining drugs that target different molecules, as well as targeting the same molecule using combinations, in an attempt to enhance cancer therapy. This applies to combinations of chemotherapeutics involving TS protein-targeting drugs, including raltitrexed or 5-FU. Raltitrexed has been used in combination with cisplatin, a DNA-binding agent that causes crosslinking of DNA (Kano *et al.*, 2000). Simultaneous administration of the two drugs, or administration of raltitrexed followed by cisplatin, results in enhanced cytoxicity *in vitro* (Kano *et al.*, 2000). 5-FU has been used clinically in combination with leucovorin (LV), which can be metabolized to $CH_2H_4$folate (Sotos *et al.*, 1994). This metabolite can form a ternary complex with FdUMP and TS and assist FdUMP in inactivating

the TS enzymatic activity resulting in enhanced cytotoxicity of 5-FU (Sotos *et al.*, 1994). Although drug combinations do provide promising results, one disadvantage is the likelihood of increased overall drug toxicity. One method to overcome this obstacle is the use of drugs targeting TS that reduce TS mRNA levels (Berg *et al.*, 2002).

### *1.3.5. Thymidylate synthase as a target in cancer therapy: antisense therapy*

In addition to the TS protein-targeting drugs already described, antisense drugs targeting TS mRNA have been studied both *in vitro* and *in vivo*. Our laboratory has previously shown that transfection of human cervical cancer cells with TS-targeting ODNs reduces TS mRNA and protein levels by 70% and 75%, respectively, compared to transfection controls (Ferguson *et al.*, 1999). Some, but not all, TS ODN sequences inhibit tumour cell proliferation upon transfection, and transfected cells become more sensitive to drugs that target TS, but not non-TS targeting drugs (Schmitz *et al.*, 2004; Ferguson *et al.*, 1999; Berg *et al.*, 2002). This finding is supported by several other studies investigating the usefulness of TS antisense *in vitro, in vivo* and in 5-FUdR-resistant cell lines (Berg *et al.*, 2001; Ferguson *et al.*, 2001; Yang *et al.*, 2006; Flynn *et al.*, 2006).

Another strategy for targeting TS in anticancer therapy is the use of multiple antisense ODNs targeting different regions of the TS mRNA transcript (Berg *et al.*, 2003). Such targeting results in greater mRNA down-regulation and

inhibition of cell proliferation, supporting the use of multiple antisense agents to enhance therapy (Berg *et al.*, 2003).

In addition to targeting TS with antisense, other key molecules involved in malignancy have been targeted with antisense simultaneously with antisense against TS. In a study using siRNA to target the pro-survival Bcl-2 mRNA along with TS mRNA, it was observed that such a combination results in unexpected, nonreciprocal antagonistic effects: Bcl-2 siRNA reduced the capacity of TS siRNA to reduce target mRNA and protein levels (Pandyra *et al.*, 2007). This emphasizes that synergy with siRNA combinations cannot be predicted *a priori* and that experimental investigations are necessary for proposed combinations. In the study reported herein, the effects of simultaneously combining TS siRNA with siRNA targeting indoleamine 2,3-dioxygenase, an enzyme involved in tumour immune tolerance, were investigated.

## *1.4. Indoleamine 2,3-dioxygenase*

### *1.4.1. Properties and biochemical function of indoleamine 2,3-dioxygenase*

Indoleamine 2,3-dioxygenase (IDO) is a heme-containing cytosolic enzyme responsible for the irreversible and rate-limiting step in the degradation of the essential amino acid L-tryptophan through the kynurenine pathway (Zheng *et al.*, 2006; Shimizu *et al.*, 1978). IDO is encoded on chromosome 8p12 and results in the production of a 407 amino acid protein with a molecular weight of 45 kDa (Ball *et al.*, 2007). Oxidative cleavage of the indole ring of tryptophan

results in the formation of *N'*-formylkynurenine (Figure 1.4.1.) (Kudo and Boyd, 2000). Subsequent enzymatic reactions result in the sequential formation of 3-hydroxyanthranilic acid, kynurenate and quinolinate.

IDO expression, as well as activity, is stimulated by several cytokines, including interferon-gamma (IFN-γ), interferon alpha and beta, tumour necrosis factor alpha (TNF-α) and platelet activating factor (Murray, 2003; Yoshida *et al.*, 1981; Pfefferkorn, 1984). Of these, IFN-γ is the most potent IDO-inducer (Yasui *et al.*, 1986). The promoter region of IDO contains two interferon-stimulated response elements (ISREs) that are essential for a 50-fold increase in human IDO mRNA levels in response to IFN-γ treatment (Konan and Taylor, 1996). In *in vitro* studies using human cell lines, IDO induction by IFN-γ was achieved at 12-18 hours after treatment, reaching a plateau at 36 – 48 hours. Induction of IDO by IFN-γ is also concentration dependent (Takikawa *et al.*, 1988) with maximal IDO enzymatic activity in the mouse model observed after approximately 48 hours (Yoshida *et al.*, 1981).

### *1.4.2. Physiological function of indoleamine 2,3-dioxygenase*

IDO is an endogenous enzyme initially thought to play an antimicrobial role against bacteria, viruses, and parasites by reducing the tryptophan availability at the site of infection and depriving infectious cells of the essential amino acid (Austin *et al.*, 2009; Pfefferkorn, 1984; Yoshida and Hayaishi, 1978; Yoshida *et al.*, 1979). More recently, IDO has been described as playing a role

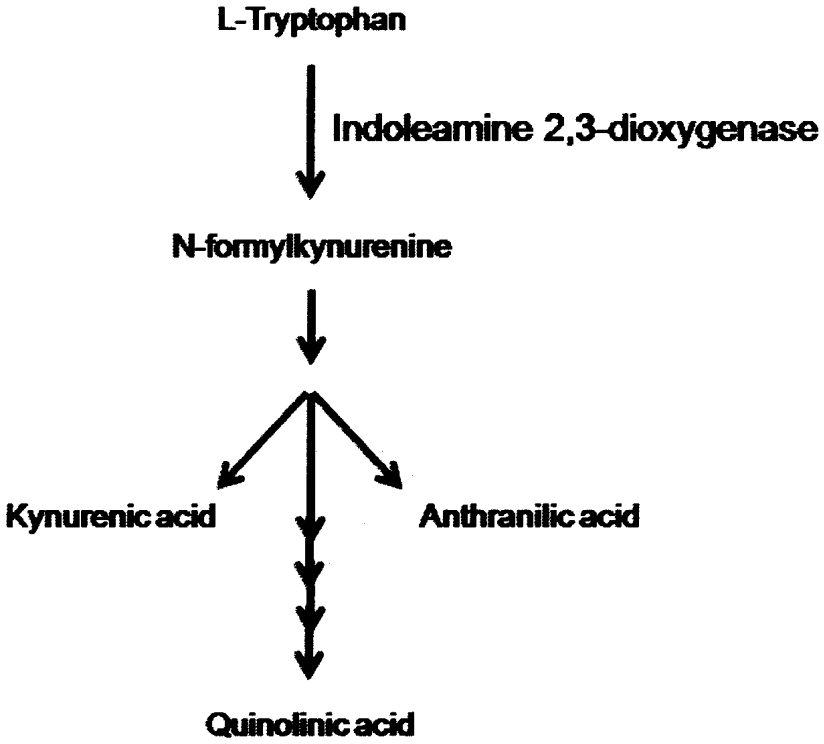


**Figure 1.4.1. Tryptophan degradation via the kynurenine pathway.** Indoleamine 2,3-dioxygenase converts L-tryptophan to N-formylkynurenine. Through subsequent steps, N-formylkynurenine is converted to kynurenic acid, quinolinic acid and anthranilic acid. Modified from Lob *et al.*, 2009a.

during fetal antigen tolerance by preventing T-cell-driven fetal rejection (Munn *et al.*, 1998; Suzuki *et al.*, 2001). In adult humans, IDO is expressed in select tissues, including some dendritic cells, the placenta, epididymis, thymus, blood mononuclear phagocytes, intestine, gut, lung, and within the endocrine and central nervous systems (Hayaishi *et al.*, 1977; Heyes and Morrison, 1997; Suzuki *et al.*, 2001). Aside from supporting early development, pregnancy and protection against infection, IDO's immunomodulatory function has been implicated in cancer (Uyttenhove *et al.*, 2003; Lob *et al.*, 2009a; Munn and Mellor, 2004). Although the effects of IDO are not entirely clear presently, IDO expression by subsets of dendritic cells, human cancer cells , and non-cancer cells located in the lymph nodes (Uyttenhove *et al.*, 2003) is proposed to induce in immune inactivation through two mechanisms: 1) a decrease in local tryptophan (Mellor and Munn, 2004) and 2) an increase in toxic metabolites (Grohmann *et al.*, 2003).

A decrease in local tryptophan can inhibit cell proliferation (MacKenzie *et al.*, 2007). Lymphocytes, particularly T cells, are extremely sensitive to local amino acid concentrations (Munn *et al.*, 1999). A decrease in amino acid levels can have a dramatic negative effect on T cell viability (Figure 1.4.2.). When tryptophan levels have decreased below the threshold concentration, <50 nM *in vitro,* T cells can no longer be activated (Munn *et al.*, 1999). This threshold must be met at a mid-G1 cell cycle check-point in order for T cells to be activated (Munn *et al.*, 1999). If this requirement is not met, the number of uncharged transfer RNA (tRNA) in T cells can rise and the cell cannot enter S phase. The

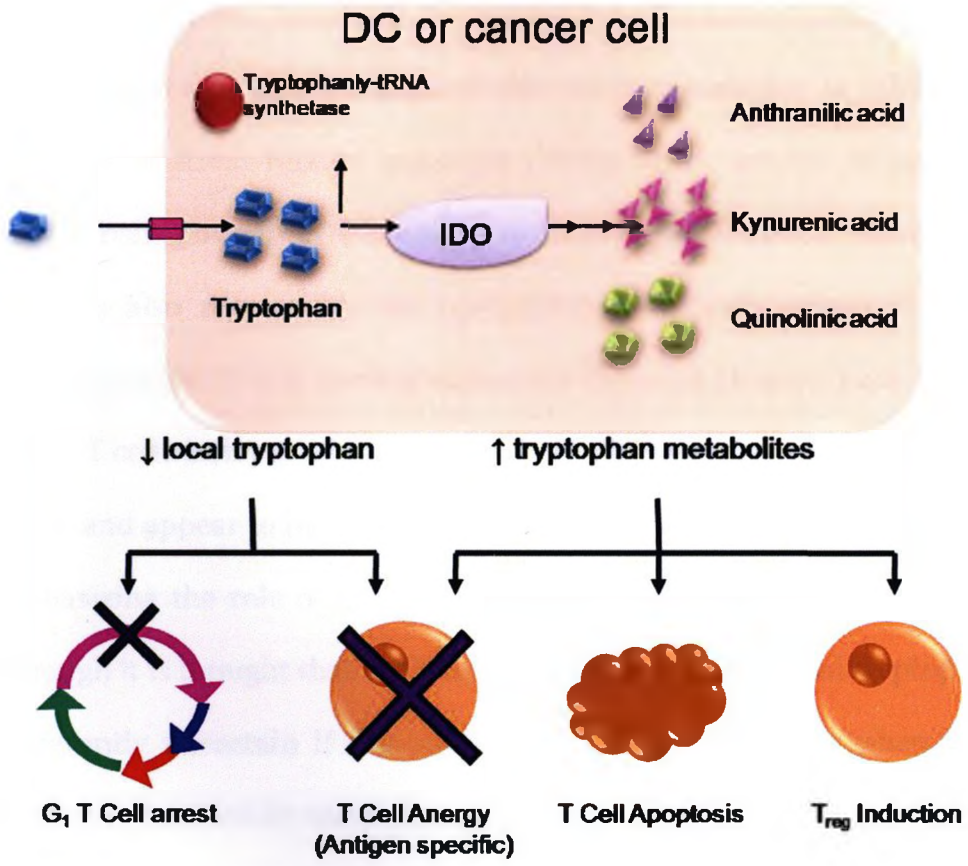


**Figure 1.4.2. The physiological effects of tryptophan degradation.** Tryptophan enters into the cytoplasm of a cell and can be used for protein synthesis by tryptophanyl-tRNA synthetase, or be degraded by indoleamine 2,3-dioxygenase (IDO) forming anthranilic acid, kynurenic acid and quinolinic acid. A decrease in local tryptophan levels can cause cell cycle arrest in T cells and T cell anergy. An increase in tryptophan metabolites can also contribute to T cell anergy, T cell apoptosis and induce T regulatory cells ($T_{reg}$).

amino acid-sensitive stress-response kinase, general control non-derepressible 2 (GCN2), is responsible for recognition of tryptophan levels by recognizing the level of uncharged tRNA and inhibits general protein translation (Dong *et al.*, 2000). GCN2 phosphorylates the eukaryotic translation initiation factor eIF-2α and it is through this mechanism that most translation is inhibited, with the exception of stress-related messages (Munn *et al.*, 2005). Mice lacking GCN2 show a resistance to IDO's ability to inhibit proliferation (Katz *et al.*, 2008). GCN2 is also responsible for up-regulating a small subset of genes causing antigen-specific T cell unresponsiveness (anergy) (Figure 1.4.2.) (Munn *et al.*, 2005). T cells isolated from the spleen of GCN2 knockout mice (which are viable, fertile, and appear to be similar to wild-type mice) cannot enter this anergic state, emphasizing the role of GCN2 in T cell responses to IDO (Munn *et al.*, 2005). Although it is thought that IDO expression can decrease local tryptophan levels, it is presently uncertain if this plays a role *in vivo,* as tryptophan levels can be quickly replenished by the surrounding tissues (Lob *et al.*, 2009a).

The decrease in local tryptophan levels is not the only proposed mechanism contributing to immune suppression caused by IDO expression. The degradation products of tryptophan (kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid) can contribute to T cell apoptosis (Figure 1.4.2.) (Uyttenhove *et al.*, 2003; Terness *et al.*, 2002; Fallarino *et al.*, 2002). These metabolites can also inhibit T cell proliferation, which cannot be re-stimulated after removal of metabolites (Figure 1.4.2) (Terness *et al.*, 2002). Additionally, it has also been observed *in vitro* that IDO expression can induce the

differentiation of $CD4^+$ T cells into regulatory T cell phenotype ($T_{reg}$), which inhibit immune responses and can facilitate tumour cell growth (Shevach, 2004; Nishikawa *et al.*, 2005; Fallarino *et al.*, 2006; Curti *et al.*, 2007); however, the mechanism by which these consequences occur is currently unknown (Lob *et al.*, 2009a).

Although T cell growth is arrested by a decrease in local tryptophan, certain cells, including cancer cells, may not be as affected. Tumour cells expressing IDO do not show proliferation arrest *in vitro* until 6 days post-IDO induction (Takikawa *et al.*, 1988). These cells lack the G1 phase tryptophan checkpoint found in T cells. Instead, cancer cells show a reduced rate of growth, and this may be due to a lower substrate affinity ($K_m$) in tryptophanyl-tRNA synthetase compared to the $K_m$ of IDO, allowing protein synthesis to take precedence over degradation (Kudo and Boyd, 2000; Praetorius-Ibba *et al.*, 2000; Jorgensen *et al.*, 2000). Addition of tryptophan to IFN-γ treated cells has the ability to partially reverse growth inhibition in cancer cells (Takikawa *et al.*, 1988).

### *1.4.3. Human and mouse IDO*

Through the use of immunocompromised mice, it is possible to explore the molecular results of IDO siRNA combined with other siRNAs targeting other mRNA transcripts *in vivo;* however, these mice lack an immune system, therefore, effects of siRNA on IDO activity and immune regulation cannot be fully

investigated. To gain a better understanding of the physiological consequences, there is a large dependence on an immunocompetent *in vivo* mouse model to study the physiological effects of IDO down-regulation. It is very important to recognize similarities and differences between structure, activity and expression of human and mouse IDO in order to properly interpret future *in vivo* results.

In humans, the IDO gene is located on the short arm of chromosome eight in the human genome (Burkin *et al.*, 1993). The gene spans 15 kb and is composed of 10 exons (Kadoya *et al.*, 1992). The gene is transcribed and spliced into a 1531 nucleotide mRNA product, which codes for a protein product of 403 amino acids and a molecular weight of 45 kDa (Dai and Gupta, 1990; Tone *et al.*, 1990; Kadoya *et al.*, 1992). In the mouse, the protein product for IDO is 407 amino acids in length, also with a molecular weight of 45 kDa (Habara-Ohkubo *et al.*, 1991).

Human and mouse IDO share approximately 62% of the mRNA sequences (Habara-Ohkubo *et al.*, 1991). The mouse IDO protein has a higher L-tryptophan binding efficiency than the human counterpart, although no significant differences in kinetic properties have been observed (Austin *et al.*, 2009). This may be due to a difference in tryptophan levels in the serum of these two systems (Austin *et al.*, 2009). Basal tryptophan levels in mouse serum are approximately 25% higher than in human serum (Fernstrom and Wurtman, 1971; Huang *et al.*, 2002). The activity of both human and mouse forms of IDO is highest at a pH between 6 and 7 (Austin *et al.*, 2009). Although there are slight differences

between these two mammalian species, it is important that we recognize them prior to conducting *in vivo* experiments.

### *1.4.4. Indoleamine 2,3-dioxgyenase-2*

Recently, an enzyme with similar structure and enzymatic activity to IDO (also referred to as IDO1) was discovered (Murray, 2007). This enzyme is referred to as indoleamine 2,3-dioxygenase-2, indoleamine 2,3-dioxygenase-like protein or proto-indoleamine 2,3-dioxygenase (IDO2, IDO-2, INDOL1, or protoIDO) (Ball *et al.*, 2009). There is approximately 43% sequence similarity between human IDO1 and IDO2 amino acid sequences (Ball *et al.*, 2009). Located on chromosome 8 in both humans and mice, the two genes are adjacent to each other (Ball *et al.*, 2009). It is thought that IDO1 arose from a gene duplication from IDO2 in mammalian evolution based on the observation that IDO1 is not present in lower vertebrates, however, an enzyme more similar to IDO2 is found in these animals (Ball *et al.*, 2007; Yuasa *et al.*, 2007; Ball *et al.*, 2009). It is possible that the two forms of IDO are not functionally redundant due to the fact that both types are expressed in different cell types and are induced under different conditions (Ball *et al.*, 2009). IDO1 is induced during an immune response and stimulation by IFN-γ, yet IDO2 levels may actually decrease with IFN-γ treatment (Ball *et al.*, 2007). Another major difference between the two forms of IDO is that IDO1 has a much higher affinity for tryptophan (approximately 500-1000 fold higher) than IDO2 (Yuasa *et al.*,

2007), suggesting that IDO2 tryptophan degradation activity may not be physiologically relevant (Ball *et al.*, 2009). High levels of IDO2 found in the tail of spermatozoa suggest that IDO2 may play an important role in fertilization (Ball *et al.*, 2007; Gutierrez *et al.*, 2003). Multiple promoters can be used to generate multiple transcripts of IDO mRNA for both IDO1 and IDO2, therefore it is important that in addition to monitoring IDO mRNA levels, the protein and activity levels of IDO be measured as well (Ball *et al.*, 2009).

Currently, the biological relevance of IDO2 is not fully understood as it is such a new discovery (Ball *et al.*, 2009). Expression patterns have been investigated and further characterization of the biological relevance is underway (Ball *et al.*, 2009; Metz *et al.*, 2007). One studied function of IDO2 is its role in cancer. A known inhibitor of IDO, 1-methyl-tryptophan (1-MT), can be found in two different forms: 1-methyl-D-tryptophan or 1-methyl-L-tryptophan. The L isoform selects for IDO1 while the D isoform is more selective for IDO2 (Metz *et al.*, 2007). This is important in that the D isoform of 1-MT has stronger anti-tumourogenic effects than the L isoform (Ball *et al.*, 2007). This strongly points to the importance and role for IDO2 in the ability of the tumour to evade the immune system, although other evidence suggests that IDO1 and IDO2 interact when suppressing the immune system (Hou *et al.*, 2007). It has also been suggested that IDO and IDO2 act in a common pathway, the latter acting upstream of IDO (Prendergast, 2008).

### *1.4.5. Indoleamine 2,3-dioxygenase in cancer*

In certain cancers, including ovarian cancer and colorectal cancer, expression of IDO is correlated with poor patient prognosis (Okamoto *et al.*, 2005; Brandacher *et al.*, 2006). Constitutive expression of IDO has been observed in a large number of human tumour cells (Uyttenhove *et al.*, 2003), suggesting a critical role in tumour immune evasion. Also, cancer cell growth can be stimulated by IDO expression *in vitro* (Hill *et al.*, 2005). Over-expression of IDO is a result of an inactivating mutation in the tumour suppressor *Bin1* (Muller *et al.*, 2005a). Our laboratory, in collaboration with Dr. Wei-Ping Min, has demonstrated that targeting IDO with siRNA results in postponed tumour formation as well as slower tumour growth in mice (Zheng *et al.*, 2006). This suggests that IDO serves an immunosuppressive function within the tumour environment.

