Determining the mechanism of double-stranded RNA-induced cell death in ovarian cancer.

Danielle Van  
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DETERMINING THE MECHANISM OF DOUBLE STRANDED RNA-INDUCED
CELL DEATH IN OVARIAN CANCER

A Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Richmond, Virginia
August 2011
Acknowledgements

It is my pleasure to thank the many people who have made this thesis possible. I would like to first thank the Department of Biochemistry and Molecular Biology, especially Dr. Sarah Spiegel, Dr. Suzanne Barbour, and Dr. Tomasz Kordula for my acceptance into the program. To my committee, thank you for all of your continued support and advice. To my mentor, Dr. Jessica Bell, thank you for patiently teaching me everything I know about science. You have been and will continue to be my role model. Thank you to all of my lab mates past and present, especially Charlotte Roberts, for contributing so much to this project. Thank you to Dr. Sandrine Lépine for being an amazing teacher and friend. You are responsible for much of the success of this project and myself.

I would like to thank my best friend, Golda Black, for being my biggest cheerleader, and her mother Mary Meyers, for inspiring me to pursue a career in cancer research. Thank you to my great-grandfather, Leonard A. Schramm, for supporting me throughout my college career. Also, thank you to my husband, Phisith Van, for being my partner in life. You have always been there to provide balance and an escape. Finally, I would like to dedicate this work to my mother, Jackie Mitts. You have been the single most influential and important person in my life. I hope that I have and will continue to make you proud.
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<tr>
<td>2-AP</td>
<td>2-aminopurine</td>
</tr>
<tr>
<td>3MA</td>
<td>3-methyladenine</td>
</tr>
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<td>AP-1</td>
<td>activator protein 1</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATG5</td>
<td>autophagy-related 5 homolog</td>
</tr>
<tr>
<td>BiP</td>
<td>binding immunoglobulin protein</td>
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<tr>
<td>BRCA1/2</td>
<td>breast cancer susceptibility gene ½</td>
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<td>CA125</td>
<td>coelomic epithelial antigen</td>
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<td>CARD</td>
<td>caspase recruitment domain</td>
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<td>eIF-2α</td>
<td>eukaryotic initiation factor-2α</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>FADD</td>
<td>FAS-associated death domain protein</td>
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<td>FAM</td>
<td>fluorescein amidite</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>IκB</td>
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<td>tudor domain containing 7</td>
</tr>
<tr>
<td>TIM</td>
<td>tumor-infiltrating macrophage</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR3</td>
<td>Toll-like receptor 3</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-----------------------------------------------</td>
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<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VISA</td>
<td>virus-induced signaling adapter</td>
</tr>
</tbody>
</table>
Abstract

DETERMINING THE MECHANISM OF DOUBLE STRANDED RNA INDUCED CELL DEATH IN OVARIAN CANCER

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

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Ovarian cancer is the most lethal of all gynecological cancers. Current ovarian cancer drug regimens, including taxanes and platinum-based agents, are susceptible to chemoresistance necessitating the development of novel chemotherapeutics. Within tumors pathogen-derived ligands, such as dsRNA, can activate pattern recognition receptors (PRRs) that are capable of inducing apoptosis. In this dissertation we have found that in ovarian cancer cell lines (DOV-13, SKOV-3, CAOV-3, and OVCAR-3), dsRNA treatment alters cell survival. When treated with dsRNA, ovarian cancer cell lines and patient...
samples could be divided into two categories, responsive which undergo significant levels of apoptosis (CAOV-3 and OVCAR-3) or non-responsive which are unaffected (DOV-13 and SKOV-3). Following dsRNA treatment, dsRNA receptor expression levels increase in responsive cell lines and patient samples only. This suggests a potential role for dsRNA receptors as biomarkers to identify dsRNA-responsive patients. Detailed investigation of the mechanism by which cell lines succumb to or avoid dsRNA-induced cell death showed that in responsive cell lines, NF-kappaB, IFN-beta and caspase 3 activation occurred. Cell death was caspase and IFN-dependent. In non-responsive cell lines, increased c-IAP2 levels and RIP1 kinase ubiquitination occurred, as well as, an increase in basal level autophagy with dsRNA stimulation. However, individual blockade of these pathways did not restore dsRNA-induced apoptosis. In a non-responsive cell line, dsRNA enhanced the action of paclitaxel, carboplatin, and vorinostat through an as yet undetermined mechanism. In a responsive cell line, dsRNA produced a synergistic effect when combined with these drugs. These novel dual therapies, innate immune ligand plus cytotoxic agent, may find application in chemoresistant ovarian cancers.
CHAPTER 1

INTRODUCTION

1.1 Overview

Ovarian cancer is the most lethal of all gynecological cancers (1). While routine screenings for breast and cervical cancer promote early detection of these diseases, ovarian cancer is asymptomatic, often going unnoticed until the later metastatic stages of the disease (2). Standard treatment calls for surgical debulking of the tumor followed by a combination of taxane and platinum-based chemotherapy (3). Despite an initial high response to therapy, the 5-year survival rate for patients diagnosed in later stages remains <30% due to recurring tumors that over time become chemoresistant (4). To target chemoresistant tumors, novel chemotherapeutic agents must be developed for targeted therapy.

1.1.1 Prevalence and etiology of ovarian cancer

Ovarian cancer affects 1 in 71 women in the United States (5). Although it accounts for only 4% of cancers in the female population, it is ranked fourth in cancer-related deaths (6). In the United States alone, approximately 15,000 women succumb to this disease annually, developing primarily in women over 45 with the median age at diagnosis of 63 (7). Studies have shown that oral contraceptives, pregnancy, lactation, tubal ligation, and hysterectomy decreased ovarian cancer risk, while early age at menarche / late age at natural menopause, incessant ovulation, hormone replacement therapy, infertility, and a family history of ovarian cancer increased risk (8). The occurrence of ovarian cancer is difficult to predict, with only 5-10% of the patients possessing a genetic predisposition (9).
Of these patients, 90% have a mutated BRCA1/2 gene, which is known to lead to a predisposition for breast cancer (10). The remaining 90-95% of cases without a genetic predisposition arise from a heterogeneous mix of mutations leading to a patient population that may respond differently to the standard treatment.

Occurrence can be identified or monitored by the serum biomarker CA125, a coelomic epithelial antigen produced by mesothelial cells that line the peritoneum, pleural cavity and pericardium (11). Although CA125 is elevated in 80% of ovarian cancer patients, levels can be elevated due to other conditions such as endometriosis, leiomyomas, congestive heart failure, cirrhosis, and menstruation (12). CA125 is monitored in patients with increased risk, but nevertheless, due to low sensitivity and specificity during the first stage of the cancer, following CA125 levels showed a very low efficacy (less than 26%) (13). Due to the lack of specificity in monitoring CA125 levels, this target alone is not an ideal biomarker for ovarian cancer. To increase the efficacy of ovarian cancer screening, panels of multiple biomarkers including CA125 are being investigated (14). In the future, biomarker panels may lead to increased detection of early stage disease.

The origin of ovarian cancer pathogenesis is unknown but multiple causes have been proposed including increased gonadotropins or androgens, inflammation such as endometriosis, proliferation of granulosa that fail to undergo apoptosis after ovulation, and incessant ovulation (15). Although ovarian cancer tumors can arise from multiple parts of the ovary (gonadal-stromal 6% and germ cell 4 %), the majority of cases (90%) begin in the ovarian surface epithelium (OSE), which covers the outside of the ovary (16). Ovarian cancer is categorized into four stages. In stage I, the cancer is confined to the ovaries only,
while stage II is localized to the ovaries and pelvic area. In the later stages, the cancer may have spread to the abdominal area (stage III), or metastasized to other organs of the body such as the liver or lungs (stage IV) (17). The five year survival rate of stage I cancer is high at 93.8%, however, it rapidly decreases at later stages (II – 72.8%, III – 28.2%, IV – 27.3%) (7). If the cancer is detected at stage I or II, surgery may cure the disease but around 25% of these patients face a recurrence (18). As the cancer spreads from the ovaries throughout the peritoneal cavity, symptoms like abdominal discomfort and bloating ensue that are usually attributed to other gastrointestinal or reproductive diseases (9). This asymptomatic nature often leads to late detection, with 75% of women being diagnosed in advanced stages III and IV where the cancer has proliferated outside of the ovary (19).

1.1.2 Ovarian cancer treatment

The customary treatment protocol begins with surgical reduction of the tumor, which allows physicians to assess stage and aggressiveness of the tumor. Subsequently, chemotherapeutic drugs are administered. Several factors are considered when determining the therapeutic regimen for each patient including histology and differentiation of the tumor cells, age, surgery type, and residual disease after primary cytoreduction (20). Following surgery, most patients receive a combination of the platinum-based drug, carboplatin, and the taxane compound, paclitaxel. Carboplatin inhibits cell division by cross-linking DNA compromising transcription and replication, while paclitaxel stabilizes microtubules stopping mitotic spindle formation which does not allow cell division (21). This regimen elicits a positive response in 65% of patients (20) and has shown effectiveness when given intraperitoneally, with patients experiencing more abdominal
discomfort but no increase in severe clinical toxicity (22). Despite the initial response, more than 70-90% of patients relapse and require second-line chemotherapy (7). If the relapse is within 1 month the patient is considered refractory to platinum treatment, within 6 months the patient is platinum-resistant, and after 6 months the patient is platinum sensitive (23). Relapsed platinum-resistant patients are currently given non-cross-resistant antineoplastic agents that have demonstrated single-agent activity against ovarian cancer (24). Some examples include the topoisomerase I inhibitor topotecan (8% response) (25), the anthracycline liposomal doxorubicin (26% response) (26), and the nucleoside antimetabolite gemcitabine (20% response) (27). Although these drugs are promising, the response rates are still low, and as a result many patients are recommended for clinical trials to aid in identification of novel individual and combination chemotherapeutics (20).

