January 2016

Overcoming Innate and Acquired Therapy Resistance by Targeting DNA Repair in Human Cancer Cells

Mateusz Rytelewski
The University of Western Ontario

Supervisor
Dr. James Koropatnick
The University of Western Ontario

Graduate Program in Microbiology and Immunology

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

© Mateusz Rytelewski 2015

Follow this and additional works at: https://ir.lib.uwo.ca/etd
Part of the Neoplasms Commons, and the Pharmaceutics and Drug Design Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/3438

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlswadmin@uwo.ca.
OVERCOMING INNATE AND ACQUIRED THERAPY RESISTANCE BY TARGETING DNA REPAIR IN HUMAN CANCER CELLS

(Thesis Format: Monograph)

By

Mateusz Rytelewski

Graduate Program in Microbiology and Immunology

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

The School of Graduate and Post-Doctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Mateusz Rytelewski 2015
Abstract:

Genomic instability and a high mutation rate lead to heterogeneity in human tumors. Mathematical modelling predicts that these characteristics promote acquired resistance to cytotoxic and targeted therapies, by increasing the likelihood that resistant subpopulations exist at the start of treatment (and promoting the accumulation of de novo resistance mutations during treatment). As a result, genome plasticity promotes increased fitness on the population level, but individual tumor cells must nonetheless maintain a level of DNA integrity that allows for continued survival, particularly in the context of DNA-damaging therapy (which DNA repair counteracts). Thus, DNA repair proteins are a source of innate resistance to many common anti-cancer drugs, and represent intriguing targets for therapeutic attack.

One way to forestall treatment resistance is to sensitize tumor cells to DNA-damaging therapy by inhibiting DNA repair and decreasing survival following drug treatment. I developed the concept of complementary lethality, defined as "potentiation of drug therapy by the inhibition of DNA repair factors responsible for resistance to that specific drug". BRCA2 is involved in homologous recombination repair (HRR) of double stranded breaks (DSBs) in DNA, and mutations in the BRCA2 gene predispose to various cancers. However, patients with BRCA2-mutated tumors respond more favourably to some types of therapy. I found that inhibition of BRCA2 with siRNA sensitized tumor cells to DNA-damaging drugs and thus overcame innate resistance to their action. Combined inhibition of BRCA2 and thymidylate synthase (TS), the enzyme responsible for de novo synthesis of
thymidylate (and the source of innate resistance to several treatments), rendered tumor cells responsive to a broader range of drugs and created a state of multi-drug sensitivity.

Based on these results, I created a novel BRCA2-targeting antisense oligodeoxynucleotide (ASO) and tested it in the context of cisplatin treatment. ASOs exhibit several advantages over siRNAs in vivo, and some ASO-based drugs have been approved by the FDA. I found that BRCA2 downregulation (with the BRCA2 ASO) enhanced the ability of cisplatin (a cytotoxic, DNA-damaging drug) to control tumor cell proliferation in vitro and metastasis in vivo, and also induced alterations in cellular metabolism.

Further studies using the PARP-1 inhibitor olaparib, which is selectively lethal in cells with HRR deficiency, led me to formulate the concept of “reciprocal positive selection for weakness”: in a population heterogeneous for HRR-proficiency, olaparib selects for HRR-proficient cells, while BRCA2 inhibition selects for HRR-deficient cells. Each individual treatment thus selects for cells ‘weak’ to the other in a reciprocal manner, and combined inhibition of both targets should prevent selection-mediated escape. This is a strategy that aims to prevent acquired resistance in a heterogeneous tumor population by nullifying enrichment of specific cell subpopulations.

I found that BRCA2 inhibition can render HRR-positive cancer cells (with innate resistance to olaparib) sensitive to PARP inhibition. Furthermore, olaparib monotherapy in a primarily HRR-deficient mixed cell population (3:1 ratio of HRR-deficient:HRR-proficient cells) induced resistance to further olaparib treatment after just one dose. This parallels clinical reports which show that patients with
BRCA2-mutated tumors can present with tumors harboring functional BRCA2 protein following olaparib therapy, presumably due to treatment-mediated selection of subclones present at the start of therapy. In my experiments, co-treatment of this same mixed population with BRCA2 ASO and olaparib prevented enrichment based on HRR-proficiency and eliminated the tumor cell population. In addition, treatment of ovarian tumor-bearing mice with BRCA2 siRNA and olaparib in vivo decreased both the number and weight of tumor nodules when compared with each treatment individually. These studies highlight the important role that DNA repair mediators such as BRCA2 play in innate and acquired resistance to treatment, and provide rationale for therapeutic targeting of DNA repair in human tumors.
ii. Keywords:

cancer therapy resistance, DNA repair, BRCA2, chemotherapy, ovarian cancer, lung cancer, olaparib, PARP1, antisense, siRNA
iii. Acknowledgments:

I would like to thank my supervisor, Dr. James Koropatnick, for providing his support, guidance, and mentorship, and for allowing me to conduct my PhD in his laboratory.

I would like to thank everyone in Dr. Koropatnick’s laboratory and all of our collaborators who aided me during my PhD studies.

In addition, I would like to thank my family, who supported me during my PhD training.

I would also like to acknowledge several entities that supported me during my PhD studies:

- The Canadian Institutes of Health Research (CIHR)
- The CIHR Banting and Best Canada Graduate Scholarship Program
- The CIHR Cancer Research and Technology Transfer (CaRTT) Program
- The Ontario Graduate Scholarship Program
- The Western Graduate Research Scholarship Program
- The Department of Microbiology and Immunology
iv. Co-authorship statement:

Chapter 3: Vincent, M.D., Ferguson, P.J., Maleki Vareki, S., Figueredo, R., Koropatnick, J.


2.2.4 Rad51 focus formation assay ................................................................. 30
2.2.5 Generation of cell stably expressing shBRCA2 and shControl ............ 31
2.2.6 Metaphase spread preparation and chromosome counting .................. 31
2.2.7 Fluorescence in situ hybridization (FISH) for whole chromosomes ....... 32
2.2.8 In vivo tumor model ............................................................................. 33
2.2.9 Statistical analysis .............................................................................. 34

Chapter 3. Inhibition of BRCA2 and thymidylate synthase creates multidrug sensitive tumor cells via the induction of combined "complementary lethality" ........................................................................... 35
3.1 Preamble ................................................................................................. 35
3.2 Results ..................................................................................................... 37
3.2.1 siRNA mediated knockdown of BRCA2 and TS reduces cancer cell growth and affects cell cycle progression ................................................................. 37
3.2.2 BRCA2 siRNA potentiates cisplatin, melphalan, and to a lesser extent 5-FUdR, but not pemetrexed ................................................................. 40
3.2.3 TS siRNA potentiates 5-FUdR and pemetrexed but not cisplatin or melphalan ......................................................................................... 43
3.2.4 Simultaneous siRNA-mediated knockdown of BRCA2 and TS induces complementary lethality to cisplatin, melphalan and both TS-targeting drugs .... 46
3.2.5 Simultaneous downregulation of BRCA2 and TS in the same cell population induces complementary lethality to two different sets of drugs and increases overall effectiveness ................................................................. 50

Chapter 4. BRCA2 Inhibition enhances cisplatin-mediated alterations in tumor cell proliferation, metabolism, and metastasis ................................................. 54
4.1 Preamble ................................................................................................. 54
4.2 Results ..................................................................................................... 55
4.2.1 Mutations in the BRCA2 gene positively impact patient prognosis ........ 55
4.2.2 BRCA2 ASO treatment decreases target mRNA, protein and RAD51 focus formation ......................................................................................... 58
4.2.3 BRCA2 maintains tumor cell proliferative capacity following cisplatin treatment ................................................................................................. 58
4.2.4 BRCA2 downregulation reverses acquired cisplatin resistance in head and neck tumor cells ................................................................................................. 62
4.2.5 BRCA2 modulates tumor cell metabolism following cisplatin treatment .... 65
4.2.6 BRCA2 inhibition decreases metastatic frequency following cisplatin treatment ................................................................................................. 71
Chapter 5. Reciprocal positive selection for weakness - overcoming olaparib resistance

5.1 Preamble

5.2 Results

5.2.1 BRCA2 inhibition overcomes innate olaparib resistance in three lung cancer cell lines

5.2.2 BRCA2 inhibition sensitizes ovarian and breast cancer cells to olaparib treatment

5.2.3 BRCA2 inhibition does not sensitize non-cancerous cells to olaparib

5.2.4 BRCA2 ASO and Olaparib treatment induces changes in copy number and chromosomal translocation frequency in ovarian cancer cells

5.2.5 Combined BRCA2 ASO and olaparib treatment can prevent resistance in a mixed cell line model with varying degrees of HRR

5.2.6 Combined BRCA2 ASO and olaparib treatment can prevent the outgrowth of resistant clones in a co-culture model of BRCA2 heterogeneity

5.2.7 Combined inhibition of BRCA2 and PARP1 prevents ovarian tumor growth in vivo

Chapter 6. Discussion

Chapter 7. References

Appendix A – Copyright permissions

Appendix B - Curriculum Vitae
vi. List of Figures:

Figure 1  Therapeutic resistance in heterogeneous tumor cell populations  p.5
Figure 2  Mutational load and "edge of viability" in tumor cells  p.9
Figure 3  Complementary lethality  p.13
Figure 4  Combined inhibition of BRCA2 and TS: combined complementary lethality  p.16
Figure 5  Reciprocal positive selection for weakness  p.21
Figure 6  Combined BRCA2 siRNA and TS siRNA inhibits A549 cell proliferation and affects cell cycle distribution  p.39
Figure 7  BRCA2 siRNA induces complementary lethality to treatment with cisplatin, melphalan and 5-FUdR, but not pemetrexed in A549 cells  p.41
Figure 8  BRCA2 siRNA induces complementary lethality to treatment with cisplatin, melphalan and 5-FUdR, but not pemetrexed in HeLa cells  p.42
Figure 9  TS siRNA induces complementary lethality to 5-FUdR and pemetrexed but not cisplatin or melphalan in A549 cells  p.44
Figure 10  TS siRNA induces complementary lethality to 5-FUdR and pemetrexed but not cisplatin or melphalan in HeLa cells  p.45
Figure 11  Simultaneous siRNA-mediated knockdown of BRCA2 and TS induces complementary lethality to cisplatin, melphalan, and both TS-targeting drugs in A549 cells  p.48
Figure 12  Simultaneous siRNA-mediated knockdown of BRCA2 and TS induces complementary lethality to cisplatin, melphalan, and both TS-targeting drugs in HeLa cells  p.49
Figure 13  Combined complementary lethality to two different drugs in the same cell population can be achieved by combined BRCA2 and TS siRNA transfection in A549 cells  p.52
Figure 14  Combined complementary lethality to two different drugs in the same cell population can be achieved by combined BRCA2 and TS siRNA transfection in HeLa cells  p.53
Figure 15  Retrospective analysis of patient survival data based on BRCA2 mutation status  p.57
Figure 16  BRCA2 ASO decreases BRCA2 mRNA, protein, and RAD51 foci formation frequency in A549 cells  p.59
Figure 17  BRCA2 regulates cisplatin-induced reduction of lung, ovarian and breast tumor proliferation  p.60
Figure 18  BRCA2 knockdown modulates cisplatin- or melphalan-induced A549 cell proliferation  p.61
Figure 19  BRCA2 does not affect cisplatin-induced proliferation changes in non-cancerous cells and cells without BRCA2 function  p.63
Figure 20  BRCA2 ASO reverses acquired cisplatin resistance in human head and neck cancer cells  p.64
Figure 21  BRCA2 ASO decreases colony forming ability of human head and neck cancer cells following cisplatin treatment  p.66
Figure 22  BRCA2 modulates tumor cell respiration response following cisplatin treatment  p.68
Figure 23  BRCA2 modulates tumor cell glucose uptake following cisplatin treatment  p.69
Figure 24  BRCA2 knockdown in HN-5a cells enhances cisplatin-induced glucose uptake but not Mitotracker-measured mitochondrial function  p.70
Figure 25  BRCA2 regulates tumor cell colony forming ability following cisplatin treatment  p.72
Figure 26  BRCA2 regulates tumor cell metastatic frequency following cisplatin treatment  p.73
Figure 27  BRCA2 inhibition overcomes innate olaparib resistance in three human lung cancer cell lines  p.78
Figure 28  BRCA2 inhibition sensitizes ovarian cancer cell lines to olaparib treatment  p.79
Figure 29  BRCA2 inhibition sensitizes MDA-MD-231 breast cancer cells to olaparib treatment, and continues to sensitize ovarian cancer cells to repeated treatment doses  p.81
Figure 30  Non-cancerous kidney and colon cell lines are not sensitized to olaparib by BRCA2 downregulation  p.83
Figure 31  Combined BRCA2 ASO and olaparib treatment increases the variability in chromosome number in ovarian and breast cancer cells  p.84
Figure 32  Combined BRCA2 ASO and olaparib treatment increases translocation frequency in ovarian cancer cells  p.86
Figure 33  Single treatment with BRCA2 ASO or olaparib has the potential to select for HRR-deficient or HRR-proficient cells, respectively  p.88
Figure 34  Combined BRCA2 ASO and olaparib treatment decreases the proliferation of both HRR-deficient and HRR-proficient cells  p.89
Figure 35  Schematic for mixed cell experiments  p.92
Figure 36  Combined BRCA2 ASO and olaparib treatment prevents outgrowth of resistant cells in a tumor cell population heterogeneous for HRR-proficiency  p.93
Figure 37  BRCA2 inhibition sensitizes ovarian cancer tumors to olaparib treatment in vivo  p.94
vii. List of Abbreviations:

2'-OMe  2'-O-methyl
5-FU   5-Fluorouracil
F-FUdR 5-Fluoro-2-deoxyuridine
ASO    antisense oligodeoxynucleotide
BRAF   serine/threonine protein kinase BRAF
BRCA1  breast cancer susceptibility protein type 1
BRCA2  breast cancer susceptibility protein type 2
CCLE   Cancer Cell Line Encyclopedia
COSMIC catalogue of somatic mutations in cancer
CTL    cytotoxic T-lymphocyte
DNA    deoxyribonucleic acid
DSB    double strand break
dTMP   deoxythymidine monophosphate (thymidylate)
FISH   fluorescence in-situ hybridization
FDA    Federal Drug Administration
HRR    homologous recombination repair
i.p.   intraperitoneal
i.v.   intravenous
miR    microRNA
mRNA   messenger RNA
NHEJ   non-homologous end joining
NSCLC  non-small cell lung cancer
PARP1  poly(ADP-ribose)polymerase 1
PBS    phosphate-buffered saline
pRB    retinoblastoma-associated protein
PRR    pattern recognition receptor
RAD51  DNA repair protein RAD51 homolog 1
RISC   RNA-induced silencing complex
RNA    ribonucleic acid
RNAi   RNA interference
RPA    replication protein A
SAC    spindle assembly checkpoint
shRNA  short hairpin RNA
siRNA  short interfering RNA
SSB    single strand break
TCGA   The Cancer Genome Atlas
TLR    Toll-like receptor
TP53   tumor protein 53
VEGF   vascular endothelial growth factor
Chapter 1. Introduction:

1.1 The hallmarks of human tumor cells

As a result of random mutagenesis and/or genetic predisposition reinforced by Darwinian selection pressures, human cells acquire a variety of interconnected biological traits (or “hallmarks of cancer”) as they progress through malignant transformation into tumor cells [1]. These characteristics, constituting complementary changes in cell physiology, result in highly plastic, immortal and invasive cells which form tumors and cause disease in the host.

According to Hanahan’s seminal review, one fundamental hallmark of a tumor cell is the ability to sustain and react to proliferative signalling [1]. This manifests through different mechanisms, including activating mutations in positive regulators of cell growth (such as the serine/threonine-protein kinase BRAF), which allow constitutive proliferative signalling [2].

By extension, cell cycle control and growth inhibition pathways which negatively regulate proliferation are also frequently mutated in tumor cells. Alterations in prototypical growth suppressors, such as tumor protein 53 (TP53) and retinoblastoma protein (pRB), facilitate an override of powerful monitoring and signalling networks that negatively regulate proliferation [1, 3]. TP53, which is mutated in a high proportion of human cancers, is normally responsible for responding to DNA damage, inappropriate growth signals, nucleotide pool availability, and other intracellular stressors, and can induce signalling events which delay cell cycle progression or promote apoptosis [4].
Apoptosis functions to prevent the survival of abnormal cells. Thus, avoiding apoptosis is another hallmark of cancer, and complements the ability to maintain uncontrolled proliferation. Tumor cells often exhibit further alterations (in addition to TP53) in pro- and anti-apoptotic mediators which collectively decrease susceptibility to programmed cell death and allow continued replication [5]. Telomeres, which function to protect chromosomes from deterioration, are normally shortened with each cell division, and serve to limit the total amount of replicative cycles in a cell. Telomerases are enzymes which can increase telomere length, and though virtually inactive in non-immortalized cells, up to 90% of tumor cells exhibit telomerase activity; this removes the normal limits on DNA replication and enables replicative immortality [1, 6].

Tumor cells develop the qualities described above, as well as other hallmarks (e.g., invasion and metastasis, evasion of the immune system, etc.) which allow for self-renewal, and support the creation of self-sustaining tumor masses. However, underlying all of these traits is a level of genomic plasticity and diversity that allows for continued mutation, adaptation, and response to selection pressures. This can be considered a ‘unifying’ hallmark of cancer, and the majority of human tumor cells exhibit a high mutation frequency and/or chromosomal instability, which facilitates the stochastic acquisition of malignant traits. On the population level, the inherent mutability of individual tumor cells creates a diverse ecosystem complete with polyclonal adaptability, phenotype and genotype variability, and constant competition for survival based on intrinsic and extrinsic selection pressures. In this context, the fit survive while the weak do not, and it is in this multifaceted biome that anti-cancer treatments must function and succeed.
1.2 Tumor heterogeneity and therapeutic resistance

A high degree of heterogeneity is a common feature of human tumors, and presents a significant obstacle for successful therapy. This complex genetic and phenotypic mosaic is determined to a large extent by accumulation of chromosome abnormalities (e.g., breaks, exchanges, duplications, etc.) and alterations in mutational load throughout the dynamic evolution of a tumor [7, 8]. Single-nucleus exome sequencing of breast cancer samples has revealed that no individual cancer cell in a tumor is genetically identical [9], and highlights one of the critical challenges to successful anti-cancer treatment: preventing and overcoming innate and acquired therapeutic resistance in a polyclonal tumor ecosystem.