### *1.4.6. Indoleamine 2,3-dioxygenase as a target in cancer therapy: protein targeting drugs*

The involvement of IDO in cancer strongly suggests that an anticancer therapy targeting IDO has excellent therapeutic potential. As discussed earlier, 1-MT has been developed and studied extensively for clinical use as an IDO inhibitor (Ball *et al.*, 2007; Lob *et al.*, 2009b; Ou *et al.*, 2008), and has recently entered phase I clinical trials for a variety of malignancies (National Institute of

Health, 2009). A racemic mixture of both the L and D isomers was previously used; however, in a study assessing the activity of each, it was observed that, although 1-methyl-L-tryptophan was a more potent inhibitor of IDO, 1-methyl-D-tryptophan showed less T cell suppression in mixed lymphocyte reactions and had greater antitumour effect and efficacy *in vivo* (Hou *et al.*, 2007). Of note, 1-MT has been studied in combination with chemotherapeutics currently used clinically, resulting in synergistic anticancer effects in a mouse *in vivo* model (Muller *et al.*, 2005b; Muller *et al.*, 2005a). One such study investigated the use of 5-FU targeting TS in combination with 1-MT targeting IDO (Muller *et al.*, 2005a). It was observed that addition of 1-MT does not enhance the efficacy of 5-FU in mice (Muller *et al.*, 2005a). It has been suggested that IDO inhibitors be used after chemotherapeutic treatment as maintenance chemotherapy to elicit an antitumour response (Hou *et al.*, 2007).

### *1.4.7. Indoleamine 2,3-dioxygenase as a target in cancer therapy: antisense therapy*

In 2006, IDO was targeted with siRNA in mice to reduce IDO mRNA levels (Zheng *et al.*, 2006). This study showed that successful reduction of IDO mRNA through siRNA-mediated knockdown results in postponed tumour formation and slower tumour growth, suggesting the stimulation of anticancer immunity (Zheng *et al.*, 2006). Another study using IDO siRNA in *in vivo* mouse tumours compared siRNA targeting IDO and siRNA targeting IDO2 in dendritic cells,

showing that IDO siRNA was more efficacious in prolonging survival compared to IDO2 siRNA, although both resulted in greater antitumour effects compared to controls (Yen *et al.*, 2009). Other groups have studied the use of siRNA targeting IDO and the implications this has on cancer, emphasizing the increasing interesting in IDO's role in cancer and IDO as a target in cancer therapy (Lob *et al.*, 2009b).

## *1.5. Thesis objectives*

Early studies have demonstrated that antisense strategies targeting TS enhances TS protein-targeting drug sensitivity and improves anti-cancer therapy *in vitro* and *in vivo*. Also, siRNA targeting IDO has the ability to postpone tumour formation and slow tumour growth *in vivo*. This study is aimed at investigating the potential for a combination of antisense drugs targeting multiple physiological events simultaneously to improve anticancer therapy. It is proposed that by combining TS and IDO antisense strategies and targeting cancer cells from multiple directions, it is possible to further enhance anticancer therapy. However, one antisense reagent might modify the effectiveness of a second. Therefore, *in vitro* effects of combined antisense drugs require investigation.

***Hypothesis:*** The degree and specificity of knockdown of TS and IDO mRNA will be the same when TS and IDO siRNAs are administered alone or together.

Furthermore, IDO siRNA will have no effect on the ability of TS siRNA to sensitize HeLa cells to TS protein-targeting drugs.

***Objectives:***

1. To determine if combining TS siRNA and IDO siRNA has an additive, synergistic, antagonistic or independent effect on the target mRNA levels.
2. To assess if combined siRNA treatment alters the ability of cancer cells to proliferate in the presence or absence of traditional TS-targeting drugs.

# CHAPTER 2: MATERIALS AND METHODS

## *2.1. Cell culture*

HeLa (human cervical carcinoma) and SK-MEL-5 (human melanoma) cell lines were used. Both cell lines were obtained from the American Type Culture Collection (Manassas, VA). These cell lines were chosen because of preliminary IDO expression data obtained using a panel of human cell lines or previous reports (Takikawa *et al.*, 1988) indicated IDO expression after cytokine induction. HeLa cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) (Invitrogen, Burlington, ON). SK-MEL-5 cells were cultured in Alpha-MEM (minimum essential medium) (Invitrogen, Burlington, ON). All media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Burlington, ON) and 50 or 100 ng/mL of human IFN-γ, unless otherwise indicated. Cells were grown in a humidified incubator at 37°C in a humidified 5% $CO_2$ atmosphere.

## *2.2. Reagents*

siRNAs were obtained from Dharmacon RNAi Technologies (Thermo Fisher Scientific., Lafayette, CO) annealed, desalted and in a 2'-hydroxyl form. Antisense strands contain a 5'-Phosphate and both strands contain 3'-UU overhangs. Two different siRNA technologies were used: siGENOME siRNA and ON-TARGET*plus* siRNA. Newer ON-TARGET*plus* reagents are modified to enhance specificity, according to Dharmacon's proprietary technology (not

provided). Two different siGENOME siRNAs were obtained to target TS and one ON-TARGET*plus* siRNA targeting IDO was used to target IDO (Table 2.2.1). As controls, two siGENOME non-targeting control siRNAs (1 and 2) and two ON-TARGET*plus* non-targeting control siRNAs (3 and 4) were used (Table 2.2.2). Control siRNA sequences contain 4 or more mismatches with all known human mRNAs. siRNAs were dissolved in 1 X siRNA buffer (300 nM KCl, 30 nM HEPES-pH 7.5, 1.0 mM $MgCl_2$ diluted 1 in 5) (Dharmacon RNAi Technologies) to a final concentration of 10 μM.

Lyophilized recombinant human Interferon-gamma (IFN-γ) was obtained from Cedarlane Laboratories Limited (Hornby, ON) with a specific activity of $2.0x10^7$ units/mg, and was reconstituted in sterile RNase-free $H_2O$ to a final working concentration of 100 μg/mL.

### 2.3. *IFN-γ treatment*

HeLa cells (untreated with inducing agents) did not have IDO mRNA levels that could be detected by qPCR during preliminary screening, consistent with findings previously published (Lob *et al.*, 2009b). Only one cell line constitutively expression IDO (Uyttenhove *et al.*, 2003), however, this cell line was not available. Despite these findings in human tumour cell lines, baseline IDO expression in most primary tumor samples is higher (Lob *et al.*, 2009b). Detecting IDO mRNA levels prior to treating human tumour cell lines with IDO-targeting siRNA was an essential prerequisite to determining the extent of IDO

| siRNA | Target Bases[a] | Antisense/Sense strand sequences | Catalog Number |
|---|---|---|---|
| TS 4 | 492 - 510 | Antisense:<br>5'-PAUAAACUGGGCCCAGUUUAUUU-3'<br>Sense:<br>5'-GGACUUGGGCCCAGUUUAUUU-3' | D-004717-04 |
| TS 5 | 332 - 350 | Antisense:<br>5'-PUUCCAGAACACACGUUUGGUU-3'<br>Sense:<br>5'-CCAAACGUGUGUUCUGGAAUU-3' | D-004717-05 |
| IDO | 858 - 876 | Antisense:<br>5'-PUUCCCAGAACCCUUCAUACUU-3'<br>Sense:<br>5'-GUAUGAAGGGUUCUGGGAAUU-3' | J-010337-11-0005 |

**Table 2.2.1 . siRNA target bases, antisense and sense strand sequences and catalog numbers for TS and IDO siRNAs.** [a] from translational start site.

| siRNA | Target sequences | Catalog Number |
|---|---|---|
| Control 1 | Target Sequence: UAGCGACUAAACACAUCAA | D-001210-01 |
| Control 2 | Target Sequence: UAAGGCUAUGAAGAGAUAC | D-001210-02 |
| Control 3 | Target Sequence: UGGUUUACAUGUUUUCUGA | D-001810-03 |
| Control 4 | Target Sequence: UGGUUUACAUGUUUUCCUA | D-001810-04 |

**Table 2.2.2. Target sequences and catalog numbers for control siRNAs.**

mRNA down-regulation after antisense treatment. In order to detect IDO mRNA by qPCR *in vitro* (Lob *et al.*, 2009b), cells are treated with IFN-γ to increase IDO expression and activity (Yasui *et al.*, 1986; Uyttenhove *et al.*, 2003). Therefore, in this study, IFN-γ was used to induce IDO mRNA to detectable levels.

To investigate the effect of IFN-γ on IDO mRNA and protein levels and cell viability, HeLa cells were treated with varying concentrations of IFN-γ. A total of $2.5x10^5$ cells were plated in 60 mm dishes in 4 mL of medium. IFN-γ was added in the following concentrations: 0, 25, 50, 100, 200 or 400 ng/mL, based on previous published studies (Takikawa *et al.*, 1988). Cell viability was assessed 24 and 48 hours after treatment by a Trypan blue exclusion assay (*Section 2.4*). Cells were harvested after 18 hours of treatment for RNA isolation (*Section 2.6.1*), and 72 hours for protein extraction (*Section 2.7.1*), as these time points for IDO mRNA and protein induction have previously been established (Takikawa *et al.*, 1988; Yoshida *et al.*, 1981).

## *2.4. Cell viability assay*

HeLa cell viability was determined using a Trypan blue exclusion assay. Trypan blue (Sigma-Aldrich, Oakville, ON) is an acidic dye that is excluded from intact cells; however, dead cells are permeable to Trypan blue and therefore appear blue. Following IFN-γ treatment, the supernatant was collected to account for cells in the medium, cells were trypsinized and resuspended in phosphate-buffered saline (PBS) and combined with supernatant. Trypan blue was mixed

with cell suspension in a 1:1 ratio and cells were counted using a hemacytometer. Viability was reported as a percent of unstained cells vs. total cells. Cell viability was normalized against the untreated control.

### *2.5. Cell transfection with siRNA*

A total of $1x10^5$ or $5x10^5$ HeLa cells were plated in a 25-$cm^2$ tissue culture flask in D-MEM + 10% FBS and 50 or 100 ng/mL IFN-γ for RNA isolation (*Section 2.7.1)* and docetaxel treatment (*Section 2.8.1)*, respectively. Different plating densities were chosen for different experiments depending on the time allowed for transfection. For 5-FUdR treatment, approximately $1.5x10^6$ HeLa cells were plated in a 75- $cm^2$ tissue culture flask in D-MEM 10% FBS and 100 ng/mL IFN-γ (*Section 2.8.1)*. Cells were incubated for 18 hours to allow IFN-γ to up-regulate IDO mRNA expression.

Transfections were performed using Oligofectamine Reagent (Invitrogen, Burlington, ON), cationic liposomal formulation, according to the manufacturer's instructions. Oligofectamine was diluted in serum-free D-MEM. Ten μL and 28 μL of Oligofectamine were used per 25-$cm^2$ and 75-$cm^2$ tissue culture flask, respectively. Concomitantly, siRNAs were diluted in serum-free D-MEM. siRNA:Oligofectamine Reagent complexes were formed by addition of diluted Oligofectamine to diluted siRNA in serum-free D-MEM to obtain a 5X siRNA concentration. These were mixed gently and incubated for 20 minutes at room temperature to allow incorporation of siRNA into cationic liposomes. Tissue

culture medium was aspirated from each flask and siRNA:Oligofectamine Reagent complexes and serum-free D-MEM were added in a 1:4 ratio to dilute siRNA to desired 1X siRNA concentrations for transfection (5, 10, 25, 50, or 100 nM siRNA). Higher siRNA concentrations were chosen based on previously published literature (Pandyra *et al.*, 2007), whereas lower siRNA concentrations were chosen based on observations throughout this study. A total transfection volume of 2.5 mL or 6 mL was used per 25-$cm^2$ or 75-$cm^2$ tissue culture flask, respectively. For combination studies, the total concentration of siRNA was kept constant at 10 nM, 25 nM, 50 nM or 100 nM, regardless of the concentration of TS or IDO siRNA, by using control siRNA (unless otherwise indicated). Cells were incubated at 37°C in a humidified 5% $CO_2$ atmosphere.

Four hours after transfection, either FBS levels were restored to 10% and 50 or 100 ng/mL IFN-γ was added for RNA isolation 24 and 48 hours later (*Section 2.6.1*) or cells were replated at $9x10^4$ cells per 25-$cm^2$ tissue culture flask or 1200 cells per well in a 96-well plate and incubated for 18 hours before 5-FUdR and docetaxel treatment, respectively (*Section 2.8.1*).

## *2.6. TS and IDO mRNA quantification*

### *2.6.1. RNA Isolation*

Total RNA was collected from cells treated with IFN-γ (*Section 2.3*) or transfected with siRNA (*Section 2.5*) to determine TS and IDO mRNA levels. Cells were lysed using TRIzol® Reagent (Invitrogen, Burlington, ON) at the

times indicated. RNA was extracted from lysed cells according to the manufacturer's protocol. Cells were lysed directly in a tissue culture dish with 1 mL of TRIzol® Reagent. Samples were incubated for 5 minutes at room temperature to allow complete nucleoprotein complex dissociation. Two hundred µL of chloroform were added to each sample followed by vigorous shaking for 10 – 15 seconds and 5 minute incubation at room temperature to allow phase separation. Samples were then centrifuged at 12 000 x g for 15 minutes at 4°C. The upper aqueous phase (~350 µL) containing RNA was removed and transferred to a new microcentrifuge tube containing 500 µL of isopropyl alcohol. Samples were incubated for 1 hour at -20°C and centrifuged at 12 000 x g for 10 minutes at 4°C. The supernatant was removed and 1 mL of 75% ethanol was used to wash the RNA pellet. Each sample was vortexed and then centrifuged again at 12 000 x g for 7 minutes at 4°C. The supernatant was removed again, and the RNA pellet was allowed to air-dry for 15 minutes and dissolved in 30 µL of RNase –free $H_2O$ by incubating for 10 minutes at 55°C. RNA concentration was quantified by UV-spectrophotometry. RNA was stored at -20°C until further use. RNA isolation protocol used was a modification of a protocol previously used by our laboratory (Berg *et al.*, 2003; Ferguson *et al.*, 1999; Jason *et al.*, 2008).

### 2.6.2. *Reverse transcription of RNA*

One µg of total cellular RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Burlington,

ON) to obtain complementary DNA (cDNA). RNase-free water was added to RNA to obtain a total volume of 10 μL. Ten μL of a reverse transcription master mix were prepared using 4 μL of 5X First-Strand Buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 2 μL 0.1 M dithiothreitol (DTT), 1 μL deoxyribonucleotide (dNTP) mix containing 10 mM dCTP, dATP, dGTP and dTTP, 1 μL random oligodeoxyribonucleotide primers (100 pmoles/μL), 1 μL RNase-free water, and 1 μL MMLV. All reagents were obtained from Invitrogen (Burlington, ON). This master mix was added to the 10 μL of total cellular RNA to prepare a final volume of 20 μL. Each sample was incubated at 25°C for 10 minutes (to allow annealing of primers), 37°C for 60 minutes (to allow the reverse transcriptase to synthesize cDNA), and 95°C for 5 minutes (to inactivate MMLV and to separate the RNA from the cDNA) using a thermal cycler (Eppendorf Mastercycler Gradient; Eppendorf, Westbury, NY). Samples were stored at 4°C or -20°C until further use. This protocol used was a modification of a protocol previously used by our laboratory (Berg *et al.*, 2003; Ferguson *et al.*, 1999; Jason *et al.*, 2008).

### *2.6.3. Quantitative PCR (qPCR)*

qPCR was used to determine the relative levels of TS mRNA or IDO mRNA compared to those of 18S ribosomal RNA (rRNA), which was used as an internal standard. cDNA from the sample containing the highest expected level of target mRNA was used to prepare the standard curves. The standard curves were

composed of serial dilutions: 2X, 1X, 1/2X, 1/4X, 1/8X, 1/16X, 1/32X and a no template control (NTC). For the 18S rRNA and TS standard curves, 4 μL of cDNA were added to 57.2 μL of RNase-free water. For the IDO standard curve, 32 μL of cDNA were added to 29.2 μL of RNase-free water. Subsequent serial dilutions were prepared by diluting 30.6 μL of the previous dilution into 30.6 μL of RNase-free water. RNase-free water alone was used for the NTC (30.6 μL). For 18S rRNA and TS samples, 1 μL of cDNA was diluted in 29.6 μL RNase-free water. For IDO samples, 8 μL of cDNA were diluted in 22.6 μL RNase-free water. A greater volume of cDNA was used for IDO detection because IDO mRNA was less abundant than 18S rRNA and TS mRNA.

Amplification of 18S and TS was performed by multiplexing, which allows assaying for multiple genes in a single well by using gene-specific probes, each with different fluorescent tags with non-overlapping emission spectra. To detect TS mRNA, a forward primer (5'-GGCCTCGGTGTGCCTTT-3'), a reverse primer (5'-GATGTGCGCAATCATGTACGT-3') (Invitrogen, Burlington, ON) and a fluorescent probe (5'-6FAM-AACATCGCCAGCTACGCCCTGC-MGBNFQ-3') (Applied Biosystems, Streetsville, ON) were used. 18S and TS master mix was composed of 35 μL of 2X TaqMan® Universal PCR Master Mix (PE Applied Biosystems, Streetsville, ON), 0.36 μL human TS forward primer (100 μM), 0.36 μL human TS reverse primer (100 μM), 0.14 μL human TS probe (100 μM), and 3.5 μL of 18S rRNA TaqMan® Pre-Developed Assay (VIC) (Applied Biosystems, Streetsville, ON). IDO master mix was composed of 35 μL of 2X TaqMan® Universal PCR Master Mix, 0.9 μL of RNase-free water, and 3.5 μL of IDO mRNA

TaqMan® Gene Expression Assay (FAM) (Applied Biosystems, Streetsville, ON). A total of 39.4 μL of qPCR master mix was added each of the diluted samples and standard curve samples to make a final volume of 70 μL.

Samples were vortexed and centrifuged to ensure all contents were mixed and collected at the bottom of the tube. For each sample, 20 μL were loaded into 3 consecutive wells in a 384-well optically clear plate (Diamed, Mississauga, ON) and the plate was covered and sealed with an optically clear adhesive cover (Diamed, Mississauga, ON). The plate was centrifuged for 5 minutes at 1000 rpm to ensure all samples were collected at the bottom of the wells, and then loaded into the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Streetsville, ON). The plate was incubated at 50°C for 2 minutes, and 95°C for 10 minutes. Following these incubations, samples underwent 40 cycles of 95°C for 15 seconds and 60°C for 1 minute and fluorescence was measured during each cycle. mRNA levels were interpolated from standard curves. TS mRNA and IDO mRNA levels were initially normalized to the corresponding 18S rRNA levels for each sample, then subsequently normalized to either untreated controls or control siRNA-treated cells.

## *2.7. IDO protein measurement*

### *2.7.1. Isolation of total protein*

Total soluble protein from HeLa and SK-MEL-5 cells was collected after 72 hours of treatment with 50 ng/mL IFN-γ to allow IDO protein up-regulation.

Cells were washed once with 5 mL of cold PBS. Five mL of cold PBS were added to each tissue culture flask and cells were removed using a cell scraper. PBS containing cells was transferred to a 15 mL conical tube. Another 5 mL of cold PBS were used to wash remaining cells from flask and were added to the conical tube. The cell suspension was centrifuged at 1 000 rpm for 10 minutes at 4°C. The supernatant was aspirated, cells were resuspended in 100 µL of a cell lysis buffer (0.02 M Tris, pH 7.6; 0.1% SDS, 1% Triton x100, 0.001% EDTA in $H_2O$) and the suspension was transferred into a 1.5 mL microcentrifuge tube. Samples underwent 3 freeze-thaw cycles of 5 minutes on dry ice and 5 minutes in a 37°C water bath, followed by sonication for 10 seconds using a Vibra-Cell™ ultrasonic processor (Sonics & Materials Inc., Newtown, CT). Cell lysates were centrifuged at 12 000 rpm for 15 minutes at 4°C to pellet the cell debris. The supernatant, containing the total soluble cellular protein fraction, was transferred to a new microcentrifuge tube. Protein was stored at -20°C until further use.

### *2.7.2. Quantification of total protein*

Concentration of total protein in cell lysates collected in *Section 2.7.1* was determined using a Bradford assay, following the manufacturer's instructions (BioRad, Hercules, CA). The Bio-Rad Bradford assay determines protein concentration based on a shift in absorbance resulting from Coomassie Brilliant Blue G-250 (CBBG) binding to aromatic amino acids and arginine residues (Bradford, 1976). A 20 µg/mL stock solution of bovine serum albumin (BSA)

(Boehringer Mannheim, Indianapolis, IN) in PBS was used to generate a standard protein concentration curve with the following concentrations of BSA: 20 μg/mL, 17.5 μg/mL, 15 μg/mL, 12.5 μg/mL, 10 μg/mL, 7.5 μg/mL, 5 μg/mL and 2.5 μg/mL. PBS alone was used as a blank. Protein samples were diluted 1:500 and 1:1 000 in PBS to a total volume of 700 μL. Three hundred fifty μL of BioRad reagent (diluted 3 mL BioRad reagent: 2 mL PBS) were added to the 700 μL in each standard curve and experimental sample. Samples were vortexed and 300 μL of each were loaded in triplicate into a 96-well plate. The plate was read at a wavelength of 595 nm using a Wallac Victor² multilabel counter (PerkinElmer Wallac, Gaithersberg, MD). Experimental protein concentrations were interpolated from the BSA standard curve.