1.1.3 Novel Chemotherapeutics

Chemoresistance stems from the ability of tumor cells to overcome the drug-induced apoptotic signal. With individual drugs often unable to eliminate all cancer cells, a combination of drugs like carboplatin and paclitaxel is more likely to induce apoptosis by targeting multiple pathways or cellular components. Although this drug combination is efficacious, it is not successful in all patients. A larger array of potential therapeutic agents are required that can be tailored to individual therapies.

Multiple cytotoxic drugs are being investigated individually and in combination with carboplatin and paclitaxel, including doxorubicin and gemcitabine (28). Additionally, considerable research is focused on molecular targets such as growth factor receptors, signal transduction pathways, cell cycle regulators, and angiogenic mechanisms.
Instead of using non-specific cytotoxic drugs, these therapies target distinct signaling cascades, which allows for greater specificity and the potential of fewer off-target effects. Bevacizumab, an antibody which blocks angiogenesis by binding to vascular endothelial growth factor (VEGF) and blocking new blood vessel formation, exemplifies this targeted therapy (29). Another promising category of drugs includes PARP inhibitors like olaparib. In tumors with BRCA mutations, olaparib has shown clinical benefit in 57.6% of patients (3). These targeted therapies provide additional treatment options to address the heterogeneous nature of ovarian cancer and thus the varied responses (or lack of response) to chemotherapeutic agents observed in patients.

This heterogeneity includes variance in immunological characteristics, expression patterns of receptors and enzymes, and metastatic ability (30). Depending on these patterns in patients, treatment could be tailored to improve the efficacy of chemotherapy. Recent findings of the Cancer Genome Atlas Research Network have characterized a catalogue of molecular aberrations that are consistent amongst ovarian cancer tumors. They have shown that many tumors exhibit defective homologous recombination and deregulated signaling pathways (31). Furthermore, with an abundance of emerging data characterizing the tumor microenvironment, the importance of the immune response in cancer has been explored (32). Ovarian cancer is immunogenic. With the ability to elicit a spontaneous anti-tumor immune response, ovarian tumors are viable targets for immunotherapy (33).

1.2 Immunotherapy in cancer

The concept of targeting the immune system in cancer treatment began long ago when Dr. William Coley linked bacterial infection and cancer regression (34). With the
perspective of identified innate immune receptors and an understanding of the adaptive immune response, we now understand that the induced bacterial infection in Coley’s patients initiated an innate immune response. In the tumor environment, this innate immune system activation occurred in the presence of tumor antigens (supplied by necrotic tumor cells) that elicited a specific, targeted response to tumor cells resulting in tumor regression.

Immunotherapy began with the idea that the immune system was responsible for regulating cancer development, a concept known as cancer immunosurveillance (35). This idea has evolved into the concept called cancer immunoediting, which consists of three phases, elimination (immunosurveillance), equilibrium, and escape (36). Elimination takes place when the immune system identifies and eradicates cancer cells. However, during the elimination phase, not all tumor cells succumb to the immunological attack. Tumor cells are genetically unstable and rapidly mutate, giving rise to subpopulations resistant to immunological elimination. This creates an equilibrium between tumor cell survival and immune system attacks. This stage lasts for an unspecified amount of time. Finally, tumor cells can escape the equilibrium phase and resume uncontrolled growth into tumors.

Tumors evade the immune system by releasing immunosuppressive agents like IL-10 and down regulating pro-inflammatory cytokines and costimulatory molecules on macrophages (37). In addition, since cancer cells arise from normal cells, they often lack danger signals needed to activate antigen-presenting cells to elicit an immune response. In order to overcome this immunosuppressive environment and restore the ability to eliminate cancer cells, research has focused on development of cancer vaccines (38).
1.2.1 Cancer vaccines and adjuvants

Vaccines are composed of an antigen molecule (to be recognized by the immune system), and an adjuvant that induces a strong immunological response. Innate immune cells known as antigen presenting cells (APCs) become activated when they encounter the antigen/adjuvant mixture. APCs then communicate to the adaptive immune system (i.e. naïve T cells) the antigen specific response required, leading to elimination of the target via cytotoxic T lymphocytes and an antibody-specific response. Cancer vaccines can prevent cancer caused by viruses (prophylactic vaccines) or treat existing tumors (therapeutic vaccines). One example of a successful prophylactic cancer vaccine is the cancer human papilloma virus (HPV) vaccine (39). This vaccine protects the host against strains of the virus that cause 70% of cervical cancer cases (40). Therapeutic cancer vaccines that target existing tumors can work in multiple ways. One method enhances the anti-tumor immune response to known tumor antigens, such as NY-ESO, an ovarian cancer rejection antigen. One vaccinated patient showed a complete response to NY-ESO-1 peptide vaccination but had a recurrence with an NY-ESO-1 negative tumor (33). Alternatively, patients can be immunized against proteins isolated from their own tumor (41).

To increase efficacy of cancer vaccines, antigens can be paired with adjuvants (42). Adjuvants are compounds that enhance the potency of the antigen-specific immune response and are an essential component of an efficacious vaccine (43). Innate immune ligands that activate innate immune receptors are potentially strong adjuvant candidates. These ligands can also directly act on cancer cells expressing these receptors (44).
addition, innate immune ligands can lead to the activation of immune cells in the tumor microenvironment such as TIMs (tumor-infiltrating macrophages) (45). Once activated, these immune cells may become sensitized to tumor antigens that were previously unrecognized due to the immunosuppressive environment. Innate immune ligands offer two potential modes of action when employed as cancer chemotherapeutics, direct cytotoxic agent on tumor cells and adjuvant to elicit an immune response to the resident tumor.

1.3 Resident innate immune receptors as chemotherapeutic targets

The innate immune system is equipped with pattern recognition receptors (PRRs), which recognize host-derived or microbial-derived danger signals known as Danger-associated molecular patterns or pathogen-associated molecular patterns (PAMPs), respectively (46). Activation of these receptors often leads to transcription of genes involved in the inflammatory response (47). Immune responses including inflammation were targeted in cancer treatment as early as 1893 when Dr. William Coley linked bacterial infection and cancer regression (34). He demonstrated that injecting live *Streptococcus pyogenes* and *Serratia marcescens* or bacterial components, known as Coley’s toxins, into tumors led to regression of sarcoma. These studies indicated that the immune system could be harnessed for the control of human cancer. It was later discovered by Shear and Turner that lipopolysaccharide (LPS) was the “haemorrhage producing fraction” of Coley’s toxin that accounted for its anti-tumor effect (48). As LPS stimulates Toll-like receptor 4, these results suggested that the anti-tumor effect of Coley’s toxin was a result of Toll-like receptor (TLR) activation.
Recently, TLR ligands have been investigated in the treatment of a variety of cancers (48). Administration of LPS has been used in Phase II clinical trials for the treatment of colorectal and lung cancer (49). Application of synthetic single-stranded RNA (TLR7 and TLR8) ligands are under investigation for the treatment of skin cancer and chronic lymphocytic leukemia (50), as is CpG, the TLR9 ligand, for the treatment of renal cancer, melanoma and lymphoma (51). Double-stranded RNA (dsRNA), the TLR3 ligand, has a long history of use in cancer therapy with clinical trials dating back to the 1970’s (52). In addition to TLR3, three other cytosolic PRRs recognize dsRNA, retinoic acid-inducible gene I (RIG-I), melanoma differentiaion-associated gene 5 (MDA5), and dsRNA-dependent protein kinase receptor (PKR). The four receptors activate multiple integrated signaling cascades including production of pro-inflammatory cytokines, type I interferons, and cell death pathways (Figure 1.1). Studies in a variety of cancer cell lines (53-56) have shown that dsRNA receptor(s), expressed by cancer cells, can induce apoptosis when stimulated making these receptors viable targets for chemotherapy.
Figure 1.1 DsRNA receptor signaling pathways. When stimulated with dsRNA, the four dsRNA receptors can activate IRF3/7 (purple), NF-κB and AP-1 (green), caspases 8 and 3 (orange) or inhibit translation via phosphorylation of eIF2α (yellow). Production of type I interferons (IFN-β) leads to the assembly of the ISGF3 complex which is composed of STAT1-STAT2 dimers and IRF9. ISGF3 binds to IFN-stimulated response elements (ISRE) in the promoters of IFN-stimulated genes to regulate their expression.
1.3.1 TLR3

Toll-like receptor 3 (TLR3) is one of ten Toll-like receptors found in humans, all of which recognize microbial components. While the other TLRs recognize ligands like bacterial products or DNA, TLR3 recognizes dsRNA of at least 45 base pairs (bp) in length (57). TLR3 is a transmembrane receptor composed of an ectodomain for ligand binding and a cytoplasmic TIR domain for cytosolic signaling. Localization in the endosome where cell components are sorted before lysosomal degradation provides the acidic environment necessary for dsRNA binding to TLR3 (58). This endosomal location exposes the receptor to “non-self” dsRNA present in cell debris entering the endosome via endocytosis and may also sequester TLR3 from “self” dsRNA in the cytosol (59, 60). Following ligand binding, the adaptor protein TRIF (Toll/interleukin-1 receptor TIR-domain-containing adapter-inducing IFN-β) mediates multiple signaling cascades (61) (Figure 1.1). TRIF can interact with a kinase complex that phosphorylates the transcription factors IRF-3 and 7, leading to the production of type I interferons (62). This interferon production can then lead to an increase in expression of TLR3 (63). Phosphoinositide 3-kinase (PI3-K) and protein kinase B (PKB also known as Akt) enhance IRF3/7 activation and broaden the transcriptional targets of IRF 3/7 (64). A second pathway can activate the IκB kinase (IKK) complex leading to the phosphorylation of inhibitor of kappa B (IκBα) and its subsequent ubiquitination and degradation. When NF-κB is released from IκBα, it is phosphorylated and is translocated into the nucleus where it activates proinflammatory cytokine gene transcription. Cell death can also be induced by TLR3 via the FAS-associated death domain protein (FADD)/caspase 8 pathway (65). The death domain of
FaDD associates with the death domain of caspase 8 leading to formation of a large complex and caspase 8 activation.