Given this high degree of intratumor diversity, it is highly likely that a population of resistant cells exists in a tumor at the start of any therapy [10, 11]. For example, hundreds of individual clones have been identified in the HCC827 lung cancer cell line with pre-existing resistance to erlotinib (EGFR inhibitor). In the same series of experiments, dozens of clones with dual resistance to both erlotinib and crizotinib (ALK-EML fusion inhibitor) were also isolated [10]. This suggests that any given tumor cell population may contain a detectable number of subclones with pre-existing resistance mutations, thus rendering the probability of treatment failure extraordinarily high, and partly explains why overall survival is rarely improved following therapy.

Alternatively, the high mutation rate present in a significant proportion of tumor cells drastically increases the likelihood of resistance through de novo mutations during the course of treatment [11]. This is more probable if the treatment is slow acting (i.e., acts over a time frame that facilitates DNA replication
and/or repair) because it allows more time for potentially deleterious mutations to develop [12]. However, these scenarios (pre-existing versus newly-arising mutations which lead to resistance) are not mutually exclusive, can both lead to therapeutic failure, and were proposed and discussed in the scientific literature as early as 1957 [13] (Figure 1).

Mathematical modeling based on melanoma patient responses to the BRAF inhibitor vemurafenib (which targets the common V600E mutation [14]), illustrate the relationship between mutation rate, tumor size, and drug resistance. According to the algorithm, it is a mathematical certainty that a tumor population will become resistant to vemurafenib if the tumor is of sufficient size and exhibits a high mutation rate [12]. This maximizes the probability that resistant subpopulations will exist at the start of treatment. If the therapy is slow acting, then a high mutation rate increases the probability of de novo resistance mutations arising during treatment. A similar relationship to that described for melanoma has been identified in the development of colon cancer cell resistance to erlotinib treatment [15]. Therefore, targeted molecular drugs such as vemurafenib and, by extension, general cytotoxic drugs such as cisplatin, are predicted to fail with a very high probability, and this prediction has proven to be true in clinical practice [16, 17]. Combination therapy decreases the likelihood of resistance, but is still a function of tumor size and the speed with which the therapy debulks the tumor mass [12].

Therefore, designing therapeutic approaches to overcome the inherent challenge of tumor heterogeneity-driven resistance is crucial to improve efficacy and patient outcome. One strategy, which will be described in the section 1.4, is to rationally (based on known function and predicted outcome) inhibit specific
Figure 1 – Therapeutic resistance in heterogeneous tumor cell populations. In a given tumor mass with a large enough size and mutation rate, it is highly likely that resistant cells pre-exist at the start of treatment. For instance, the resistant cells may express a DNA repair protein like BRCA2 at high levels, which renders them less responsive to DNA-damaging therapy. The sensitive cells (e.g., BRCA2 deficient), which make up the majority of the population, are eliminated by treatment and the resistant cells re-populate the tumor, leading to therapeutic failure. However, it is also possible that stochastic emergence of resistant cells occurs as a result of random mutagenesis during the treatment process. This is more likely to occur if the treatment is slow acting.
mediators of DNA repair and combine these treatments with chemotherapeutic drugs that kill or inhibit tumor cell replication by damaging DNA. The aim is to sensitize tumor cells to treatment by overcoming DNA repair-mediated, innate resistance to therapy, and decrease the probability of tumor cell survival following treatment. A second approach, described in section 1.5, is to combine inhibition of specific DNA repair mediators in a manner that exploits their functional interdependence and renders acquired resistance more unlikely in a heterogeneous tumor population by selecting for ‘weakness’ instead of ‘fitness’.

1.3 Genome plasticity, DNA repair, and cancer treatment

A significant proportion of solid tumors exhibit a high mutational rate and/or chromosomal instability (CIN), which contribute to tumor heterogeneity and thus increase the fitness of a tumor cell population and the incidence of therapeutic resistance [18]. However, these same characteristics can act as an “Achilles’ heel” for individual tumor cells which mutate past a tolerable threshold and reach a level of DNA or chromosomal instability that is no longer compatible with survival [19].

It is well established that mutations in DNA repair proteins such as BRCA1 or BRCA2 lead to an increased risk of developing breast, ovarian, and other cancers [20]. Compromised homologous recombination repair (HRR) capacity leads to higher mutational load and chromosomal instability that promotes malignant transformation and tumor development [21]. However, even though individuals with BRCA2 mutations exhibit increased cancer incidence, patients with BRCA2-mutated tumors respond more favourably to therapy than those with sporadic (non-BRCA2 related) cancers [22, 23]. This may occur due to a higher
sensitivity of BRCA2-mutated tumors to DNA damaging chemotherapy (as a result of decreased ability to repair DNA following treatment).

Mathematical simulations combined with genome sequencing data have shown that increased levels of passenger mutations (generally considered innocuous with respect to direct impact on tumor cell survival) can actually be deleterious to tumor viability during therapy, and that treatments which either increase mutational load or increase the impact of mutations already present in cells can lead to tumor regression. [24]. A sufficiently high number of passenger mutations may contribute to chromosome instability (which can negatively impact cell replication and viability), as well as increased immune recognition of tumor cells (due to higher production of immunostimulatory neo-antigens) [25]. In addition, analysis of The Cancer Genome Atlas (TCGA) data on the basis of high versus low mutation load and survival, has shown that patients with tumors that exhibit a high level of mutation have better overall and progression-free survival compared to patients with tumors that harbour fewer mutations [26].

Therefore, vulnerability due to high mutational load and an inability to counteract the effects of DNA damaging anti-cancer treatment are characteristics of tumor cells with reduced or non-functional DNA repair pathways. Actively inhibiting DNA repair modulators is a promising therapeutic avenue to recapitulate these traits in all tumor cells, regardless of baseline DNA repair proficiency.

In other words, inhibiting DNA repair in tumor cells may sensitize (decrease the innate resistance) to specific types of DNA-damaging therapy and render cancer cells more responsive to the action of commonly used drugs. It is possible this type of approach may also be cancer cell specific, by virtue of the highly de-
regulated genomic landscape which renders tumor cells closer to the proverbial "edge of viability" [27] (Figure 2). This concept is based on the premise that cells must maintain a minimum level of genomic fidelity to survive (the edge of viability). Mutations and chromosomal aberrations present in tumor cells decrease the baseline level of genome fidelity and thus render cancer cells closer to the theoretical edge, past which survival is not possible. Non-cancerous cells, which are further away from the edge of survival due to a low mutation load, also exhibit rigidly controlled DNA replication and repair mechanisms that ensure that they do not progress through the cell cycle when damage occurs. A comparatively high proportion of tumor cells exhibit compromised cell cycle and DNA replication control, which may render them vulnerable to therapeutic attack that increases mutation load and/or DNA damage, pushing them over the edge of viability [27].

In addition, actively inhibiting DNA repair in cancer cells may reduce acquired resistance by preventing therapy-mediated selection of clones that exhibit high DNA repair ability within a heterogeneous population of cells. In a polyclonal tumor environment, subpopulations with more proficient repair mechanisms may survive treatment. Actively inhibiting DNA repair will help to prevent selection based on DNA repair ability, because these processes will be (in theory) impaired in all tumor cells (irrespective of baseline repair proficiency).

1.4 Antisense molecules as therapeutic agents

Two major classes of antisense molecules being explored for therapeutic use are short interfering RNAs (siRNAs) (20-25 mer RNA duplexes) and antisense
Figure 2 – Mutational load and “edge of viability” in tumour cells. In this theoretical graph, cancer cells are thought to be closer to the “edge of viability” due to a higher than normal mutational load. Thus, treatments that increase DNA damage and mutation may preferentially affect cancer cells, which usually exhibit compromised cell cycle control and/or apoptotic pathways. Normal cells, which have a lower mutational load and exhibit tightly controlled DNA replication and repair mechanisms, are further from that edge and will not accumulate mutations or genome abnormalities as readily. This phenomenon is supported by evidence showing that patients with tumours that exhibit a higher mutational load and/or DNA repair defects, respond more favourably to therapy.
oligodeoxynucleotides (ASOs) (20-25 mer single-stranded DNA molecules) [28]. siRNAs bind to complementary RNA within the cell and induce Argonaute 2-mediated degradation of the target transcript via the RNA-induced silencing complex (RISC) [29]. Eukaryotic cells utilize RISC for post-transcriptional gene regulation (via endogenous RNA-interference molecules such as microRNAs (miRs)), and exogenous siRNAs exploit these naturally occurring pathways for both experimental and therapeutic applications. ASOs, unlike siRNAs, mediate target RNA destruction via the RNase H pathway, which normally degrades RNA-based DNA primers to ensure efficient DNA synthesis [30]. ASOs for therapeutic or experimental use exploit this naturally occurring phenomenon by forming DNA-RNA hetero-duplexes with target RNA and inducing their degradation via RNase H [31].

The use of antisense-based therapeutic agents holds significant promise because it can target pathways considered “undruggable” by conventional approaches (e.g., small molecule inhibitors and therapeutic antibodies). Antisense molecules can induce degradation of target mRNA via complementary base pairing and, in theory, any RNA product can be silenced by antisense approaches [32].

Another important advantage of antisense based drugs is high specificity and the relative lack of toxicity. Small molecule inhibitors, such as those targeting enzymatic active sites, can often have significant off-target effects due to agent promiscuity. However, antisense molecules are sequence specific and can be engineered to limit toxicity or off-target mRNA degradation by specific chemical modification. For instance, the addition of a 2’-OMe group has been shown to decrease off-target transcript degradation by 80% [33]. Oligodeoxynucleotides also
have the potential to stimulate pattern recognition receptors (PRRs) (such as Toll-Like Receptors (TLRs) and cytoplasmic receptors like PKR or RIG-I) and thus induce an innate immune response (e.g., type I interferon). The occurrence of this can be limited by replacing immunostimulatory bases such as guanosine and uridine, with relatively non-stimulatory bases such as adenosine [33].

One of the barriers to successful antisense clinical application has been the lack of an efficient in vivo delivery system. However, the chemical characteristics of ASOs (which, as single-stranded DNA molecules, lack the labile, alkali-sensitive hydroxyl group on carbon 2 of the pentose sugar present in antisense RNA molecules and are of lower molecular weight than double-stranded antisense RNA) have greatly increased uptake and stability in vivo without the need for a delivery vehicle [34]. In addition, different types of liposomal delivery vehicles have been designed for siRNA use to protect siRNA from cleavage and to enhance their delivery into target cells [32, 35], and some of these are being tested clinically [36]. As a result, both classes of antisense molecules described herein have been utilized successfully in animal models in vivo, and have also made the transition to various stages of human application (e.g., clinical trials and FDA approval).

Mipomersen is one FDA approved ASO drug which targets apolipoprotein B and is used for the treatment of familial hypercholesterolemia. It is administered on a weekly basis without a delivery vehicle [37, 38]. In the cancer treatment sphere, a number of ASO-based drugs developed by different companies are at varying stages in the clinical pipeline [28, 29]. Custirsen (OGX-011) is an ASO agent that has been investigated in Phase I and II clinical trials for use in prostate cancer, and has demonstrated survival and palliative benefit [39, 40]. Phase III
trials of this drug are currently ongoing for both castration-resistant prostate cancer (second line) as well as advanced or metastatic non-small cell lung cancer (NSCLC) [41].

siRNA molecules have been delivered *in vivo* to downregulate a multitude of targets in tumor xenografts [42, 43] and also in clinical trials [35, 44, 45]. In one trial, siRNA was used to downregulate VEGF and KSP in patients with liver metastases, and showed anti-tumor activity while inducing minimal side-effects [36]. These studies highlight the promise of antisense based drugs and show that the true potential of this technology is still untapped clinically.

1.5 Overcoming innate resistance through complementary lethality – sensitizing cancer cells to therapy

The effect of DNA-damaging therapy can be attenuated by functioning DNA repair pathways in tumor cells [46]. Thus, intact DNA repair pathways are a source of intrinsic (or innate) resistance to common types of anti-cancer therapy and inhibition of DNA repair mediators is an avenue to sensitize cancer cells to treatment. I termed this concept “complementary lethality” and defined it as the enhancement of therapeutic efficacy by inhibition of factors important for cellular resistance to the action of that specific therapy (Figure 3) [47].

BRCA2 is intimately involved in DNA repair and replication in human cells. It functions as a scaffold by displacing replication protein A (RPA) on the single-stranded DNA tails which flank sites of double-stranded breaks (DSBs), and promotes RAD51 loading onto DNA to initiate the process of HRR [48]. Furthermore, BRCA2 prevents the degradation of stalled replication forks by
**Figure 3 – Complementary lethality.** Factor X is involved in the amelioration of Drug X-induced effects in the cell. For instance, Factor X can be a DNA repair protein and Drug X may be inducing DNA DSBs. This limits the efficacy of the drug because the function of Factor X is to repair the damage that Drug X promotes. Inhibiting Factor X will eliminate Factor X-mediated resistance to Drug X in a complementary manner.
stabilizing RAD51 filament formation via its C-terminal domain, in a process separate from the functions of BRCA2 in HRR [49]. In addition, BRCA2 is active during the spindle assembly checkpoint (SAC) and promotes the acetylation of BuBR1 (a crucial mediator of the SAC) by recruiting the PCAF acetyltransferase [50]. As a result, BRCA2 represents a source of innate resistance to a variety of chemotherapeutic drugs, particularly DNA alkylators and platinators which induce damage that lead to DSBs [51]. This role, combined with the more favourable prognosis of patients with BRCA2 mutated tumors [22, 23], render BRCA2 an intriguing target for direct therapeutic intervention in combination with other specific treatments. Such a strategy contrasts with simply identifying the individuals with BRCA2-mutated cancers and tailoring treatment accordingly [52], which though potentially valuable, has the potential to aid only a small proportion of patients.

The cytotoxic agent cisplatin is commonly used to treat a variety of solid human tumors, including lung and head and neck cancers [16, 53]. Cisplatin induces cytotoxicity by causing DSBs in cellular DNA, leading to replication collapse and apoptosis [54]. Resistance to cisplatin develops with high frequency via several mechanisms [51, 55], and in most cases treatment fails to cure patients of disease. Given the role of BRCA2 in DSB repair through HRR, inhibiting BRCA2 in the context of cisplatin treatment may be a method to induce complementary lethality and increase tumor cell sensitivity (decrease innate tumor cell resistance) to cisplatin.

In addition, due to the high degree of heterogeneity present in most solid tumors and the correspondingly high probability of resistance to treatment, it may be favourable to induce complementary lethality to another class of drugs by
targeting a separate pathway simultaneously. Theoretically, this should decrease the probability of a pre-existing resistant subpopulation being present at the start of treatment, which is favourable in terms of patient outcome and preventing therapeutic failure [12].

One potential candidate for this type of combination treatment is thymidylate synthase (TS). TS is responsible for de novo synthesis of cellular thymidylate (dTMP) and is the target of several anti-cancer drugs, including fluoropyrimidines and folate analogs (e.g., 5-FU, 5-FUdr, pemetrexed, etc.) [56]. Inhibition of TS or the TS pathway results in decreased pools of dTMP available for DNA synthesis, which negatively impacts tumor cell replication and growth. However, similar to the case of cisplatin, resistance to this class of drugs is common, and TS has been well-studied in our laboratory as a potential target to prevent drug resistance [57]. Previous studies have shown that downregulation of TS sensitizes tumor cells to TS-targeting drugs and can render resistant cells sensitive to treatment [56, 58, 59]. Therefore, both BRCA2 and TS appear to function as innate sources of resistance to two different types of chemotherapy, which target DNA replication and repair from separate angles. It is possible, therefore, that combined inhibition of both BRCA2 and TS may be an avenue to increase tumor cell susceptibility to two distinct drug classes at the same time (Figure 4A&B).
Figure 4 – Combined inhibition of BRCA2 and TS: combined complementary lethality. (A) DNA damaging drugs such as cisplatin induce DSBs in cellular DNA, while TS-targeting agents inhibit the function of TS; they are involved in largely separate pathways inside the cell. However, the effectiveness of these drugs can be decreased, and the cell can be rescued from their action if it expresses functional BRCA2 and maintains residual TS activity (innate sources of resistance to these drugs). (B) When DNA damaging drugs and TS-targeting drugs are administered in combination with antisense molecules targeting both resistance factors (BRCA2 and TS), the cell will be more susceptible to drug induced effects. This type of “combined complementary lethality” which targets innate sources of resistance, may also be a way to prevent the development of acquired resistance in heterogeneous tumor cell populations, by decreasing the likelihood of cancer cell survival following treatment.
Such a strategy may more effectively eliminate cancer cells by overcoming innate resistance, and decreasing the probability of acquired resistance appearing in the population: a tumor cell would have to develop counter measures not only to two separate classes of drugs, but also to sensitization to those drugs. For instance, drug efflux pumps decrease the concentration of drug in the cytoplasm, but a sensitized cell may still be negatively affected despite the lower drug concentration (relative to an un-sensitized cell).

Our group has previously developed and tested a TS-targeting ASO in pre-clinical *in vitro* and *in vivo* models [56, 58, 60]. A significant portion of this thesis is devoted to testing a novel BRCA2-targeting ASO that I aim to use in conjunction with chemotherapeutic drugs such as cisplatin, as well as combining it with our TS-targeting ASO to create multi-drug sensitive tumor cells.