### *2.7.3. Electrophoresis and blotting*

Twenty μg of total protein were separated using 12% SDS-polyacrylamide electrophoresis gel then transferred onto nitrocellulose membranes (GE Healthcare, Baie-d'Ufre, QC), as previously described (Pandyra *et al.*, 2007). Membranes were blocked using 5% skim milk powder in PBS containing 0.05% Tween 20 for 1 h at 4°C, then probed using one of two primary antibodies: a mouse anti-human IDO antibody (Alexis Biochemicals, Plymouth Meeting, PA) diluted 1:2000, or a rabbit anti-actin antibody (Sigma-Aldrich, Oakville, ON) diluted 1:1000, for one hour at room temperature. Membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies

(GE Healthcare, Baie-d'Ufre, QC) diluted 1:2000 for mouse anti-human IDO antibody, and rabbit anti-actin antibody. A 15-minute wash, followed by two 5-minute washes, was performed between each antibody step using PBS containing 0.05% Tween 20.

### *2.7.4. Immunodetection*

ECL (enhanced chemiluminescence) Plus (GE Healthcare, Buckinghamshire, UK) and X-ray film (Eastman Kodak, Rochester, NY) were used to detect horseradish peroxidase activity. Film was developed and images of film were acquired using a FluorChem 8800 digital image system (Alpha Innotech Corp., San Leandro, CA). Band intensities were quantified by densitometry using Alpha-Ease FC software (Alpha Innotech, San Leandro, CA). Relative IDO protein levels in cell lysates were calculated by dividing IDO densitometric band intensities by those of β-actin.

## ***2.8. Drug sensitivity assay***

### *2.8.1. Drug Treatment*

HeLa cells transfected with TS, IDO or non-targeting siRNAs (*Section 2.5)* were treated with 5-FUdR (dissolved in $H_2O$) (Sigma Chemical Co., St. Louis, MA) or docetaxel (Aventis; purchased from the LRCP Pharmacy, London, ON) and drug sensitivity was determined.

A total of $9x10^4$ previously transfected cells were plated in a 25- $cm^2$ tissue culture flask in D-MEM containing 10% FBS and 100 ng/mL IFN-γ. Cells were incubated for 18 hours at 37°C in a 5% $CO_2$ atmosphere, and then treated with 5-FUdR at concentrations of 0.25, 0.5, 1, 2, 5, 10, 20, and 40 nM. Concentrations were chosen based on preliminary experiments involving 5-FudR concentrations between 0.25 nM and 10 μM. Cell number was measured using a cell proliferation assay 4 days after drug treatment (*Section 2.8.2*).

Twelve hundred previously transfected cells were plated in each well of a 96-well plate in D-MEM containing 10% FBS and 100 ng/mL IFN-γ. Cells were incubated for 18 hours at 37°C in a humidified 5% $CO_2$ atmosphere, and then treated with docetaxel at concentrations of 0.25, 0.5, 0.75, 1, 2 and 3 nM. Docetaxel concentrations were chosen based on previously published data (Pandyra *et al.*, 2007). Cell density was measured using an alamarBlue™ assay 4 days after docetaxel treatment (*Section 2.8.2*).

### *2.8.2. Cell proliferation assay*

Cells were counted on day 0 and day 4 of 5-FUdR treatment using a particle counter (Coulter Electronics, Hialeah, FL) to determine drug sensitivity. Fold increase in cell number, reflecting cell proliferation, was calculated by subtracting the values obtained on day 0 from those from day 4, then dividing by the value on day 0. Cell proliferation was normalized to cells not treated with 5-FUdR and a dose response curve was generated. The protocol used was a

modification of a protocol previously used by our laboratory (Pandyra *et al.*, 2007; Ferguson *et al.*, 1999; Jason *et al.*, 2008).

### *2.8.3. AlamarBlue™ fluorescence assay*

Cell density was determined after 4 days of continuous docetaxel treatment using an alamarBlue™ (Biosource Europe, Nivelles, Belgium) fluorescence assay. Medium was removed from the 96-well plate, leaving 100 µL of medium in each well. alamarBlue™ was diluted in D-MEM + 10% FBS 1:11. One hundred µL of diluted alamarBlue™ was added to each well, resulting in a final concentration of 4%. The plate was incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 3.5 hours. Samples were excited at a wavelength of 544 nm and fluorescence was measured at a wavelength of 580 nm using a Wallac Victor² multi-label counter (PerkinElmer Wallac, Gaithersberg, MD). Resulting fluorescence reflects cell density and was used as an estimation of cell proliferation. Values were normalized to no docetaxel treatment for each transfection and a dose response curve was generated. The protocol used was a modification of a protocol previously used by our laboratory (Berg *et al.*, 2003; Pandyra *et al.*, 2007; Ferguson *et al.*, 1999).

### *2.8.4. $IC_{50}$ determination*

From the dose response curves generated in *Sections 2.8.2* and *2.8.3*, $IC_{50}$ (concentration of drug that inhibited proliferation by 50%) was determined by interpolation of the plotted data (Ferguson *et al.*, 1999).

## *2.9. Statistical analysis*

Data from experiments where $n$ data points were measured is presented as means of $n \pm$ S.E. (calculated using Student $t$-tests). One way ANOVA analysis and Tukey's tests were used to determine significant differences among means. A level of significance for all analyses was chosen to be $p<0.05$ prior to statistical analysis.

# CHAPTER 3: RESULTS

## *3.1. Effects of IFN-γ on IDO and TS mRNA and protein levels and HeLa cell viability*

### *3.1.1. Requirement of IFN-γ-induced IDO*

IFN-γ was used to induce IDO mRNA to detectable levels. To confirm that IDO mRNA levels prior to IFN-γ treatment were not sufficient to detect and quantify by qPCR, IDO mRNA as analyzed by qPCR using cDNA derived from untreated HeLa cells (Figure 3.1.1). Amplification of cDNA did not occur until late cycle numbers, confirming that IDO mRNA is expressed at relatively low levels without IFN-γ induction. To confirm that RNA was present and that mRNA was detectable by qPCR, 18S rRNA was amplified in parallel to IDO mRNA (Figure 3.1.1).

### *3.1.2. IFN-γ induction of IDO mRNA and protein*

IFN-γ-induced IDO expression in HeLa cells has been well established and studied in the literature (Takikawa *et al.*, 1988; Lob *et al.*, 2009b). To confirm that IFN-γ increased IDO mRNA and protein levels in HeLa cells, these were measured in cells treated with 50 ng/mL of IFN-γ. A second cell line, human melanoma cancer (SK-MEL-5), was used to establish if IFN-γ had the same effect on IDO mRNA and protein levels in other models. qPCR analysis of IDO mRNA

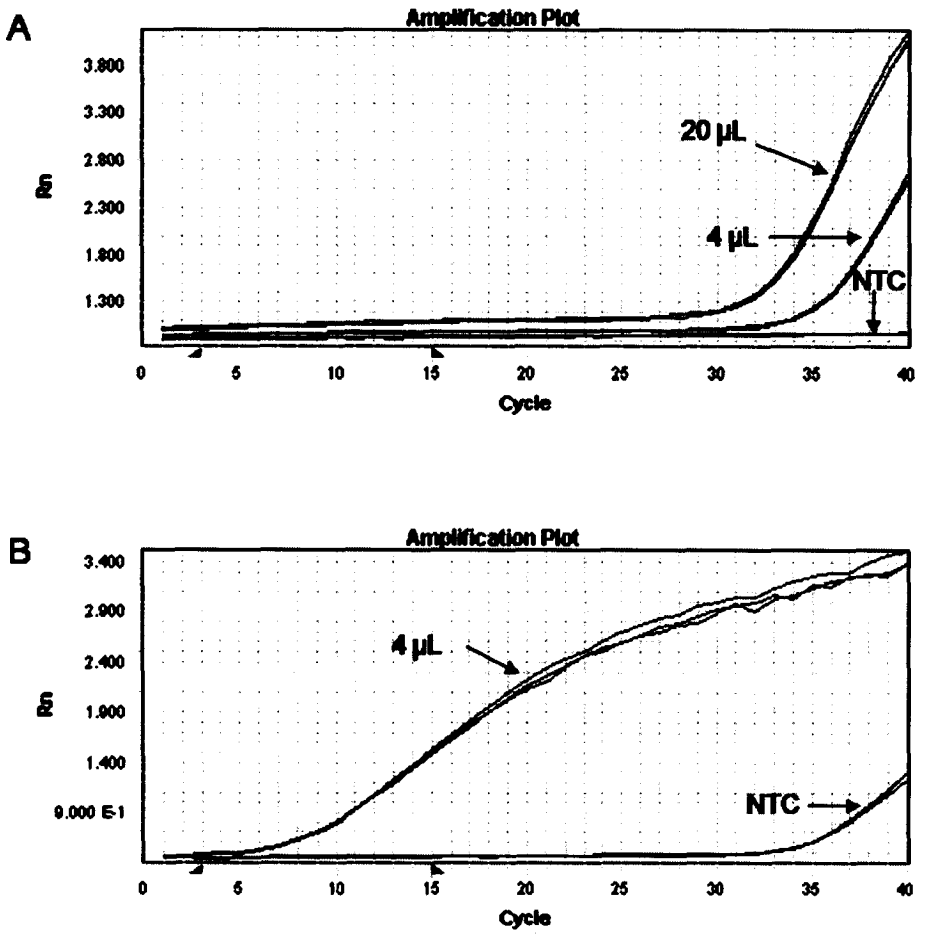


**Figure 3.1.1. qPCR amplification of IDO mRNA and 18S rRNA in untreated HeLa cells.** RNA was extracted from cells, and converted to cDNA. (A) IDO mRNA levels and (B) 18S rRNA levels were estimated by reverse transcription followed by qPCR using 20 µL or 4 µL of cDNA or no template control (NTC), as described in Materials and Methods. The coloured lines represent amplification of cDNA. $R_n$ (the normalized reporter) is the fluorescence emission intensity.

levels in HeLa and SK-MEL-5 cells after IFN-γ (50 ng/mL) treatment showed that IDO mRNA levels increased to detectable levels 48 hours after treatment (Figure 3.1.2). Prior to IFN-γ treatment, IDO mRNA levels were undetectable in HeLa cells; however, IFN-γ significantly increased IDO mRNA levels ($p<0.05$). In SK-MEL-5 cells, baseline IDO mRNA levels were detectable; however, IFN-γ significantly up-regulated IDO mRNA levels by 5 times ($p<0.05$). Immunoblot analysis of the lysates from IFN-γ treated HeLa and SK-MEL-5 cells demonstrated that 72 hours of IFN-γ (50 ng/mL) treatment were sufficient to increase IDO protein to detectable levels ($p<0.05$; Figure 3.1.3). Therefore, IFN-γ treatment can be used to increase IDO mRNA and protein levels prior to targeting with IDO siRNA.

To determine the extent of IDO mRNA up-regulation with increasing concentrations of IFN-γ, HeLa cell IDO and TS mRNA levels were analyzed by qPCR after 18 hours of IFN-γ at 25, 50 and 100 ng/mL and a dose-response curve was generated. IDO mRNA levels increased by IFN-γ treatment and this effect was concentration dependent (Figure 3.1.4A). Higher concentrations of IFN-γ (100-400 ng/mL) also resulted in a similar trend (Figure 3.1.4B). As IFN-γ concentrations were increased from 25 ng/mL to 400 ng/mL, IDO mRNA levels also increased.

qPCR analysis of TS mRNA revealed that they remained unchanged after 18 hours of IFN-γ treatment ($p>0.05$; Figure 3.1.5A). Consistent with these results, higher IFN-γ concentrations did not alter TS mRNA levels after 18 hours of treatment with IFN-γ ($p>0.05$; Figure 3.1.5B). A concentration of 50 ng/mL or

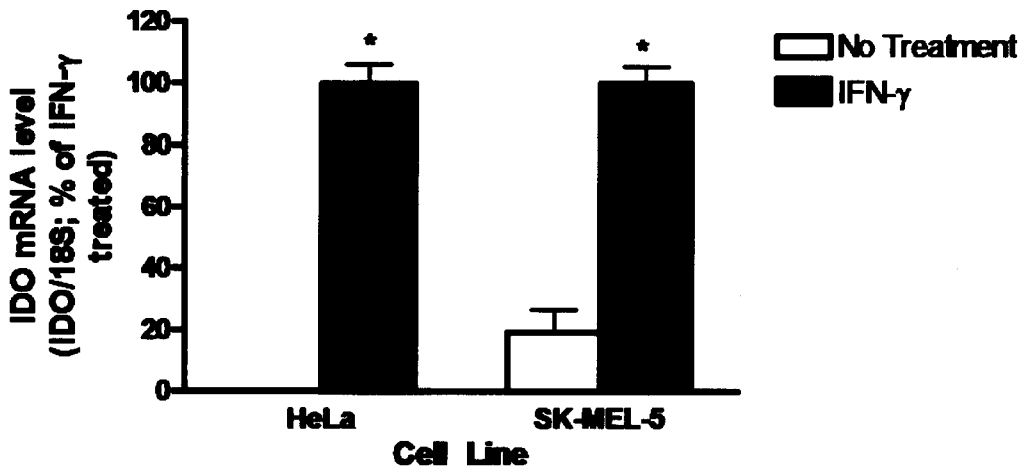


**Figure 3.1.2. IDO mRNA levels are up-regulated with IFN-γ treatment in HeLa and SK-MEL-5 cells.** Cells were treated with IFN-γ (50 ng/mL) for 48 hours. RNA was extracted, and converted to cDNA. IDO mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. White represents untreated cells, black represents IFN-γ treated cells. Results are plotted as a percentage ± SEM of IFN-γ treated cells (n=3). * significantly different from untreated cells ($p<0.05$). The experiment was performed three times in triplicate.

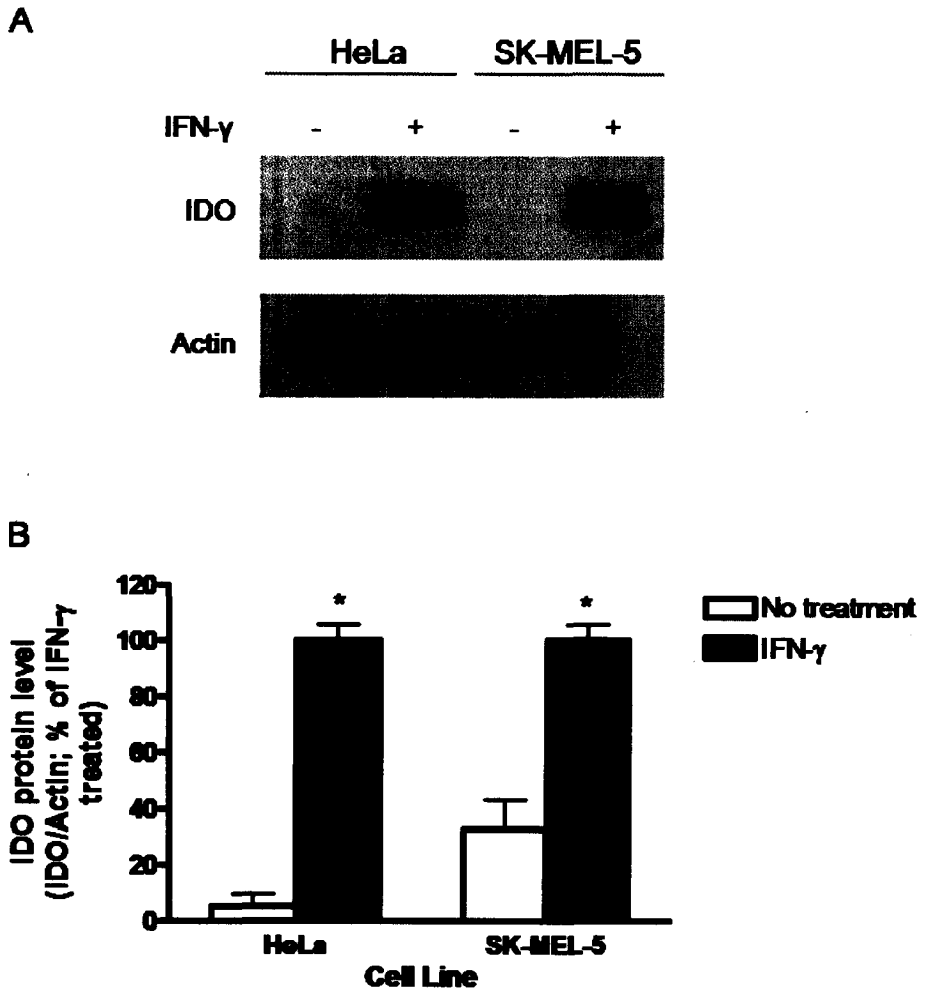


**Figure 3.1.3. IDO protein levels are up-regulated with IFN-γ treatment in HeLa and SK-MEL-5 cells.** Cells were treated with IFN-γ (50 ng/mL) for 72 hours. Protein was released from cells by sonication and immunoblotting was performed to determine IDO levels using anti-IDO antibody. (B) Data from (A) immunoblot was quantified by densitometry as IDO signal/Actin signal, as described in Materials and Methods. White represents untreated cells, black represents IFN-γ treated cells. Results are plotted as a percentage ± SEM of IFN-γ treated cells (n=2). * significantly different from untreated cells ($p<0.05$). The experiment was performed two times in triplicate.

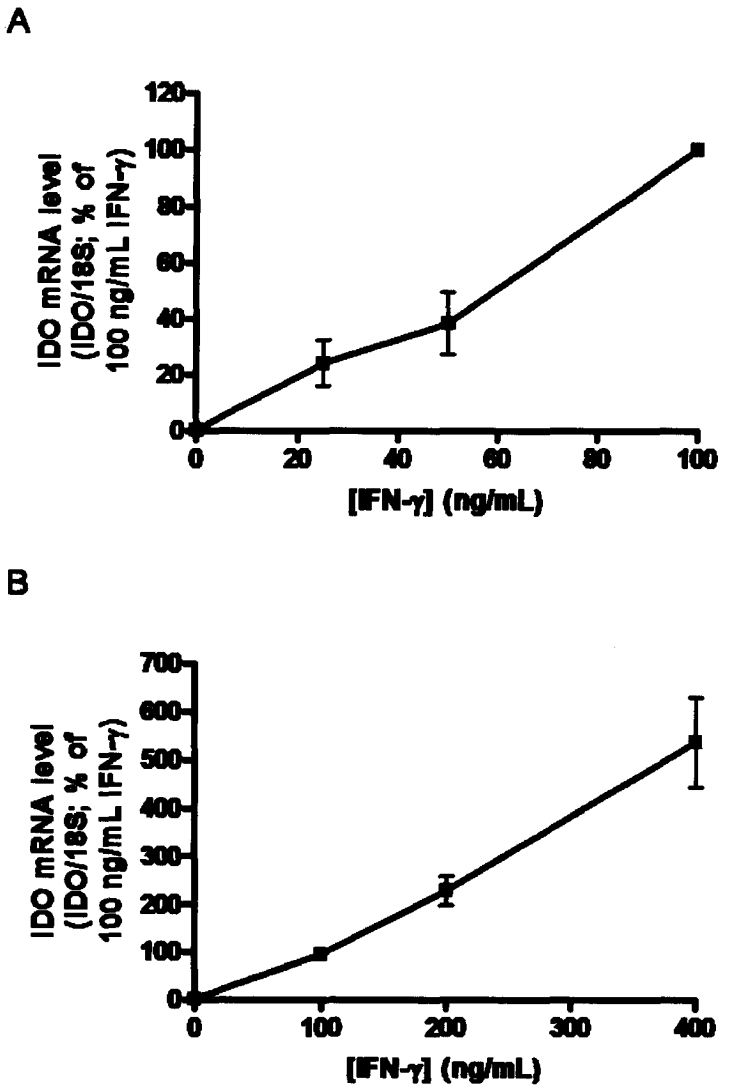


**Figure 3.1.4. IDO mRNA levels are up-regulated in HeLa cells with increasing IFN-γ concentrations.** HeLa cells were treated with IFN-γ at (A) 25, 50 or 100 ng/mL or (B) 100, 200 or 400 ng/mL for 18 hours. IDO mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of IDO mRNA expression at 100 ng/mL (n=3). The experiment was performed three times in triplicate.