1.3.2 RIG-I and MDA5

RIG-I (also known as DDX58) and MDA5 (also known as Ifih1 or Helicard) are helicases that recognize dsRNA present in the cytosol. Their expression is ubiquitous and, like TLR3, is increased by type I interferon (66). Their domain consists of two caspase recruitment domains (CARD) and a C-terminal RNA helicase domain (67). MDA5 recognizes dsRNA that is at least 1 kbp in length, while RIG-I recognizes dsRNA with a 5’ triphosphate moiety between 1 kbp and ~70 bp (68, 69). This allows RIG-I to recognize “self” and “non-self” dsRNA based on the 5’ end of the RNA as viral genomes maintain their 5’-triphosphate whereas the 5’-triphosphate of host RNA is usually either removed or masked by capping (70). RIG-I and MDA5 share similar signaling cascades (Figure 1.1). When stimulated, their CARD domains associate with the CARD domain of adaptor IFN-β promoter stimulator (IPS-1, also known as Cardif, MAVs, and VISA), which is anchored to the outer membrane of the mitochondria (71). Similar to RIG-I and MDA5, IPS-1 contains a CARD domain and a non-CARD domain. The non-CARD domain can interact with TRAF3, initiating signaling cascades via the TRAF family member associated NF-κB activator (TANK)/TANK binding kinase-1 (TBK1)/ IκB kinase epsilon (IKKe) kinase complex and FADD. Analogous to TLR3, these pathways can lead to the induction of IRF-3, NF-κB and caspase 8 activation (72).

1.3.3 PKR
PKR, a serine-threonine kinase composed of a kinase domain and two dsRNA binding domains, was the first of the dsRNA receptors to be identified and characterized. Located in the cytoplasm, PKR monomers bind their ligand dsRNA leading to dimerization and autophosphorylation (73). PKR can bind to dsRNA that is at least 33 bp in length (74). The dimer can then dissociate from the dsRNA. Much of PKR’s antiviral activity is attributed to it’s phosphorylation of eukaryotic translation initiation factor alpha (eIF2α), inhibiting translation (75) (Figure 1.1). PKR can also induce cell death via the FADD/caspase 8 pathway (76).

1.4 Summary

Many viruses have developed the ability to subvert these pathways necessitating the redundancy in this system. This is why the receptors are slightly unique in their ligand recognition. Targeting these dsRNA receptors could lead to the activation of an apoptotic response in cancer cells. Ovarian cancer is a lethal gynecological malignancy, with less than 30% of women with advanced stage disease surviving long-term (77). Current chemotherapeutic regimens, although promising, must be improved to target chemoresistance and recurrent disease. Alternatives include innate immune agonists that trigger signaling cascades within the cell that can lead to cell death. This work will explore the utility of the innate immune ligand, dsRNA, as a single and dual chemotherapeutic.
CHAPTER 2

MECHANISM OF DSRNA-INDUCED CELL DEATH AND RESISTANCE

2.1 INTRODUCTION

Virally-derived double stranded RNA or its synthetic analogs, polyinosinic-polycytidylic acid (pI:pC) or polyadenylic-polyuridylic acid (pA:pU), activate at least four known innate immune receptors, Toll-like receptor 3 (TLR3) (78) retinoic acid-inducible gene I (RIG-I) (79) melanoma differentiation factor 5 (MDA5) (80) and dsRNA-dependent protein kinase receptor (PKR) (81). Once stimulated, these dsRNA receptors initiate signaling pathways that lead to the production of pro-inflammatory cytokines, type I interferons, apoptosis and translational inhibition (Figure 1.1.). Clinical trials for breast (82) and gastric (83) cancer that included dsRNA in combination with standard care showed improved overall survival and progression-free survival. In melanoma (54, 84) breast (53), prostate (56) and hepatoma (85) cancer cell lines, dsRNA has been shown to trigger a caspase-dependent apoptotic response. Each study implicated a different subset of dsRNA receptors as essential for dsRNA responsiveness and shown type I interferon (53), increased autophagy (54) and PKC-α activation (56) contributed to dsRNA-induced cell death. Predictive biomarkers for a dsRNA responsive patient population have been difficult to identify due to these varied requirements for dsRNA responsiveness. In breast cancer, a retrospective analysis of clinical trial tissue samples suggested that TLR3 expression could serve as a predictive biomarker for responsive patient populations (86).
Ovarian surface epithelial cells that were normal, benign, or malignant express Toll-like receptors suggesting innate immune ligands could trigger inflammatory responses (87). Recent studies have shown that RIG-I activation induced apoptosis in ovarian carcinoma. In monocyte-derived dendritic cells, phagocyted cells increased costimulatory and MHCII molecules suggesting that dsRNA may enhance immunotherapy in ovarian cancer (88). We initiated our studies in ovarian carcinoma to identify essential dsRNA receptors and associated signaling pathways that mediated the dsRNA-induced apoptotic response.
2.2 MATERIALS AND METHODS

2.2.1 Reagents

2-aminopurine, anti-IgG antibody, pA:pU, and pI:pC were purchased from Sigma-Aldrich. Anti-phospho-NF-κB p65 (Ser536), anti-RIG-I, anti-STAT1, anti-phospho-STAT1 (Tyr701), anti-caspase 3, anti-c-IAP2, anti-ATG5, anti-phospho-eIF2α (Ser51), anti-eIF2α, anti-PARP, anti-Bax, anti-phospho-MTOR (Ser2448), anti-MTOR, anti-beclin and anti-β-tubulin antibodies were purchased from Cell Signaling Technology, Inc. Anti-NF-κB p65, anti-PKR, anti-IFN-α/βR2, anti-Bax(6A7), anti-BiP/GRP78 and anti-actin antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-TLR3 antibody and anti-IFN-α/βR2 was purchased from R&D Systems, Inc. Anti-LC3 antibody was purchased from Novus Biologicals. Anti-MDA5 antibody was a gift from Dr. Paul Fisher’s laboratory. Anti-keratin 18 (Clone DC-10) antibody was purchased from Thermo Scientific. Anti-RIPI kinase and anti-cytochrome C antibodies were purchased from BD Biosciences. Anti-NOXA antibody was purchased from Alexis Biochemicals.

2.2.2 Cell culture

Ovarian cancer cell lines OVCAR-3, CAOV-3, and SKOV-3, breast cancer cell line CAMA-1, renal cancer cell line 786-O, and human embryonic kidney (HEK) 293 cells were purchased from ATCC. The ovarian cancer cell line DOV-13 was a gift from Dr. Xianjun Fang’s laboratory. Cells were cultured in RPMI 1640 medium supplemented with 10% low endotoxin FBS, 20 mM L-glutamine, 100 mM HEPES, 10 mM sodium pyruvate and 1X non-essential amino acids solution at 37°C in 5% CO₂. Anonymized patient
samples provided by TDAAC (Tissue Data Acquisition and Analysis Core), were isolated from ascites fluid and cultured in 50% MCDB 105 (Sigma) / 50% M199 (Invitrogen) medium pH 7.2, 10% low endotoxin FBS, 2 mM L-glutamine, 50 U penicillin 50 µg/mL streptomycin (89). Immortalized non-tumorigenic ovarian epithelial cell line IOSE 385 was a gift from the Canadian Ovarian Tissue Bank. Cells were cultured in 50% MCDB 105 (Sigma) / 50% M199 (Invitrogen) medium pH 7.2, 5% low endotoxin FBS, 2 mM L-glutamine, and 50 µg/mL gentamycin.

2.2.3 Delivery of dsRNA via PEI

Cells were plated in 24-well plates and allowed to adhere overnight. When cells reached 60-80% confluency, they were stimulated for 24, 48, or 72 hours with jetPEI (Polyplus Transfection) according to manufacturer’s protocol, delivering 0.02, 0.2, or 2 µg/mL of pI:pC.

2.2.4 IFN-β and IFN-γ treatment

Human-IFN-β (1000 units/µL) was a gift from Dr. Andrew Larner’s laboratory. IFN-β in media at 1000 units/mL was added directly to cells for specified time points. IFN-γ (R&D Systems Inc.) in media (5 ng/mL) was added to cells for 0-60 minutes.

2.2.5 Long-term exposure to dsRNA

DOV-13 cells were plated in 6-well plates and allowed to reach 60-80% confluency. They were then cultured in complete media or complete media plus 50 µg/ml pI:pC. Every 48 hours, one well per treatment was passaged while a second duplicate well was monitored for apoptosis via Hoechst and propidium iodide staining.
2.2.6 Western blotting

Cells, plated at 1x10^6 cells per 100x20 mm tissue culture dish, were harvested and lysed (100 μL of 0.02 M Tris; pH 8, 0.15 M NaCl, 1 mM DTT, 1% NP-40, 1 X protease inhibitor cocktail (Roche complete EDTA-free), 25 mM NaF, 10 X PhosSTOP (Roche)) at specified time points following stimulation. Lysates were separated by SDS-PAGE (20-50 μg/lane, Bio-Rad assay), transferred to nitrocellulose and immunoblotted with specified antibodies. For TLR3, RIG-I, and MDA5 Western blots, 8% gels were used. For LC-3 Western blots, 15% 18.5 X 20 cm gels were used. All other Western blots were performed using 10% Tris-Glycine gels. Western blots were developed with chemiluminescent reagents from Thermo Scientific (Supersignal West Dura) or (GE Healthcare ECL Plus).