**1.6 Preventing and overcoming acquired resistance – reciprocal positive selection for weakness**

Anti-cancer treatment acts as a powerful positive selection tool which destroys susceptible cells and enriches the population of cells resistant to a particular therapy. In a clinical setting, this can lead to an increase in progression free survival, but rarely an appreciable difference in overall survival since the tumors almost inevitably recur as a result of acquired resistance [18, 61].

As described above, one method that may prevent or delay the onset of therapeutic failure is to induce complementary lethality by inhibiting a target like BRCA2 or TS to sensitize tumor cells to the specific action of a drug or combination
of drugs. Such a strategy attempts to increase the likelihood that drug treatment will affect the largest proportion of tumor cells, and decrease the mathematical probability of pre-existing resistance based on tumor size and mutation rate.

However, a second method to minimize the probability of resistance and increase the duration of therapeutic response is to employ a treatment regimen that selects for weakness in the tumor cell population instead of fitness. This can seem contrary to the principles of Darwinian evolutionary thought, but I hypothesize it can be achieved using a carefully orchestrated combination therapy regimen involving simultaneous inhibition of BRCA2 and PARP1.

PARP1 is an enzyme involved in a variety of cellular processes, including DNA repair and replication. More specifically, PARP1 regulates single strand break (SSB) DNA repair which is essential for normal DNA replication [62]. If SSBs are left unresolved they can cause replication fork collapse, which results in DSBs that must be repaired by HRR or the error prone non-homologous end joining (NHEJ) pathway [63]. In addition, PARP1 is directly involved in the maintenance of stalled replication forks by preventing MRE11-mediated degradation of DNA. When a replication fork is stalled due to base damage or other obstacles that hinder the progression of DNA polymerase, MRE11 acts as an endonuclease which degrades the DNA, causing fork collapse and replication failure. PARP-1 prevents this occurrence and maintains replication fork integrity, providing the time necessary for damage to be repaired [64].

Given the role of PARP1 in DNA repair and replication, the PARP1 inhibitor olaparib is synthetically and selectively lethal in cells with HRR defects, but does not affect HRR-proficient cells [65-67]. In the absence of functional HRR, the cell
is unable to repair the DSBs that result from PARP-1 inhibition (via unresolved SSBs), which results in lethal DNA damage. This ability to spare non-cancerous, HRR-proficient cells was the basis for much of the enthusiasm surrounding PARP1 inhibition and spawned a large effort by the biotechnology industry to identify, test, and market a gamut of PARP1-inhibiting drugs [68]. After several clinical trials with mixed results and an FDA rejection for accelerated drug status, Olaparib was approved by the FDA for use in advanced ovarian cancer patients with validated BRCA gene mutations [69]. Another PARP1 inhibitor called Veliparib is currently undergoing a Phase III clinical trial as a first-line therapy in combination with chemotherapy for BRCA-mutation-positive breast cancer [70].

However, the same characteristics and circumstances that render PARP1 inhibition so attractive in oncology (selective killing of tumor cells with HRR defects) is also part of what ultimately nullifies its effectiveness. For one, this limits the applicability and usefulness of PARP1 inhibitors to the treatment of HRR-deficient tumor cells, which represents only a subset of all cancer patients [26, 71]. Furthermore, selective killing in a heterogeneous tumor population may rapidly lead to the outgrowth of HRR-proficient, resistant clones and therapy failure.

At least five separate PARP1 inhibitor resistance mechanisms have already been identified in in vitro experiments and in clinical studies, including upregulation of drug efflux pumps that decrease drug concentration inside the cell, and 53BP1 mutations that reactivate HRR pathway functionality in BRCA1 deficient cells [72-74]. However, the most striking resistance mechanism is the reported reversion of BRCA2-mutated tumors to functional BRCA2 following olaparib treatment [75]. The implications of this finding are twofold: 1) BRCA2 mutation status (and by extension
HRR-proficiency) is heterogeneous, even in a tumor population primarily composed of BRCA2 mutated cells and; 2) The selection pressure for HRR proficiency is so great during PARP1 inhibitor treatment, that tumor cells with functional HRR have a distinct survival advantage and will eventually overtake the HRR-deficient population.

PARP1 inhibitor resistance displays the need for a new combinatorial approach to their application in the clinic. Given the unique relationship between PARP1 and HRR status, it is inevitable that PARP1 inhibition alone will simply select for a small group of subclones in the population that are proficient for HRR. I hypothesize, therefore, that resistance based on HRR function can be forestalled or even eliminated by combining inhibition of PARP1 and BRCA2.

In a tumor population with heterogeneous HRR proficiency, olaparib treatment will eliminate the HRR-deficient cells and select for the HRR-proficient clones. BRCA2 inhibition, on the other hand, will affect cells with intact HRR and select for HRR-deficient cells. Therefore, in this scenario, PARP1 inhibition selects for cells that are susceptible to BRCA2 inhibition, and BRCA2 inhibition selects for cells that are sensitive to PARP1 inhibition. I termed this concept “reciprocal positive selection for weakness”, since each treatment selects for cells which will be sensitive (weak) to the other treatment in a reciprocal manner. I believe this may be a mechanism to prevent or delay the development of acquired resistance in a heterogeneous tumor cell population, by forestalling the enrichment of a particular group of subclonal variants with pre-existing resistance to treatment (Figure 5).
Figure 5 – Reciprocal positive selection for weakness. In a population heterogeneous for HRR-proficiency, BRCA2 inhibition will impair the growth of HRR-proficient cells and select for HRR-deficient cells. PARP-1 inhibition will accomplish the reciprocal of that by impairing the growth of HRR-deficient cells while selecting for HRR-proficient cells. Therefore, each treatment selects for cells that are susceptible to the other treatment. When BRCA2 and PARP1 inhibition is combined, both HRR-proficient and HRR-deficient cells are affected, nullifying selection based on HRR-proficiency.
1.7 Hypothesis

Inhibiting DNA repair pathways will overcome innate drug resistance and prevent acquired drug resistance in human cancer cell populations.

1.8 Objectives:

1) To investigate whether BRCA2 siRNA can sensitize cancer cells \(i.e., A549\) and HeLa\) to specific DNA-damaging chemotherapeutic drugs \(i.e.,\) cisplatin and melphalan\) related to BRCA2 function.

2) To determine whether combined BRCA2 siRNA and TS siRNA treatment can render cancer cells \(i.e., A549\) and HeLa\) sensitive to multi-drug treatment \(i.e.,\) cisplatin or melphalan and 5-FUdR or pemetrexed.

3) To develop and test a BRCA2 targeting ASO and determine effects on tumor cell proliferation \(via\) cell counting and colony formation assays, metastasis \(via\) in vivo chick cam assays\) and metabolic response \(respiration and acidification\) when combined with cisplatin treatment.

4) To determine whether BRCA2 ASO treatment can overcome innate resistance to the PARP1 inhibitor olaparib in a variety of cancer cell lines \(i.e., A549, H2052, SKOV-3, CaOV-3, MDA-MB-231\)

5) To investigate whether combined BRCA2 and PARP1 inhibition can prevent therapeutic resistance in a tumor cell population heterogeneous for HRR-proficiency, using a 3:1 co-culture model of HRR-proficient and HRR-deficient cells \(\text{SKOV-3}^{\text{shcontrol}}\) and \(\text{SKOV-3}^{\text{shBRCA2}}\)\) to monitor long term response to treatment.
6) To determine whether combined BRCA2 siRNA and olaparib treatment can decrease ovarian tumor growth *in vivo* (number of nodules and total tumor weight) using an orthotopic model of intraperitoneal SKOV3-1P1 cell injection, and whether the magnitude of tumor growth suppression is higher than with either treatment alone.
Chapter 2. Materials and Methods:

2.1.0 - Cell lines

Human lung (A549), cervical (HeLa), mesothelioma (211H & H2052), ovarian (SKOV-3 & CaOV3), breast (MCF-7 & MDA-MB-231) and BRCA2-mutated pancreatic (CAPAN-1) cancer cells, as well as non-tumorigenic kidney (HK-2) and colon (CCD-841) cells were obtained from ATCC (Manassas, Virginia, USA) and maintained in AMEM, DMEM, or Iscove’s medium (Wisent, St. Bruno, Quebec, Canada) supplemented with 10 or 20% fetal bovine serum (FBS) (GIBCO - ThermoFisher Scientific, Waltham, MA, USA), or Keratinocyte Serum Free medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor (GIBCO – ThermoFisher Scientific) under standard conditions (37°C in a humidified 5% CO₂ atmosphere). Parental human head and neck squamous carcinoma HN-5a cells and cisplatin-resistant HN-5a/carbo15a cells [76] were maintained in AMEM medium with 10% FBS under standard conditions. Cell culture plastic-ware was obtained from Invitrogen, Fisher Scientific (Unionville, Ontario), and VWR International (Mississauga, Ontario).

2.1.1 - siRNA transfection

siRNA transfection was performed according to the following protocol: the concentrations of siRNAs targeting human BRCA2 (OnTarget Plus BRCA2 siRNA #4 or OnTarget Plus SMARTPool BRCA2 [Dharmacon RNAi Technologies, Lafayette, CO, USA]) and human TS (OnTarget Plus TS siRNA #2; [Dharmacon])
that reduced target mRNAs by approximately 70% at 24 h post-transfection were determined (10 nM for BRCA2 siRNA, and 2.5 nM for TS siRNA). To apply equal amounts of siRNA to A549 and HeLa cells in studies where BRCA2 and TS were knocked down individually or collectively, control non-targeting siRNA (Dharmacon control siRNA #2 or control SMARTPool) was added to BRCA2 siRNA or TS siRNA so that the total siRNA concentration applied in every case was 12.5 nM. BRCA2 siRNA (10 nM plus 2.5 nM control non-targeting siRNA), TS siRNA (2.5 nM plus 10 nM control non-targeting siRNA), for BRCA2 siRNA plus TS siRNA (10 nM plus 2.5 nM, respectively) were diluted in serum-free AMEM and incubated with diluted Lipofectamine 2000 (LFA2K, Invitrogen – ThermoFisher Scientific) for 20 min. The siRNA:LFA2K mix was then added to cells seeded, in triplicate, at a density of 2.0 x 10^5 cells in 25 cm^2 flasks 24 hours prior to transfection. Medium was exchanged for fresh AMEM plus 10% FBS 4 hours post-transfection and the effects of siRNA treatments on target mRNA levels and sensitivity to cytotoxic drugs were assessed as described below.

2.1.2 - Antisense oligonucleotide (ASO) transfection

Oligonucleotide transfection was performed according to the same protocol as siRNA transfection (above), with some modifications [47]: BRCA2 ASO or control ASO were diluted in serum-free medium and incubated with Lipofectamine 2000 (Invitrogen - ThermoFisher Scientific) for 20 min, then added to cells to a final concentration of 20 nM. After 4 hours, transfection medium was replaced or cells were harvested for subsequent treatment as described.
2.1.3 – mRNA quantification

Twenty-four hours post-transfection of siRNA or ASO, total cellular RNA was isolated from cells using Trizol® reagent (Ambion - ThermoFisher Scientific) according to manufacturer’s instructions and reverse-transcribed to generate cDNA using M-MLV RT enzyme (Invitrogen - ThermoFisher Scientific). cDNA (1 μg) was used in conjunction with BRCA2, TS, and 18S or GAPDH RNA qPCR probe and primers and Taqman master mix (Applied Biosystems – ThermoFisher Scientific) to generate fluorescently labelled target cDNA. Quantification of cDNA to infer levels of TS and BRCA2 mRNAs and 18S or GAPDH RNA was performed using an Applied Biosystems ViiA 7 Real-time PCR System (ThermoFisher Scientific). TS and BRCA2 mRNA levels were determined relative to cellular 18S or GAPDH RNA levels.

2.1.4 – Protein immunoblot

BRCA2 immunoblotting was performed according to the following protocol: cells were transfected with ASO and cell lysates were collected 72 hour post-transfection. Following a Bradford assay, total cellular protein (40 μg per well) was loaded on a 4-15% gradient gel (Bio-Rad, Hercules, California, USA) and electrophoresed (1.5 h, 100 V), then transferred to PVDF membrane using a BioRad Turbo Transfer Pack (Bio-Rad). The membrane was blocked with TBS-T + 5% BSA for 1 hour and then incubated with BRCA2 MAb (1:500, 16 hours) (Cell Signaling #9012S) and actin MAb (1:1000, 1 h) (Sigma-Aldrich Co., St. Louis, MO, USA)
2.1.5 - Cell cycle analysis

Cells were transfected with siRNA as described previously. Forty-eight and 72 hours post transfection they were harvested, washed with PBS, and fixed with 70% ethanol for at least 2 hours. Cells were washed with PBS and re-suspended in 1 ml of a propidium iodide (20µg/ml) (Sigma Aldrich) and 0.1% Triton X-100 (BDH Chemicals, Toronto, ON Canada) staining solution with RNAse A (Bioshop Canada, Inc., Burlington, ON) for 15 minutes at 37°C. Cell cycle enumeration was performed using a BD FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada) and analyzed using Flow Jo software (Tree Star, Inc., Ashland, OR, USA).

2.1.6 - cBioPortal Analysis of TCGA data

The cBioPortal website client (http://www.cbioportal.org/public-portal/) was utilized to analyze the survival differences in patients with wild type or mutated BRCA2 or BRCA1, in both ovarian and endometrial cancer. The analysis was performed using the Ovarian Serous Cystadenocarcinoma (Cancer Genome Atlas Research Network, 2011) dataset and the Uterine Corpus Endometroid Carcinoma [77] dataset.

2.1.7 - Cell proliferation following drug treatment

Cells were trypsinized, counted, and re-plated in triplicate at a density of 2.0-5.0x10^4 cells per well in a six well plate 24 h post transfection. Cytotoxic drugs
(e.g., cisplatin [Sigma Aldrich], melphalan [Sigma Aldrich], 5-FUdr [Sigma-Aldrich], pemetrexed [Sigma Aldrich], olaparib [Selleckchem, Boston, USA]) were added in triplicate wells for each drug concentration. Cells were trypsinized 76 h after addition of drug and counted (twice per sample) using a Coulter Z-1 Particle Counter (Beckman-Coulter, Mississauga, ON). Relative proliferation was calculated using the starting cell number at seeding, and normalized to the no drug ASO treatment condition (e.g., control ASO + drug treatment was normalized to control ASO alone).

2.1.8 - Colony formation assay

Forty-eight hours post transfection the cells were exposed to different concentrations of drug for a total of 6 hours. The cells were collected and plated in triplicate at a density of 500 cells per well in a 6 well dish. In the case of irradiation treatment, cells were harvested immediately post treatment and plated at a density of 500 cells per well in a 6 well dish. Colonies were fixed with a Protocol® Hema-3 kit (ThermoFisher Scientific) 7-10 days later and counted.

2.1.9 - Chicken chorioallantoic membrane (CAM) metastasis assay

Forty-eight hours post transfection cells were exposed to 6 µM of cisplatin for 6 hours. The cells were harvested, washed three times, and adjusted to a final concentration of $1.0 \times 10^6$ cells per ml in PBS. $1.0 \times 10^5$ cells (100 µl) were injected into the venous circulation of 9 day old chicken embryos. Seven to 9 days post
injection the metastatic foci were counted using an Axio Zoom V16 (Carl Zeiss AG, Germany) microscope at 20x magnification [78].

2.2.0 - Bionas Discovery metabolic measurement

Four hours post-transfection cells were collected and seeded onto Bionas biosensor chips (Bionas GmbH, Rostock, Germany) at a density of 180,000 cells per chip. The medium was changed to AMEM+0.2% FBS and the biosensor chips were loaded into the Bionas system (Bionas GmbH) 24 hours later. Using a 4µl/min flow rate, the cells were exposed to AMEM+0.2% medium for 6 hours and then 6 µM cisplatin for 24 hours. The cells were exposed to medium for 48 hours, at which point the cells were lysed with 0.1% Triton-X to determine baseline readings [78].

2.2.1 - Glucose uptake assay

Twenty-four hours post-transfection cells were treated with 6µM of cisplatin. Cells were washed with PBS and then incubated with glucose free DMEM (GIBCO) + 10% FBS for 20 mins, 48 h post drug treatment. The cells were incubated in glucose free DMEM+10% FBS+29µM 2-NBD fluorescent glucose (Molecular Probes, ThermoFisher Scientific) for 1 h. Cells were collected, washed with PBS, and glucose uptake was enumerated using a FACSCalibur2 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).
2.2.2 - Mitotracker assay

Twenty-four hours post-transfection cells were treated with 6µM of cisplatin. To enumerate the frequency of cells with functional mitochondria, cells were harvested and stained with 75nM MitoTracker Red CMXROS (ThermoFisher Scientific) [79] according to manufacturer’s instructions, 48 hours post drug treatment. MitoTracker staining was enumerated using flow cytometry and expressed as mean fluorescence intensity (geometric mean).

2.2.3 - AlamarBlue assay for cell proliferation

Cells were transfected with ASO as described and re-plated in 96 well plates (7.5 X 10^3 cells per well). Twenty-four hours post transfection cells were treated with different concentrations of cisplatin, melphalan, 5-FUdR, or pemetrexed (Sigma Aldrich, Co.). Seventy-two hours later medium was removed and a 1:12 dilution of AlamarBlue (ThermoFisher Scientific) was added for 3 hours. Following incubation, the turnover of AlamarBlue reagent was quantified using a Victor2 plate reader (Wallac, Perkin-Elmer, USA).