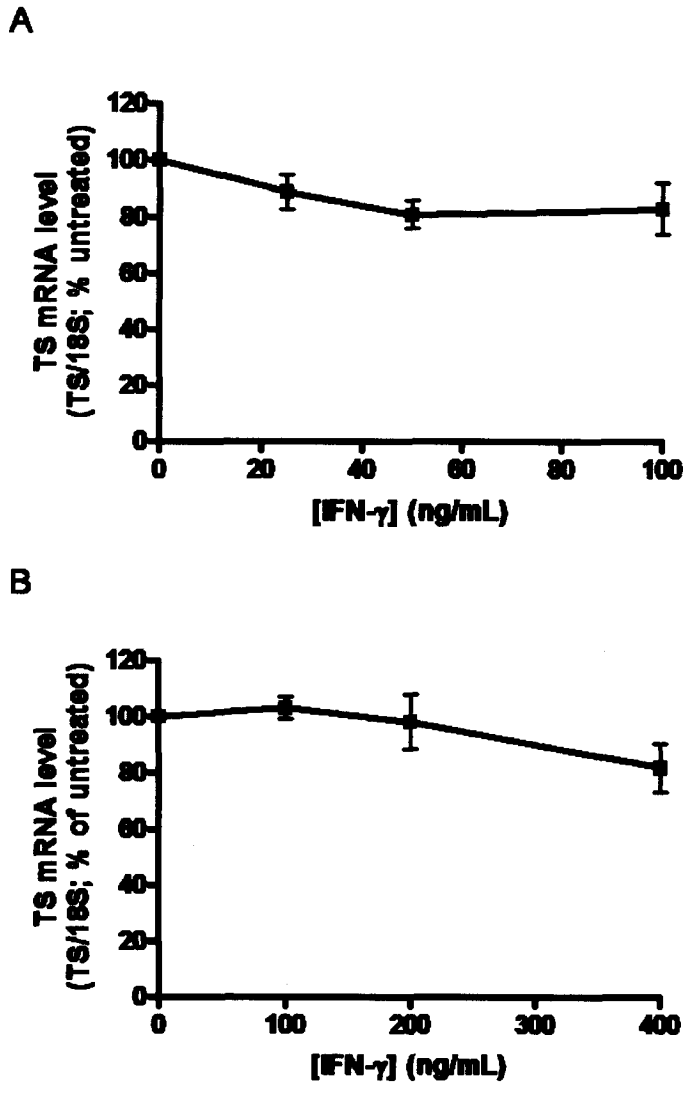


**Figure 3.1.5. TS mRNA levels are not affected by increasing IFN-γ concentrations.** HeLa cells were treated with IFN-γ at (A) 25, 50 or 100 ng/mL or (B) 100, 200 or 400 ng/mL for 18 hours. TS mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). The experiment was performed three times in triplicate.

100 ng/mL of IFN-γ was chosen for subsequent experiments because these concentrations increased IDO mRNA levels to those detectable by qPCR but had no apparent effect on TS mRNA levels in HeLa cells after 18 hours.

A time course experiment was carried out to determine the duration of IFN-γ-induced-IDO mRNA and at approximately which time point IDO mRNA was maximally up-regulated without further addition of IFN-γ. qPCR was used to estimate IDO mRNA levels in HeLa cells treated with 50 ng/mL of IFN-γ for 0, 4, 8, 12, 24, and 48 hours after treatment. IDO mRNA levels were significantly increased by 4 hours after IFN-γ treatment ($p<0.05$; Figure 3.1.6). At the experimental time points, IDO mRNA levels peaked at approximately 24 hours after IFN-γ treatment (Figure 3.1.6), consistent with previous reports of maximal IDO mRNA up-regulation occurring 12-18 hours after IFN-γ treatment (Takikawa *et al.*, 1988). Maximal up-regulation significantly decreased by 48 hours after IFN-γ treatment, however, IDO mRNA levels were still significantly higher than untreated cells ($p<0.05$; Figure 3.1.6). From this it was determined that IFN-γ exerted its effect within 12-24 hours and that IFN-γ levels must be maintained every 24-48 hours. TS mRNA levels were not affected between 0-24 hours of 50 ng/mL IFN-γ treatment; however, after 48 hours of treatment, TS mRNA levels were significantly lower than TS mRNA levels in cells not treated with IFN-γ ($p<0.05$; Figures 3.1.7A and 3.1.7B ).

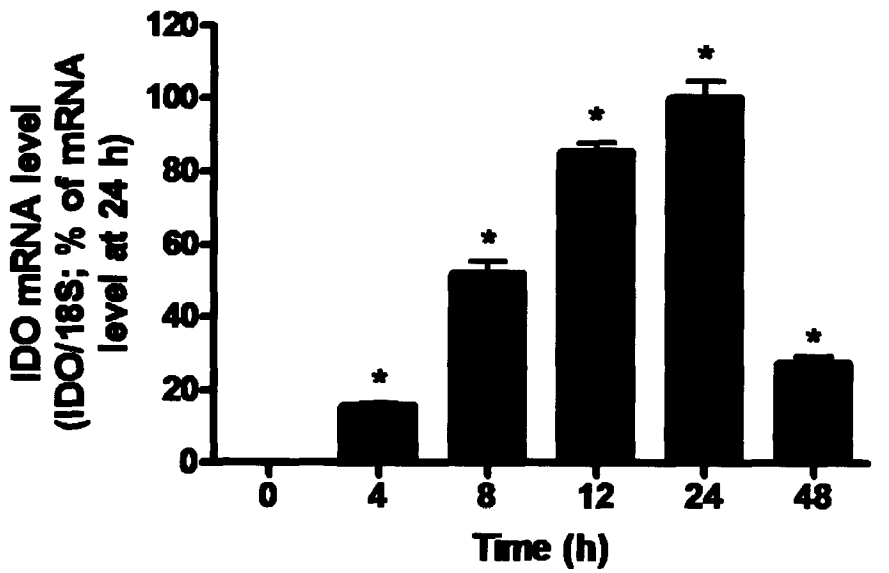


**Figure 3.1.6. Time course of IDO mRNA levels upon treatment with IFN-γ in HeLa cells.** Cells were treated with IFN-γ (50 ng/mL) for up to 48 hours. IDO mRNA levels were estimated in cells at the times indicated on the abscissa. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of the mRNA level at 24 h (n=3). * significantly different from untreated cells ($p<0.05$). The experiment was performed three times in triplicate.

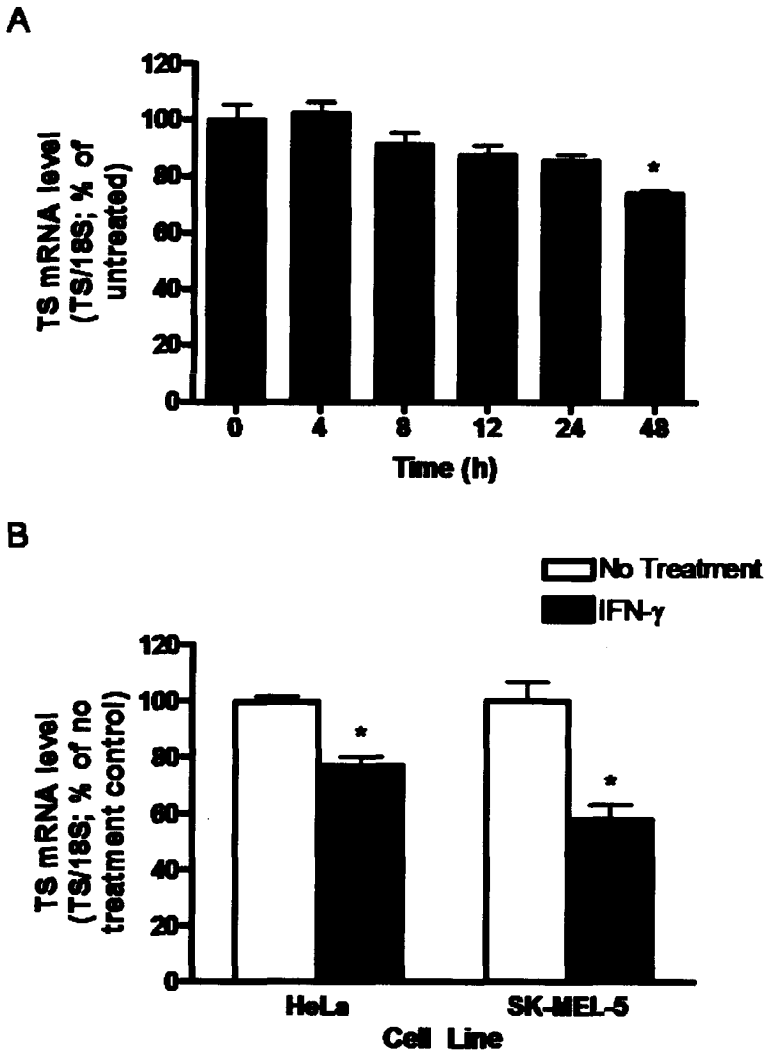


**Figure 3.1.7. Effects of IFN-γ on TS mRNA levels in HeLa and SK-MEL-5 cells.** Cells were treated with IFN-γ (50 ng/mL) for up to 48 hours. TS mRNA levels were estimated (a) in HeLa cells at times between 0 and 48 hours and (B) in HeLa and SK-MEL-5 cells at 48 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. White represents untreated cells, black represents IFN-γ treated cells. Results are plotted as a percentage ± SEM of the mRNA level of untreated cells (n=3). * significantly different from untreated cells ($p<0.05$). The experiment was performed three times in triplicate.

### *3.1.3. Effect of IFN-γ on HeLa cell viability*

It was important to determine if IFN-γ treatment had adverse side effects on cell viability. HeLa cell viability was assessed using a Trypan blue exclusion assay 24 and 48 hours after IFN-γ treatment with concentrations of 25, 50 and 100 ng/mL IFN-γ. There was no apparent affect of IFN-γ on HeLa cell viability at 24 and 48 hours, regardless of the IFN-γ concentration used ($p>0.05$; Figure 3.1.8). The number of cells after IFN- γ treatment (used to calculate fractional changes) was also not significantly different from controls (data not shown). From this it was determined that a concentration of IFN-γ between 25 and 100 ng/mL could be used without affecting cell viability. Concentrations of 50 and 100 ng/mL were used in subsequent experiments.

### *3.1.4. IDO siRNA can prevent and knock down IFN-γ-induced IDO mRNA levels*

To confirm that IDO siRNA can prevent IFN-γ-mediated induction of IDO mRNA, cells were transfected with IDO siRNA and subsequently treated with IFN-γ. qPCR was performed to analyze IDO mRNA levels. HeLa cells pretreated with IDO siRNA showed a 93% smaller increase in IDO mRNA levels upon IFN-γ treatment ($p<0.05$; Figure 3.1.9A). Therefore, treatment with IDO siRNA prior to IFN-γ treatment prevented the IFN-γ-induced increase in IDO mRNA levels ($p<0.05$; Figure 3.1.9A). Of note, transfecting HeLa cells with non-targeting

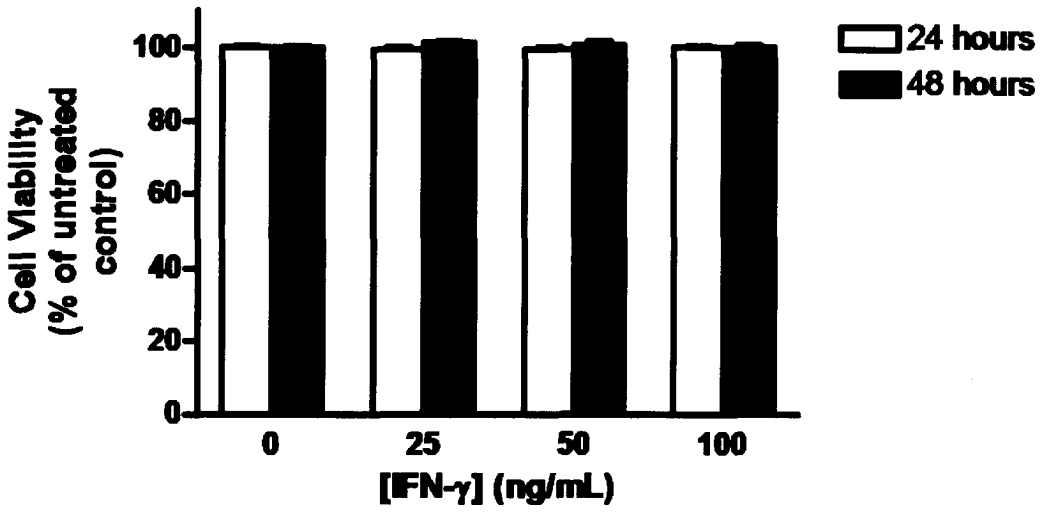


**Figure 3.1.8. IFN-γ treatment at concentrations between 0 ng/mL and 100 ng/mL does not affect HeLa cell viability after 24 and 48 hours.** HeLa cells were treated with 25, 50 or 100 ng/mL of IFN-γ and cell viability was assessed at 24 and 48 hours using a Trypan blue exclusion assay, as described in Materials and Methods. White represents 24 hours, black represents 48 hours. Results are plotted as a percentage ± SEM of no treatment control (n=3). There was no significant decrease in cell viability with 0 to 100 ng/mL of IFN-γ at 24 and 48 hours. The number of cells after IFN-γ treatment (used to calculate fractional changes) was also not significantly different from controls. The experiment was performed three times in triplicate.

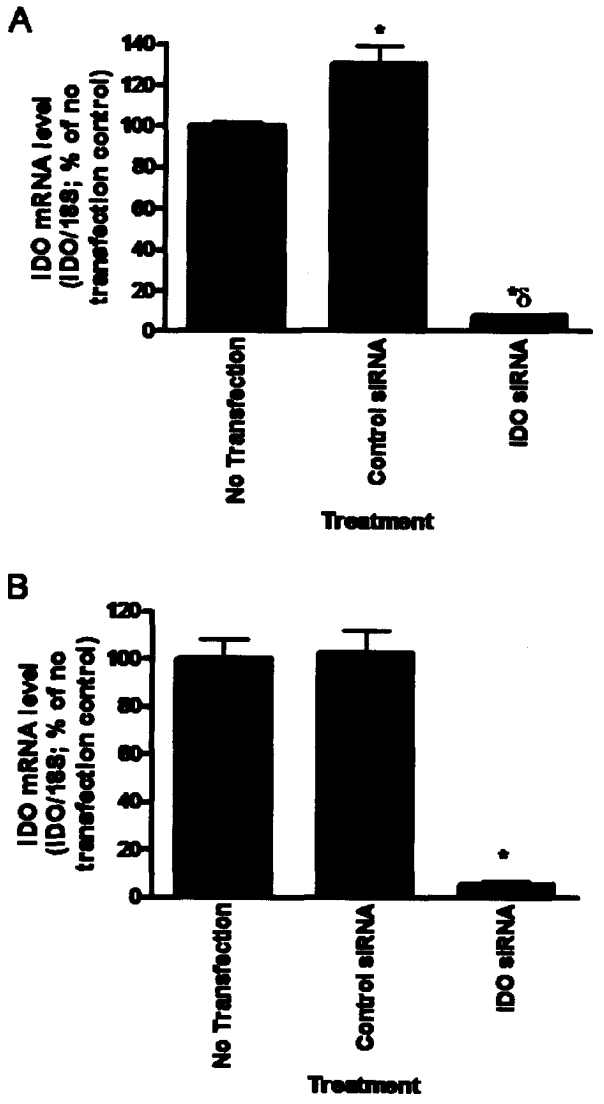


**Figure 3.1.9. IDO siRNA can (A) prevent and (B) knock-down IFN-γ-induced IDO mRNA in HeLa cells.** Cells were (A) untreated or (B) pre-treated with IFN-γ (100 ng/mL) for 18 hours. Cells were then transfected with 25 nM IDO siRNA for 24 hours. IDO mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no transfection control (n=3). * significantly different from untreated cells ($p<0.05$). δ significantly different from control siRNA treated cells ($p<0.05$). The experiment was performed three times in triplicate.

control siRNA prior to IFN-γ treatment resulted in a 30% increase in IDO mRNA levels ($p<0.05$; Figure 3.1.9A).

To determine if IDO siRNA could knock down IFN-γ-induced IDO mRNA, HeLa cells were treated with IFN-γ to up-regulate IDO mRNA levels prior to transfection with IDO siRNA. qPCR analysis of IDO mRNA levels revealed that transfecting HeLa cells with IDO siRNA after IFN-γ treatment also resulted in a 93% decrease in IDO mRNA levels, compared to untransfected cells ($p<0,05$; Figure 3.1.9B).

## 3.2. *Capacity of TS and IDO siRNAs to reduce target mRNA levels*

### *3.2.1. Effect of control siRNAs on TS and IDO mRNA levels in HeLa cells*

To determine the effect of non-targeting control siRNAs on TS and IDO mRNA levels in HeLa cells, cells were transfected with 5 or 10 nM of non-targeting control siRNAs 1, 2, 3 and 4. Controls 1 and 2 are siGENOME technology, whereas controls 3 and 4 are ON-TARGET*plus* technology. Newer ON-TARGET*plus* reagents are modified to enhance specificity, according to Dharmacon's proprietary technology; however, these modifications were not provided. qPCR analysis of TS and IDO mRNA levels 24 hours after transfection suggested that different non-targeting control siRNAs had different effects on mRNA levels. TS mRNA levels were significantly reduced by approximately 30% with both 5 and 10 nM control siRNA 1 compared to untreated cells ($p<0.05$;

Figure 3.2.1A). Although control siRNA 3 at 5 nM did not affect TS mRNA levels, 10 nM control siRNA 3 reduced TS mRNA levels by 30% compared to untreated cells ($p<0.05$; Figure 3.2.1A). Control siRNA 2 and 4 at both 5 and 10 nM did not affect TS mRNA levels ($p>0.05$; Figure 3.2.1A).

Determination of IDO mRNA levels by qPCR also revealed that different non-targeting siRNAs had different effects on IDO mRNA levels in HeLa cells. Control siRNA 1 significantly increased IDO mRNA levels by 50%, compared to untreated cells ($p<0.05$; Figure 3.2.1B). Cells transfected with control siRNA 2 at both 5 and 10 nM siRNA resulted in a trend towards a decrease in IDO mRNA levels; however, this was not significant ($p>0.05$; Figure 3.2.1B). Control siRNAs 3 and 4 did not affect IDO mRNA levels 24 hours after transfection ($p>0.05$; Figure 3.2.1B).

### *3.2.2. Effect of TS siRNA on TS mRNA levels in HeLa cells*

To decrease any off-target effects, non-specific toxicities, and concentration-dependent effects of siRNA, it was important to determine the lowest practical siRNA concentration able to reduce target mRNA levels. Initial experiments were done to test the ability of the siRNA to down-regulate the target mRNA in HeLa cells. TS siRNA alone significantly reduced TS mRNA levels by 90% relative to cells treated with control siRNA, 24 hours after transfection ($p<0.05$; Figure 3.2.2A). The capacity of TS antisense to reduce mRNA levels at varying concentrations was also assessed. TS siRNA

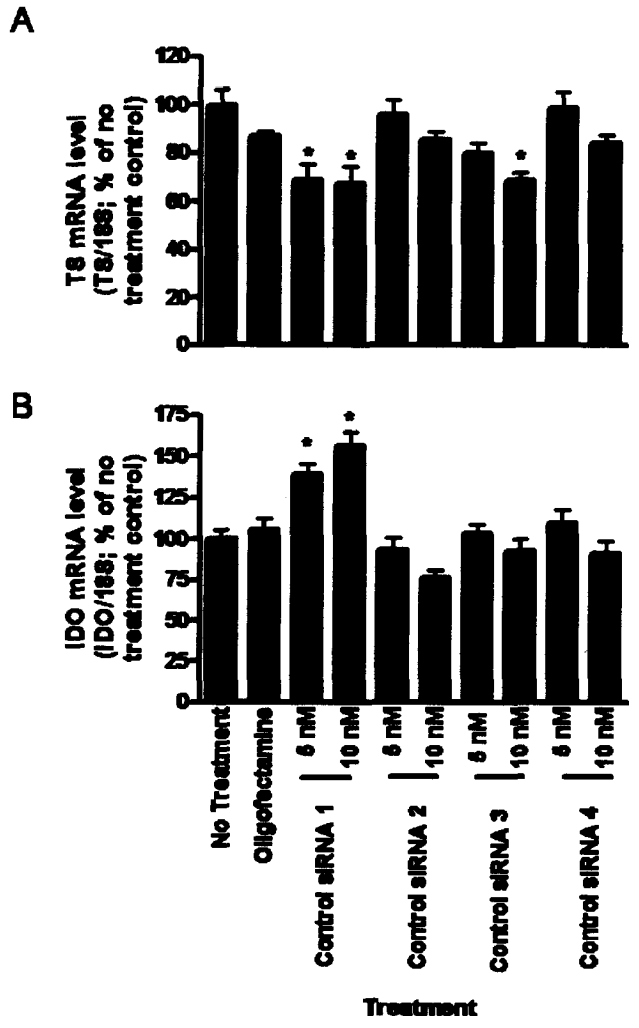


**Figure 3.2.1. TS and IDO mRNA levels in HeLa cells transfected with non-targeting siRNAs.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with control non-targeting siRNAs 1, 2, 3 and 4. Cells were incubated for 24 hours. (A) TS and (B) IDO mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of untreated cells (n=3). * significantly different from cells treated with control siRNA ($p<0.05$). The experiment was performed three times in triplicate.