2.2.7 Cell fractionation for cytochrome C analysis

Cells were plated at 1x10^6 cells per 100x20 mm tissue culture dish and (un)treated with 50 μg/mL pI:pC for 4, 10, and 24 hours. Cells were then washed and resuspended in an appropriate amount of fractionation buffer (75 mM NaCl, 8 mM Na2HPO4, 1 mM NaH2PO4, 1 mM EDTA, protease inhibitor). Digitonin was added (0.05%) and the lysate was mixed vigorously by pipeting. The lysate was centrifuged at 12,000 rpm for 5 min. The supernatant represented the cytosolic fraction. The pellet was resuspended in an appropriate amount of RIPA (radio-immunoprecipitation assay buffer) buffer (50 mM Tris, pH 8.0, 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and sonicated. This fraction was centrifuged at 14,000 rpm for 10 minutes and the supernatant represented the mitochondria rich fraction (90).

2.2.8 Quantitation of cell death
Cells were (un)stimulated with 5 or 50 µg/mL pI:pC and monitored for apoptotic / other cell death at 24, 48 and 72 hour time points by Hoechst / propidium iodide (PI) staining of nuclear DNA. DNA was visualized under a Nikon TE300 Eclipse microscope equipped with a Hg-lamp and blue excitation fluorescence filter (excitation 330-380 nM / emission 420 long pass). Percentage of apoptotic cells was determined by counting normal (diffuse nuclear DNA) versus apoptotic or other cell death in three fields per well of >100 cells per field. Each experiment contained three wells per condition and the experiment was repeated three times.

2.2.9 Reverse Transcriptase PCR

Cells were plated at 1.5x10^5 cells/mL in 6-well plates and (un)stimulated with 50 µg/mL pI:pC 24 hours prior to harvest. Total RNA was extracted from cell lysates using the Qiagen RNeasy Mini Kit as per the manufacturer’s protocol. RNA was separated on a 1.2% formaldehyde agarose gel to assess purity. Using Qiagen’s OneStep RT-PCR kit, RT-PCR reactions were completed for each sample using primers specific to each dsRNA receptor and GAPDH spanning ≥2 exons and give a product of ~400 bp (Table 1). Reactions were separated on a 2% agarose gel.
Table 1. DsRNA receptor and GAPDH primers for RT-PCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ to 3’</th>
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<tr>
<td>TLR3 5’</td>
<td>CCTTCTGCACGAATTTGACTG</td>
</tr>
<tr>
<td>TLR3 3’</td>
<td>CTCAGAGACAGATTCGGAATG</td>
</tr>
<tr>
<td>MDA5 5’</td>
<td>CCAGAGTGCTGTATACATTG</td>
</tr>
<tr>
<td>MDA5 3’</td>
<td>CTGAGGAAGGGGAATCACTGG</td>
</tr>
<tr>
<td>RIG-I 5’</td>
<td>GCGAATCAGATCCCAGTGATG</td>
</tr>
<tr>
<td>RIG-I 3’</td>
<td>GACGCATCAAGAGAAGCAGACAC</td>
</tr>
<tr>
<td>PKR 5’</td>
<td>CACTACAATGGCTGTGGGAT</td>
</tr>
<tr>
<td>PKR 3’</td>
<td>GTTCTGCTGCTGTATCGCA</td>
</tr>
<tr>
<td>GAPDH 5’</td>
<td>AGGTGAAGGCTCGGAGTCAAC</td>
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<td>GAPDH 3’</td>
<td>GCTCTGGAAGATGCTGATG</td>
</tr>
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</table>

Note: Primers span ≥2 exons and are specific to dsRNA receptors TLR3, MDA5, RIG-I, and PKR. GAPDH primers were used as a positive control. Component amplified for each receptor was ~400 bp.
2.2.10 Determination of RNA integrity

Clean gel apparatus: wash with a mild detergent, rinse well with dH₂O,
dilute H₂O₂ to 3% (For 400 mL, 24 mL 50% H₂O₂ bring up to 400 mL with dH₂O),
put 3% H₂O₂ into apparatus with comb and let sit for 10 min, rinse very well with dH₂O,
rinse with small amount of DEPC H₂O. Make and pour gel: *Do this in the chemical hood
because formaldehyde is toxic. Add 1.2 g agarose in 81 mL dH₂O and dissolve in
microwave, cool agarose to 55°C in water bath, take apparatus, agarose, 37%
formaldehyde, and 10X MOPS to hood, add 10 mL 10X MOPS (21 g MOPS, 3.4 g sodium
acetate (MW 136.08), 10mL 0.5M EDTA (11.4 mL 0.44 M EDTA), 400 mL H₂O, pH to
7.0 w/ NaOH, bring up to 500mL with dH₂O. Sterile filter, wrap in foil, and keep in the
dark.) and 5.8 mL formaldehyde to agarose and swirl to mix, pour gel and let harden for ~1
hour, take gel out of hood and to the lab bench. Prepare RNA samples by putting 5-10µg
RNA in a tube (total volume of 23 µL). Add RNA to RNAse free tube and bring up to 40
µL with DEPC H₂O. Add: 10 µL formamide (deionized by passage through mixed bed
resin), 4 µL 37% formaldehyde, 2 µL 10X MOPS buffer, 2 µL 0.4 mg/mL EtBr (Dilute lab
stock which is 10 mg/mL with DEPC H₂O), 1 µL dye (for RNA)). Heat sample at 65°C for
10 minutes in water bath. Spin briefly to collect condensate. Place sample on ice (load gel
within ~10 min of this step). To load and run gel, make up 1X MOPS from 10X stock (For
small apparatus need 350 mL), remove comb from gel carefully, pour 1X MOPS buffer
into the apparatus, if using RiboLadder Short, add 4 µL of marker to 4 µL of dye (for
RNA), add marker for RNA, load the 8 µL of marker in the first lane, load the 23 µL sample(s) in the other lane(s), run at 25 V for 3 hours.

2.2.11 Quantitative RT-PCR

Cells were stimulated with 50 µg/mL pI:pC for 24 hours and total RNA was extracted using the Qiagen RNeasy Mini Kit as per the manufacturer’s protocol. Using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), 1 µg of total RNA was reverse-transcribed as per manufacturer’s protocol. For real-time PCR, premixed primer-probes sets (FAM-labeled) and TaqMan Gene Expression Master Mix were purchased from Applied Biosystems and cDNAs were amplified using ABI-7900HT cycler. ABI Prism software was used to construct a calibration curve, plotting the threshold cycle ($C_T$) versus the logarithm of the calibrator concentration. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were purchased from Applied Biosystems (TLR3 - Hs00152933_m1, MDA5 (IFIH1) - Hs00223420_m1, PKR (EIF2AK2) - Hs00169345_m1, RIG-I (DDX58) - Hs01061434_m1, IFNB1 - Hs02621180_s1, ATG5 (APG5L) - Hs00169468_m1, OAS1 - Hs00242943_m1, STAT1 - Hs01013998_m1, IFIT1 - Hs00356631_g1, CXCL10 - Hs00171042_m1, IFI44 - Hs00197427_m1, GAPDH - Hs99999805_m1).

2.2.12 Exposure of non-responsive cell lines to supernatant from stimulated responsive cell lines

Responsive cell line CAMA-1 was plated in 6-well plates at 5 x 10^4 cells/mL and allowed to adhere overnight. They were then stimulated with 50 µg/mL pI:pC. Target cell lines
(786-O, SKOV-3, DOV-13, and HEK293) were plated in 24-well plates and allowed to adhere overnight. After a total of 48 hours of stimulation, the media was pulled from the CAMA-1 cells and centrifuged to remove cell debris. Media was then pulled from the target cells and replaced with fresh media or the CAMA-1 supernatant. The target cells were incubated with this supernatant for 24, 48, and 72 hours.

2.2.13 Microarray analysis

Cells were plated at 1x10^6 cells per 100x20 mm tissue culture dish and (un)treated with 50 µg/mL pI:pC for 24 hours. RNA was extracted using the TRIzol method. The ovarian cell line RNA samples were analyzed on HG-U133A 2.0 arrays following the standardized Affymetrix protocol, as described elsewhere (91). Briefly, starting with 10 µg of total RNA from every sample, we generated double-stranded cDNA using a 24-mer oligodeoxythymidylic acid primer with a T7 RNA polymerase promoter site added to the 3’ end (Superscript cDNA Synthesis System; Life Technologies Inc, Rockville, MD). After second-strand synthesis, in vitro transcription was performed using the Enzo BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) to produce biotin-labeled cRNA. cDNA and cRNA synthesis products were prepared and rigorously evaluated for quality to ensure generation of good microarray data using a sample processing method previously established by the Dumur laboratory (92). Fifteen micrograms of labeled cRNA were fragmented and 10 µg of the fragmented product were hybridized for 16 hours into HG-133A 2.0 microarrays, containing 22,277 probe sets. The arrays were washed and stained with streptavidin phycoerythrin (SAPE; Molecular Probes, Eugene, OR) in the Affymetrix fluidics workstation. Every chip was scanned at a high
resolution, with pixelations ranging from 2.5 µm down to 0.51 µm, by the Affymetrix GeneChip® Scanner 3000 according to the GeneChip® Expression Analysis Technical Manual procedures (Affymetrix, Santa Clara, CA). After scanning, the raw intensities for every probe were stored in electronic files (in .DAT and .CEL formats) by the GeneChip® Operating Software (GCOS v1.4) (Affymetrix, Santa Clara, CA). The overall quality of each array was assessed by monitoring the 3′/5′ ratios for a housekeeping gene (GAPDH) and the percentage of “Present” genes (%P); where arrays exhibiting GAPDH 3′/5′ < 3.0 and %P > 40% were considered good quality arrays. Statistical analysis (background correction, normalization, and estimation of probe set expression summaries), were performed using the log-scale robust multiaarray analysis (RMA) method (93). Identification of altered gene expression on pI:pC treated cells compared to untreated cells was assessed by using the Significance score (S-score) method (94). The S-score method uses an error-based model to determine the variances for probe pair signals and follows a normal standard distribution. The procedure produces scores centered around ‘0’ (no change) with a standard deviation of 1. Thus, scores >2 or <–2 from a single comparison have, on average, a 95% chance of being significant hybridization changes, at a univariate level, corresponding to a p-value of < 0.05. As this approach does not take into account the number of multiple comparisons inherent to any microarray experiment, we used the Benjamini-Hochberg (95) correction method to correct for multiple testing and obtained adjusted alpha-levels for each probe set.