2.2.4 - Rad51 focus formation assay to examine BRCA2 function

Cells were transfected with ASO as described previously, except that they were grown on microscope slide cover slips in 12 well plates prior to transfection. Cells were treated with cisplatin (6 µM, 6 hours) starting 48 hours post-transfection, washed with PBS and fixed (4% paraformaldehyde, 30 min), permeabilized (TBS
+ 0.3% Triton X-100, 10 min), and blocked (TBS + 3% BSA, 3% goat serum, 1 h). Anti-Rad51 MAb (Calbiochem - EMD Millipore, Germany, 1:500) in blocking solution was added and incubated at 4°C for 16 h. Cells were washed in TBS and incubated with secondary antibody (Cell Signaling, 1 h, 25°C), washed again, then stained and mounted with DAPI SlowFade Gold anti-fade reagent (Molecular Probes, ThermoFisher Scientific) [80].

2.2.5 - Generation of cells stably expressing shBRCA2 and shControl

SKOV-3 cells (ATCC) were transfected with linearized plasmids expressing the shControl and the 4 shBRCA2 constructs (Cat. No. 336312 Qiagen) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. Seventy-two hours post transfection, the cells were treated with hygromycin (Sigma Aldrich) for seven days. Over 40 individual colonies were isolated using glass isolation rings and then expanded. Sensitivity to olaparib was tested using cell counting proliferation assays as described above.

2.2.6 - Metaphase spread preparation and chromosome counting

Four hours post transfection, cells were collected and plated on microwell containing glass slides. Drug treatment commenced 24 hours post transfection and the cells were allowed to grow for a further 24 hours. Colcemid (Sigma Aldrich) was added to the medium for the last 2 hours of culture. The cells were washed with PBS and then treated with pre-warmed (37°C) KCL (75 mM) for 20 minutes.
Cold (4°C) fixative (3:1 methanol:acetic acid solution) was added to the cells for 2 minutes. The fixative solution was then replaced with fresh solution and incubated for a further 20 minutes. The fixative solution was replaced a final time for another 20 minutes and the slide was allowed to air-dry at room temperature. The slides were then mounted with DAPI or used for subsequent FISH. DAPI mounted slides were used to determine chromosome number using an inverted fluorescent microscope (Olympus, Tokyo, Japan).

2.2.7 - Fluorescence in situ hybridization (FISH) for whole chromosomes

Following metaphase preparation, the slides were washed in 2X SSC (pH 7.0) for 2 minutes at 73°C. The slides were transferred into a 0.005% pepsin solution for 10 minutes at 37°C and then washed with PBS for 5 minutes at room temperature. The slides were fixed in 1% formaldehyde for 5 minutes at room temperature and then washed with PBS. The slides were sequentially dehydrated by immersion in 70%, 85% and 100% ethanol. Diluted FISH probes (Empire Genomics, Buffalo, NY, USA) were applied to the slide, covered with glass coverslips and sealed with rubber cement. The probes and chromosomal DNA were co-denatured on a hot plate at 68°C for 5 minutes. Hybridization was performed at 43°C in a humidified chamber for 4 hours. Following hybridization, the slides were washed with 2X SSC +0.1% Igepal (Sigma Aldrich) at room temperature to remove the cover slips and rubber cement. The slides were washed at 65°C with 0.4X SSC + 0.3% Igepal for five minutes, rinsed briefly with ddH₂O.
and then air dried. The slides were mounted with DAPI mounting medium and visualized using an inverted fluorescent microscope.

2.2.8 - In vivo tumor model

Female athymic nude mice were purchased from the National Cancer Institute (Frederick, MD) and all mouse studies were approved and supervised by the MD Anderson Cancer Center Institutional Animal Care and Use Committee. All animals used were between 8 and 12 weeks of age at the time of injection. To establish the tumors, SKOV3ip1 ovarian cancer cells (1.0 x 10^6) were trypsinized and suspended in 200 μl of Hanks balanced salt solution (HBSS; Gibco) and injected into the intraperitoneal cavity (i.p.) of mice on day 1. Seven days after cell injection, mice were randomly divided into 4 groups: 1) Control siRNA/DOPC or 2) BRCA2 siRNA/DOPC or 3) Control siRNA/DOPC + olaparib or 4) BRCA2 siRNA/DOPC + olaparib (n=10 mice per group). Treatment began by injecting siRNA/DOPC nanoparticles twice weekly (150 μg/kg body weight) and olaparib (5 mg/kg body weight; 5 days a week) through i.p. injection. Mice were monitored daily for adverse effects of therapy and were sacrificed when they became moribund (6-7 weeks after cell injection) [81]. At the time of sacrifice, mouse and tumor weight was recorded. Tumor tissue was harvested and either fixed in formalin for paraffin embedding, or frozen in optimum cutting temperature medium (OCT; Miles, Inc., Elkhart, IN, USA) to prepare frozen slides, or snap-frozen in liquid nitrogen for lysate preparation. The individuals who performed the
necropsies, tumor collections, and tissue processing were blinded to the treatment group assignments.

2.2.9 - Statistical analysis

For measures of drug sensitization Student’s t tests were employed to compare means between groups and test the a priori null hypothesis that there was no difference between specific means. A p-value of less than 0.05 was selected a priori as the benchmark for rejection of the null hypothesis. For determination of increased variation in chromosome number following treatment, a statistical analysis of standard deviation was performed via the Bartlett’s test to evaluate the null hypothesis that the standard deviation between groups was the same.
Chapter 3. Inhibition of BRCA2 and thymidylate synthase creates multidrug sensitive tumor cells via the induction of combined “complementary lethality”

3.1 Preamble:

Genomic instability and cellular heterogeneity are among the strongest drivers of cancer progression, malignancy, and recurrence [82, 83]. A high mutation level and chromosomal instability enhances the rate at which malignant characteristics are generated in human cells to produce progressively more aggressive tumors [27, 84]. Next-generation sequencing (NGS) technology has highlighted the extreme degree of genomic variability and mutation present in human malignancies [83, 85, 86]. This underscores one of the reasons human cancers are difficult to treat effectively – genomic plasticity and phenotypic heterogeneity allow for rapid adaptation and differential response to environmental selection pressures [27].

However, continuing high mutation frequency and lack of genomic fidelity also leads to increased rates of cell death [87]. Consequently, cancer cells may be closer to a maximum tolerated threshold of mutation than normal cells and are highly dependent for continued survival on cellular molecules that maintain at least some integrity of genetic information. This phenomenon has led to the concept of tumor cell ‘addiction’ to some mediators of DNA repair [88]. Therefore, targeting factors critical to DNA maintenance may be a useful strategy to promote an increase in DNA damage from which cancer cells cannot recover, thus decreasing innate resistance to DNA damaging therapy.
As mentioned above, BRCA2 promotes repair of DSBs [48, 89], and mutations in the BRCA2 gene induce a highly penetrant, autosomal dominant predisposition to a variety of cancers [20, 90, 91]. However, tumors with BRCA2 mutations respond better to chemotherapy than BRCA2-intact tumors in patients with sporadic cancer [23, 92, 93]. Thus, BRCA2 constitutes a source of innate resistance to DNA-damaging drugs. TS is the only de novo source of cellular thymidylate and is a well-established target of many approved anti-cancer drugs including fluoropyrimidines (e.g., 5-FU) and folate analogs (e.g., pemetrexed) [57]. My group previously reported that TS antisense oligonucleotides (ASOs) can overcome innate resistance to TS-targeting drugs in human cancer cells [58, 59].

This led me to formulate and test the concept of ‘complementary lethality’, defined as the enhancement of drug efficacy by downregulation of factors important for cellular resistance to the action of that specific drug. In other words, I propose to potentiate the effectiveness of chemotherapeutic drugs by targeting DNA repair mediators, such as BRCA2, which are functionally involved in the amelioration of specific types of drug-induced effects (e.g., DSBs produced by platinating or alkylating agents).

I hypothesized that BRCA2 could be downregulated in combination with TS to induce complementary lethality to a wider and different spectrum of drugs, thus creating multi-drug sensitive tumors. The association of both BRCA2 and TS with different aspects of DNA integrity suggests that combined downregulation of both targets could lead to enhanced cell death and potentiation of both TS-targeting and other drugs that induce or enhance accumulation of DNA damage.
In this chapter, I demonstrate that actively targeting DNA repair pathways is potentially useful therapeutically and decreases innate resistance to specific chemotherapeutic drugs, a phenomenon I labelled complementary lethality. In particular, I show that BRCA2 is valuable as a therapeutic target in addition to TS, and that combined downregulation of BRCA2 and TS can sensitize cells to a panel of chemotherapeutic drugs with different mechanisms of action. I used siRNAs targeting TS and BRCA2 to promote sensitization to separate classes of chemotherapeutics in the same human tumor population, and induced a state of combined complementary lethality resulting in decreased tumor cell proliferation.

3.2. Results:

3.2.1 – siRNA-mediated knockdown of BRCA2 and TS reduces cancer cell growth and affects cell cycle progression

siRNA-mediated target mRNA downregulation was confirmed by RT-qPCR 24 hours post transfection. There were no synergistic or inhibitory effects in mRNA levels when both targets were downregulated at the same time (Figure 6A). Given the potential for baseline DNA damage normally accrued by mammalian cells and repaired by complexes in which BRCA2 is important, it was hypothesized that BRCA2 siRNA would have an effect on cell growth even in the absence of an exogenous damaging agent. Ninety-six hours post-transfection, A549 cells treated with BRCA2 siRNA (10 nM) exhibited a significant decrease in cell number (20% ± 9%, p=0.036) compared to cells treated with control siRNA (10 nM) (Figure 6B). Cells transfected with TS siRNA (2.5 nM) did not appear to be negatively affected.
by this treatment compared to cells transfected with control, non-targeting siRNA. Combination treatment with BRCA2 siRNA (10 nM) and TS siRNA (2.5 nM) decreased A549 cell number to a similar level as BRCA2 siRNA alone (29.6% ± 7.0%, p=0.04 vs control siRNA; no difference vs BRCA2 siRNA) (Figure 6B), suggesting that these are separate and non-overlapping pathways. In HeLa cells tested under the same conditions, both BRCA2 and TS siRNA, as well as their combination, induced decreases in cell number (Figure 6C).

To assess the possibility that the difference in cell number following siRNA treatment was due to cell cycle changes, A549 cells were transfected with BRCA2 and TS siRNA and then stained with propidium iodide (PI) for cell cycle analysis. Forty-eight hours post transfection, BRCA2 siRNA induced a small increase in the number of cells in G<sub>0</sub>/G<sub>1</sub> phase (8.15% ± 2.14%, p=0.007), and a concomitant decrease in the number of cells in S phase (14.8% ± 3%, p=0.009) compared to treatment with non-targeting siRNA (Figure 6D).

TS siRNA treatment exhibited the opposite effect, with an accumulation in S phase (26.7% ± 1.1%, p=0.0002) and a decrease in G<sub>0</sub>/G<sub>1</sub> (32% ± 2.21%, p=0.00004). Combined downregulation of both TS and BRCA2 led to a cell cycle profile that reflected the opposite contributions of the individual siRNA treatments in terms of G<sub>0</sub>/G<sub>1</sub> and S phase frequency which, as a result, yielded cell cycle frequencies that were intermediate when compared to control siRNA treatment (G<sub>0</sub>/G<sub>1</sub> change: -19.8% ± 4.9%, p=0.005; S change: +13.8% ± 1.9%, p=0.01). However, the G<sub>2</sub>/M phase frequency in BRCA2 and TS siRNA treated cells was increased compared to control (10.4% ± 3%, p=0.04) (Figure 6D).
Figure 6 – Combined BRCA2 siRNA and TS siRNA inhibits A549 cell proliferation and affects cell cycle distribution. A) A549 cells were transfected with control siRNA, TS siRNA and/or BRCA2 siRNA. mRNA was isolated 24 hours later. TS mRNA (black bars) and BRCA2 mRNA (white bars) levels were measured relative to 18S endogenous control. B) Effect of siRNA treatment on A549 cell proliferation, 96 hours post-transfection. *Different from treatment with control siRNA (p<0.05). Representative data from one of three independent experiments is shown (mean ± SD). C) Effect of siRNA treatment on HeLa cell proliferation, 96 hours post-transfection. *Different from treatment with control siRNA (p<0.05). Representative data from one of two independent experiments is shown (mean ± SD).
3.2.2 - BRCA2 siRNA potentiates cisplatin, melphalan, and to a lesser extent 5-FUdR, but not pemetrexed

I investigated whether BRCA2 siRNA altered the capacity of A549 cells to proliferate after treatment with DNA-damaging chemotherapeutic drugs such as cisplatin. Cisplatin binds to DNA and induces inter- and intra-strand crosslinks which progress to double stranded breaks (DSBs) and are repaired by homologous recombination (HR) [21]. In A549 cells treated with BRCA2 siRNA and cisplatin, drug treatment was 43% ± 3% (p=0.0001) more effective when compared to cells transfected with non-targeting control siRNA (Figure 7A).

I determined whether potentiation of cisplatin treatment by BRCA2 siRNA was due to the induction of DNA strand crosslinking and subsequent DSBs, by using another anti-cancer agent with a related mechanism of action. Melphalan is a DNA alkylator but not a platinum based agent and is therefore structurally distinct from cisplatin [94]. A549 cells transfected with BRCA2 siRNA 24 hours prior to melphalan treatment were 63% ± 0.3% (p=9.5x10^-7) more sensitive to melphalan-mediated growth inhibition than cells treated with control non-targeting siRNA (Figure 7B).

I tested whether siRNA-mediated knockdown of BRCA2 potentiated the effects of a folate antimetabolite (pemetrexed) and a fluoropyrimidine (5-FUdR), both approved for use in treatment of a broad range of human tumors [95, 96]. These drugs are not DNA alkylators and do not share a mechanism of action with cisplatin and melphalan. Rather, they inhibit the action of TS and consequently starve cells of the thymidylate necessary for DNA replication and repair [57]. Cells
Figure 7 – BRCA2 siRNA induces complementary lethality to treatment with cisplatin, melphalan and 5-FUdR, but not pemetrexed in A549 cells. A549 cells were transfected with control siRNA (black bars) or BRCA2 siRNA (white bars) and treated with (A) cisplatin, (B) melphalan, (C) 5-FUdR, or (D) pemetrexed. Effects on cell growth were assessed 96 h post transfection, as a percentage of cells treated with control non-targeting siRNA alone. *Different from treatment with control siRNA alone (p<0.05). **Different from treatment with drug plus control siRNA (p<0.05). Representative data from one of three independent experiments is shown (mean ± SD).
Figure 8 – BRCA2 siRNA induces complementary lethality to treatment with cisplatin, melphalan and 5-FUdR, but not pemetrexed in HeLa cells. HeLa cells were transfected with control siRNA (black bars) or BRCA2 siRNA (white bars) and treated with (A) cisplatin, (B) melphalan, (C) 5-FUdR, or (D) pemetrexed. Effects on cell growth were assessed 96 h post transfection, as a percentage of cells treated with control non-targeting siRNA alone. *Different from treatment with control siRNA alone (p<0.05). **Different from treatment with drug plus control siRNA (p<0.05). Representative data from one of two independent experiments is shown (mean ± SD).
transfected with BRCA2 siRNA (10 nM) were 31.2% ± 3.1% (p=0.001) more sensitive to 5-FUdR than cells transfected with control siRNA 96 hours post transfection (Figure 7C). The majority of this effect was driven by the growth inhibitory action of the BRCA2 siRNA itself, however a statistically significant difference still exists even after normalization for the effect of siRNA on its own. BRCA2 siRNA treatment did not sensitize A549 cells to pemetrexed and cell numbers were similar to control siRNA transfected cells 96 hours post-transfection (Figure 7D). These results were broadly reproducible in HeLa cells tested under the same conditions, and BRCA2 inhibition induced complementary lethality to cisplatin, melphalan, and to a lesser extent 5-FUdR (Figure 8A-D).

3.2.3 - TS siRNA potentiates 5-FUdR and pemetrexed but not cisplatin or melphalan

A549 cells were transfected with control and/or TS siRNA, and then treated with 5-FUdR 24 hours later. As predicted, TS siRNA sensitized cells to 5-FUdR, reducing cell proliferation by 55.3% ± 4.9% (p=0.0006) relative to cells treated with control siRNA (Figure 9A). In addition, TS siRNA also induced complementary lethality to pemetrexed 96 hours post-transfection and decreased cell number by 56.0% ± 3.5% relative to cells treated with 5-FUdR and control siRNA (p=0.0003) (Figure 9B).

I determined whether TS siRNA sensitized A549 cells to drugs which do not target the TS pathway. A549 cells transfected with TS siRNA (2.5 nM) did
Figure 9 – TS siRNA induces complementary lethality to 5-FUdR and pemetrexed but not cisplatin or melphalan in A549 cells. A549 cells were transfected with control siRNA (black bars) or TS siRNA (white bars) and treated with (A) 5-FUdR, (B) pemetrexed, (C) cisplatin, or (D) melphalan. Effects on cell growth were assessed 96 h post transfection, as a percentages of cells treated with control non-targeting siRNA alone. *Different from cells treated with control non-targeting siRNA alone (p<0.05). **Different from cells treated with control non-targeting siRNA plus drug. Representative data from one of three independent experiments is shown (mean ± SD).
Figure 10 – TS siRNA induces complementary lethality to 5-FUdR and pemetrexed but not cisplatin or melphalan in HeLa cells. HeLa cells were transfected with control siRNA (black bars) or TS siRNA (white bars) and treated with (A) 5-FUdR, (B) pemetrexed, (C) cisplatin, or (D) melphalan. Effects on cell growth were assessed 96 h post transfection, as a percentages of cells treated with control non-targeting siRNA alone. *Different from cells treated with control non-targeting siRNA alone (p<0.05).**Different from cells treated with control non-targeting siRNA plus drug. Representative data from one of two independent experiments is shown (mean ± SD).
not exhibit increased sensitivity to cisplatin (Figure 9C) or melphalan (Figure 9D) compared to cells treated with drug and control siRNA. This suggested that TS siRNA-mediated potentiation of drug efficacy is limited to drugs that target the TS pathway and not drugs that induce specific types of DNA damage. These results were reproduced in HeLa cells under the same experimental conditions (Figure 10A-D), and though TS siRNA induced a statistically significant difference in cell number following cisplatin and melphalan treatment, this was an additive response driven by the action of TS siRNA alone on cell number. After normalization for the effect of siRNA alone, there was no statistically significant difference relative to control siRNA.