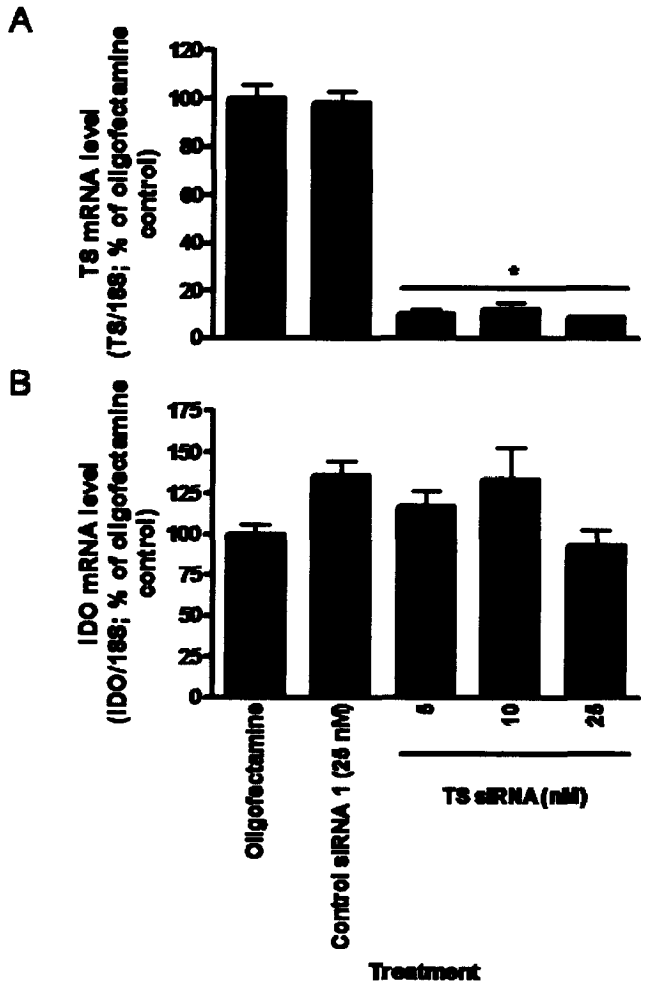


**Figure 3.2.2. siRNA targeting TS at 5, 10 and 25 nM (A) down-regulates TS mRNA to the same extent and (B) does not down-regulate IDO mRNA.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with TS siRNA 5 at varying concentrations. The total concentration of all siRNAs in transfection medium always totaled 25 nM using control siRNA 1. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR. Results are plotted as a percentage ± SEM of cells treated with transfection reagent alone (n=3). * significantly different from cells treated with control siRNA ($p<0.05$). The experiment was performed three times in triplicate.

concentration between 5 and 25 nM reduced TS mRNA levels by 90% and all to the same extent ($p>0.05$; Figure 3.2.2A). TS siRNA successfully decreased TS mRNA in HeLa cells, and 5 nM TS siRNA was sufficient to significantly reduce target mRNA levels. IDO mRNA levels were not affected by TS siRNA treatment, compared to mock transfected cells ($p>0.05$; Figure 3.2.2B).

### *3.2.3. Effect of IDO siRNA on IDO mRNA levels in HeLa cells*

It was also important to establish a low IDO siRNA concentration sufficient to reduce IDO mRNA levels in HeLa cells with minimal off-target effects. IDO siRNA significantly reduced IDO mRNA levels by 90% in HeLa cells compared to cells treated with control siRNA, 24 hours after transfection ($p<0.05$; Figure 3.2.3B). All concentrations tested between 5 and 25 nM reduced IDO mRNA levels to the same extent ($p>0.05$; Figure 3.2.3B). From this experiment, it was determined that 5 nM IDO siRNA was sufficient to significantly reduce IDO mRNA levels. TS mRNA levels were not affected by IDO siRNA treatment, compared to mock transfected cells ($p>0.05$; Figure 3.2.3A).

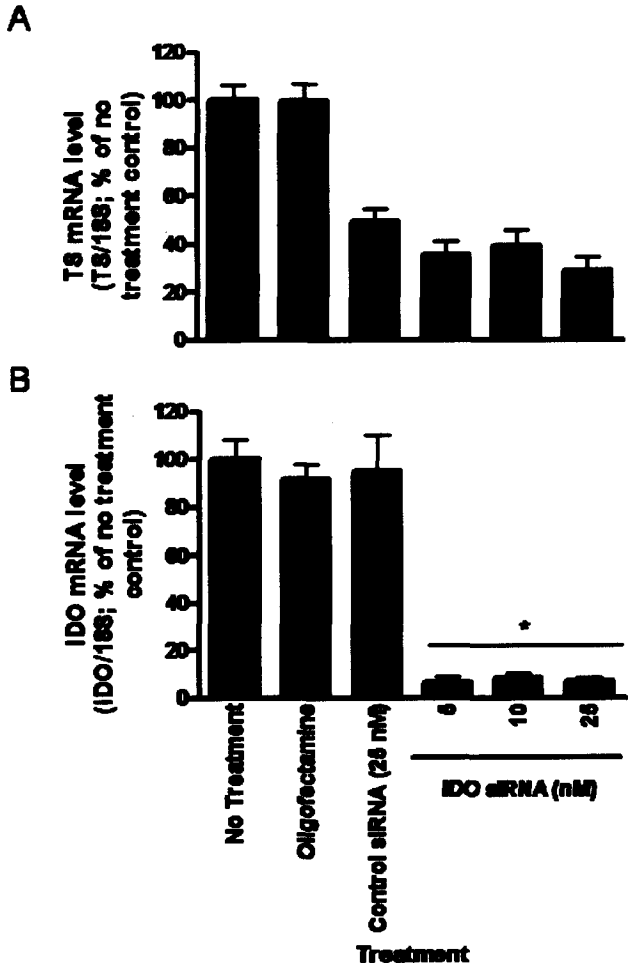


**Figure 3.2.3. siRNA targeting IDO at 5, 10 and 25 nM (A) down-regulates IDO mRNA to the same extent and (B) does not down-regulate TS mRNA.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with IDO siRNA at varying concentrations. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Material and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). * significantly different from cells treated with control siRNA ($p<0.05$). The experiment was performed three times in triplicate.

## *3.3. Capacity of TS and IDO siRNAs to reduce target mRNA levels when used in combination*

### *3.3.1. IDO siRNA does not affect TS mRNA levels or TS siRNA-mediated down-regulation of TS mRNA*

To determine if IDO siRNA affects TS siRNA-mediated knock-down of TS mRNA, qPCR analysis was performed using the cDNA from HeLa cells transfected with different combinations of TS, IDO and non-targeting control siRNAs. Two different constructs of TS siRNA (TS siRNA 4 and TS siRNA 5) at a concentration of 5 nM, administered either alone (Figure 3.3.1) or in combination with 5 nM of control siRNAs 3 or 4 (Figures 3.3.2A or 3.3.2B, respectively), reduced TS mRNA levels by 90-95% 24 hours after transfection ($p<0.05$). Likewise, TS siRNA administered concurrently with 5 nM IDO siRNA (Figures 3.3.1, 3.3.2A and 3.3.2B) still significantly reduced TS mRNA levels to the same extent 24 hours post-transfection ($p>0.05$). Treatment of HeLa cells with IDO siRNA combined with control siRNAs 3 and 4 at lower siRNA concentrations (10 nM total) did significantly reduce TS mRNA levels ($p<0.05$; Figures 3.3.2A and 3.3.2B). In both conditions, this was by approximately 22 - 30%, compared to control siRNAs alone. At the concentrations used, IDO siRNA did not affect TS mRNA levels and TS mRNA knock-down by TS siRNA.

When control siRNA 1 was used to maintain the total siRNA concentration at 10 nM, control siRNA and IDO siRNA had no effect on TS mRNA levels; however, raising the total siRNA concentration to 25 or 50 nM total reduced TS

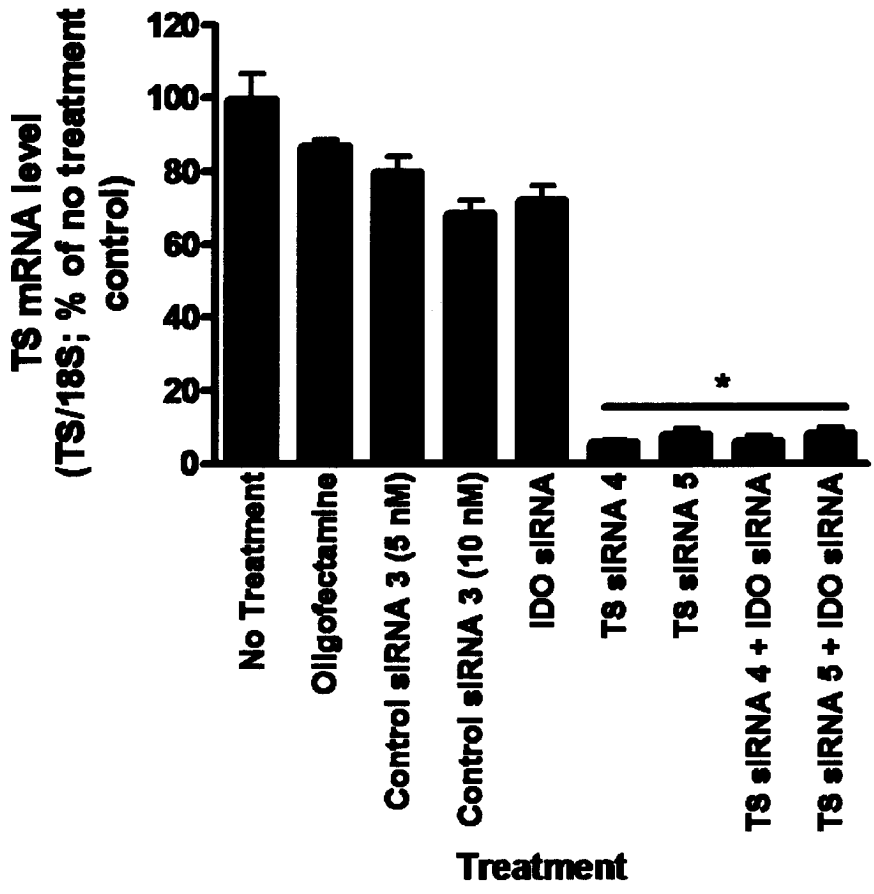


**Figure 3.3.1. siRNA targeting IDO does not affect TS mRNA levels or TS siRNA-mediated down-regulation of TS mRNA.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with IDO siRNA and/or 2 different TS siRNAs. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). * significantly different from cells treated with control siRNA 3 at 10 nM ($p<0.05$). The experiment was performed three times in triplicate.

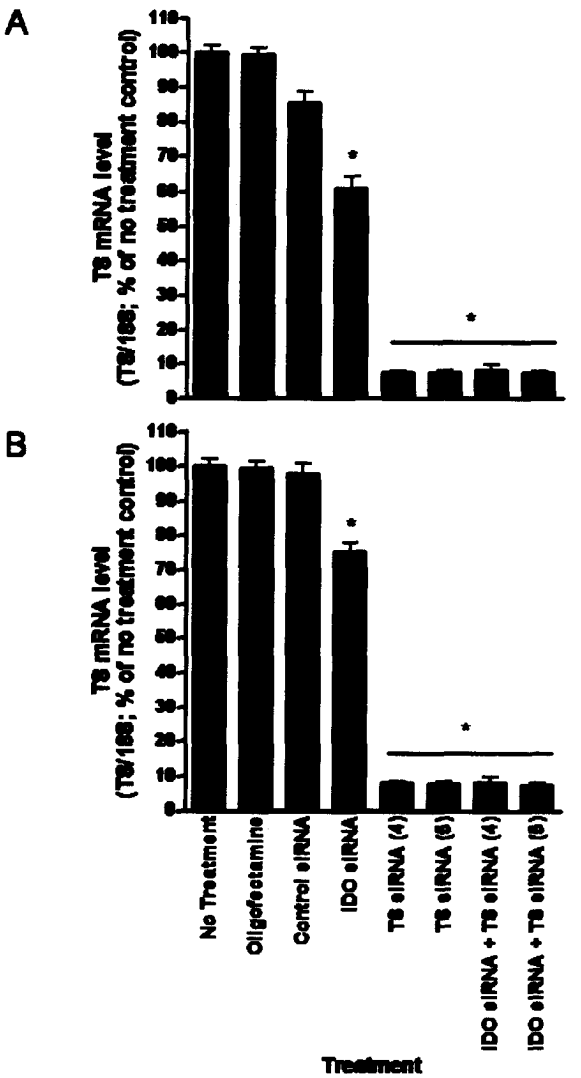


**Figure 3.3.2. IDO siRNA does not affect the capacity of TS siRNA to reduce TS mRNA levels in HeLa cells.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with combinations of non-targeting siRNAs, IDO siRNA or TS siRNAs. (A) Control siRNA 3 or (B) control siRNA 4 were used to maintain the total siRNA concentration at 10 nM. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). * significantly different from cells treated with control siRNA ($p<0.05$). The experiment was performed three times in triplicate.

mRNA levels by 20-40% compared to untransfected cells ($p<0.05$; Figure 3.3.3). There was no significant difference between control siRNA 1 alone or control siRNA 1 combined with IDO siRNA ($p>0.05$), consistent with previous results regarding the effects of control siRNA 1 (Figure 3.2.1), which suggests that the reduction in TS mRNA levels was a result of control siRNA 1 transfection. Regardless of the total siRNA concentration, TS siRNA, when combined with control siRNA, reduced TS mRNA levels by 80-90% ($p<0.05$; Figures 3.2.1, 3.2.2 and 3.3.3). Additionally, combining IDO siRNA with TS siRNA (when total siRNA concentration was 10, 25 and 50 nM) did not affect the ability of TS siRNA to down-regulate TS mRNA ($p>0.05$), consistent with previous results (Figures 3.3.1 and 3.3.2). From this, it was also determined that IDO siRNA did not affect the ability of TS siRNA to decrease TS mRNA levels, and there was a general tendency of siRNAs, when used in concentrations of 25-50 nM, to decrease TS mRNA; however this was not unique to IDO siRNA.

### 3.3.2. *TS siRNA decreases the capacity of IDO siRNA to reduce IDO mRNA levels in HeLa cells*

To determine if TS siRNA affects IDO siRNA-mediated knock-down of IDO mRNA, qPCR analysis was performed using the cDNA from HeLa cells transfected with different combinations of TS, IDO and non-targeting control siRNAs. IDO siRNA at a concentration of 5 nM, when used alone (Figure 3.3.4) or in combination with 5 nM of two non-targeting control siRNAs (control siRNA

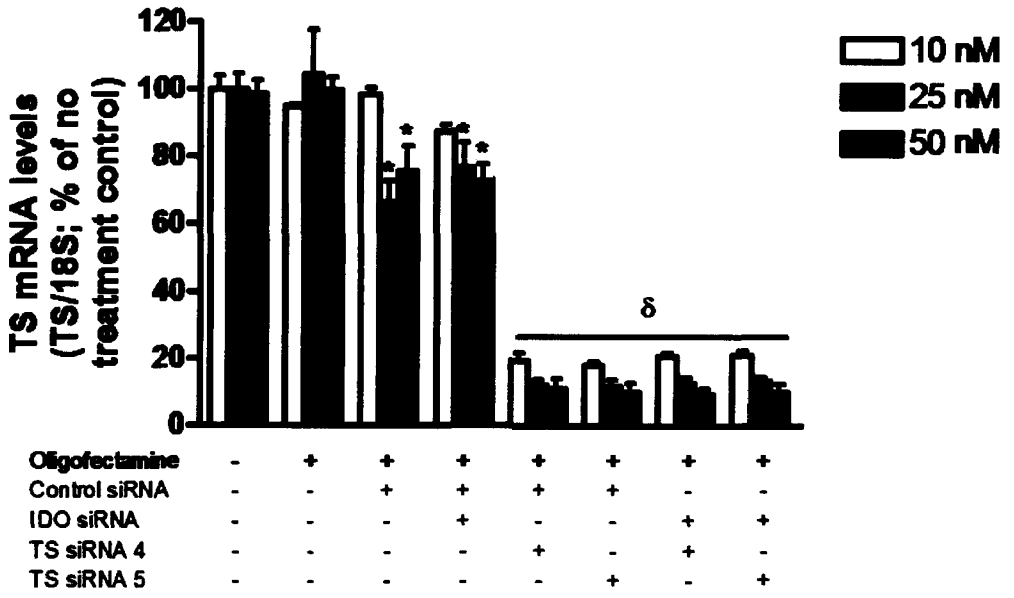


**Figure 3.3.3. IDO siRNA does not affect the capacity of TS siRNA to reduce TS mRNA levels in HeLa cells.** HeLa cells were transfected with combinations of non-targeting siRNA 1, IDO siRNA or TS siRNAs. Control siRNA 1 was used to maintain the total siRNA concentration at 10, 25 or 50 nM. Cells were incubated for 48 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). * significantly different from untreated cells. δ significantly different from untreated cells and cells treated with control siRNA ($p<0.05$). The experiment was performed three times in triplicate.

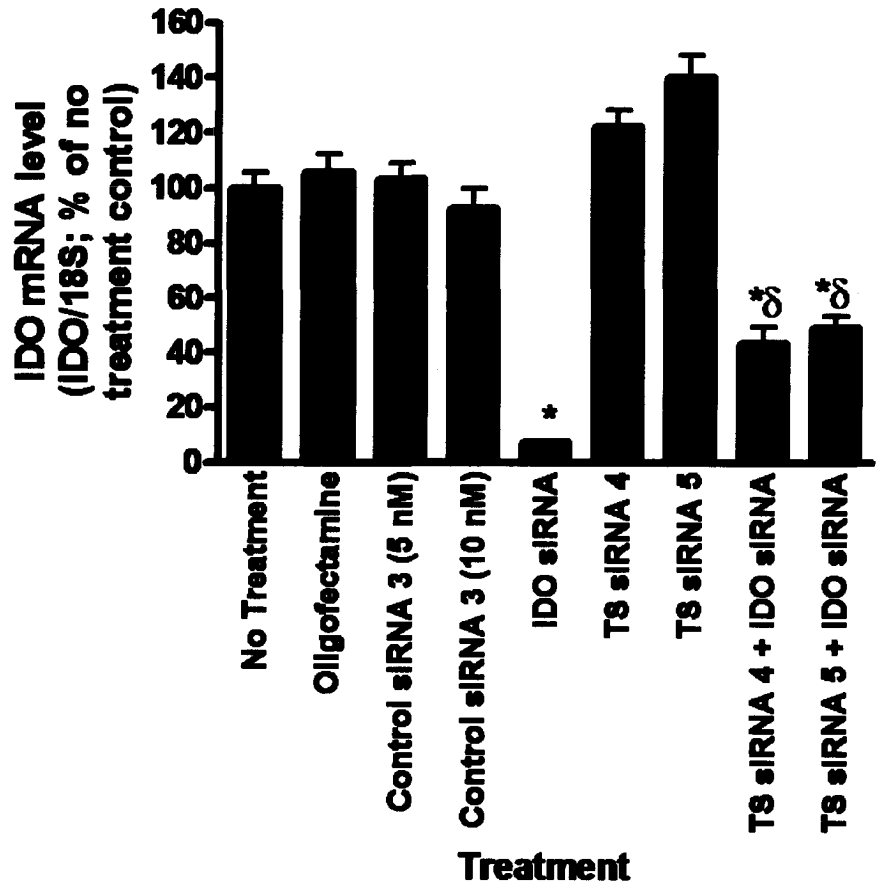


**Figure 3.3.4. TS siRNA antagonizes the capacity of IDO siRNA to reduce IDO mRNA levels in HeLa cells.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with 5 nM or IDO siRNA and/or 5 nM of 2 different TS siRNAs. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). * significantly different from cells treated with control siRNA 3 at 10 nM ($p<0.05$). δ significantly different from cells treated with IDO siRNA ($p>0.05$). The experiment was performed three times in triplicate.

3 and control siRNA 4) (Figures 3.3.5A and 3.3.5B) reduced IDO mRNA levels by 90% ($p<0.05$). However when IDO siRNA was delivered in combination with two different TS targeting siRNAs, IDO mRNA was reduced by only ~60%, resulting in approximately 3-4 times the amount of IDO mRNA in HeLa cells compared to IDO siRNA treatment alone (Figures 3.3.4, 3.3.5A and 3.3.5B). This was significantly different from IDO siRNA administration alone ($p<0.05$). Nevertheless, there was still a significant reduction in IDO mRNA levels compared to control siRNA treatments ($p<0.05$; Figures 3.3.4, 3.3.5A and 3.3.5B). Treatment of HeLa cells with TS siRNA combined with control siRNAs 3 and 4 significantly increased IDO mRNA levels ($p<0.05$, Figures 3.3.5A and 3.3.5B). In both conditions, this was by approximately 60% compared to control siRNAs alone. From this, it was determined that both TS siRNA constructs used decreased the capacity of IDO siRNA to down-regulate IDO mRNA levels.