2.2.14 ELISA

Cell lines (OVCAR-3, CAOV-3, SKOV-3, DOV-13, and IOSE 385) were treated with 50
µg/mL pI:pC for 24 hours. Supernatants were harvested and pre-cleared of cell debris via centrifugation. ELISA for IFN-β was performed via manufacturer’s protocol (R&D Systems, Inc.).

For the chemokine/cytokine experiments performed by Jessica Schreiter, the Invitrogen human 25-plex Luminex kit was used as per manufacturer’s protocol. Precleared supernatants from cells (un)treated with 50 µg/mL pI:pC for 24 hours were used.

2.2.15 IFNα/βR2 inhibition
CAOV-3 and OVCAR-3 cells were plated in 24-well plates and when they reached 60-80% confluency, were treated with IgG or IFN-α/βR2 neutralizing antibody (2.5 µg/well), followed 6 hours later by stimulation with 50 µg/mL pI:pC for 24 hours.

2.2.16 Caspase inhibitor / activation assays
Cells were (un)treated with 25 µM pan-caspase inhibitor Z-VAD-FMK, caspase 9 inhibitor Z-LEHD-FMK, caspase 8 inhibitor Z-IETD-FMK, or caspase 4 inhibitor Z-YVAD-FMK, (R&D Systems, Inc.), followed 6 hours later by 50 µg/mL pI:pC for 24 hours. Caspase 3/7 activity assay was performed according to manufacturer’s protocol (Promega).

2.2.17 SMAC mimetic
DOV-13 and SKOV-3 cells were plated in 24-well plates and when 60-80% confluent they were treated with 100 nM SMAC mimetic (provided by Dr. Sarah Spiegel’s laboratory), for 4 hours. Cells were then treated with 50 µg/mL pI:pC for 48 hours.

2.2.18 RIP1 kinase immunoprecipitation
Cell lysates were prepared in 20 mM HEPES pH 7.4 containing 150 mM NaCl, 10 mM β-
glycerophosphate, 1.5 mM MgCl₂, 10 mM NaF, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EGTA, 1 mM PMSF, 0.5% Triton X-100, 1:500 protease inhibitor cocktail (Sigma-Aldrich), and 1 mg/mL of N-ethylmaleimide. RIP1 kinase was complexed from 750 µg of cell lysate with 1 µg anti-RIP antibody overnight at 4 °C. Complexes were captured with protein A/G-plus agarose beads (Santa Cruz Biotechnology Inc.). After extensive washing, bound proteins were released by boiling in SDS-PAGE sample buffer and polyubiquitination of RIP1 kinase determined by Western blotting (96).

2.2.19 siRNA knockdown

siRNA for ATG5 (ON-TARGET plus® SMARTpool, Thermo Scientific Dharmacon), nontarget control siRNA (ON-TARGET plus® Control Pool, Thermo Scientific Dharmacon) (2.25 µg), or GFP plasmid (2 µg) was transfected into DOV-13 cells (1x10⁶ cells) with Amaxa Cell Line Nucleofector Kit V (Lonza) using program V-005 on Nucleofector™ II (Amaxa Biosystems). The transfected cells were cultured in 12-well plates and allowed to rest for 24 hours before receiving fresh media followed by another 24 hour rest period. The cells were then (un)treated with 50 µg/mL pI:pC for 24 or 48 hours.

2.2.20 Inhibition of autophagy

To inhibit autophagy, DOV-13 cells were plated in 6-well plates and at 60-80% confluency were treated with 10 mM 3-methyladenine (3-MA) for 4 hours followed by treatment with 50 µg/mL pI:pC for 24 and 48 hours. 3-MA was prepared by dissolving 30 mg of 3-MA in 1 X PBS in a 1.5 mL tube. The solution was heated to dissolve at 95 °C and then sterile filtered in hood. The filtered solution was immediately applied to cells.
2.2.21 Quantification of autophagy via GFP-LC3 expression

Cells were plated at 1x10^5 cells/mL in 6-well plates including coverslips coated with poly-L-lysine (Sigma). pCDNA3.1 LC3-eGFP was transfected into cells using Amaxa Cell Line Nucleofector Kit V (Lonza) using program V-005 on Nucleofector™ II (Amaxa Biosystems). The transfected cells were cultured in 12-well plates and allowed to rest for 24 hours before receiving fresh media followed by another 24 hour rest period. The cells were then (un)treated with 50 µg/mL pI:pC for 24 hours. Cells were then washed with PBS and fixed with 4% paraformaldehyde. Coverslips were then mounted on glass slides and examined with a Zeiss LSM 510 Meta laser confocal microscope (1 mm section). All images were exported directly using Zeiss LSM Image Examiner (v. 3.2.0.70) software to 8-bit tagged image file format (TIFF) files without compression, contrast, or gamma adjustments. Cells that contained more than 5 punctate GFP structures were considered LC3-GFP positive for induced autophagosome formation. Experiments were done in duplicate and for each coverslip, ≥100 cells were assessed for autophagosome formation.

2.2.22 DsRNA receptor over expression

SKOV3 or DOV13 cells (2x10^4 cells/well) were plated in 24-well plates. After 24 hours, the cells received fresh media and were then transfected with 500 ng of receptor DNA (MDA5, RIG-I, or TLR3), 500 ng of empty vector DNA (pCDNA3.1), or a combination of receptor and empty vector at a combined total of 500 ng DNA. The pCDNA3-hMDA5 and pCDNA3-hRIG-I plasmids were a gift from the Fisher laboratory and the pUNO-hTLR3 plasmid was purchased from Invivogen. To determine transfection efficiency, GFP plasmid was transfected into cells at 5, 50, or 500 ng. All transfections were performed
using TransIT LT1 (Mirus Bio Corp.) as per manufacturer’s protocol. Following 24 hours of transfection, the cells received media alone or media with 50 µg/mL pI:pC.

2.2.23 Lentiviral infections

CAOV-3 and OVCAR-3 cells (5x10^4 or 6 x10^4 cells/well, respectively) were plated into a 24-well plate and allowed to adhere for 24 hours. Cells were at 50% confluency. Multiplicity of infections, 1:1 and 1:2, were calculated from a single-well cell count. Lentiviral particle gene silencers (Santa Cruz Biotechnology, Inc.) targeting MDA-5 or PKR or RIG-I or TLR3 were added to cells as per manufacturer’s protocol. 24 hours after infection, cells received fresh media and were given a 24 hour rest period. The cells were then split 1:3 and stable cell lines selected with 4 µg/mL puromycin containing media.

2.2.24 Statistical analysis

One-Way ANOVA or Student’s t test was used for statistical analyses using JMP 8.0 software (SAS Institute Inc.). A p-value ≤ 0.05 was considered significant.
2.3 RESULTS

2.3.1 DsRNA stimulation can induce ovarian cancer cell death.

To determine the effect of dsRNA on ovarian cancer cell lines, four cell lines, CAOV-3, OVCAR-3, DOV-13, and SKOV-3 were tested along with an immortalized ovarian surface epithelium cell line IOSE 385. HEK293 cells, which are not responsive to dsRNA, were used as a negative control. The effect of dsRNA was tested via Hoechst and propidium iodide staining to measure the level of apoptosis after treatment with the synthetic dsRNA pI:pC (50 µg/mL) for 24, 48, and 72 hours. DOV-13 and SKOV-3 did not respond to the treatment, however, CAOV-3 and OVCAR-3 underwent significant levels of cell death (Figure 2.1). The IOSE 385 cell line showed a moderate response to the treatment, however, much less than the responsive cancer cell lines. This data led us to refer to DOV-13 and SKOV-3 as “non-responsive,” and CAOV-3 and OVCAR-3 as “responsive.” In these experiments, pI:pC was delivered directly in the media. In this case, pI:pC is bound to the Scavenger Receptor Class-A (97) on the cell surface and then is taken in via endocytosis accessing TLR3 which is localized to endosomes. To target cytosolic receptors more directly, pI:pC can be delivered via polyethyleneimine (PEI), allowing for more efficient escape from the endosome to the cytosol. The cell lines showed similar responsiveness when dsRNA was delivered with PEI (Figure 2.2). DOV-13 and SKOV-3 remained non-responsive while OVCAR-3 and CAOV-3 underwent apoptosis at an even higher level at a much lower pI:pC concentration of 0.2 µg/mL.