3.2.4 - Simultaneous siRNA-mediated knockdown of BRCA2 and TS induces complementary lethality to cisplatin, melphalan and both TS-targeting drugs

Since single siRNA treatment promoted the efficacy of different sets of drugs (e.g., TS siRNA and pemetrexed, BRCA2 siRNA and cisplatin), I assessed whether transfecting BRCA2 and TS siRNA simultaneously sensitized A549 cells to the entire panel of 4 chemotherapeutic drugs. My aim was to investigate whether combination siRNA treatment could be utilized to induce complementary lethality to both alkylating drugs and TS targeting agents, and whether the magnitude of sensitization would exceed that induced by each individual siRNA to its "non-complementary" drug partner (e.g., TS siRNA and cisplatin).
A549 cells were transfected with control siRNA, BRCA2 siRNA and/or TS siRNA and subsequently treated with cisplatin 24 hours later. Ninety-six hours post transfection the combination siRNA treatment potentiated the effects of cisplatin by 46.3% ± 9.9% (p=0.002) compared to control siRNA treatment, and was 41.2% ± 10.9% (p=0.002) more effective at growth inhibition following drug treatment than TS siRNA alone (Figure 11A).

In addition, simultaneous BRCA2 and TS siRNA treatment sensitized human A549 cancer cells to melphalan treatment by 60% ± 0.8% (p=2.4x10⁻⁷) more than control siRNA treatment, and 56% ± 1% (p=1.0x10⁻⁶) more than TS siRNA (Figure 11B).

Combination siRNA treatment sensitized A549 cells to 5-FUdR by 59.2% ± 5.1% (p=0.0001) and was 43.0% ± 7.1% (p=0.0005) more effective at sensitization than single BRCA2 siRNA treatment (Figure 11C), even though BRCA2 siRNA also potentiated 5-FUdR toxicity on its own as shown previously.

Furthermore, combined BRCA2 and TS siRNA transfection potentiated the efficacy of pemetrexed by 56% ± 3.3% (p=0.0004) more than control siRNA treatment, and was 56.4% ± 3.3% (p=1.0x10⁻⁵) more effective at drug sensitization than BRCA2 siRNA alone (Figure 11D). The above experiments were repeated in HeLa cells and yielded results of similar magnitude and scope (Figure 12A-D). These results show that combination BRCA2 and TS siRNA treatment induces complementary lethality to a broader panel of chemotherapeutic agents and creates multi-drug sensitive tumors. They also suggest that combination siRNA treatment and combination drug treatment may be an effective way to maximize chemotherapeutic efficacy of different classes of drugs.
Figure 11 - Simultaneous siRNA-mediated knockdown of BRCA2 and TS induces complementary lethality to cisplatin, melphalan, and both TS-targeting drugs in A549 cells. A549 cells were transfected with control, TS and/or BRCA2 siRNA and, at 24 h post-transfection, treated with (A) cisplatin, (B) melphalan, (C) 5-FUdR or (D) pemetrexed. Effects on cell proliferation were determined by direct cell counting at 96 h and calculated as a percentage of non-drug treated, control siRNA transfected cells.*Different from cells treated with control non-targeting siRNA and drug (p<0.05). **Different from cells treated with single siRNA and drug (p<0.05). Representative data from one of three independent experiments is shown (mean ± SD).
Figure 12 - Simultaneous siRNA-mediated knockdown of BRCA2 and TS induces complementary lethality to cisplatin, melphalan, and both TS-targeting drugs in HeLa cells. HeLa cells were transfected with control, TS and/or BRCA2 siRNA and, at 24 h post-transfection, treated with (A) cisplatin, (B) melphalan, (C) 5-FUdR or (D) pemetrexed. Effects on cell proliferation were determined by direct cell counting at 96 h and calculated as a percentage of non-drug treated, control siRNA transfected cells.*Different from cells treated with control non-targeting siRNA and drug (p<0.05). **Different from cells treated with single siRNA and drug (p<0.05). Representative data from one of two independent experiments is shown (mean ± SD).
Simultaneous downregulation of BRCA2 and TS in the same cell population induces complementary lethality to two different sets of drugs and increases overall treatment effectiveness.

Combined treatment with TS-targeting and platinum-based anticancer drugs is standard of care for a variety of common and important human cancers [97, 98]. Therefore, antisense-mediated enhancement of these drug combinations is desirable and potentially valuable therapeutically. Given that complementary lethality was separately induced to cross-linking agents and TS targeting drugs by combined TS and BRCA2 siRNA treatment, I next determined whether combined complementary lethality to both types of drugs could be induced simultaneously in the same cell population and if the magnitude of drug potentiation by this regimen could further decrease cancer cell proliferation.

A549 cells were transfected with control siRNA and combination BRCA2 and TS siRNA. Twenty-four hours post transfection, the cells were treated with either cisplatin, 5-FUdR or both drugs simultaneously, and effects on proliferation were enumerated 96 hours post transfection.

Combination siRNA treatment potentiated the effects of combination cisplatin and 5-FUdR treatment by $64.1\% \pm 7\%$ ($p=0.0001$) relative to control siRNA treatment. This showed that complementary lethality to two different drugs could be induced in the same cell population with combination siRNA treatment. Furthermore, the overall magnitude of this approach was more effective at reducing overall cell number compared to non-drug treated control than combination siRNA transfection and treatment with any single drug ($p<0.05$), suggesting that there is
potential therapeutic benefit to sensitizing to cisplatin and 5-FUdR in the same cell population (Figure 13A).

To determine whether the same phenomenon could be reproduced with another drug whose toxicity was potentiated by BRCA2 siRNA, the experiment was repeated with melphalan substituted for cisplatin. Again, combined siRNA treatment effectively sensitized to combined drug treatment relative to control siRNA (47.4% ± 4.6%, p=0.0006) and the final outcome in terms of cell proliferation was superior to treatment with combination siRNA and any single drug (p=<0.05) (Figure 13B).

Interestingly, though combination siRNA transfection still sensitized to combination cisplatin and pemetrexed treatment (52.8% ± 2.9%, p=5.6x10^{-5}), it did not result in a larger decrease in cell proliferation than combination siRNA transfection and pemetrexed treatment alone (Figure 13C). Concomitant cisplatin and pemetrexed treatment also failed to induce an additive effect on cell growth, unlike that seen with cisplatin and 5-FUdR, and melphalan and 5-FUdR.

The experiments with cisplatin plus 5-FUdR, and melphalan plus 5-FUdR were repeated in HeLa cells to establish the generalizability of the phenomenon. The results obtained using HeLa cells mirrored those in A549 cells (Figure 14A&B) and again showed that combined complementary lethality to two separate drugs in the same cell population is of potential therapeutic benefit.
Figure 13 - Combined complementary lethality to two different drugs in the same cell population can be achieved by combined BRCA2 and TS siRNA transfection in A549 cells. A549 cells were transfected with control, TS and/or BRCA2 siRNA and treated with (A) cisplatin, 5-FUdR, or cisplatin plus 5-FUdR, (B) melphalan, 5-FUdR or melphalan plus 5-FUdR, and (C) cisplatin, pemetrexed, or cisplatin plus pemetrexed. Black bars = cells untreated with drugs (A,B,C), white bars = treatment with cisplatin (A,C) or melphalan (B). Patterned white bars = cells treated with 5-FUdR (A,B) or pemetrexed (C); dark grey bars = cells treated with combinations of drugs (A,B,C). Cell proliferation was measured 96 h post-transfection and is shown as a percentage of cells treated with control non-targeting siRNA alone. **Different from cells treated with drug combination and control non-targeting siRNA (p<0.05). *Different from cells treated with combined siRNA, and one drug or no drug (p<0.05). Representative data from one of two independent experiments is shown (mean ± SD).
Figure 14 - Combined complementary lethality to two different drugs in the same cell population can be achieved by combined BRCA2 and TS siRNA transfection in HeLa cells. HeLa cells were transfected with control, TS and/or BRCA2 siRNA and treated with (A) cisplatin, 5-FUdR, or cisplatin plus 5-FUdR, (B) melphalan, 5-FUdR or melphalan plus 5-FUdR. Black bars = cells untreated with drugs (A,B), white bars = treatment with cisplatin (A,C) or melphalan (B). Patterned white bars = cells treated with 5-FUdR (A,B); dark grey bars = cells treated with combinations of drugs (A,B). Cell proliferation was measured 96 h post-transfection and is shown as a percentage of cells treated with control non-targeting siRNA alone. **Different from cells treated with drug combination and control non-targeting siRNA (p<0.05). *Different from cells treated with combined siRNA, and one drug or no drug (p<0.05). Representative data from one of two independent experiments is shown (mean ± SD).
Chapter 4. BRCA2 Inhibition enhances cisplatin-mediated alterations in tumor cell proliferation, metabolism, and metastasis

4.1 Preamble:

A high rate of mutation and genetic instability can be considered fundamental hallmarks of cancer that distinguish tumor cells from their normal counterparts [83, 85, 86]. Lack of integrity in the cancer genome is often precipitated by compromised or incomplete DNA repair, reliance on error-prone repair, and/or defects in cell cycle control [1]. This renders cancer cells susceptible to DNA damage-induced toxicity, and is at least partly responsible for the difference between non-tumor cells and cancer cells in sensitivity to DNA-damaging therapeutic agents.

However, genomic heterogeneity and mutability also underlie tumor cell adaptability and acquired resistance to chemo- and/or radiotherapy [7]. Thus, the same traits that render cancer cells more susceptible to DNA-damaging therapeutics can also lead to treatment failure. Tumor cells must preserve a level of DNA repair sufficient to maintain survival in the face of selection pressures posed by treatment, but sufficiently limited to permit enhanced adaptability [99]. This dichotomy can be exploited for therapeutic benefit. Inhibiting DNA repair may push tumor cells past a tolerable threshold of DNA damage and induce cell death, thus preventing further heterogeneity and the development of acquired drug resistance.

Cisplatin is a platinating drug commonly used to treat various types of solid malignancies [100]. However, the associated toxicities and both inherent and
acquired resistance can limit the effectiveness of these drugs in the clinic [55]. Strategies to render cancer cells more sensitive to platinum agents are therefore of high clinical importance.

Patients with tumors harboring BRCA2 mutations have an improved prognosis following treatment with platinum-containing drugs, compared to patients with tumors with intact BRCA2 genes [101, 102]. In addition, BRCA2 mutation status leads to differences in metastatic frequency in patients [22, 103]. This suggests that actively targeting BRCA2 in cancer cells with functional HRR may render cisplatin treatment more effective.

Based on proof-of-concept studies described in chapter 3 [47], I developed a second generation BRCA2-targeting antisense oligodeoxynucleotide (ASO). I investigated the potential therapeutic value of the BRCA2 ASO in a setting with concomitant cisplatin treatment by determining the effects on tumor cell proliferation, metabolic response, and metastasis. In this chapter I show that BRCA2 ASO plus cisplatin treatment is potentially valuable clinically, and describe a new role for BRCA2 in the maintenance of human tumor cell metabolic response and metastasis frequency following cisplatin treatment.

4.2. Results:

4.2.1 - Mutations in the BRCA2 gene positively impact patient prognosis

Using the cBioPortal for Cancer Genomics I analyzed the effect of mutations in the BRCA2 gene on cancer patient survival [104]. Both endometrial and ovarian cancers were investigated to assess the consequences of naturally-occurring
BRCA2 mutations on survival in patient populations, as BRCA2 mutations were reasonably common in these tumor types and the availability of TCGA was relatively high. A query of the TCGA Uterine Endometrial Carcinoma (UEC) dataset [77] revealed a higher disease-free survival (logrank $p = 0.032$) and a trend to higher overall survival (logrank $p = 0.10$) in patients with BRCA2 alterations (Figure 15A). However, survival of endometrial carcinoma patients with wild type BRCA2 was relatively high, diminishing the power to detect survival differences.

BRCA2 mutations were queried against survival using the TCGA Ovarian Serous Cystadenocarcinoma dataset (Cancer Genome Atlas Research Network, [105]) in which overall 5 year survival was 40% or less. As opposed to analyzing the combined effect of BRCA1 and BRCA2 mutations on outcome (Cancer Genome Atlas Research Network, [105]), my analysis correlated changes in BRCA2 alone with improved overall (72.9 vs 43.3 months, logrank $p = 0.002$) and disease-free (26.2 vs 15.4 months, logrank $p = 0.014$) survival (Figure 15B). No such correlation was found for BRCA1 gene mutations (Figure 15C). Overall, naturally-occurring BRCA2 mutations in at least two tumor types (of the endometrium and ovary) trend toward or are significantly associated with increased survival. That association supports the hypothesis that BRCA2 may be a therapeutic target to enhance anticancer therapy.
Figure 15 - Retrospective analysis of patient survival data based on BRCA2 mutation status. The TCGA Uterine Endometrial Carcinoma dataset was queried using the cBio Portal to determine changes in disease-free and overall survival (A) in patients with alterations in BRCA2 (BRCA2 WT N = 215, BRCA2 mut N = 24). The TCGA Ovarian Serous Cystadenocarcinoma dataset was queried to determine changes in disease-free and overall survival in patients with alterations in BRCA2 (WT N = 276, mut N = 39) (B) or BRCA1 (WT N = 277, mut N = 38) (C). Red line = mutant BRCA1 or BRCA2, Blue line = WT BRCA1 or BRCA2
4.2.2 - BRCA2 ASO treatment decreases target mRNA, protein and RAD51 focus formation

Transfection of A549 non-small cell lung cancer cells with BRCA2 ASO decreased BRCA2 mRNA by 78% and reduced BRCA2 protein levels (Figure 16A&B). BRCA2 ASO also negatively impacted BRCA2 function on a cellular level, as shown by decreased RAD51 focus formation induced by subsequent cisplatin treatment (Figure 16C). Thus, the degree of BRCA2 ASO-induced target downregulation was sufficient to reduce at least one BRCA2 function important in HRR.

4.2.3 - BRCA2 maintains tumor cell proliferative capacity following cisplatin treatment

To determine the effect of BRCA2 knockdown on tumor cell response to cisplatin, I transfected A549 cells with BRCA2 ASO and treated with cisplatin. The effectiveness of cisplatin was increased as much as 50 ± 3% (p<0.05) in cells transfected with BRCA2 ASO compared with control (Figure 17A and 18A).

BRCA2 ASO sensitized A549 cells to melphalan, another DNA cross-linking agent, by 45 ± 2% (p<0.05) compared to cells transfected with control ASO (Figure 17B and 18B). BRCA2 ASO treatment also rendered cisplatin more effective in SKOV-3 ovarian cancer cells (55% ± 3%, p<0.05) and in MDA-MB-231 breast cancer cells (26% ± 8%, p<0.05), confirming that this phenomenon is reproducible in human tumor cell lines of differing origin (Figure 17C&D).
Figure 16 - BRCA2 ASO decreases BRCA2 mRNA, protein, and RAD51 focus formation frequency in A549 cells. A) A549 cells were transfected with 20 nM BRCA2 ASO and total cellular RNA was isolated 24 hours later. Relative BRCA2 mRNA levels (mean ± SD) were assessed with RT-qPCR. B) BRCA2 protein levels were assessed 72 hours after BRCA2 ASO transfection. C) RAD51 focus formation (mean ± SD) was quantified 48 hours after BRCA2 ASO transfection (p<0.05).
Figure 17 - BRCA2 regulates cisplatin-induced reduction of lung, ovarian and breast tumor proliferation. A549 cells were treated with varying concentrations of cisplatin (A) or melphalan (B) and control (black circles) or BRCA2 (white circles) ASO. Cells were counted 96 hours post transfection to assess effects of BRCA2 knockdown. SKOV-3 cells (C) and MDA-MB-231 cells (D) were treated with different concentrations of cisplatin and control or BRCA2 ASO, and counted 96 hours post transfection. *Different from cells treated with control ASO (p<0.05). Representative results from one of two experiments.
Figure 18 - BRCA2 knockdown modulates cisplatin- or melphalan-induced A549 cell proliferation. A549 cells were treated with varying concentrations of A) cisplatin or B) melphalan, following transfection with control (black circles) or BRCA2 (white circles) ASO. Effects on cellular proliferation were determined using the turnover of AlamarBlue reagent as the readout. *Different from cells treated with control ASO using a Student's t-test (p<0.05).
Somatic BRCA2 mutations predispose human cells to malignant transformation [20]. In addition, since BRCA2 knockdown may enhance toxicity of DNA-damaging drugs to normal, non-tumor cells, I investigated the effect of BRCA2 ASO on cisplatin toxicity in human non-tumor HK-2 kidney cells. Treatment of HK-2 cells with BRCA2 ASO induced only a small increase in sensitivity to cisplatin (12% ± 6%) (Figure 19A).

Human pancreatic CAPAN-1 cells, which lack a functional BRCA2 protein [106], were subjected to the same BRCA2 ASO/cisplatin treatment. There was no increase in cisplatin sensitivity in CAPAN-1 cells following BRCA2 ASO transfection (Figure 19B), suggesting that the effects of BRCA2 knockdown with BRCA2 ASO seen in tumor cell lines are due to BRCA2 downregulation and not off-target events.

4.2.4 - BRCA2 downregulation reverses acquired cisplatin resistance in head and neck tumor cells

To assess the effectiveness of BRCA2 downregulation in reversing acquired cisplatin resistance in human tumors, I tested cisplatin sensitivity in three human head and neck squamous carcinoma cell lines [76]: HN-5a, HN-5a/carbo-10a, and HN-5a/carbo-15a (Figure 20A).