When control siRNA 1 was used to maintain the total siRNA concentration at 10 nM, control siRNA 1 and TS siRNA had no effect on IDO mRNA levels; however, raising the total siRNA concentration to 25 or 50 nM increased IDO mRNA levels by 2-3.5 times compared to untransfected cells ($p<0.05$; Figure 3.3.6). There was no significant difference between control siRNA 1 alone or control siRNA 1 combined with TS siRNA at high concentrations ($p>0.05$), suggesting that the increase in IDO mRNA levels was a result of control siRNA 1 transfection (consistent with previous conclusions from Figure 3.2.1). Regardless of the total siRNA concentration, cells transfected with IDO siRNA combined with control siRNA 1 displayed a reduction in IDO mRNA levels compared to

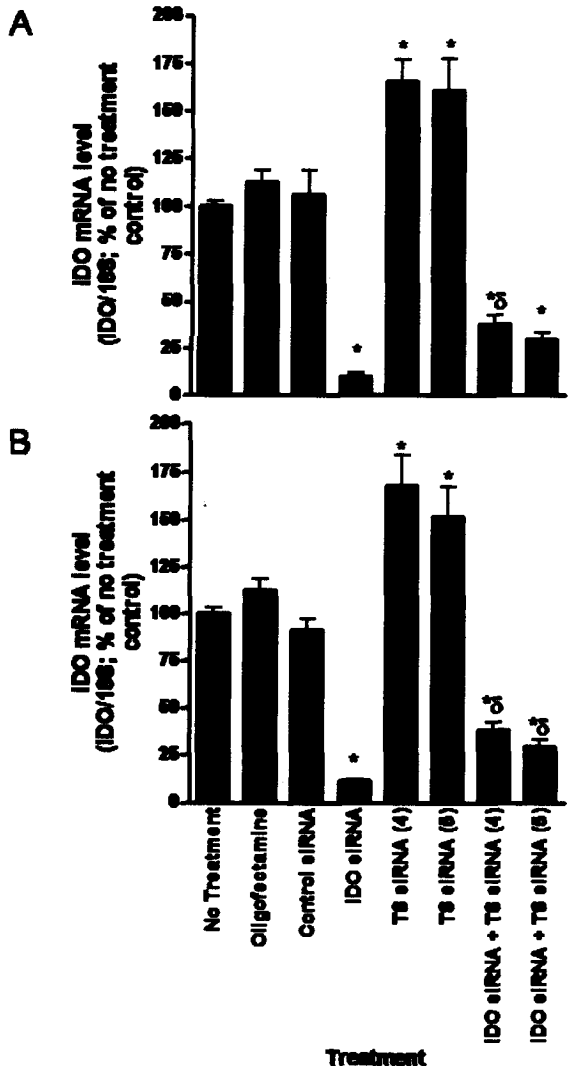


**Figure 3.3.5. TS siRNA antagonizes the capacity of IDO siRNA to reduce IDO mRNA levels in HeLa cells.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with combinations of non-targeting siRNAs, IDO siRNA or TS siRNAs. (A) Control siRNA 3 or (B) control siRNA 4 were used to maintain the total siRNA concentration at 10 nM. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). * significantly different from cells treated with control siRNA ($p<0.05$). δ significantly different from cells treated with IDO siRNA ($p>0.05$). The experiment was performed three times in triplicate.

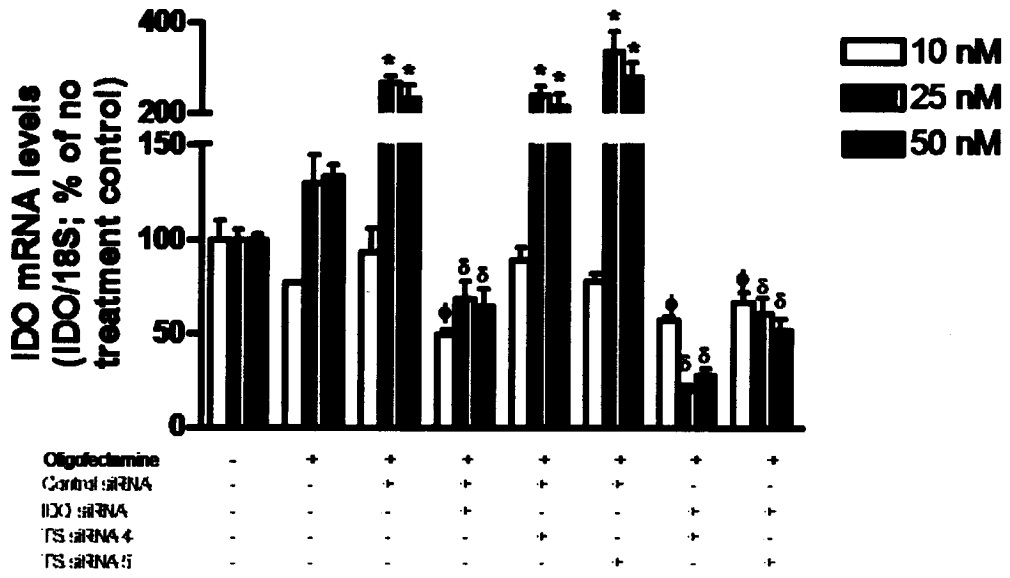


**Figure 3.3.6. IDO siRNA does not affect the capacity of TS siRNA to reduce TS mRNA levels in HeLa cells.** HeLa cells were transfected with combinations of non-targeting siRNA 1, IDO siRNA or TS siRNAs. Control siRNA 1 was used to maintain the total siRNA concentration at 10, 25 or 50 nM. Cells were incubated for 48 hours. mRNA levels were estimated by reverse transcription followed by qPCR, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=3). * significantly different from cells treated with control siRNA ($p<0.05$). δ significantly different from cells treated with control siRNA ($p<0.05$). Φ significantly different from untreated cells and cells treated with control siRNA ($p<0.05$). The experiment was performed three times in triplicate.

mock transfected cells ($p<0.05$), although this was only by 40-50% compared to untransfected cells and was not significantly different ($p>0.05$; Figure 3.3.6). Addition of TS siRNA to IDO siRNA did not prevent IDO siRNA from down-regulating target mRNA compared to IDO siRNA combined with control siRNA 1, regardless of the total siRNA concentration ($p>0.05$; Figure 3.3.6). These results suggest that an increase in the total siRNA concentration and the type of siRNA technology used can result in greater off-target effects. From previous experiments, it was observed that IDO siRNA can reduce IDO mRNA levels by 90% when used alone (Figure 3.1.9). Combining IDO siRNA with control siRNA 1 resulted in a reduction of only 40-50% in IDO mRNA levels. From these results, it was determined TS siRNA did not decrease the capacity of IDO siRNA to down-regulate IDO mRNA levels compared to IDO siRNA combined with control siRNA 1; however, the previously determined effects of TS siRNA may be masked by the effects of control siRNA 1 and required further investigation.

Analysis of IDO mRNA levels over time after siRNA transfection (25 nM total siRNA using control siRNA 1) revealed that, although IDO mRNA levels are only down-regulated ~40–50% by 24 hours, allowing the transfected cells to incubate for 48 or 72 hours resulted in a 75-90% reduction in IDO mRNA levels (Figure 3.3.7). Also, IDO mRNA levels did not begin to recover by 72 hours after siRNA transfection.

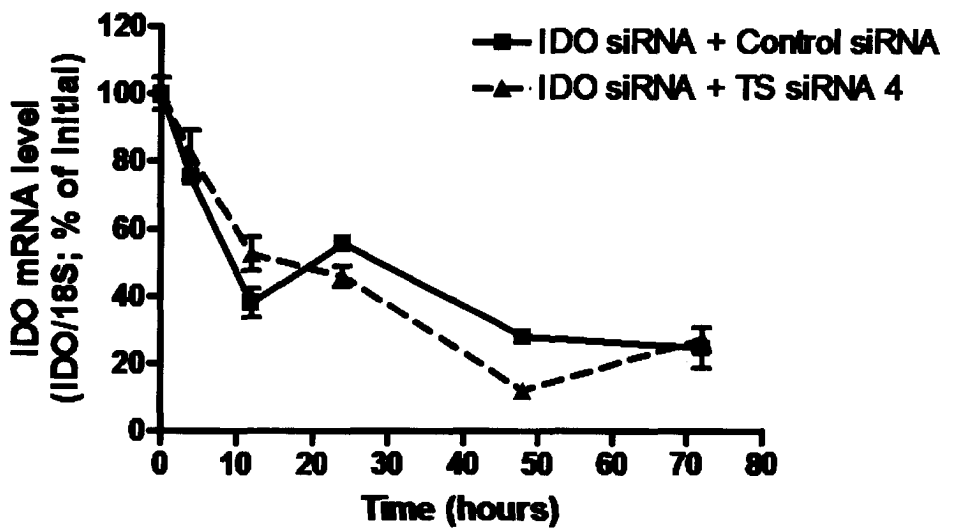


**Figure 3.3.7. IDO mRNA reduction after IDO siRNA and TS siRNA transfection in HeLa cells.** HeLa cells were transfected with combinations of non-targeting siRNA 1, IDO siRNA or TS siRNAs. Control siRNA 1 was used to maintain the total siRNA concentration at 25 nM. Cells were incubated for 48 hours. mRNA was extracted and levels were estimated by reverse transcription followed by qPCR at indicated times, as described in Materials and Methods. Results are plotted as a percentage ± SEM of no treatment control (n=1). The experiment was performed once in triplicate.

## *3.4. Effects of non-targeting siRNAs on TS and IDO mRNA levels in HeLa cells*

### *3.4.1. Non-targeting siRNAs also antagonize the capacity of IDO siRNA to reduce IDO mRNA levels in HeLa cells*

It was important to determine whether the decreases observed when TS siRNA was added to IDO siRNA was a consequence of a reduction in TS mRNA levels or an effect of a non-IDO-targeting siRNA. To test this, cells were transfected with IDO siRNA with or without control siRNAs 1 and 2. Interestingly, non-targeting control siRNAs 1 and 2 also decreased the capacity of IDO siRNA to reduce IDO mRNA levels in HeLa cells ($p<0.05$; Figures 3.4.1A and 3.1.4B, respectively). IDO siRNA alone reduced IDO mRNA levels by approximately 90% (Figures 3.1.9A, 3.1.9B, 3.2.3B, 3.3.4, 3.3.4A, 3.3.5B, 3.4.1.A, 3.4.1B, 3.4.2A and 3.4.2B). Addition of control siRNAs 1 and 2 to IDO siRNA resulted in only a 40% decrease in IDO mRNA levels compared to untransfected cells (Figures 3.4.1A and 3.4.1B). This was consistent with results previously shown (Figure 3.3.6). Varying the ratio of IDO siRNA to non-targeting control siRNAs 1 and 2 revealed that as the ratio of IDO siRNA: control siRNA decreased, so did the capacity of IDO siRNA to reduce IDO mRNA levels (Figures 3.4.2A and 3.4.2B). This suggests that the effect on IDO mRNA levels observed when combining IDO siRNA and TS siRNA may not be a direct result of TS knockdown. Instead, it may be a result of the antisense technology.

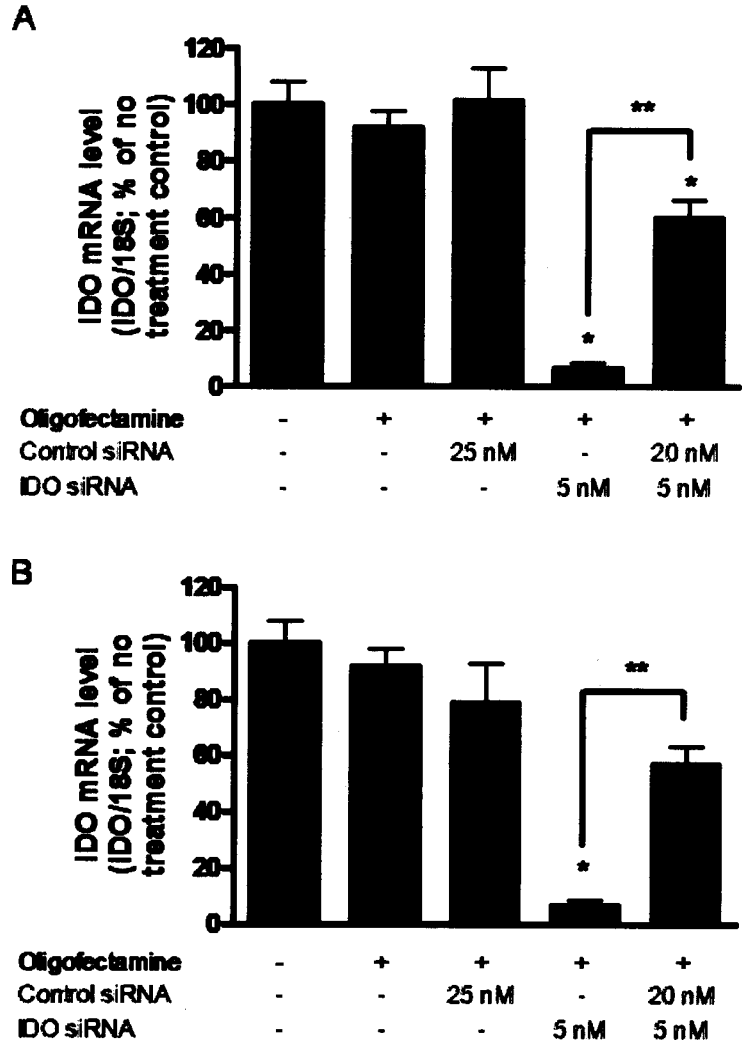


**Figure 3.4.1. Non-targeting siRNA antagonizes the capacity of IDO siRNA to down-regulate IDO mRNA levels in HeLa cells.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with IDO siRNA at 5 nM. Using control siRNA (A) 1 or (B) 2, control siRNA was added to IDO siRNA to maintain the total siRNA concentration at 25 nM. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR. Results are plotted as a percentage ± SEM of untreated control (n=3). * significantly different from cells treated with control siRNA ($p<0.05$). ** significantly different ($p<0.05$). The experiment was performed three times in triplicate.

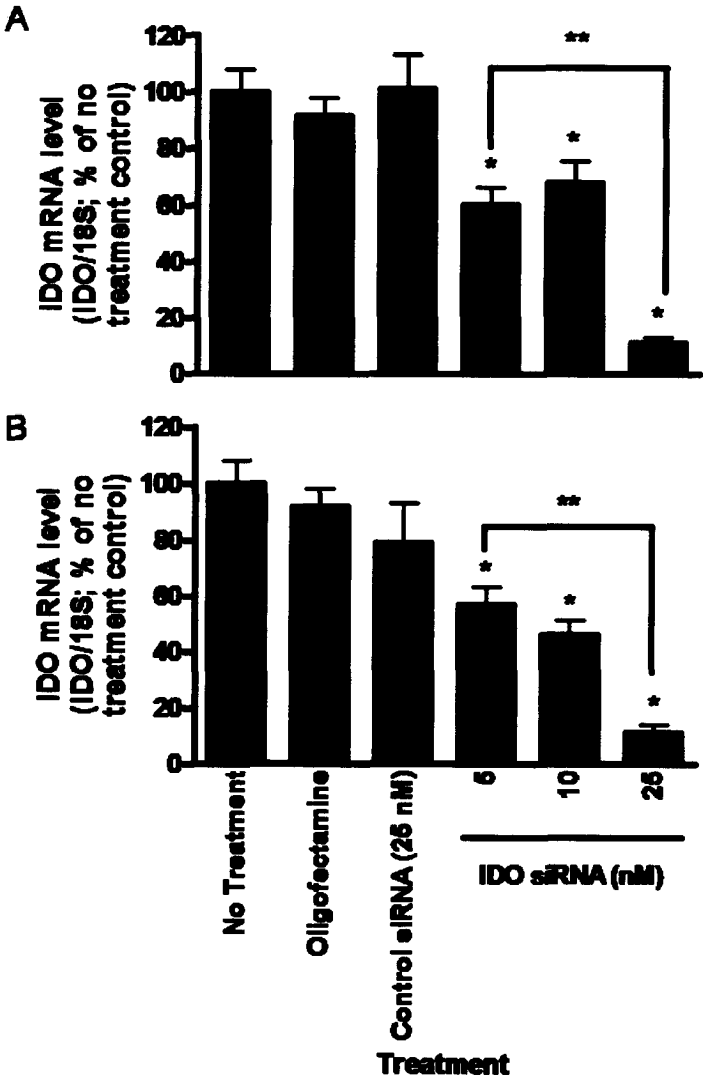


**Figure 3.4.2. Non-targeting siRNA also antagonizes the capacity of IDO siRNA to down-regulate IDO mRNA levels at various ratios of IDO siRNA:Control siRNA in HeLa cells.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with IDO siRNA at various concentrations using control siRNA (A) 1 or (B) 2 to maintain the total siRNA concentration at 25 nM. Cells were incubated for 24 hours. mRNA levels were estimated by reverse transcription followed by qPCR. Results are plotted as a percentage ± SEM of untreated control (n=3). * significantly different from cells treated with oligofectamine ($p<0.05$). ** significantly different ($p<0.05$). The experiment was performed three times in triplicate.

## *3.5. Effects of TS and IDO siRNA transfection on HeLa cell growth and response to TS protein-targeting chemotherapeutics*

### *3.5.1. Effects of siRNA on HeLa cell number*

To determine the effects of TS, IDO and non-targeting siRNAs on HeLa cell growth, cell proliferation after siRNA transfection was analyzed. HeLa cells transfected with either non-targeting siRNA, IDO siRNA or TS siRNA displayed no significant changes in cancer cell number ($p>0.05$; Figure 3.5.1). However, HeLa cells transfected with TS siRNA combined with IDO siRNA (5 nM each) did show a slight increase in cell number by approximately 27% ($p<0.05$; Figure 3.5.1). Thus, TS and IDO siRNA alone did not affect HeLa cell proliferation; however, combining TS and IDO siRNA accelerated HeLa cell proliferation.

### *3.5.2. Effects of TS and IDO siRNA on 5-FUdR and Docetaxel toxicity*

To determine the effects of TS, IDO and non-targeting siRNAs on 5-FUdR (a TS protein-targeting chemotherapeutic) sensitivity, and if IDO siRNA alters TS siRNA-induced 5-FUdR sensitivity, cell proliferation was assessed after siRNA transfection followed by drug treatment. As expected, TS siRNA sensitized HeLa cells to 5-FUdR, resulting in a lower concentration range of 5-FUdR (0.25-5 nM vs. 2-40 nM) necessary in order to determine $IC_{50}$ (Figures 3.5.2A and 3.5.2B). IDO siRNA had no effect on HeLa cell sensitivity to 5-FUdR compared to both no siRNA treatment and control siRNA 3 and 4 treated cells ($p>0.05$; Figure

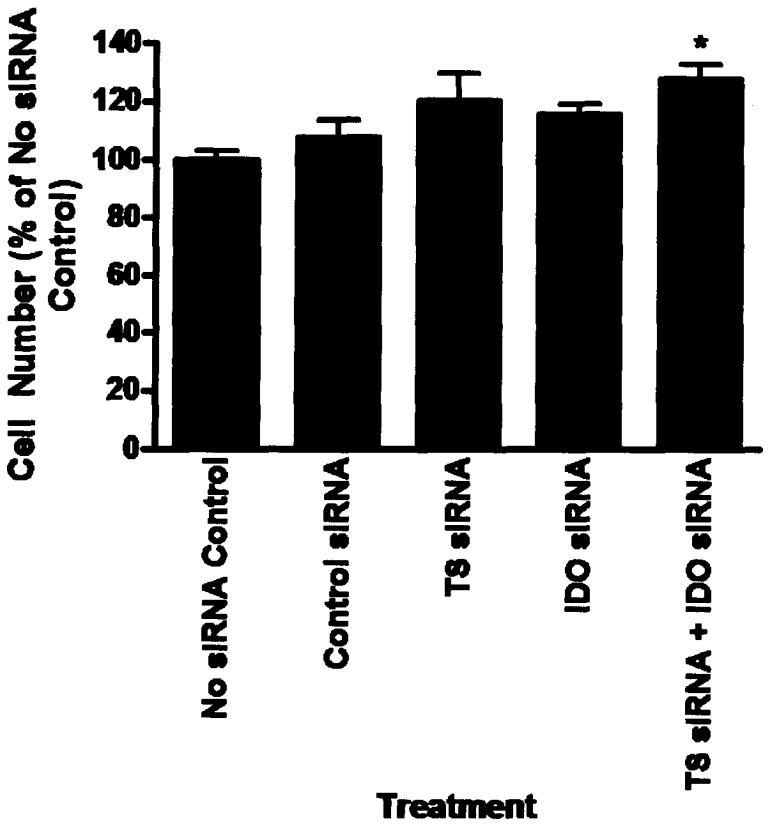


**Figure 3.5.1. Combining TS siRNA with IDO siRNA affects HeLa cell number.** HeLa cells were pre-treated with 100 ng/mL IFN-γ before being transfected with combinations of control siRNAs, IDO siRNA or TS siRNAs. Control siRNAs 3 and 4 were used to maintain the total siRNA concentration at 10 nM. Cells were replated 4 hours post-transfection. Cell number was determined 4 days after plating, as described in Materials and Methods. Proliferation was determined as fold change and was normalized to no siRNA treatment control. Results a presented as mean ± SEM (n=3). * significantly different from control siRNA treated cells ($p<0.05$). The experiment was performed three times in duplicate.