To verify the effect of pI:pC on patient samples, we acquired ascites-derived
patient samples from Virginia Commonwealth University’s Tissue Data Acquisition and Analysis Core (TDAAC). We received two malignant samples and one benign sample, which were kept in culture until they showed the cobblestone-like morphology characteristic of epithelial cells (Figure 2.3 A). The presence of keratin in the patient samples confirmed the epithelial origin of the cell lines and patient samples (Figure 2.3 B). The benign patient sample did not respond to the treatment, however, the two malignant samples underwent significant levels of cell death (Figure 2.3 C). This data shows that pI:pC is effective not only in ovarian cancer cell lines, but patient samples as well. To confirm the similarity with in the sub-groups of responsive and non-responsive cell lines, a microarray analysis was performed with the help of Dr. Dumur’s laboratory. The analysis shows that before stimulation with dsRNA, responsive and non-responsive cell lines cluster separately confirming two distinct sub-groups (Figure 2.4).
Hoechst staining of ovarian cancer cell lines by Dr. Sandrine Lépine.

Figure 2.1. DsRNA induces ovarian cancer cell death.
Ovarian cancer cell lines CAOV-3, OVCAR-3, DOV-13, and SKOV-3 were stimulated with 50 µg/mL pI:pC for 48 hours. IOSE 385 and HEK293 (ctrl) cell lines were also tested. Apoptosis was assessed via Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. Student’s t test was used to determine statistical significance, * indicates significant difference (p≤0.05).
**OVCAR-3**

**CAOV-3**

**DOV-13**

**SKOV-3**
Figure 2.2. PEI delivery of dsRNA induces ovarian cancer cell death. OVCAR-3, CAOV-3, DOV-13 and SKOV-3 cell lines were treated with pI:pC alone at 2 µg/mL or with PEI delivery at 2, 0.2, and 0.02 µg/mL. Cell death was assessed after 48 hours with Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. Student’s t test was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 2.3. DsRNA induces cell death in ascites-derived patient samples. A. Ascites-derived malignant sample 1 (left panel), 2 (middle panel), and a benign sample (right panel) at passage 0. B. Whole cell lysates were collected from cell lines and patient samples. Expression of keratin 18 to determine epithelial origin of cells was determined by Western blotting. β–tubulin was used as a loading control. C. Ascites-derived samples Malignant 1, Malignant 2, and a benign sample as a control, were stimulated with 50 µg/ml pI:pC for 48 hours. Hoechst / PI staining was used to assess apoptosis. The mean ± SD of 3 independent experiments is shown. Student’s t test was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 2.4. Gene cluster analysis of unstimulated ovarian cancer cell lines. Two-dimensional hierarchical clustering of samples and genes using Pearson (centered) correlation and average linkage, based on 75 probe sets that were significantly (p < .001; FDR < 5%) different between non-responsive and responsive cell lines. Each of the 75 rows in the heat map located beneath the dendrogram shows the relative expression for that specific gene in four separate cell lines (columns). The relative gene expression levels (0-fold increase to 23-fold increase) are plotted according to the color scale at the bottom of the heat map, where red and green areas correspond to genes over expressed and under expressed, respectively, compared to the median intensity across the 4 samples.
2.3.2 DsRNA receptor levels determine responsiveness.

To verify the presence of the dsRNA receptors in our cell lines, we completed reverse transcriptase PCR. All receptor mRNAs are present in the cell lines before and after pI:pC treatment, with the exception of PKR mRNA in SKOV-3 cells, whose mRNA expression is induced by dsRNA treatment (Figure 2.5). We then quantified the level of dsRNA receptor mRNA via quantitative PCR using FAM-labeled primers. Fold induction, \(2^{\Delta\Delta Ct}\), represents dsRNA receptor expression in specified cell line, normalized to GAPDH, relative to the normalized dsRNA receptor expression in a control cell line. Initially, the renal carcinoma cell line 786-O was used to normalize the data (Figure 2.6 and Table 2), however, we then switched to a non-cancerous cell line HEK293 (Figure 2.7 A and Table 3). The responsive cell lines greatly increase their receptor mRNA levels following stimulation with pI:pC. Although all dsRNA receptor mRNAs increase following stimulation, cytosolic receptors show the highest increase (~100 fold) after treatment. As in the responsive cell lines, both malignant patient samples increase their dsRNA receptor mRNA level after dsRNA treatment (Figure 2.7 B and Table 3). Western blotting confirmed that the upregulation of receptor mRNA in responsive cell lines translated to an increased receptor expression (Figure 2.8). This data shows that responsive cell lines and patient samples increase the level of their dsRNA receptors after pI:pC stimulation.

To examine changes in mRNA levels following dsRNA stimulation that would further delineate non-responsive and responsive cell lines, we examined mRNA expression via microarray data (-/+ pI:pC for 24 hours) in collaboration with the Dumur group. The
data showed that the non-responsive cell lines again clustered separately from the responsive cell lines (Figure 2.9). Responsive cell lines showed alterations in many interferon-inducible genes while non-responsive cell lines exhibited very few gene alterations. Consequently, we analyzed the known signaling cascades to establish how pathway activation determines responsiveness to dsRNA.
Figure 2.5. Ovarian cancer cell lines express dsRNA receptor mRNAs. Ovarian cancer cell lines were (un)treated with 50 µg/mL pI:pC for 24 hours. Cells were harvested and total RNA was extracted. Gene segments for each target receptor were amplified from the RNA using standard RT-PCR techniques. Primers (Table 1) for each gene amplified an ~400 bp segment spanning at least 2 exons.
Figure 2.6. Determination of dsRNA receptor expression levels using 786-O as the control cell line. Cells were treated with 50 µg/mL pl:pC for 24 hours and total RNA was extracted. cDNA was prepared, and receptor levels were quantitated from TaqMan qPCR reactions using FAM-labeled primers for each dsRNA receptor and the housekeeping gene, GAPDH. Receptor mRNA expression is reported as a fold induction normalized to GAPDH, relative to the normalized receptor expression in control cell line 786-O.
Table 2. DsRNA receptor levels following dsRNA stimulation using 786-O as the control cell line.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>pl:pC</th>
<th>MDA5</th>
<th>PKR</th>
<th>RIGI</th>
<th>TLR3</th>
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<tbody>
<tr>
<td>CAOV3</td>
<td>-</td>
<td>1.3</td>
<td>10.5</td>
<td>2.9</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>55.8</td>
<td>59.3</td>
<td>76.2</td>
<td>9.7</td>
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<tr>
<td>OVCAR3</td>
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<td>7.3</td>
<td>4.4</td>
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</tr>
<tr>
<td></td>
<td>+</td>
<td>15.9</td>
<td>28.9</td>
<td>81.1</td>
<td>0.6</td>
</tr>
<tr>
<td>DOV13</td>
<td>-</td>
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<td>8.2</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.7</td>
<td>11.1</td>
<td>3.5</td>
<td>0.3</td>
</tr>
<tr>
<td>SKOV3</td>
<td>-</td>
<td>0.3</td>
<td>11.4</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.0</td>
<td>24.8</td>
<td>5.4</td>
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<tr>
<td>786-O</td>
<td>-</td>
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<tr>
<td></td>
<td>+</td>
<td>1.0</td>
<td>1.0</td>
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</table>

*Note: *Values indicate relative dsRNA receptor mRNA induction (fold).
Figure 2.7. Levels of dsRNA receptor mRNAs increase following dsRNA stimulation in responsive cell lines and patient samples. A. Ovarian cancer cell lines and B. ascites-derived malignant cells were treated with 50 µg/mL pI:pC for 24 hours and RNA was extracted. cDNA was prepared, and receptor levels were quantitated from TaqMan qPCR reactions using FAM-labeled primers for each dsRNA receptor and the housekeeping gene, GAPDH. Receptor mRNA expression is reported as a fold induction normalized to GAPDH, relative to the normalized receptor expression in control cell line HEK293. The mean ± SD of 3 independent experiments is shown.
Table 3. DsRNA receptor levels in ovarian cancer cell lines and patient samples following dsRNA stimulation using HEK293 as a control cell line.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>pI:pC</th>
<th>MDA5</th>
<th>PKR</th>
<th>RIG-I</th>
<th>TLR3</th>
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<tr>
<td>CAOV-3</td>
<td>-</td>
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<td></td>
<td>+</td>
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<td>4.8</td>
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<td>DOV-13</td>
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<td>0.7</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.0</td>
<td>1.5</td>
<td>5.3</td>
<td>0.9</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>-</td>
<td>1.3</td>
<td>1.6</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.2</td>
<td>2.3</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Malignant 1</td>
<td>-</td>
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<td></td>
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<td>19.2</td>
<td>2.1</td>
<td>50.7</td>
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<td>Malignant 2</td>
<td>-</td>
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<td>1.1</td>
<td>5.5</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>199.1</td>
<td>12.8</td>
<td>388.1</td>
<td>120.4</td>
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</tbody>
</table>

*Note: *Values indicate relative dsRNA receptor mRNA induction (fold).
Figure 2.8. DsRNA receptor protein expression increases following stimulation in responsive cell lines. Cells were (un)treated with 50 µg/mL pI:pC for 24 hours and whole cell lysates were collected. Receptor expression levels were determined by Western blotting, * indicates a non-specific band. Actin was used as a loading control.
Experiment by the Dumur laboratory.

Figure 2.9. Gene cluster analysis of ovarian cancer cell lines. Two dimensional hierarchical clustering of samples and genes using Pearson (centered) correlation and average linkage, based on 75 probe sets that were significantly (p <.001; FDR < 5%) different between non-responsive and responsive cell lines. Each of the 75 rows in the heat map located beneath the dendrogram shows the relative expression for that specific gene in the cell lines -/+ pl:pC for 24 hours (columns). The relative gene expression levels (0-fold increase to 23-fold increase) are plotted according to the color scale at the bottom of the heat map, where red and green areas correspond to genes over expressed and under expressed, respectively, compared to the median intensity across the 4 samples.
2.3.3 NF-κB activation does not determine responsiveness.