BRCA2 ASO treatment potentiated cisplatin in parental HN-5a cells by 63 ± 5% and cisplatin-resistant HN5a-carbo-15a cells by 57 ± 2% (Figure 20B&C). The resulting sensitivity generated by BRCA2 ASO transfection was similar to that of parental HN5a cells (Figure 20C). Although HN-5a/carbo-15a cells exhibit
Figure 19 - BRCA2 does not affect cisplatin-induced proliferation changes in non-cancerous cells and cells without BRCA2 function. A) Non-tumor HK-2 kidney cells were treated with two concentrations of cisplatin and control or BRCA2 ASO, and relative proliferation was quantified using cell counting 96 hours post transfection. B) BRCA2 deficient CAPAN-1 cells were exposed to varying concentrations of cisplatin and control or BRCA2 ASO, and counted 96 hours post transfection. *Different from cells treated with control ASO (p<0.05). Representative results from one of two experiments.
Figure 20 - BRCA2 ASO reverses acquired cisplatin resistance in human head and neck cancer cells. A) HN-5a, HN5a/carbo-10a, and HN-5a/carbo-15a cells were exposed to cisplatin and effects on proliferation were assessed 72 hours after drug treatment. HN-5a cells (B) and HN-5a/carbo-15a cells (C) were transfected with control (black circles) or BRCA2 (white circles) ASO, treated with varying concentrations of cisplatin and then counted 96 hours post transfection. *Different from cells treated with control ASO using a Student's t-test (p<0.05). Representative results from one of two experiments.
decreased intracellular accumulation of cisplatin compared to the parental HN-5a cell line [76], BRCA2 downregulation was still able to effectively sensitize cells to cisplatin.

An assay of *in vitro* colony formation was used as a more stringent measure of the long term effects of treatment on seeding potential [107]. Treatment with BRCA2 ASO and cisplatin decreased colony formation ability in both HN-5a and HN-5a/carbo-15a cells (Figure 21A&E), suggesting that BRCA2 inhibition can enhance the ability of cisplatin to limit cancer cell proliferation and colony forming potential.

### 4.2.5 - BRCA2 modulates tumor cell metabolism following cisplatin treatment

Given the dependence of DNA maintenance and repair on functional metabolic processes [108], it was possible that at part of the BRCA2 ASO-mediated increase in cisplatin cytotoxicity was due to alterations in cellular metabolism. In addition, cisplatin has been shown to preferentially target mitochondrial DNA in tumor cells [109]. To investigate, I measured changes in cell impedance, acidification and oxygen consumption associated with BRCA2 ASO and cisplatin treatment to determine monolayer integrity, cellular glycolytic activity, and respiration [54].

After 24 h exposure to cisplatin, A549 tumor cells pre-treated with BRCA2 ASO had 39% less respiratory activity than cells pre-treated with control ASO. Furthermore, in BRCA2 ASO treated cells, the respiration decrease was evident 10 h after addition of cisplatin and 15 h earlier than in cells treated with control ASO.
Figure 21 - BRCA2 ASO decreases colony forming ability of human head and neck cancer cells following cisplatin treatment. HN5a cells (A) and HN5a cells (B) were transfected with control or BRCA2 ASO, treated with different concentrations of cisplatin for 6 hours, and then re-plated at a density of 500 cells per well to determine colony forming ability. *Different from cells treated with control ASO using a Student’s t-test (p<0.05).
(Figure 22A). Respiration began to decrease in response to cisplatin in BRCA2-treated cells 10 h prior to observable reduction in adhesion, suggesting that respiration reduction occurred independent of changes in cell number or viability (Figure 22B). However, no difference in acidification (a measure of glycolysis) was observed between the BRCA2 ASO and control ASO groups treated with cisplatin (Figure 22C).

Changes in cellular respiration induced by BRCA2 ASO in conjunction with cisplatin suggested BRCA2 ASO-mediated inhibition of mitochondrial function. I used mitochondria-specific dye accumulation to determine the frequency of functional mitochondria in A549 and HN-5a cells, and changes in those parameters induced by BRCA2 ASO plus or minus cisplatin. Cisplatin treatment induced a 63% increase in Mitotracker staining in both A549 and HN5a cells. However, there was no difference in staining between cells treated with BRCA2 ASO plus cisplatin and control ASO plus cisplatin (Figure 23A and 24A).

Cellular glucose uptake is modulated by cisplatin treatment and DNA damage [110]. I observed that cisplatin treatment of A549 cells increased glucose uptake by 60%. Pretreatment with BRCA2 ASO increased that response to cisplatin by a further 17% (Figure 23B and 24B). This raised the possibility that increased glucose entry induced by cisplatin after BRCA2 reduction might be a cellular response to generate additional energy necessary for increased DNA repair. However, decreased acidification induced by cisplatin (a measure of reduced glycolysis [54]) was approximately the same regardless of ASO treatment (Figure 22C). This suggests that the BRCA2 ASO-induced increase in glucose uptake in response to cisplatin was not sufficient to enhance glycolytic activity.
Figure 22 - BRCA2 modulates tumor cell respiration following cisplatin treatment. A549 cells were exposed to cisplatin (6 µM, 24 hours) following a six hours of incubation in medium to determine baseline metabolic levels. At 24 hours after addition of cisplatin, medium was exchanged for free medium without cisplatin. Measurements of oxygen consumption (A), impedance (B), and changes in medium pH (C) were conducted. Representative results from one of three experiments.
**Figure 23 - BRCA2 modulates tumor cell glucose uptake following cisplatin treatment.** A549 cells were transfected with control or BRCA2 ASO and then treated with cisplatin (6µM). Mitotracker staining (A) and glucose uptake (B) were determined using flow cytometry. *Different from cells treated with control ASO using a Student’s t-test (p<0.05). Representative results from one of two experiments.
Figure 24 - BRCA2 knockdown in HN-5a cells enhances cisplatin-induced glucose uptake but not Mitotracker-measured mitochondrial function. HN-5a cells were transfected with control or BRCA2 ASO and then treated with cisplatin (6µM). Mitotracker staining (A) and glucose uptake (B) were determined using flow cytometry. *Different from cells treated with control ASO using a Student's t-test (p<0.05).
Furthermore, the loss of adhesion (indicative of a decrease in cell monolayer integrity [54]) in response to cisplatin in cells treated with BRCA2 ASO indicates that increased glucose uptake was not capable of rescuing cells from BRCA2 ASO-mediated sensitization to cisplatin (Figure 22B).

4.2.6 - BRCA2 inhibition decreases metastatic frequency following cisplatin treatment

The majority of cancer patients do not die from primary disease, but instead succumb to metastatic tumors. I therefore investigated whether concomitant BRCA2 ASO and cisplatin treatment would decrease the number of metastatic foci to a higher degree than cisplatin alone, since there is some evidence linking BRCA2 mutation status to metastasis in cancer patients [22, 103].

To ensure that the long term effects of treatment were reproducible in A549 cells before investigating metastasis directly, I conducted an in vitro colony formation assay. Colony formation by A549 cells treated with BRCA2 ASO and cisplatin (6 µM) was reduced by 48 ± 4% (p<0.05) compared to cells treated with control ASO and cisplatin (Figure 25A). BRCA2 downregulation also decreased the ability of A549 cells to form colonies following gamma irradiation (Figure 25B), in accord with previous reports [110].

To extend my findings to a metastatic context I utilized the chicken chorioallantoic membrane (CAM) invasion assay, and determined the effects of treatment on metastatic focus formation [111, 112]. A549-GFP cells were treated with control or BRCA2 ASO in the presence of absence of cisplatin, and then
Figure 25 - BRCA2 regulates tumor cell colony forming ability following cisplatin treatment. A549 cells were transfected with control (black) or BRCA2 (white) ASO and then exposed to varying concentrations of cisplatin (A) or different doses of ionizing radiation (B). Six hours after treatment, the capacity of cells to form colonies in vitro was determined. * Different from cells treated with control ASO (p<0.05). Representative results from one of two experiments.
Figure 26 - BRCA2 regulates tumor cell metastatic frequency following cisplatin treatment in vivo. A549-GFP cells were transfected with control or BRCA2 ASO, treated with 6μM cisplatin for 6 hours, then injected i.v. into the CAM (Left and Center panel). Metastatic foci were counted 7-9 days following injection (Right panel) (n≥6 per group). * Different from cells treated with control ASO (p<0.05).
injected i.v. into CAM veins. Treatment of A549-GFP cells with BRCA2 ASO alone produced a trend toward decreased metastatic focus frequency. Combined BRCA2 ASO and cisplatin treatment reduced the frequency of metastatic focus formation by 77 ± 7% (p<0.05) relative to cisplatin alone (Figure 26). These data indicate that intact BRCA2 function limits the ability of cisplatin to modulate tumor cell metastatic frequency in the CAM model.

Chapter 5. Reciprocal positive selection for weakness - overcoming olaparib resistance

5.1 Preamble:

Tumor heterogeneity, which is a feature of most solid human tumors, renders therapeutic resistance a mathematical certainty due to the presence of minute numbers of resistant cells at the start of therapy; this phenomenon has been described using experiment models in vitro and has also been modelled in silico using data from clinical studies [10, 12]. Single nucleus genome sequencing of breast cancer specimens has shown that no two cancer cells in a tumor are exactly the same [9], highlighting the challenge to effective and long-term anti-cancer therapy.

Cytotoxic and targeted therapies impose powerful selection pressure on the polyclonal and diverse tumor ecosystem. As a result, this promotes the survival of cells with the highest fitness and destroys the weak (or susceptible cells), which leads to eventual therapeutic failure and is in line with classical Darwinian
evolutionary theory [113]. It is necessary therefore, to design a treatment regimen which reverses Darwinian positive selection and, instead of positively selecting for strength in the population, selects for weakness.

PARP-1 inhibitors such as olaparib are selectively effective in tumors with homologous recombination repair (HRR) deficiency [52]. This occurs primarily due to the inability of HRR-deficient tumor cells to repair double-strand DNA breaks (DSBs) that result when PARP1 inhibition prevents resolution of single-strand breaks (SSBs) [63]. However, the selective killing of tumor cells with HRR defects, an attractive therapeutic goal for PARP inhibitors, ultimately contributes to reduced effectiveness [74]. First, HRR-deficient tumors, against which PARP1 inhibitors are most useful, are present in only a subset of cancer patients [65]. Second, selective killing in a heterogeneous tumor population leads to outgrowth of resistant clones and therapy failure, a phenomenon already described for PARP-1 inhibitors [74].

Combining PARP1 inhibition with BRCA2 inhibition may be an avenue to prevent resistance, via a mechanism I termed “reciprocal positive selection for weakness”. In a heterogeneous tumor population, BRCA2 inhibition will select for cells with deficient HRR, while concomitant olaparib treatment will eliminate those cells. The reciprocal is also true: olaparib treatment will select for HRR-proficient cells, which will then be susceptible to BRCA2 inhibition. I hypothesize that such a strategy will prevent the outgrowth of resistant lesions and extend the time that a tumor is responsive to treatment.

In this chapter, I show that BRCA2 inhibition sensitized lung, ovarian and breast tumor cell lines to the PARP-1 inhibitor olaparib. Importantly, BRCA2 ASO treatment did not increase the susceptibility of non-cancerous HK-2 kidney and
HCC-841 colon cell lines to PARP-1 inhibition. Furthermore, combined BRCA2 ASO and olaparib treatment in a tumor cell population with varying degrees of HRR-proficiency prevented the outgrowth of resistant clones. In addition, I found that combined inhibition of BRCA2 and PARP1 in vivo delayed the growth of ovarian cancer tumors. This work provides rationale for combining BRCA2 ASO and olaparib treatment and also extends the applicability of olaparib clinically, which up until now has been used primarily in the context of BRCA1/2 mutated ovarian cancers.

5.2. Results

5.2.1 BRCA2 inhibition overcomes innate olaparib resistance in three lung cancer cell lines

Olaparib has limited efficacy in cancer cells with intact HRR [114]. The majority of lung tumors do not exhibit mutations in BRCA1/2 genes (BRCA1/2 is mutated in 1.8%-11.2% of cases depending on the data set and tumor type) [104]. Thus, olaparib may have little utility in lung cancer treatment on its own. To determine whether BRCA2 inhibition could overcome innate olaparib resistance, I tested control or BRCA2 ASO treatment with increasing concentrations of olaparib in BRCA2-proficient A549 lung adenocarcinoma and H2052 and 211H mesothelioma cell lines.

All three cell lines harbor mutations (513 coding + 616 non-coding mutations in A549 cells; 402 coding + 509 non-coding mutations in 211H cells; and 80 coding
+ 76 non-coding mutations in H2052 cells) (Figure 27A), suggesting heterogeneity in each population. BRCA2 downregulation increased olaparib sensitivity by as much as 34.5% ± 2.8%, 31.9% ± 8.5%, and 44.1%± 7.8% (p<0.05) in A549, 211H, and H2052 cells, respectively (Figure 27B-D). BRCA2 ASO treatment sensitized all three lung cancer cell lines to olaparib across the entire range of drug concentrations regardless of mutational signature and load, suggesting that BRCA2 inhibition may render lung tumors with disparate backgrounds amenable to PARP inhibition.

5.2.2 BRCA2 inhibition sensitizes ovarian and breast cancer cells to olaparib treatment

Olaparib is approved by the FDA for treatment of BRCA1/2-mutated ovarian cancers [69]. However, only a fraction of ovarian tumors exhibit BRCA1/2 mutations [104] and most ovarian cancer patients are not eligible for olaparib treatment. Overcoming innate olaparib resistance in ovarian cancer cells with WT BRCA1/2 is potentially valuable clinically.

I tested whether BRCA2 downregulation could sensitize two different ovarian cancer cell lines to olaparib treatment. BRCA2 ASO treatment sensitized SKOV-3 cells to PARP1 inhibition by as much as 52.3% ± 2.7% (p<0.05) (Figure 28A) and CaOv3 cells by 41.3% ± 9.9% (p<0.05) (Figure 28B). Although SKOV-3 cells were sensitized more than CaOV3 cells, the amount of antisense-mediated BRCA2 knockdown was greater than 90% in both cell lines, similar to the amount of BRCA2 reduction in H2052 and 211H mesothelioma cells (Figure 28C).
Figure 27 – BRCA2 inhibition overcomes innate olaparib resistance in three human lung cancer cell lines. A mutation heat map for each cell line was generated using the COSMIC CCLE database interface (A). A549 (B), 211H (C), and H2052 (D) cells were transfected with control ASO (●) or BRCA2 ASO (○) and then treated with three different concentrations of olaparib. Proliferation was determined by cell counting 96 hours post-transfection (*p<0.05). Means ± SD from representative experiments are shown. All experiments were repeated at least once.
Figure 28 – BRCA2 inhibition sensitizes ovarian cancer cell lines to olaparib treatment. SKOV-3 (A) and CaOV3 (B) cells were transfected with control ASO (●) or BRCA2 ASO (○) and then treated with three different concentrations of olaparib. Proliferation was determined by cell counting 96 hours post-transfection (*p<0.05). C) BRCA2 mRNA levels were measured by qPCR 24 hours following BRCA2 ASO transfection in SKOV-3, CaOV3, 211H and H2052 cell lines. Cell counts were performed 96 hours post transfection (*p<0.05). Means ± SD from representative experiments are shown. All experiments were repeated at least once.
Furthermore, I tested whether BRCA2 ASO could sensitize triple-negative (estrogen receptor (ER), progesterone receptor (PR), and Her2/neu [9]) MDA-MB-231 human breast cancer cells to olaparib. Treatment options for triple-negative breast cancer are limited [115] and decreasing innate olaparib resistance by BRCA2 downregulation could reveal a new path to more effective treatment. MDA-MB-231 cells were rendered as much as 28.0% ± 5.1% (p<0.05) more sensitive to olaparib compared to cell treated with control ASO (Figure 29A).

To determine whether cells that survived the initial BRCA2 ASO and olaparib treatment remain sensitive to subsequent treatment, I treated the same cells with BRCA2 ASO and olaparib a second time. I found that single BRCA2 ASO + olaparib treatment hindered the proliferation of cells which were re-seeded without any additional olaparib treatment. In addition, a second round of BRCA2 ASO + olaparib treatment decreased SKOV-3 cell proliferation by 67.0% ± 12.4% (p<0.05) compared to cells which did not receive this second olaparib treatment (Figure 29B). This suggests that cells which survive the first round of BRCA2 ASO + olaparib treatment are still amenable to a second treatment, which will continue to decrease their proliferation.

5.2.3 BRCA2 inhibition does not sensitize non-cancerous cells to olaparib

An important question when inhibiting BRCA2 in the context of olaparib treatment is whether non-cancer cells are affected to the same degree as tumor cells. It would be undesirable to increase sensitivity to olaparib in all host tissues.

Non-cancer, BRCA2-positive HK-2 kidney proximal tubule epithelial cells were transfected with either control ASO or BRCA2 ASO and treated with three
Figure 29 – BRCA2 inhibition sensitizes MDA-MB-231 breast cancer cells to olaparib treatment, and continues to sensitize ovarian cancer cells to repeated treatment doses. A) MDA-MB-231 breast cancer cells were transfected with control or BRCA2 ASO, treated with olaparib, and proliferation determined as described above. B) SKOV-3 cells were transfected with control or BRCA2 ASO and then treated with olaparib 24 hours post transfection. Ninety-six hours post transfection, cells were counted, plated, and re-transfected with control ASO or BRCA2 ASO and re-treated with olaparib. Cell counts were performed 96 hours post transfection (*p<0.05). Means ± SD from representative experiments are shown. All experiments were repeated at least once.
concentrations of olaparib. BRCA2 inhibition did not sensitize HK-2 cells to olaparib at the tested concentrations (Figure 30A). BRCA2 mRNA downregulation was confirmed by qPCR to ensure that lack of sensitization was not due to inadequate transfection (Figure 30B). The experiment was repeated using non-cancer CCD-841 colon epithelial cells and BRCA2 ASO treatment did not increase the potency of olaparib at any tested concentration (Figure 30C).