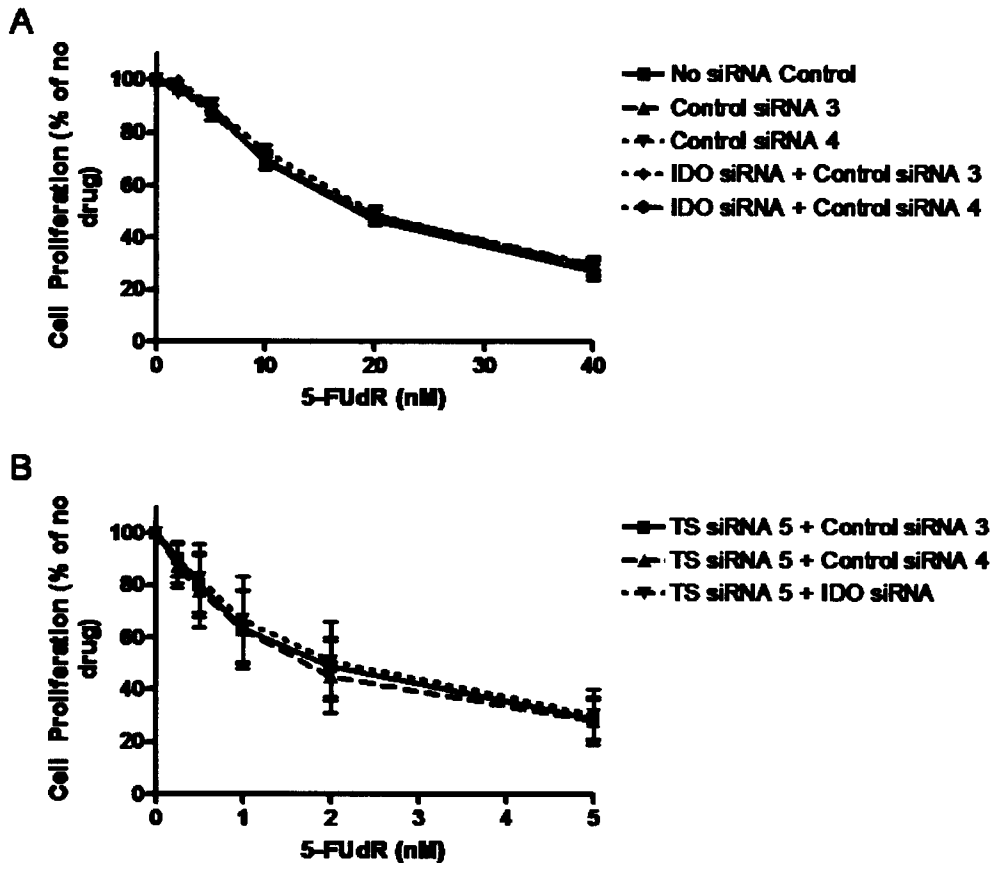


**Figure 3.5.2. IDO siRNA does not prevent TS siRNA from sensitizing HeLa cells to TS-targeting chemotherapeutics.** HeLa cells were pretreated with (A) control siRNAs 3 and 4 or IDO siRNA alone or (B) TS siRNA alone or TS siRNA combined with IDO siRNA. Control siRNAs were used to maintain the total siRNA concentration at 10 nM. Cells were treated with varying concentrations of 5-FUdR 24 hours after transfection. Proliferation rate was determined as fold change after a 4-day continuous drug exposure and was normalized to no drug treatment for each transfection condition, as described in Materials and Methods. Results are plotted as mean ± SEM (n=3). The experiment was performed three times in duplicate.

3.5.2A). Addition of IDO siRNA to cells transfect with TS siRNAs 4 and 5 had no effect on the increased sensitivity to 5-FUdR provided by TS siRNA ($p>0.05$; Figure 3.5.2B). The $IC_{50}$ (half maximal inhibitory concentration) for each treatment was also determined (Table 3.5.1). There were no significant differences in the $IC_{50}$ of those cells left untreated or transfected with non-targeting siRNAs or IDO siRNA ($p>0.05$). TS siRNA significantly reduced the 5-FUdR $IC_{50}$, regardless of whether TS siRNA was administered alone or co-administered with IDO siRNA ($p<0.05$). Therefore, IDO siRNA did not prevent TS siRNA from sensitizing HeLa cells to TS protein-targeting chemotherapeutics.

To confirm enhanced drug sensitivity was specific to TS-targeting chemotherapeutics, cell number after transfection followed by docetaxel (a non-TS-targeting anti-mitotic chemotherapeutic) was determined using an alamarBlue™ assay. Neither control siRNA, TS siRNA nor IDO siRNA affected the sensitivity of HeLa cells to docetaxel (Figure 3.5.3; $p>0.05$; Table 3.5.2). Therefore, neither TS nor IDO siRNAs affected docetaxel sensitivity, confirming that enhanced chemotherapeutic sensitivity mediated by TS siRNA was specific to TS-targeting chemotherapeutics.

| | Treatment | 5-FUdR $IC_{50}$ (nM) |
|---|---|---|
| (A) | No siRNA Control | 18.3 ± 0.817 |
| (B) | Control siRNA 3 | 18.4 ± 0.933 |
| (C) | Control siRNA 4 | 20.2 ± 1.42 |
| (D) | IDO siRNA + Control siRNA 3 | 19.7 ± 1.83 |
| (E) | IDO siRNA + Control siRNA 4 | 18.7 ± 0.693 |
| (F) | TS siRNA 5 + Control siRNA 3 | 2.38 ± 0.885* |
| (G) | TS siRNA 5 + Control siRNA 4 | 2.11 ± 0.992* |
| (H) | TS siRNA 5 + IDO siRNA | 2.71 ± 1.04* |

**Table 3.5.1. IDO siRNA does not prevent TS siRNA from sensitizing HeLa cells to TS-targeting chemotherapeutics.** $IC_{50}$ was determined for each treatment from Figure 3.5.2. Data is presented as mean ± SEM (n=3). * significantly different from no siRNA control ($p<0.05$). [a] Total siRNA concentration was 10 nM for all treatments (B-F). The experiment was performed three times in duplicate.

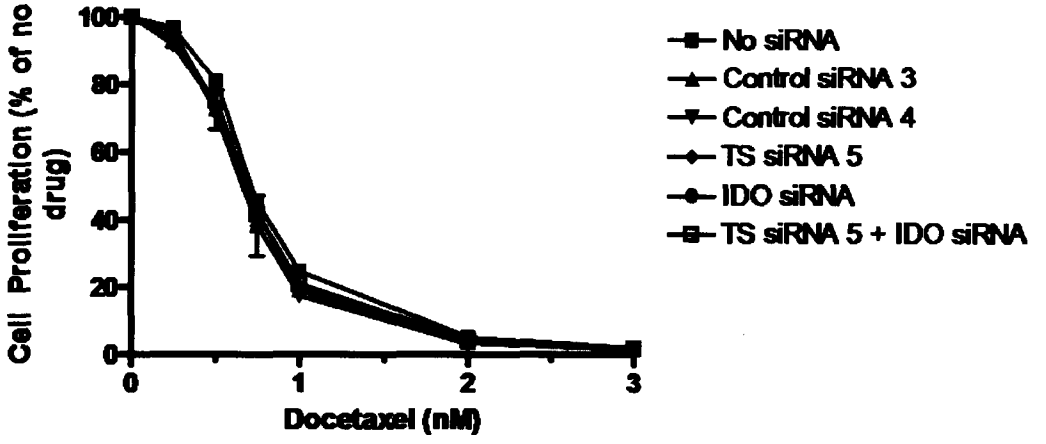


**Figure 3.5.3. TS and IDO siRNA do not affect the sensitivity of HeLa cells to non-TS-targeting chemotherapeutics.** HeLa cells were pretreated with control siRNA 3, IDO siRNA, TS siRNA or combinations of siRNAs. Control siRNAs were used to maintain the total siRNA concentration at 10 nM. Cells were treated with varying concentrations of docetaxel 24 hours after transfection. Proliferation rate was determined after a 4-day continuous drug exposure and was normalized to no drug treatment for each transfection condition, as described in Materials and Methods. Results are plotted as mean ± SEM (n=3). The experiment was performed three times in triplicate.

| | Treatment | Docetaxel $IC_{50}$ (nM) |
|---|---|---|
| (A) | No siRNA Control | 0.70 ± 0.027 |
| (B) | Control siRNA 3 | 0.68 ± 0.030 |
| (C) | Control siRNA 4 | 0.68 ± 0.030 |
| (D) | IDO siRNA | 0.69 ± 0.026 |
| (E) | TS siRNA 5 | 0.70 ± 0.026 |
| (F) | TS siRNA 5 + IDO siRNA | 0.68 ± 0.030 |

**Table 3.5.2. TS and IDO siRNA do not affect the sensitivity of HeLa cells to non-TS-targeting chemotherapeutics.** $IC_{50}$ was determined for each treatment from Figure 3.5.3. Data is presented as mean ± SEM (n=3). [a] Total siRNA concentration was 10 nM for all treatments (B-F). The experiment was performed three times in triplicate.

# CHAPTER 4: DISCUSSION

Antisense therapy is becoming an increasingly viable option to use in a variety of different clinical settings, including in combination with chemotherapy for cancer treatment (National Institute of Health, 2009). Currently, antisense therapy is used in clinical trials to treat Duchenne Muscular Dystrophy, age-related macular degeneration, Chrohn's Disease, and Rheumatoid Arthritis (National Institute of Health, 2009). Extensive research has been done in order to investigate the effect of using antisense agents to target TS in human cancer cells to enhance chemosensitivity, and results thus far have been very promising (Ferguson *et al.*, 2001; Ferguson *et al.*, 1999; Flynn *et al.*, 2006; Berg *et al.*, 2001; Pandyra *et al.*, 2007; Schmitz *et al.*, 2004). In addition, acquired immunosuppressive abilities of cancer cells through expression of IDO can be reversed and studies have shown that antisense agents targeting IDO enhance immune function both *in vitro* and *in vivo* (Zheng *et al.*, 2006; Lob *et al.*, 2009b). If it were possible to treat patients with both antisense technology targeted towards TS (to increase chemosensitivity) and IDO (to stimulate anticancer immunity), this could prove to be extremely beneficial for cancer patients. Currently, little is known about the effects of combining multiple antisense agents; however, data from previous studies suggests that these effects be explored and considered when antisense agents are used in combination (Pandyra *et al.*, 2007).

The concept of combining drugs in anticancer therapy is commonly used and is very successful (Fisher *et al.*, 1993; Van Cutsem *et al.*, 2009). It has been shown that tumour cells can be sensitized to chemotherapeutic agents by combining antisense agents which target molecules responsible for the chemotherapy-mediated cell death responses, such as Mdm2 and p21WAF1/CIP1 (Sato *et al.*, 2000). Another study has shown that simultaneous antisense treatment targeting different growth factors results in greater than additive growth inhibitory effects (Normanno *et al.*, 1996). Furthermore, it has been shown that combining bcr-abl and c–myc antisense agents has led to synergistic antiproliferative effects both *in vitro* and *in vivo* (Skorski *et al.*, 1995; Skorski *et al.*, 1996). While these studies have shown that there is great potential for combination antisense therapy, it is important to recognize that this may not always be the result. In fact, Pandyra *et al.* (2007) showed that combining antisense *in vitro* can produce antagonistic responses at the molecular level and at the functional level. For example, when Bcl-2 siRNA was used in conjunction with TS siRNA, the result was that Bcl-2 siRNA antagonized the capacity of TS siRNA to reduce TS mRNA and protein levels and to sensitize tumour cells to traditional TS-targeting chemotherapeutics (Pandyra *et al.*, 2007). Clearly, it is essential to investigate the molecular and functional effects of combining antisense agents prior to therapeutic intervention. Therefore, the purpose of this study was to explore the effectiveness of combining antisense agents targeting TS and IDO in immortalized human tumour cell lines. Ultimately, if it were possible to combine antisense agents targeting both TS and IDO mRNA simultaneously,

cancer cells could be sensitized to traditional chemotherapeutics and immune recognition of the tumour could be enhanced. By targeting cancer cells from multiple directions using antisense agents, it is possible to further enhance anticancer therapy.

## *4.1. Effects of IFN-γ on IDO and TS mRNA and protein levels and HeLa cell viability*

IDO expression in cancer is associated with suppression of the immune system, which has detrimental effects on the cancer patient. Therefore, it is beneficial to determine how IDO expression can be reduced using antisense therapy. It is estimated that nearly 100% of primary human tumours express IDO at both the mRNA and protein levels (Lob *et al.*, 2009b; Uyttenhove *et al.*, 2003); however, most cell lines do not (Lob *et al.*, 2009b). This is most likely due to the fact that cells in culture do not encounter the exact same environment as cells *in vivo* (Uyttenhove et al., 2003). The presence of IFN-γ in the tumour microenvironment may contribute to induction of IDO (Muller *et al.*, 2005a). Therefore, it was not surprising that neither the HeLa nor the SK-MEL-5 cell lines used in the present study expressed IDO mRNA levels that were detectable by RT-qPCR. It is known, however, that when cells in culture are stimulated with IFN-γ, they begin to express IDO mRNA which translates into IDO activity (Lob *et al.*, 2009b; Takikawa *et al.*, 1988; Zheng *et al.*, 2006). Since it was necessary to detect IDO mRNA levels prior to treatment of cells with IDO-targeting siRNA in

order to determine the extent of IDO mRNA down-regulation by the IDO siRNA, both HeLa and SK-MEL-5 cell lines were treated with IFN-γ prior to performing siRNA experiments. In both cases, IFN-γ treatment caused a dose-dependent up-regulation of IDO mRNA levels (Figures 3.1.2, 3.1.4, and 3.1.6) and an up-regulation of IDO protein levels (Figure 3.1.3), which is consistent with previously published findings (Yasui *et al.*, 1986; Takikawa *et al.*, 1988). Therefore, IDO mRNA levels were quantifiable prior to siRNA-mediated knock-down when they were treated with IFN-γ 12-24 hours prior to siRNA transfection, which is also consistent with previously reported data (Takikawa *et al.*, 1988).

While IFN-γ treatment was essential in order to detect IDO mRNA levels, it was not known how IFN-γ treatment would affect TS mRNA levels, or how it would affect HeLa cell viability. IFN-γ has been shown to produce strong negative effects on HeLa cell growth 6 days post-treatment (Takikawa *et al.*, 1988). It is possible that, since high TS levels are associated with cell proliferation (Ferguson *et al.*, 1999), a decrease in TS mRNA levels 48 hours after IFN-γ treatment reflects a decrease in cell proliferation. In support of this, when mice were treated with IFN-γ in combination with 5-FU, TS activity was significantly decreased compared to mice treated with 5-FU alone (Ishii and Marumo, 2004). This suggests that IFN-γ may effect TS expression. The results of the present study, however, demonstrate that there is no adverse effect of IFN-γ on TS mRNA until 48 hours post-treatment and that IFN-γ does not affect HeLa cell viability and cell number (Figures 3.1.5, 3.1.7, and 3.1.8). Since IDO mRNA levels increase

to detectable levels within 12-24 hours post- IFN-γ treatment, then it is possible to use IFN-γ without any negative effects on TS mRNA levels or on cell viability.

As previously discussed, decreasing IDO levels in cancer cells would be beneficial in anticancer therapy through enhancement of immune function (Lob *et al.*, 2009a; Ou *et al.*, 2008; Curti *et al.*, 2007). For this reason, previous studies have focused on targeting IDO mRNA with siRNA (Lob *et al.*, 2009b). However, earlier studies have focused on the prevention of IFN-γ-induced IDO mRNA up-regulation with the use of siRNA. After IDO siRNA transfection in mouse and human tumour cells, IDO mRNA levels do not increase upon IFN-γ treatment (Reddy *et al.*, 2008; Zheng *et al.*, 2006; Lob *et al.*, 2009b). The results presented in this thesis not only confirm those initial findings, but also demonstrate that IFN-γ can induce IDO mRNA and protein levels in two different human tumour cell lines (HeLa and SK-MEL-5) prior to transfection. Furthermore, after IDO mRNA up-regulation by IFN-γ, IDO siRNA transfection can significantly down-regulate IDO mRNA levels (Figures 3.1.9). To our knowledge, this is the first report to show that IDO siRNA can knock-down IFN-γ-induced IDO mRNA in human cells.

The experiments presented here emphasize the importance of treating cells with IFN-γ to up-regulate IDO in order to more effectively mimic the normal physiological conditions of the tumour in order to specifically address knocking down IDO mRNA levels *in vitro*. Furthermore, IDO siRNA can effectively reduce IDO mRNA, making it an excellent candidate for therapeutic use.

### *4.2. Capacity of TS and IDO siRNAs to reduce target mRNA levels*

In order to determine the effects of siRNA targeted to either TS or IDO mRNA, it is essential to determine proper siRNA controls for the transfection experiments. Ideally, controls that do not affect the mRNAs of interest (namely TS and IDO mRNA) can be used to ensure that the effects seen with combination treatments are a result of target down-regulation and not off-target effects of the siRNA. The results of these experiments suggest that different control siRNAs have different effects on TS and IDO mRNA levels, with some resulting in a higher degree of off-target effects than others (Figure 3.2.1). In particular, control siRNA 1 had the greatest degree of off-target effects, resulting in a decrease in TS mRNA levels and an increase in IDO mRNA levels.

Initially, an increase in IDO mRNA levels was not expected because it was previously thought that siRNA does not elicit an IFN response (which can contribute to an increase in IDO mRNA levels) (Takikawa *et al.*, 1988; Kurreck, 2009). However, other studies have shown that non-targeting siRNAs (21 nucleotides in length) have the potential to induce IFN-γ expression through toll-like receptor 3 (TLR3) activation (Kleinman *et al.*, 2008). TLR3 normally recognizes dsRNAs and plays a critical role in the IFN-γ-related dsRNA response (Negishi *et al.*, 2008; Alexopoulou *et al.*, 2001); however, it can also recognize 21-nucleotide siRNAs and can induce IFN-γ protein (Kleinman *et al.*, 2008). TLR-

mediated immune responses can also vary depending on the siRNA sequence and structural motif (Kurreck, 2009; Judge *et al.*, 2005). In this case, it seems logical to hypothesize that control siRNA 1 induced an IFN-γ response, which, in turn, increased IDO mRNA levels; however, this was not tested. Conversely, control siRNAs 2, 3, and 4 seem to be ideal controls to use since very little change was seen in TS and IDO mRNA levels when cells were treated with these siRNAs (Figure 3.2.1). It is possible, at least for control siRNAs 3 and 4, that the new ON-TARGET*plus* technology (Dharmacon), with modified siRNAs to enhance specificity, is the reason that these controls are superior to control siRNA 1. How exactly these modifications changed the siRNAs is unclear as the specifications have not yet been released by Dharmacon; however, we are continuing to pursue the company for further information regarding these modifications. Therefore, the response to control non-targeting siRNAs observed in this study may be a result of a TRL-mediated response to dsRNA. Also, those siRNAs with greater off-target effects would be ideal to determine if effects seen with combination experiments are a result of target down-regulation.

The concept of off-target effects mediated by high siRNA concentrations is not new, and has been reported in other studies (Fedorov *et al.*, 2006; Persengiev *et al.*, 2004) and also by suppliers of siRNA (AppliedBiosystems, 2009). Interestingly, siRNAs are concentration-dependent in their ability to affect off-target transcripts, with concentrations as low as 25 nM being sufficient to repress non-targeted genes (Persengiev *et al.*, 2004), and 10 nM sometimes sufficient to induce a toxic phenotype through altering cell viability, which can be diminished

by using lower siRNA concentrations, such as 1 nM (Fedorov *et al.*, 2006). siRNA treatment can alter expression of a variety of genes; genes involved in cytokine signaling, cytoskeletal rearrangement, apoptosis, metabolism, and cell adhesion are stimulated, whereas other genes involved in RNA synthesis, chromosome organization transcription, metabolism and cell adhesion are repressed (Persengiev *et al.*, 2004). Therefore, it is important to determine the lowest possible effective siRNA concentrations in order to minimize or eliminate potential off-target effects, for example, increased IDO expression due to an off-target or IFN response. Typically, siRNA concentrations of 25-100 nM are used to down-regulate their target mRNA, and this is true for previous work done with both TS mRNA (Pandyra *et al.*, 2007) and IDO mRNA (Lob *et al.*, 2009b).

Maximal down-regulation for both TS and IDO mRNA with higher siRNA concentrations (25-100 nM) is approximately 80-90%. In support of these observations, Yang *et al.* (2006) found that infecting HeLa cells with a plasmid directing the synthesis of a TS siRNA, it was also possible to achieve 80-95% reduction in TS mRNA levels. The results of this study, however, suggest that siRNA concentrations as low as 5 nM are sufficient to induce maximal down-regulation (~90%) by TS and IDO mRNA levels (Figures 3.2.2 and 3.2.3). Additionally, Grunweller *et al.* (2003) showed that 0.1 nM siRNA targeting the vanilloid receptor subtype 1 (VR1), was sufficient to induce a 90% down-regulation of the target mRNA (Grunweller *et al.*, 2003). Therefore, it seems as though lower siRNA concentrations can still achieve sufficient down-regulation of target mRNA, and this could potentially eliminate any off-target effects on

other mRNA. This information could be extremely valuable for future studies involving the use of siRNA to down-regulate target mRNA.