The dsRNA receptor signaling pathway can lead to removal of IκBα from NF-κB and the phosphorylation of the p65 subunit of NF-κB and the subsequent translocation of NF-κB to the nucleus (98). To test if this pathway was activated in our cancer cell line panel, we probed for phosphorylated (S536) p65 subunit of NF-κB by Western blot (Figure 2.10). This phosphorylation site was chosen because it is phosphorylated following activation of IKKβ (99), which is included in a kinase complex activated by all four dsRNA receptors (Figure 1.1). All four cell lines increased the phosphorylation of p65, following dsRNA treatment. Analysis of supernatants collected 24 hours post-stimulation for chemokine/cytokine expression showed all cell lines increased IL-6, IL-8, and MCP-1 (Figure 2.11 and Table 4). These data show that NF-κB activation occurs in all cell lines after dsRNA stimulation. CXCL-10, which requires not only activated NF-κB but also requires interferon-inducible STAT1, only increased in responsive cell lines. This data as well as the microarray data indicate that only responsive cell lines induce interferon stimulated genes, suggesting a dichotomy in the interferon response of responders and non-responders.
Figure 2.10. DsRNA stimulation leads to NF-κB activation in ovarian cancer cell lines. Cells were (un)treated with 50 μg/mL pI:pC for 10, 20, 30, 60, or 120 minutes and whole cell lysates were collected. Expression of total NF-κB p65 and NF-κB activation (S536 phosphorylation of p65 subunit) were determined by Western blotting. Actin was used as a loading control. Densitometry for cell lines OVCAR-3 and DOV-13 are shown in the bottom panel represented as fold change of phosphorylated NF-κB compared to total NF-κB relative to actin.
Figure 2.11. Chemokine/cytokine levels increase following dsRNA stimulation in ovarian cancer cell lines. Ovarian cancer cell lines were (un)treated with 50 µg/mL pI:pC for 24 hours. Supernatants were collected and cleared of cell debris. Relative cytokine release was measured in pg/mL using the Invitrogen human 25-plex Luminex kit. The mean ± SD of 2 independent experiments shown.
Table 4. Chemokine/cytokine concentrations following dsRNA stimulation in ovarian cancer cell lines.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>untreated</td>
</tr>
<tr>
<td>CAOV-3</td>
<td>2449</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>4.2</td>
</tr>
<tr>
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*Note: Values indicate concentrations in pg/mL. * Values did not fall within the linear range of the standard curve for the given cytokine / chemokine. Values were extrapolated or set to minimum/maximum detectable for assay. Graph shown in Figure 2.10. Data collected by Jessica Schreiter.*
2.3.4 Activation of the type I IFN response contributes to cell death.

Microarray data compiled with the help of Dr. Catharine Dumur’s laboratory showed that many of the genes exhibiting a fold change in responsive cell lines are known to be upregulated during an interferon response (Table 5 and Figure 2.12). DsRNA receptor signaling pathways can activate a type I interferon response, which can lead to cell death (98). To determine if this pathway is activated following pI:pC treatment, we measured levels of interferon inducible genes (chosen based on the microarray data), CXCL10, IFI44, IFIT1, OAS1, and STAT1 via qPCR in the ovarian cancer cell lines. Responsive cell lines increased these genes to much higher levels than non-responsive cell lines in response to treatment (Figure 2.13 and Table 6).

We then examined if supernatant from responsive cell lines, containing secreted chemokines / cytokines, could induce cell death in non-responsive cell lines. The dsRNA responsive cell line chosen was the breast cancer cell line CAMA-1, which has been shown to undergo apoptosis partially dependent on type I interferons (53). CAMA-1 cells were stimulated with pI:pC for 24 hours causing ~50% apoptosis (Figure 2.14). The media, cleared of cell debris, was then added to multiple non-responsive cell lines. The non-responsive cell lines did not undergo apoptosis suggesting that the responsive cells do not release sufficient quantities, or the non-responsive cell lines do not possess the ability to respond to those components.

As part of the interferon response, IFN-β is produced and secreted where it can act in an autocrine or paracrine manner on its target receptor, IFNAR. When IFN-β levels were measured, responsive cell lines increased their IFN-β mRNA (Figure 2.15 A) and
protein (Figure 2.15 B). However, the two malignant patient samples did not increase their IFN-β mRNA after dsRNA treatment.

To determine if the non-responsive phenotype is caused by lack of IFN-β production, we stimulated the cells with exogenous IFN-β at 1000 units/mL for 48 hours and measured cell death. Only responsive cell lines underwent cell death following treatment with IFN-β (Figure 2.16). This shows that the non-responsive cell lines do not produce IFN-β or respond to exogenous IFN-β. To determine if the interferon response was essential for the dsRNA-induced apoptotic response, we blocked the IFNα/βR with a neutralizing antibody to the β chain for 6 hours followed by 24 hours of dsRNA treatment. Both responsive cell lines show significantly lower cell death when treated with the neutralizing antibody prior to addition of pI:pC (Figure 2.17). However, cell death did not return to basal levels showing that this pathway contributes to dsRNA-induced apoptosis.

As expected pI:pC induced Y701 STAT1 phosphorylation in responsive cell lines, as these cell lines produce IFN-β which can activate the IFNAR. In non-responsive cell lines, to determine whether components downstream of the IFNAR are activated following pI:pC (Figure 2.18 A) and IFN-β (Figure 2.18 B) treatment, cells were probed via Western blot for phosphorylation of STAT1 at Y701. Neither pI:pC nor exogenous IFN-β led to the phosphorylation of STAT1 in non-responsive cell lines. This led us to question whether the IFNα/βR is functional, or if downstream pathway components were non-functional. To test the downstream components we stimulated non-responsive cell lines with IFN-γ. IFN-γ uses a different receptor than IFN-β, but both ligands activate similar pathway components
including phosphorylation of STAT1. When the cells were stimulated with IFN-γ, STAT1 was phosphorylated within 5 minutes, showing these cells have functioning signaling pathways (Figure 2.19). To examine the IFNAR, we used immunoblot to probe for expression (Figure 2.20). According to the manufacturer’s data sheet, the soluble receptor is found at 55 kDa and a dimer can be found at around 95-100 kDa. Bands for both these species are found on the immunoblot indicating IFNAR is present in all cell lines. However, these data are concerning for multiple reasons: 1) Why would a “dimer” be present after reducing SDS-PAGE? 2) This receptor is known to be glycosylated (100) and therefore our bands should be wide or even laddered due to this post-translational modification. At this point, we are unable to interpret if IFNAR expression is altered between responsive or non-responsive cell lines.
Table 5. Genes altered in responsive cell lines following dsRNA stimulation.

<table>
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Note: *Numbers represent fold change of stimulated (pI:pC 24 hours) cell lines compared to unstimulated. Data collected by the Dumur laboratory.
Experiment by the Dumur laboratory.

Figure 2.12. Venn diagram shows common and unique genes altered in responsive cell lines following dsRNA stimulation. Numbers in the individual circles represent altered genes specific to that cell line as determined by microarray analysis. Numbers between circles represent genes common between two cell lines. The number in the three overlapping circles represents altered mRNA expression common to all 3 cell lines. These genes are listed in Table 5.
Figure 2.13. Responsive cell lines increase IFN-inducible genes following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 µg/mL pI:pC for 24 hours. Cells were harvested and total RNA extracted. cDNA was prepared from RNA extracted from cells. Levels of CXCL10, IFI44, IFIT1, OAS1, and STAT1 were quantitated from TaqMan qPCR reactions using FAM-labeled primers for each receptor and GAPDH. Expression is reported as a fold induction normalized to GAPDH, relative to the normalized expression in control cell line IOSE-385. The mean ± SD of 2 independent experiments is shown.
Table 6. IFN-inducible gene levels following dsRNA stimulation in ovarian cancer cell lines.