5.2.4 BRCA2 ASO and Olaparib treatment induces changes in copy number and chromosomal translocation frequency in ovarian cancer cells

Failure of the spindle assembly checkpoint (SAC) results in abnormal chromosomal segregation and can lead to fatal chromosome gain or loss in daughter cells [116]. Both BRCA2 and PARP1 support SAC in mitotic cells [50] and I hypothesized that the decreased proliferation following inhibition of both targets may be due to perturbation of SAC.

I investigated the effect of BRCA2 ASO and olaparib treatment on bulk chromosome number using metaphase spreads of SKOV-3 and MDA-MB-231 cells. Twenty-four hours following olaparib or vehicle treatment, I identified a significant increase in the variance of the chromosome number in cells treated with BRCA2 ASO + olaparib (Figure 31A&B). This is consistent with the hypothesis that combined BRCA2 and PARP1 inhibition negatively affects the SAC and allows for the mis-segregation of chromosomes, leading to altered aneuploidy in daughter cells.
Figure 30 – Non-cancerous kidney and colon cell lines are not sensitized to olaparib by BRCA2 downregulation. Non-tumor HK-2 kidney proximal tubule epithelial cells (A) and CCD-841 fetal colon epithelial cells (B) were transfected with control or BRCA2 ASO and then treated with three different concentrations of olaparib. Proliferation was determined by cell counting 96 hours post-transfection (*p<0.05). C) BRCA2 mRNA levels in HK-2 cells were measured by qPCR following transfection of BRCA2 ASO. Means ± SD from representative experiments are shown. All experiments were repeated at least once.
Figure 31 – Combined BRCA2 ASO and olaparib treatment increases the variability in chromosome number in ovarian and breast cancer cells. SKOV-3 (A) and MDA-MB-231 (B) cells were treated with control ASO or BRCA2 ASO in the presence or absence of olaparib. Forty-eight hours following olaparib treatment, cells were fixed and processed to yield metaphase spreads. The number of chromosomes in individual metaphase cells is shown. A’, B’: Mean chromosome number ± SD after each treatment, calculated from the data shown in panels A and B (*Difference in SD, p<0.05, Bartlett’s Test).
To determine whether BRCA2 ASO and olaparib treatment had an effect on genome stability I used whole chromosome FISH probes to label chromosomes X, 3, and 16, and quantify the incidence of random translocations following treatment. Combined BRCA2 ASO + olaparib treatment led to 1.18 mean translocations per metaphase, compared to 0.1, 0.25 and 0.14 for other treatments (*p<0.05) (Figure 32).

5.2.5 Combined BRCA2 ASO and olaparib treatment can prevent resistance in a mixed cell line model with varying degrees of HRR

Human tumors exhibit a high degree of heterogeneity [10, 18, 61] which can lead to olaparib resistance [74]. Resistance can occur through a variety of mechanisms [73] including reversion to HRR-proficiency in tumors that were predominantly HRR-deficient prior to treatment [74, 75]. Due to the functional linkage between BRCA2 and PARP-1, I hypothesized that combined BRCA2 ASO and PARP inhibition would prevent reversion to HRR proficiency and the appearance of olaparib resistance.

To test this hypothesis, I used three human tumor cell lines with varying degrees of HRR proficiency: SKOV-3 (BRCA2 WT [117]), MCF-7 (HRR deficient [118]), and CAPAN-1 (BRCA2 mutant [117]).

When these three cell lines were treated with BRCA2 ASO (20 nM) simultaneously, the proliferation of HRR-proficient SKOV-3 cells was decreased by 35% ± 10% (p<0.05) (Figure 33A). Thus, BRCA2 inhibition effectively reduced growth of HRR-proficient tumor cells. BRCA2 knockdown in HRR-deficient MCF-7 and CAPAN-1 cells, on the other hand, had no negative effect on proliferation.
Figure 32 – Combined BRCA2 ASO and olaparib treatment increases translocation frequency in ovarian cancer cells. SKOV-3 cells were transfected with control ASO alone or with olaparib or BRCA2 ASO alone or with olaparib. Forty-eight hours post-olaparib, cells were processed to yield metaphase spreads. FISH was performed for chromosomes X, 3, and 16. The number of translocation events in these chromosomes was counted and graphed. Mean numbers of translocations for each treatment are shown as bars (−). *Mean number of translocations were significantly different (p<0.05, Welch’s t-test). Data from two pooled experiments are shown.
These data support the hypothesis that BRCA2 downregulation in a mixed population of HRR-proficient and HRR-deficient cells would lead to an increased fraction of HRR-deficient, BRCA2 ASO-resistant cells (Figure 33A). When the cell fraction of a theoretical mixed population was calculated on the basis of relative proliferation after treatment with BRCA2 ASO, HRR-deficient MCF-7 and CAPAN-1 cells increased in proportion from a total of 66% to 77% relative to SKOV-3 cells (Figure 33B). Thus, BRCA2 downregulation can select for HRR-deficient cells.

On the other hand, treatment of each of the 3 cell lines with a single round of olaparib treatment decreased the proliferation of HRR-deficient MCF-7 and CAPAN-1 cells by 39% ± 6.8% and 94% ± 4.6% (p<0.05), respectively, but had no effect on SKOV-3 proliferation (Figure 33C). Based on these numbers, the fraction of HRR-proficient SKOV-3 cells in a theoretical mixed population after olaparib treatment increased from 33% to 61%. Thus, a single round of olaparib treatment can select for HRR-proficient cells, which is the reciprocal of the effect of BRCA2 ASO.

HRR-proficient SKOV-3 cells are resistant to olaparib relative to HRR-deficient MCF-7 cells (Figure 34A). Combined treatment with BRCA2 ASO and olaparib abolished that relative resistance and led to a decrease in proliferation in both cell lines of 39% ± 0.1% and 40% ± 3.4% (p<0.05), respectively (Figure 34B). This suggests that simultaneous inhibition of BRCA2 and PARP-1 in heterogeneous tumor cell populations can prevent selection events and forestall emergence of treatment-resistant populations.
Figure 33 – Single treatment has the potential to select for HRR-deficient or HRR-proficient cells, respectively. A) BRCA2-wild type SKOV-3 cells (black bars), HRR-deficient MCF-7 cells (white bars), and BRCA2-mutated CAPAN-1 cells (grey bars) were treated simultaneously but separately with BRCA2 ASO (20 nM). Post-transfection (96 h), cells were counted and proliferation determined (% of proliferation after control ASO treatment). B) The theoretical proportions of a mixed cell population (HRR-proficient SKOV-3 + MCF-7, and HRR-deficient CAPAN-1) following BRCA2 ASO treatment were calculated using the data shown in panel A. C) SKOV-3, MCF-7 and CAPAN-1 cells were treated with two different concentrations of olaparib for 96 hours. After drug treatment they were counted and proliferation was determined as a percent of that of vehicle-treated cells. D) The theoretical proportions of a mixed cell population following BRCA2 ASO treatment were calculated based on the experimental data shown in panel C. Data from representative experiments are shown. All experiments were repeated at least once.
Figure 34 – Combined BRCA2 ASO and olaparib treatment decreases the proliferation of both HRR-deficient and HRR-proficient cells. BRCA2-wild type SKOV-3 cells (A) and HRR-deficient MCF-7 cells (B) were transfected with control ASO (black bars) or BRCA2 ASO (white bars) and treated with vehicle or olaparib (1 μM). Proliferation was determined using cell counting 96 hours post-transfection. Data from representative experiments are shown. All experiments were repeated at least once.
5.2.6 Combined BRCA2 ASO and olaparib treatment can prevent the outgrowth of resistant clones in a co-culture model of BRCA2 heterogeneity

To evaluate the effects of BRCA2 ASO and olaparib on population dynamics and resistance to treatment over time, I devised a co-culture model of SKOV-3 ovarian cancer cells stably expressing either control shRNA or shRNA targeting BRCA2. This emulated a tumor population with different proportions of cells of varying HRR-proficiency.

I determined how olaparib monotherapy would impact population dynamics in the context of varying HRR proficiency. To mimic a heterogeneous tumor cell population that is predominantly HRR-deficient, I co-cultured SKOV-3<sup>shBRCA2</sup> (low BRCA2) and SKOV-3<sup>shcontrol</sup> (high BRCA2) in a 3:1 ratio. The mixed cell population, along with unmixed SKOV-3<sup>shBRCA2</sup> and SKOV-3<sup>shcontrol</sup> populations, was treated with olaparib (1<sup>°</sup> Olaparib), then counted, re-seeded and treated with olaparib a second time (2<sup>°</sup> Olaparib), according to the experimental schematic (Figure 35). The mixed cell population, though sensitive to initial treatment with olaparib (Bar 9 vs 10), was completely unresponsive to a second dose (Bar 11 vs 12) (Figure 36A). The unmixed SKOV-3<sup>shBRCA2</sup> population remained sensitive to olaparib even after two doses (Bar 7 vs 8) (Figure 36A). This suggests that 1<sup>°</sup> olaparib treatment of the mixed cell population selected for HRR-proficient cells and allowed them to outgrow HRR-deficient cells.

To determine if combined BRCA2 ASO and olaparib treatment could forestall the development of resistance among mixed SKOV-3<sup>shBRCA2</sup> and SKOV-3<sup>shControl</sup> cells, the mixed and unmixed cells were treated with either control ASO or BRCA2 ASO, in the presence or absence of drug treatment. BRCA2 ASO
treatment sensitized the mixed cell population to olaparib (Bar 11 and 12), and the proliferation level of the mixed population following BRCA2 ASO and olaparib treatment was similar to that of the SKOV3shBRCA2 cells treated similarly (Bar 7 and 8) (Figure 36B).

When mixed and unmixed SKOV-3 populations treated with olaparib and either control ASO or BRCA2 ASO were re-seeded without any further treatment, the mixed cell population that had received BRCA2 ASO + olaparib was unable to proliferate (Figure 36C). This suggests that simultaneous inhibition of both BRCA2 and PARP1 can prevent the outgrowth of resistant cells in a tumor population with HHR heterogeneity.

5.2.7 Combined inhibition of BRCA2 and PARP1 decreases ovarian tumor growth in vivo

I determined whether combined BRCA2 and PARP1 inhibition could prevent or delay growth of ovarian tumors in vivo. Female athymic nude mice were injected with SKOV3-IP1 cells i.p and treated 7 days later with control siRNA or BRCA2 siRNA in the presence or absence of olaparib. Following 7 weeks of treatment, mice were weighed (Figure 37A), euthanized, and dissected to determine the number and combined weight of tumor nodules in the peritoneal cavity. BRCA2 siRNA + olaparib treatment decreased both the number (Figure 37B) and weight (Figure 37C) of tumors relative to BRCA2 siRNA or olaparib treatment alone (p<0.05), suggesting that combing BRCA2 reduction with PARP1 inhibition may be useful therapeutically to limit tumor growth.
Figure 35 – Schematic for mixed cell experiments. The mixed cell population, as well as the unmixed populations were treated according to the experimental schematic. The experiment was conducted in a serial and continuous fashion, such that all treatment groups and cell populations were in culture for the same amount of time, and all controls were exposed to the same conditions.
Figure 36 – Combined BRCA2 ASO and olaparib treatment prevents outgrowth of resistant cells in a tumor cell population heterogeneous for HRR-proficiency. A) SKOV-3<sup>shBRCA2</sup> cells were mixed with SKOV-3<sup>shControl</sup> cells at a 3:1 ratio, resulting in a primarily HRR-deficient mixed cell population. Parental and mixed populations were treated for the first time with olaparib (1° olaparib, 2.5 μM) or vehicle. Cells were re-plated at equal density 96 hours post-treatment. Parental and mixed populations were treated a second time with olaparib (2° olaparib, 2.5 μM). Ninety-six hours post-treatment, proliferation for all groups was determined based on cell counts and seeding density following 1° olaparib or vehicle treatment. White bars: 2° olaparib. Black bars: no 2° olaparib. B) SKOV-3<sup>shBRCA2</sup> cells, SKOV-3<sup>shControl</sup> cells, and a mixed cell population (3:1 as above) were transfected with control ASO or BRCA2 ASO followed by treatment with vehicle or olaparib (2.5 μM). Proliferation (percent of control ASO-treated cells) was determined 96 hours post-transfection. White bars: 2° olaparib. Black bars: no 2° olaparib. C) SKOV-3<sup>shBRCA2</sup> cells, SKOV-3<sup>shControl</sup> cells, and a mixed cell population (3:1 as above) previously treated with ASO (control or BRCA2) and olaparib (2.5 μM) were re-plated at the same density and allowed to proliferate without further treatment. Data from two pooled experiments are shown.
Figure 37 – BRCA2 inhibition sensitizes ovarian cancer tumors to olaparib treatment in vivo. Female athymic nude mice were injected with 1.0x10⁶ SKOV3-IP1 cells i.p. Mice were treated 7 days later with olaparib (5 mg/kg 5 days a week i.p.) and either control or BRCA2 siRNA twice per week encapsulated in DOPC-liposomes (150 µg/kg) (N=40, 10 animals per group). Once the mice in any group were moribund, the animals were weighed (A) and euthanized. The tumour weight (B) and number of tumour nodules (C) were determined (p*<0.05, Student’s t-test).
Chapter 6. Discussion

Genomic instability, mutation, and the accompanying heterogeneity represent common traits among most human cancers [119]. However, and despite the fact that a high mutation rate is a driving factor behind malignant progression, lack of genomic fidelity can act as an 'Achilles heel' for tumor cells [28]. In the face of selection pressure (e.g., cytotoxic DNA-damaging therapy), all cancer cells must preserve a level of DNA repair that is sufficiently active to maintain their survival, but also sufficiently limited or error prone to allow continued mutability, adaptability, and heterogeneity on a tumor (population) level. Consequently, although cancer cells often exhibit compromised or inefficient DNA repair or cell cycle control, which in many cases is a driver of their malignant evolution and etiology [71, 120, 121], the level of DNA maintenance they continue to harbour is (by definition) adequate to keep the population alive -- the idea of cancer cell reliance on a limited number of DNA repair mediators for continued survival has important implications for cancer treatment.

Thus, functional DNA repair machinery in a cancer cell constitutes an intriguing target for novel therapeutic intervention, and this is an active and rapidly expanding field of investigation. Inhibiting cancer cell ability to repair DNA in combination with chemo or radio-therapy treatment may render cancer treatment more potent; it may push tumor cells past a tolerable point or threshold of DNA damage and induce cell death, preventing further mutation, heterogeneity and the generation or outgrowth of resistant clones which leads to resistance on a tumor level.
In addition, the same level of DNA repair which decreases the efficacy of cancer treatment can, through its inherently limited and often error prone nature, lead to continued mutation and increased genome variation [122]. Therefore, DNA repair activity in tumor cells is not only a direct source of resistance to chemo- and radio-therapy, but this same DNA repair functionality can promote continued mutation and heterogeneity as a direct result of its limited scope, and thus indirectly lead to more robust and acquired drug resistance.

I propose that inhibiting proteins involved in DNA repair and maintenance (such as BRCA2) is a method to induce complementary lethality to DNA damage-inducing chemotherapeutic drugs and enhance the probability of catastrophic and lethal toxicity in tumor cells. In the third chapter of this thesis I show that the induction of complementary lethality with combined targeting of DNA repair factors involved in cellular response to the mechanism of action of different drugs may be a potential strategy to improve cancer therapy (by overcoming innate resistance to treatment).

I hypothesized that BRCA2 knockdown with siRNA would potentiate cisplatin and melphalan treatment because both drugs induce DSBs [52], and BRCA2 is an integral part of the complex responsible for HRR [123, 124]. My data support this assertion, which is in agreement with another study highlighting increased sensitivity to alkylating agents in BRCA2 knockout mice [21], and a report describing glioma cell sensitization to temozolomide following downregulation of BRCA2 and RAD51 [125].

I was surprised to find that BRCA2 knockdown potentiated 5-FUdR toxicity, albeit to a much smaller degree than cisplatin and melphalan, but enough to fulfill
my definition of complementary lethality. I expected that BRCA2 knockdown would have no effect on 5-FUdR toxicity given the lack of effect with pemetrexed, and the apparently limited involvement of 5-FUdR in pathways that correspond with BRCA2 function. BRCA2 does not appear directly related to the mechanism of action of 5-FUdR since the drug targets TS. However, upsetting the balance of intracellular nucleotide pools through treatment with drugs like 5-FU can stall replication fork progression [126]. BRCA2 has been shown to maintain stability of stalled replication forks [49, 127] which may explain why BRCA2 knockdown potentiates the activity of 5-FUdR. This, however, may not be the primary function of BRCA2 or 5-FUdR in the cell, which may explain why the potentiation of 5-FUdR was relatively minor.

As expected from previously-published work, TS downregulation potentiated the effects of both 5-FUdR and pemetrexed. This may in large part be due to the fact that knockdown of TS mRNA in combination with pharmacological inhibition of TS protein are complementary functions [58, 128]. TS siRNA, however, did not appear to induce complementary lethality to either cisplatin or melphalan, suggesting that TS is not intimately involved in cellular response to those drugs.

Cumulatively, these results suggest that BRCA2 and TS are involved in resistance to different classes of drugs, and that they may be involved in largely separate pathways within the cell. The cell cycle data further supports this assertion because it shows that BRCA2 and TS downregulation differentially affects cell cycle progression and that simultaneously targeting both factors has the potential to induce two discrete blocks in cell cycle. This renders an anti-BRCA2 and anti-TS combination therapy particularly intriguing from an acquired resistance
perspective, since mathematical modelling and clinical data has established that
the probability of resistance decreases with the number of targets and drugs in a
particular treatment [12]. It would be interesting to determine mathematically what
the probability of resistance to combined siRNA and combined drug treatment
would be, and is something I hope to do on a collaborative basis in the future.