For the purposes of this study, 5 nM siRNA was used to target either TS or IDO mRNA; however, for the future, it would be beneficial to investigate if even lower concentrations could be used to target mRNA levels with similar efficiency. It has been demonstrated that siRNA concentrations can exhibit a dose-dependent effect on target down-regulation when lower concentrations are used (Grunweller *et al.*, 2003). Furthermore, additive or synergistic effects may be difficult to detect if mRNA levels are fully down-regulated by siRNA. In these cases, down-regulation of TS and IDO mRNA levels by only ~50% may be sufficient in order to assess any potential additive or synergistic effects when combining siRNAs.

TS is an excellent candidate for siRNA-mediated knock-down as its mRNA has a relatively long half-life in rodents (8 hours) (Jenh *et al.*, 1985), and TS activity is more reliant upon cell cycle changes in translation than on changes in transcription (Navalgund *et al.*, 1980; Johnson, 1994). This last point emphasizes the relative importance of TS mRNA compared to the protein; therefore, targeting TS mRNA is expected to have a major impact on the cell (Kitchens *et al.*, 1999). In fact, reducing TS mRNA levels by 90% has been shown to have multiple effects on cells, including sensitizing cells to TS protein-targeting drugs (as discussed in *Section 4.4*) (Schmitz *et al.*, 2004; Pandyra *et al.*, 2007; Ferguson *et al.*, 2001; Ferguson *et al.*, 1999; Berg *et al.*, 2003). With regards to IDO, the mRNA also appears to be a good candidate for siRNA-mediated down-

regulation. Although IDO's mRNA and protein half-lives are not known, experiments have shown that siRNA down-regulated IDO mRNA for a minimum of 72 hours (Figure 3.3.7). Previous studies have shown that reducing IDO mRNA in rodent cancer cells by siRNA decreased IDO *in vitro*, reduced tumour-induced T cell apoptosis *in vitro* and *in vivo*, delayed onset of cancer *in vivo*, and slowed tumour growth *in vivo* (Belladonna *et al.*, 2009; Zheng *et al.*, 2006). This suggests that IDO mRNA reduction by siRNA can enhance antitumour immunity (Zheng *et al.*, 2006).

Finally, in order to determine combinatorial effects of target-specific down-regulation, it is extremely important to show that TS and IDO siRNAs only affect their respective mRNAs. The results from this study show that TS siRNA alone did not affect IDO mRNA levels (Figure 3.2.2) and IDO siRNA alone did not affect TS mRNA levels (Figure 3.2.3) when compared to control siRNA transfected cells.

## *4.3. Capacity of TS and IDO siRNAs to reduce target mRNA levels when used in combination*

Studies have shown that the use of multiple antisense agents simultaneously can result in a reduced level of both targets' proteins to the same extent as when each antisense is used as a single agent (Skorski *et al.*, 1995). For example, combining bcr-abl antisense with c-myc antisense results in a reduction in both target mRNA and protein levels to a similar extent as when each

antisense is used independently (Skorski *et al.*, 1995). However, previous work by our group has shown that the use of multiple antisense agents concurrently can result in reduced siRNA-mediated knock-down abilities compared to when each antisense is used as a single agent (Pandyra *et al.*, 2007). For example, combining siRNA targeting Bcl-2 in human cancer cells interfered with the ability of TS siRNA to reduce TS mRNA levels (Pandyra *et al.*, 2007). It is important that we acknowledge that it cannot be predicted that siRNAs used in combination will down-regulate target mRNA to the same extent as when used independently. In addition, it cannot automatically be predicted which siRNAs, when used concurrently, will work synergistically, antagonistically, or have no effect on each other. Therefore, it was essential to determine the effects of combining both TS and IDO siRNA on target mRNA down-regulation.

### *4.3.1. IDO siRNA affect on TS siRNA action*

In this study, it was shown that addition of siRNA targeting IDO in human cancer cells had no effect on the ability of TS siRNA to down-regulate target mRNA levels, regardless of the total siRNA concentration used, time allowed for transfection, or presence of control siRNA (Figures 3.3.1, 3.3.2, and 3.3.3). This provides evidence that IDO siRNA did not directly antagonize TS siRNA efficiency. It should be noted here, however, that when IDO siRNA was used in conjunction with control siRNAs 3 and 4 at 10 nM total siRNA concentration, TS mRNA levels were reduced by 20% compared to HeLa cells treated with only

control siRNAs (Figure 3.3.2). With higher siRNA concentrations (25-50 nM total siRNA), there was no difference in TS mRNA levels between cells transfected with control siRNA only or control siRNA with IDO siRNA (Figure 3.3.3), with both conditions causing a 20-40% decrease in TS mRNA levels. These results suggest that high siRNA concentrations may produce mass effects, further supporting the literature with regards to off-target effects mediated by high siRNA concentrations (>25 nM), as previously discussed (*Section 4.2*). As little as 25 nM siRNA treatment can alter expression of a variety of genes, including those involved in RNA synthesis (Persengiev *et al.*, 2004). This suggests that genes involved in DNA synthesis may also be indirectly altered, and provides a potential explanation as to why high siRNA concentrations resulted in a decrease in TS mRNA levels, however this should be further investigated. Lowering the siRNA concentrations, as discussed earlier, may reduce the off-target effects such that it would be possible to observe a biological effect of IDO mRNA down-regulation. It would also be informative to down-regulate TS mRNA by only 50% to determine if IDO siRNA can enhance the ability of TS siRNA to down-regulate TS mRNA.

Overall, these results indicate that IDO siRNA does not interfere with TS siRNA action more than control siRNAs. As a result, concurrent addition of IDO siRNA to TS siRNAs would not be expected to result in any negative, off-target interactions on the respective target mRNAs. Also, combined antisense therapy is not predicted to have negative physiological consequences to enhanced TS-targeting chemosensitivity and might have potential therapeutic benefits in overall anticancer therapy.

*4.3.2. TS siRNA affect on IDO siRNA action*

Analysis of IDO mRNA levels in cells transfected with IDO siRNA and TS siRNA at 10 nM total siRNA concentrations revealed that TS siRNA substantially reduced the ability of IDO siRNA to down-regulate target mRNA levels from 90% down-regulation to 60-75% (Figures 3.3.5). Furthermore, when TS siRNA was combined with control siRNA, the result was an increase in IDO mRNA levels in HeLa cells (Figures 3.3.5 and 3.3.6). The observation that TS siRNA may antagonize IDO siRNA was not initially expected. Direct interaction between the siRNAs could result in the observed antagonism; however, this was not expected to play a role in these results as the siRNAs used in these experiments showed little to no hybridization complementarity. Additionally, the siRNA used was double-stranded until the sense strand was removed by Ago2 in the RISC complex, preventing inter-siRNA binding. Finally, the converse antagonism (IDO siRNA antagonizing TS siRNA) was not observed. The increase in IDO mRNA observed when using TS siRNA might possibly oppose the down-regulatory activity of IDO siRNA, contributing to the antagonism observed.

Interestingly, it seems that the type of siRNA used, or the total amount of siRNA used, may play a role in siRNA antagonism. When IDO siRNA was combined with some, but not all, control siRNAs, the capacity of IDO siRNA to reduce target mRNA levels was significantly hindered (Figures 3.3.6, 3.4.1, and 3.4.2). Therefore, the antagonism observed when TS siRNA was added to IDO siRNA did not appear to be a consequence of a reduction in TS mRNA levels.

Instead, it appears to be an effect of some non-IDO targeting siRNAs or the antisense technology used. There is presently very little known about the RISC complex and its components with regards to siRNA preference or saturation, although it has been speculated previously (de Vries and Berkhout, 2008; Morris, 2008). Anderson *et al.* (2005) provided evidence that virus-associated RNAs can saturate RISC and compete with dsRNA as a Dicer substrate (Andersson *et al.*, 2005). Furthermore, they were able to show that there may be strand biases of competing siRNAs loading in to RISC (Andersson *et al.*, 2005). Further evidence suggests that TRBP, a necessary component of the activated RISC complex, can be saturated by the HIV-1 expressed RNA, TAR, resulting in diminished RISC activity (Bennasser *et al.*, 2006). Therefore, it is possible that in this case, either RISC itself, or the RISC components may favour TS siRNA, or some non-targeting siRNAs, over IDO siRNA. Additionally, the effectiveness of the siRNA may be mediated through Dicer, TRBP, Ago2, or the entire RISC. Saturation of RISC with siRNA in a non-selective fashion (*i.e.*, where RISC associates with siRNA withoutsequence preference) is not likely as IDO siRNA does not affect TS siRNA. However, it is suspected that RISC or the RISC components may favor particular siRNAs over others. The possibility that some siRNAs may be favoured over others when used in combination, as the results of this thesis suggest, should be considered when combining multiple siRNAs both therapeutically as well as in basic research.

When higher total siRNA concentrations (25-50 nM total) were used with non-IDO-targeting siRNAs, greater off-target effects were observed which may

contribute to the antagonism of IDO siRNA by non-IDO-targeting siRNA (Figure 3.3.6). Work done by Persengiev *et al.* (2004) showed that siRNA concentrations as low as 25 nM can stimulate gene expression, including cytokines and interferon stimulated genes such as ISG20; however, this is not thought to be a true IFN response as off-target effects of siRNAs are overlapping, but not identical, to IFN responses (Persengiev *et al.*, 2004). Therefore, high concentrations of non-IDO-targeting siRNA could potentially induce a reaction similar to an IFN response, ultimately resulting in increased IDO mRNA levels. This observation is consistent with other experiments involving high concentrations of non-IDO targeting siRNAs (Figures 3.3.6, 3.4.1, and 3.4.2). At high concentrations, control siRNA either alone or in combination with 2 different TS-targeting siRNAs increased IDO mRNA levels as high as 3 times the IDO mRNA levels observed in untransfected cells (Figure 3.3.6). Conversely, when lower siRNA concentrations were used (5-10 nM), IDO mRNA levels were increased by only 1-1.75 times those of untransfected cells, further emphasizing the importance of using low siRNA concentrations (Figures 3.3.4, 3.3.5, and 3.3.6). In fact, for future experiments, it would be useful to explore even lower doses of siRNA since it is known that off-target effects of siRNA can be concentration dependent (Persengiev *et al.*, 2004; Fedorov *et al.*, 2006).

Although IDO siRNA retains some capacity to down-regulate IDO mRNA levels when other siRNAs are present, the observation that some siRNAs at commonly studied concentrations up-regulate IDO mRNA is important. The implication is that siRNAs intended to be therapeutic may unexpectedly increase

levels of untargeted mRNAs that enhance malignant characteristics (for example, capacity to evade T cell cytotoxicity). In this thesis, only IDO mRNA levels in response to several siRNAs were studied; other pro-carcinogenic or oncogenic messages, however, could also be affected. This phenomenon may be a limitation of siRNA-based therapies in general. Off-target effects that may increase the ability of tumour cells to survive and grow, such as up-regulation of IDO, should be assessed and avoided in anti-cancer therapy.

Regardless of the cause(s) of the antagonism observed when combining IDO siRNA with other non-IDO-targeting siRNAs, it is essential that these effects are considered when designing potential siRNA anticancer combinations. Specifically, in regards to IDO down-regulation, determining the extent of IDO mRNA down-regulation is necessary to produce physiological effects is crucial, and will be discussed further in *Section 4.5*.

## *4.4. Effects of TS and IDO siRNA transfection on HeLa cell growth and response to TS protein-targeting chemotherapeutics*

Treatment of HeLa cells with TS siRNA did not affect tumour cell growth in this study. Although previously published literature from our group has shown that TS antisense can slow tumour cell growth (Berg *et al.*, 2003; Berg *et al.*, 2001; Pandyra *et al.*, 2007; Jason *et al.*, 2008; Ferguson *et al.*, 1999), recently published data has shown that antisense targeting the 5' portion in the coding region of the TS mRNA transcript did not have an antiproliferative effect on HeLa

cells (Jason *et al.*, 2008). Each of the TS siRNAs used in these experiments also target the 5' portion of the coding region, further supporting the hypothesis that targeting different regions of TS mRNA can result in highly variable effects on cell proliferation (Berg *et al.*, 2003; Jason *et al.*, 2008; DeMoor *et al.*, 1998). These results also support the hypothesis that a reduction in TS mRNA level does not necessarily correlate with a reduction in cell proliferation (Jason *et al.*, 2008).

IDO siRNA alone also did not affect HeLa cell growth. Up-regulation of IDO activity by IFN-γ treatment has previously been shown to negatively affect HeLa cell growth *in vitro*; however, this effect was not apparent until 6 days after IFN-γ treatment (Takikawa *et al.*, 1988). Another study reported that an increase in MCF-7 human breast cancer cell proliferation was observed when IDO was inhibited with 1-MT (Hill *et al.*, 2005). Reducing IDO mRNA levels by means of siRNA would be expected to reverse the anti-proliferative activity of IDO, although such effects may not be evident after only 4 days. Simultaneous addition of TS siRNA with IDO siRNA did not alter HeLa cell growth when compared to IDO siRNA-transfected cells; however, double knock-downs did show an increase in cell growth compared to untransfected cells. In this case, the increase in HeLa cell growth could conceivable be due to a hormetic effect, wherein when low doses of a drug result in the opposite effect of what is expected with a high dose, and may actually benefit organisms (Kaiser, 2003). It is possible that the low siRNA concentrations induced a stress-response to the damage, thus potentially stimulating cell growth. In addition, these additive effects observed when

combining TS and IDO siRNA may be a result of hormesis combined with a decrease tryptophan degradation and kynurenine metabolite production, which can also increase cell growth (Takikawa *et al.*, 1988).

Down-regulation of TS mRNA and protein with antisense agents has resulted in enhanced sensitivity of human tumour cells to TS protein-targeting chemotherapeutics, such as 5-FU, 5-FUdR and raltitrexed (Schmitz *et al.*, 2004; Pandyra *et al.*, 2007; Ferguson *et al.*, 2001; Ferguson *et al.*, 1999; Berg *et al.*, 2003), with as little as 30% reduction in TS mRNA levels sufficient to enhance sensitivity to 5-FUdR (Ferguson *et al.*, 1999). Enhanced sensitivity of cells is specific to TS-targeting chemotherapeutics, as TS antisense agents do not enhance tumour cell sensitivity to non-TS-targeting chemotherapeutics, such as docetaxel and cisplatin (Ferguson *et al.*, 1999; Pandyra *et al.*, 2007). The work presented here provides further evidence that down-regulating TS using siRNA can enhance tumour cell cytotoxicity specifically to TS protein-targeting drugs (Figures 3.5.2 and 3.5.3, Tables 3.5.1 and 3.5.2). This study also demonstrated that down-regulation of IDO by siRNA did not affect either 5-FUdR or docetaxel sensitivity of HeLa cells. Additionally, the combination of IDO siRNA and TS siRNA did not affect the ability of TS siRNA to sensitize HeLa cells to TS-targeting chemotherapeutics. These results support the use of combining TS and IDO siRNA with TS-targeting chemotherapeutics, as IDO siRNA did not interfere with TS siRNA-mediated enhanced cytotoxicity of 5-FUdR. Although IDO does not enhance sensitivity of cells to TS-targeting chemotherapeutics, synergy and enhancement in anticancer therapy are not necessary when combining

therapeutic agents. Additivity at the physiological level is of great significant and maintains potential providing the drugs do not interfere with one another and this should be further evaluated in the future.

## *4.5. Conclusions*

This study was aimed at investigating the potential for a combination of antisense drugs targeting multiple physiological events to improve anticancer therapy. In this study, it was demonstrated that IDO siRNA treatment or TS siRNA treatment, when administered as a single agent, each effectively reduced their mRNA targets specifically and without effects on non-target mRNAs. When TS siRNA and IDO siRNA were used in combination, there was no effect observed on TS siRNA's ability to reduce TS mRNA levels. The presence of TS siRNA, however, appeared to reduce the ability of IDO siRNA to down-regulate IDO mRNA, possibly due to an up-regulating effect of non-IDO-targeting siRNAs or RISC saturation. IDO siRNA did not affect TS siRNA-induced specific drug sensitivity. Targeting multiple mRNAs simultaneously may not have the effect predicted by studies involving single siRNAs. Despite the antagonism observed with respect to IDO mRNA down-regulation when IDO siRNA is combined with TS siRNA, it is still possible that combining TS siRNA with IDO siRNA may potentially be beneficial in anticancer therapy, and further experiments exploring immune cell responses are necessary. These results are highly valuable in determining potentially beneficial drug combinations for anticancer therapy.

## *4.6. Future Directions*

It has become increasingly evident that combining siRNAs for therapeutic benefit is much more complex than initially considered, such as the present case of combining TS and IDO siRNAs. In previous sections, several experiments have been suggested in order to further understand the complexities of siRNA combinations. In addition to those suggested, investigating the effects of varying the rations of IDO siRNA to TS siRNA may provide further insight into how they function in combination. This strategy has been employed to investigate Bcl-2 siRNA's antagonism of TS siRNA, and showed that an increased ratio of TS siRNA to Bcl-2 siRNA results in greater down-regulation of TS (Pandyra *et al.*, 2007). Furthermore, it would be interesting to investigate sequential siRNA transfections. In this study, we were able to show that by targeting IDO mRNA with siRNA for 24 h, it is possible to down-regulate IDO mRNA levels by ~90%. Following IDO siRNA treatment, transfecting those IDO knock-down cells with TS siRNA may potentially result in 90% down-regulation of both targets. Using lower siRNA concentrations may also be beneficial in determining if there are additive effects on TS down-regulation when IDO siRNA is used in combination with TS siRNA.

In addition to varying concentrations of siRNA and the timing of the transfections, another important aspect to consider is the effect of simultaneous TS and IDO siRNA transfections on TS and IDO protein levels. Measuring protein levels would have been ideal for this study; however, the IDO protein

assays used in these experiments did not yield consistent results. The only 2 successful immunoblots that were performed were for the effects of IFN-γ on IDO protein levels. In the future, establishing a reliable IDO protein assay in order to validate the protein levels would be of enormous benefit.

Additionally, the experiments performed in this study, along with any future work, should be done using multiple siRNAs targeting the same mRNA transcript to determine if targeting different regions results in consistent observations. Furthermore, it would be interesting to perform these experiments in other human cancer cell lines in order to determine if the effects seen in HeLa cells are reproducible in other cancer models.

The construction of a plasmid containing the human IDO gene, which can subsequently be transfected into human tumour cell lines, may be valuable for future studies by eliminating the requirement of IFN-γ treatment to induce IDO mRNA expression. Furthermore, the use of primary human tumours would be beneficial, as IDO is constitutively expressed in a variety of primary tumours, also eliminating the need for IFN-γ. Through the use of immunocompromised mice, it would be possible to validate the *in vitro* results in an *in vivo* model. It should be noted here, however, that since these mice lack an immune system, the effects of siRNA on IDO and immune regulation cannot be fully investigated.

As a result of IDO's involvement in immune regulation, determining the biological effects of IDO down-regulation combined with TS down-regulation is of great importance. The results presented here suggest that targeting TS and IDO simultaneously results in antagonism of IDO siRNA; however, IDO mRNA

levels are still significantly reduced compared to untransfected cells. Previous work has shown that a 50% reduction in IDO mRNA and 80% reduction in IDO protein levels in mice (by 1-MT) correlated with an 85% inhibition of IDO activity (Okamoto *et al.*, 2007). Other studies have shown that 50% reduction in mouse IDO mRNA levels by siRNA correlated with an 87.5% reduction in IDO activity and that IDO siRNA delivery *in vivo* resulted in increased T cells, delayed tumour growth and increased survival (Yen *et al.*, 2009; Zheng *et al.*, 2006). Determining the extent of human IDO mRNA and protein knock-down necessary to prevent immune suppression *in vitro* and to elicit an anti-tumour response *in vivo* is essential. In order to investigate this *in vitro*, cell medium tryptophan and kynurenine metabolite levels can be measured by high performance liquid chromatography (HPLC) and T cell response by mixed lymphocyte reaction (MLR) (Takikawa *et al.*, 1988; Zheng *et al.*, 2006; Lob *et al.*, 2009b). In order to determine if down-regulation of IDO mRNA is sufficient in cells that are transfected with both TS and IDO siRNA *in vivo*, we rely heavily on mouse models. These models involving IDO siRNA *in vivo* have previously been established (Zheng *et al.*, 2006; Yen *et al.*, 2009), and are ready to be used in future experiments. Evaluating *in vivo* anti-tumour immunity can be done through measurement of tumour volume , number of tumour-infiltrating T cells and T cell apoptosis (measure via annexin V and CD8 staining) (Zheng *et al.*, 2006; Yen *et al.*, 2009; Li *et al.*, 2008).

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