Simultaneous downregulation of BRCA2 and TS potentiated the effects of
all four tested drugs, thus creating multi-drug sensitive tumors. These experiments
demonstrated that tumors can be sensitized to different classes of
chemotherapeutics by targeting separate factors critical to cellular response to
those drugs. An important determination was that there were no antagonistic
effects observed during combined siRNA treatment. These findings are particularly
germane in the context of chemotherapy, because drugs are rarely administered
in isolation [129].

I tested whether combined complementary lethality to two drugs could be
achieved simultaneously in the same cell population, because this would be most
closely related to current and future clinical scenarios. An important point to
consider is that differential pharmacokinetic and pharmacodynamic factors
relevant to different drugs result in unequal uptake into tumor cells; that is, cells in
different locations within tumors, and at different sites in the body, are likely to be
accessible to different drugs in different ways [130, 131]. Since tumor cells may
take up drugs at rates different from those administered (and some drugs may
accumulate in tumors at low concentrations that are difficult to predict or plan for)
then sensitizing tumor cells to all drugs in a treatment cocktail can be an advantage.
The proposed treatment with combined siRNAs targeting multiple mRNAs to induce complementary lethality to different drugs may have this desirable affect.

Interestingly, I observed some apparent antagonism when cisplatin and pemetrexed were used in a combined treatment, and antisense mediated complementary lethality was not as effective in this treatment group. These results have potential implications for therapy because cisplatin and pemetrexed combinations are currently used to treat a variety of different cancers, including non-small cell lung cancer (NSCLC) [132].

The induction of complementary lethality in the context of DNA repair provides an alternative perspective to the focus on personalized medicine in cancer therapy. It contrasts with the idea of synthetic lethality, which relies upon identifying pre-existing deficiencies in cancer cell genomes and exploiting them for therapeutic benefit [67].

A prototypical example of synthetic lethality with therapeutic applications is the discovery and development of PARP1 inhibitors, many of which have and are currently undergoing clinical trials with varying degrees of success [114, 133]. The key to this strategy however, is the identification of a pre-existing weakness (e.g., BRCA deficiency) in a cancer cell, and tailoring a therapy designed to exploit or exacerbate that vulnerability. In addition, the application of synthetic lethality in a heterogeneous tumor cell context renders resistance to therapy highly likely, because by definition only a certain subset of tumor cells with a particular mutation or alteration will be affected.

Induction of complementary lethality via targeting DNA repair theoretically nullifies the need to understand the distinct genotype of a targeted tumor. Instead,
this concept relies on a unifying principle: the fact that tumors need to maintain a minimum level of genomic stability in order to survive, and that this must occur in the face of chemotherapy and radiation. Therefore, complementary lethality not only overcomes innate resistance to treatment, it may help to prevent the outgrowth of resistant clones by sensitizing all cells in a tumor to a particular therapy, instead of relying on pre-existing vulnerabilities within a heterogeneous population.

As a result of my studies into complementary lethality using siRNA in Chapter 3, I developed a BRCA2-targeting ASO candidate drug to specifically sensitize cancer cells to the effects of the DNA damaging agent cisplatin. Many candidate antisense oligodeoxynucleotide (ASO) based agents are currently undergoing various phases of clinical trials [134, 135]. Importantly, ASOs also exhibit several advantages over siRNAs for in vivo use, including increased stability in serum and decreased need for a delivery or carrier vehicle [29]. My rationale was based on the known role of BRCA2 in DNA repair and clinical data that individuals with BRCA2-mutated tumors respond more favorably to platinum-based chemotherapy. In addition, high levels of BRCA2 mRNA have been associated with poor patient prognosis [136]. Retrospective analysis of RNA-sequencing data revealed that BRCA2 mRNA is highly expressed in different types of human cancers (personal communication, Dr. Sherry Y. Wu), suggesting that BRCA2 may be a good target for an antisense-based drug directly targeting BRCA2 mRNA.

In order to better understand the role that BRCA2 inhibition may play in human patients, I performed TCGA database analysis which revealed that BRCA2 mutations in endometrial and ovarian cancer provide a better survival benefit than BRCA1 mutations. Although the lack of correlation does not rule out possible
therapeutic advantages after inhibition of BRCA1 in patients with functional BRCA1 genes, these data suggest that BRCA2 may be a more useful target for reduction. My analysis of human tumor sample data is in accord with previous reports that BRCA2 mutations may be beneficial for patient prognosis following drug treatment and/or radiotherapy [92, 102] and supports the potential of BRCA2 reduction to enhance patient survival.

I showed that combining BRCA2 ASO and cisplatin treatment is an effective strategy to render different types of cancer cells more sensitive to the effects of DNA-damaging drugs. In particular, the ability of BRCA2 ASO treatment to reduce acquired cisplatin resistance in head and neck tumor cells is an important clinical finding, since a proportion of cancer patients eventually develop resistance to platinum-based chemotherapy [51].

The fact that BRCA2 ASO did not greatly potentiate cisplatin treatment in non-cancerous HK-2 cells suggests that tumor cells may be more sensitive to this type of treatment regimen. I hypothesize this is due to differences in DNA integrity, however it remains to be determined whether this is the case, or if specific, common mutations in tumor cells predispose them to BRCA2 ASO mediated sensitization to cisplatin.

Furthermore, an emerging phenomenon is the development of treatment resistance in previously treatment-responsive BRCA2-mutated tumors. Such tumors can undergo reversion mutations that re-activate BRCA2 function and lead to treatment failure [75, 137]. The implications are twofold. First, it highlights the importance of BRCA2 function for tumor cell survival in the face of strong selection pressures such as cytotoxic drug treatment. Second, it suggests that BRCA2 ASO
treatment could be of benefit even in individuals with tumors composed primarily of cells harboring inactivating BRCA2 polymorphisms.

For the first time, I linked BRCA2 with cellular metabolic response following cisplatin treatment and showed that cellular respiration is negatively affected in cells treated with BRCA2 ASO and cisplatin. I was unable to detect a difference in mitochondrial function by MitoTracker staining, suggesting that the defect in respiration is either mitochondria-independent, or not related to changes in the number of functioning mitochondria per cell. Furthermore, I identified an increase in glucose uptake that did not result in increased glycolysis, nor did it prevent a decrease in cell adhesion. Therefore, this may be a marker of cell stress and a response by affected cells to generate additional energy necessary to deal with the results of this stress. Overall, these data suggest that BRCA2 helps to maintain cellular metabolic processes following cisplatin treatment and, after reduction of BRCA2, cisplatin induces respiratory collapse and a non-productive increase in glucose uptake.

Due to the clinical importance of metastatic disease, I determined the effect of my treatment regimen on metastatic frequency. I found that ASO-mediated reduction of BRCA2 combined with cisplatin treatment led to decreased human tumor cell metastatic frequency in a chick CAM model. Cisplatin may be particularly effective at preventing metastasis by cells without active BRCA2, which may be due to an enhanced capacity to reduce tumor cell proliferation/viability (as suggested by the in vitro experiments). However, the results of the experiments presented here do not distinguish among three possibilities: that the lower number of metastatic foci present in the CAM following combined BRCA2 ASO and
cisplatin treatment was due to decreased tumor cell viability, that the combined treatment decreased the ability of tumor cells to extravasate and survive in tissue surrounding blood vessels without decreasing tumor cell viability, or a combination of the two. Regardless, inhibition of BRCA2 may be a useful strategy to decrease metastatic burden in patients treated with cisplatin, because it decreases the innate resistance of cancer cells to this cytotoxic drug. In addition, cisplatin is not the only anti-cancer agent that BRCA2 ASO treatment may improve, and the PARP1 inhibitor olaparib holds intriguing promise for combination treatment with BRCA2 knockdown (from both an innate resistance and acquired resistance perspective).

Olaparib is approved for treatment of BRCA1/2-mutated ovarian tumors. However, this represents only a subset of cancer patients [69] and resistance can occur even in this population [75]. I tested the BRCA2 ASO in combination with olaparib to determine whether the combination could: 1) overcome innate resistance and increase the potential usefulness of olaparib by rendering HRR-proficient, BRCA2-positive tumors sensitive to the drug, and 2) prevent acquired resistance in cell populations with mixed HRR-proficiency.

I showed, using human lung, ovarian, and breast cancer cell lines, that BRCA2 ASO treatment can overcome innate resistance to olaparib in these cell lines. None are reported to harbour BRCA2 or BRCA1 mutations (COSMIC CCLE database) [117] and, with functional BRCA2 capable of mediating HRR, are relatively resistant to the therapeutic effects of PARP1/2 inhibition by olaparib. Therefore, BRCA2 ASO treatment has the potential to render a higher proportion of tumor cells sensitive to olaparib treatment, which may extend the usefulness and applicability of this drug in the clinic.
The fact that olaparib primarily targets HRR-deficient tumors is also a potential problem in terms of positive selection for resistant clones in a heterogeneous tumor ecosystem. Most solid human tumors exhibit complex polyclonal variability and data from single nucleus sequencing of breast tumors suggests that no two tumor cells are identical [9]. This renders resistance to targeted therapy and chemotherapy inevitable mathematically [12], and very common biologically [11, 18]. Several olaparib resistance mechanisms have already been described in this context, including the outgrowth of tumors with re-activation mutations in BRCA2 which render olaparib ineffective [73]. In addition, BRCA1-mutated tumors cells with a concomitant mutation in 53BP1 are no longer HRR-deficient and also exhibit resistance to PARP1 inhibition [138]. It therefore appears that olaparib treatment will fail at high frequency without any corresponding positive selection pressure for cells with HRR-deficiency.

Combining BRCA2 inhibition with PARP1 inhibition can achieve a state where each individual treatment positively selects for cells with unique susceptibility to the other treatment, thus preventing or delaying resistance: this is the essence of the concept which I have termed reciprocal positive selection for weakness. The data from my mixed cell experiments suggests that simultaneous inhibition of BRCA2 and olaparib treatment has the ability to limit the proliferation of tumor cells heterogeneous for HRR-proficiency, thus preventing positive selection of resistant cells based on their underlying ability to repair DNA.

An important consideration for the future is whether it is possible to develop resistance to simultaneous BRCA2 and PARP1 inhibition (either through a primary mechanism related to HRR, or a secondary mechanism unrelated to HRR-
proficiency). It may be possible to address this question using a barcoded shRNA library to downregulate a large assortment of genes in the context of BRCA2 deficiency and olaparib treatment. The shRNA barcode could be used to determine which gene or genes were down-regulated in any surviving cells. This experiment would divulge whether resistance to combined BRCA2 and PARP1 inhibition is possible, and if so, identify a subset of targets for further study and development of strategies to prevent or overcome this potential resistance mechanism.

The in vivo data suggest that it is possible to combine BRCA2 inhibition and olaparib treatment for potential therapeutic benefit. The mice which received combination treatment exhibited the fewest tumor nodules, and the lowest tumor weight relative to control and each of the single treatments. In particular, the i.p model recapitulates several hallmarks of later stage ovarian cancer, and is a model to explore the potential of therapy to prevent the establishment of metastatic lesions at secondary sites in the peritoneal cavity [139]. This data suggests that combined BRCA2 downregulation and olaparib treatment may prevent spread and growth of ovarian tumours to secondary sites. Further experiments are needed to determine the capacity of BRCA2 ASO and olaparib treatment to extend the survival of mice harbouring ovarian tumors, since the experiment described in this thesis was designed only to asses tumor growth, and did not determine overall or progression-free survival in the host mouse.

Furthermore, to address the potential for treatment-mediated toxicity in vivo it will be necessary to use a mouse homograft model and inhibit BRCA2 using a sequence that targets mouse BRCA2. The in vivo experiments conducted during this thesis work utilized a human xenograft model, and the siRNA targeted human
BRCA2 (but not mouse BRCA2). As a result, the potential adverse effects of combined BRCA2 inhibition with olaparib treatment could not be investigated.

In the future, to directly address the ability of BRCA2 ASO and olaparib treatment to prevent selection based on HRR proficiency \textit{in vivo}, I propose utilizing a mixed tumor model that incorporates secreted luciferases to identify changes in population proportion. For this experiment, HRR-proficient (shControl) and deficient (shBRCA2) cells would be engineered to express secreted luciferases of different wavelengths, and this would enable real time monitoring of tumor proportion from blood draw [140]. Such an experiment would show, for the first time, whether resistance to olaparib can be prevented in a heterogenous tumor population by simultaneous inhibition of BRCA2 \textit{in vivo}.

In addition, immunotherapy is a burgeoning field of research in oncology, and numerous novel strategies to increase immune-mediated destruction of cancer cells are being developed. Tumors with a high mutational load produce immunogenic neo-antigens, which can potentially facilitate their elimination by the immune system [25, 141]. Lack of efficient DNA repair mechanisms can increase mutation load, and thus promote cytotoxic T-lymphocyte (CTL) recognition of cancer cells [142]. A logical extension of this thesis research would be to test whether inhibition of BRCA2 function could lead to an increase in antigenic mutational load, and whether this increase could make tumors more immunogenic. It would be illuminating to combine BRCA2 inhibition with immune checkpoint blockade (either in the form of anti-CTLA4 or anti-PD1 antibodies) in an immunocompetent mouse model of breast or ovarian cancer, to determine whether anti-tumor CTL responses are increased.
Chapter 7. References:

28. Cresce, C., et al., Antisense Technology: From Unique Laboratory Tool to Novel Anticancer Treatments


41. Industries, T.P., *Comparison of Docetaxel/Prednisone to Docetaxel/Prednisone in Combination With OGX-011 in Men With Prostate Cancer (SYNERGY)*. 2014, National Institutes of Health.


114.


Appendix A – Copyright Permissions

Portions of this thesis are based in whole or in part on the following published articles:


Used with permission – Creative Commons License (Attribution – Noncommercial)


Used with permission – License number: 3737681282656
Appendix B – Curriculum Vitae

Education:

PhD Candidate – Microbiology and Immunology  
*University of Western Ontario*  
Sept. 2011- Dec. 2015

Master of Science – Microbiology and Immunology  
*University of Western Ontario*  
Sept. 2009 – Aug. 2011

Bachelor of Health Sciences (Honors) Program  
*McMaster University*  

Scholarships and Awards:

New Zealand Society for Oncology (NZSO) Travel Award  
Oct. 2014

Drs. Madge and Charles Macklin Fellowship for Teaching and Research  
- *Schulich School of Medicine Award*  
  Jun. 2014

Oral Presentation Award - 10th Annual Research and Education Day - Department of Oncology  
Jun. 2013

CIHR Banting and Best Canada Graduate Scholarship (CIHR CGS-D)  
- *National level external award*  
  May 2013 - 2016

Ontario Graduate Scholarship (OGS) (Declined)  
- *Provincial level external award*  
  May 2013 – Apr. 2014

Vanier award nominee  
Sept. 2012

Queen Elisabeth II Graduate Scholarship in Science And Technology (QEIIGSST)  
- *Provincial level external award*  
  May 2012 – Apr. 2013

CIHR-STP Cancer Research and Technology Transfer (CaRTT) Studentship  
- *Institutional level competitive award funded by CIHR*  
  Sept. 2012 – 2013

Vanier award nominee (placed 63rd out of 250)  
Sept. 2011

CIHR-STP Cancer Research and Technology Transfer  
Sept. 2011 – 2012
(CaRTT) Studentship
- *Institutional level competitive award funded by CIHR*

Translational Breast Cancer Studentship from the London Regional Cancer Program
- *Institutional level competitive award*

Microbiology and Immunology Department Travel Award
- *Departmental level award based on academic merit*

Alexander Graham Bell Canada Graduate Scholarship (NSERC CGS-M) - *National level external award*

Ontario Graduate Scholarship Master’s Award (OGS) (Declined)
- *Provincial level external award*

Department of Microbiology & Immunology Fellowship - *Awarded to the top 4 students of incoming class*

Western Graduate Research Scholarship
- *Awarded based on academic merit*

McMaster University Dean’s Honor List
- *Awarded based on academic merit*

McMaster President’s Award Entrance Scholarship
- *95%+ entrance average*

**Peer-reviewed Grant support as Co-applicant**

“Targeting CD5 and Fas for Ovarian and Breast Cancer Immunotherapy” – Lawson Internal Research Fund - July 2015 – 2017

**Patents and Peer-reviewed Research Publications**


**Books and Book Chapters**

Di Cresce, C., Way, C., Rytelewski, M., Maleki Vareki, S., Nilam, S., Vincent, M.D., Koropatnick, J., Ferguson, P.J. “Antisense Technology: From Unique Laboratory Tool to Novel Anticancer Treatments.” Chapter 12 - From Nucleic Acids to Nuclear Medicine; Springer Vanier. (June 2012)

**Published Abstracts & Research Presentations**


**Rytelewski, M.**, Tong, J., Buensuceso, A., Maleki Vareki, S., Ferguson, P.J., Figueredo, R., Vincent, M., Shepherd, T., Deroo, B.J., Koropatnick, J. "A novel BRCA2 targeting antisense oligonucleotide sensitizes human tumor cells to chemotherapy and radiotherapy-the induction of 'complementary lethality'by targeting DNA repair". *Poster Presentation* - AACR Molecular Targets Meeting, Boston, USA. October 2013 - Published in Molecular Cancer Therapeutics


PJ Ferguson, MD Vincent, R Figueredo, M Rytelewski, J Koropatnick. "Enhancement of Cisplatin Cytotoxicity by Antisense Oligonucleotides Targeting DNA Repair Protein BRCA2: Creation of Synthetic Lethality to Improve Selectivity"


Rytelewski, M., Garg, N., Harding, M., Mazzuca, D., Haeryfar, S.M. “Tryptophan Metabolism and CD8+ T-cell Function.” **Poster Presentation:** Canadian Society for Immunology (CSI) Meeting April 2011

Maleki-Vareki, S., Harding, M., Rytelewski, M., Haeryfar, S.M. “Rapamycin differentially regulates antiviral and allogenic CD8+ T-cell responses elicited in the same host.” **Poster Presentation:** Canadian Society for Immunology (CSI) Meeting 2011