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Note: *Values indicate fold induction of IFN-inducible genes on a linear scale.
Figure 2.14. Exposure of non-responsive cell lines to supernatant from a responsive cell line does not increase cell death. Responsive CAMA-1 cells were treated with 50 µg/mL pI:pC for 48 hours. Supernatant, cleared of debris, was added to non-responsive cell lines (786-O, DOV-13, HEK293, and SKOV-3) for 24, 48, and 72 hours. Cell death was assessed by Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown.
Figure 2.15. Responsive cell lines increase levels of IFN-β mRNA following dsRNA stimulation. A. Cell lines and patient samples Malignant 1 (M1) and Malignant 2 (M2) were treated with 50 µg/ml pl:pC for 24 hours and RNA was extracted. cDNA was prepared and levels of IFN-β were quantitated from TaqMan qPCR reactions using FAM-labeled primers for IFN-β and GAPDH. Expression is reported as a fold induction normalized to GAPDH, relative to the normalized expression in control cell line IOSE. The mean ± SD of 2 independent experiments is shown. B. Cells were (un)treated with 50 µg/mL pl:pC for 24 hours and supernatants were harvested and cleared of cell debris. Level of IFN-β in cleared supernatants was determined in duplicate via an IFN-β specific ELISA as per manufacturers protocol. IFN-β concentration was calculated from a standard curve. Cell lines with readings less than ~10 pg/mL (dashed line) effectively do not produce measurable IFN-β. IOSE-385 cell line was used as a control. The mean ± SD of 3 independent experiments is shown. Student’s t test was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 2.16. Exogenous IFN-β does not induce cell death in non-responsive cell lines. Cells were (un)treated with 1000 units/mL IFN-β for 48 hours. Apoptotic cells were assessed via Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. Student’s t test was used to determine statistical significance, * indicates (p≤0.05).
Figure 2.17. IFN-β pathway inhibition reduces dsRNA-induced cell death. CAOV-3 and OVCAR-3 cell lines were treated with anti-IgG or anti-IFNAR2 neutralizing antibody followed 6 hours later by stimulation with 50 µg/mL pI:pC. Hoechst / PI staining was used 24 hours later to monitor apoptosis. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance,* indicates significant difference between anti-IFNAR2 + pI:pC and the pI:pC alone (p≤0.05).
Figure 2.18. Responsive cell lines phosphorylate STAT1 in response to dsRNA and IFN-β. A. Cells were (un)treated with 50 µg/mL pl:pC for 0, 2, 4, 6, 18 and 24 hours or B. 1000 units/mL IFN-β for 0, 10 and 60 minutes, and whole cell lysates were collected. Expression of phosphorylated STAT1 (Y701) and total STAT1 were determined by Western blotting. Actin was used as a loading control.
Figure 2.19. Pathway components downstream of the IFNα/βR are functional in non-responsive cell lines. SKOV-3 and DOV-13 cells were (un)treated with IFN-γ for 5, 10, 20, or 60 minutes and whole cell lysates were collected. Levels of phosphorylated STAT1 (Y701) and STAT1 were determined by Western blot. Actin was used as a loading control.
Figure 2.20. Expression of IFNAR2 in ovarian cancer cell lines. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 24 hours and whole cell lysates were collected. Expression of IFNAR2 was determined by Western blotting. Soluble (as per manufacturer’s data sheet) IFNAR2 is 55 kDa and the IFNAR2 dimer is 95-100 kDa. Actin was used as a loading control.
2.3.5 Caspase activation is necessary for dsRNA-induced cell death.

The type I interferon response did not induce the same level of cell death observed with pI:pC treatment, suggesting additional cell death pathways contribute to the dsRNA-induced apoptosis. Caspase 3 activation was assessed 24 hours after pI:pC stimulation by Western blot. Caspase 3 cleavage was observed in the responsive cell lines and malignant patient samples (Figure 2.21 A). In the IOSE cell line which shows a minor response to pI:pC, caspase 3 cleavage was also detected. Caspase 3 activation in the cell lines was confirmed via an activity assay specific for caspase 3/7 (Figure 2.21 B) and Western blot for PARP cleavage (Figure 2.21 C). To determine which activator caspase was responsible for the cleavage of caspase 3, we utilized multiple, specific caspase inhibitors. The pan caspase inhibitor completely blocked the dsRNA-induced cell death response in CAOV-3 (Figure 2.22 top panel) and OVCAR-3 (Figure 2.22 bottom panel), showing that caspase activation is necessary for dsRNA-induced cell death. The caspase 9 and 8 inhibitors decreased the cell death response in both cell lines, but not to basal levels indicating both caspases may contribute to dsRNA-induced cell death. Inhibition of caspase 4, also known to cleave caspase 3, had no effect on cell death. To confirm caspase 9 activation, cytochrome C release was monitored in mitochondria rich and cytosolic fractions via Western blot. In both responsive cell lines, cytochrome C decreased in the mitochondria rich fraction and increased in the cytosol after stimulation with pI:pC consistent with caspase 9 activation (Figure 2.23).

Cytochrome C release can be caused by pore formation in the mitochondria due to activation of pro-apoptotic proteins (101). When pro-apoptotic proteins like NOXA
sequester anti-apoptotic Bcl-2 family proteins, they no longer block fellow pro-apoptotic proteins Bax and Bak (102). This allows Bax to oligomerize and form pores in the mitochondria, releasing cytochrome C which associates with caspase 9 to form the apoptosome (103). NOXA has been shown to be induced by dsRNA (104) and to be necessary for dsRNA-induced cell death in melanoma cells (54, 84). Surprisingly in the ovarian cancer cell lines, NOXA is increased in both responders and non-responders (Figure 2.24). Bax has been connected to the dsRNA pathway via IRF-3. IRF3 activation has been shown to activate Bax, which can then lead to cell death induced by cytochrome C release from the mitochondria (105). In responsive and non-responsive cell lines, levels of activated Bax, Bax 6A7, increased following pl:pC stimulation (Figure 2.25). Both NOXA levels and Bax activation increased in all cell lines, suggesting that activation of these proteins could play a role in dsRNA-induced cell death in responsive cell lines. In non-responsive cell lines, these levels of activation may not be sufficient or downstream components may be inactivated.

In the dsRNA signaling pathway that activates caspase 8, the upstream kinase RIP1 kinase, can activate two signaling pathways, caspase 8 and NF-κB. If RIP1 kinase is ubiquitinated by cIAP1/2, the NF-κB pathway is activated, but if RIP1 kinase is not ubiquitinated, caspase 8 can be cleaved (106). Of our cell lines, only non-responsive cell lines increased c-IAP2 levels, with SKOV-3 increasing c-IAP2 as early as 2 hours post stimulation (Figure 2.26). RIP1 kinase ubiquitination was also shown to increase following pl:pC stimulation in SKOV-3, suggesting that this cell line required activated NF-κB (Figure 2.27). To determine the importance of c-IAP2 activation, c-IAP2 was blocked
using a SMAC mimetic. The SMAC mimetic resulted in increased cell death in SKOV-3 but cell death did not increase when SMAC treated SKOV-3 were stimulated with pI:pC (Figure 2.28). DOV-13 were unaffected by the SMAC mimetic consistent with the reduced RIP1 kinase ubiquitination. These data show that SKOV-3 cells utilize c-IAP2 to ubiquitinate RIP1 kinase to activate NF-κB. With a later increase of c-IAP2 expression and less RIP1 kinase ubiquitination, DOV-13 may not be as dependent on RIP1 kinase for survival.

Overall, dsRNA treatment leads to activation of caspases 8 and 9 that then activate the effector caspase, caspase 3. This caspase response is required for dsRNA induced cell death. Non-responsive cells do not activate caspases but do increase their expression of c-IAP2 and their RIP1 kinase ubiquitination levels in response to dsRNA treatment.
**Figure 2.21. Caspase 3 is activated in responsive cell lines.** A. Ovarian cancer cell lines and malignant patient samples were (un)treated with 50 µg/mL pl:pC for 24 hours and whole cell lysates were collected. Expression of full length (35 kDa) and cleaved (19 and 17 kDa) caspase 3 were determined by Western blotting. Actin was used as a loading control. B. Cells were (un)treated with 50 µg/mL pl:pC for 24 hours and caspase 3/7 activity was measured. The mean ± SD of 3 independent experiments is shown. C. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 24 hours and whole cell lysates were collected. Expression of full length and cleaved PARP were determined by Western blotting. Actin was used as a loading control.
Figure 2.22. Caspase activation is necessary for dsRNA-induced cell death. CAOV-3 (top panel) and OVCAR-3 (bottom panel) cell lines were (un)treated with 25 μM of pan or caspase 9 or caspase 8 or caspase 4 specific inhibitor. 6 hours later, cells were (un)treated with 50 μg/mL pI:pC for 48 hours. Hoechst / PI staining was used to assess apoptosis. The mean ± SD of 3 independent experiments is shown. Student’s *t* test was used to determine statistical significance, * indicates significant difference (p ≤ 0.05).
Figure 2.23. Responsive cell lines release cytochrome C following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 0, 4, 10, or 24 hours. Mitochondrial rich and cytosolic fractions were collected. Expression of cytochrome C (Cyt C) was determined by Western blotting. Actin was used as a loading control.
Figure 2.24. NOXA increases in ovarian cancer cells following stimulation with dsRNA. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 24 hours and whole cell lysates were collected. Expression of NOXA was determined by Western blotting. Actin was used as a loading control.
Figure 2.25. Bax is activated in ovarian cancer cells following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 μg/mL pl:pC for 24 hours and whole cell lysates were collected. Expression of Bax and activated Bax(6A7) were determined by Western blotting. Actin was used as a loading control.
Figure 2.26. Non-responsive cell lines increase expression of c-IAP2 following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 0, 2, 4, 6, 18, or 24 hours and whole cell lysates were collected. Expression of c-IAP2 was determined by Western blotting. β–tubulin was used as a loading control.
Immunoprecipitation and Western blot by Dr. Hari Kuzhuvelil.

Figure 2.27. Non-responsive cell lines ubiquitinate RIP1 kinase following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 10 minutes and whole cell lysates were collected. RIP1 kinase was immunoprecipitated and probed for via Western blot analysis.
Figure 2.28. Inhibition of c-IAP2 does not increase cell death following dsRNA stimulation in non-responsive cell lines. Non-responsive ovarian cancer cell lines DOV-13 and SKOV-3 were (un)treated with a SMAC mimetic at 100 nM for 4 hours followed by treatment with 50 µg/mL pI:pC for 48 hours. Hoechst / PI staining was used to assess apoptosis. The mean ± SD of 3 independent experiments is shown.
2.3.6 Non-responsive cell lines increase autophagy in response to dsRNA.

It is well known that cancer cells can use autophagy as a cell survival mechanism (107). To determine if the non-responsive cell lines utilize this system, LC3-I and II levels were monitored via Western blot. When autophagy takes place, LC3-I is lipidated forming LC3-II which is incorporated into autophagosomes. Non-responsive DOV-13 and SKOV-3 show increased LC3-II levels after stimulation with pI:pC, but responsive OVCAR-3 and CAOV-3 do not (Figure 2.29). This was confirmed by transfecting SKOV-3 and DOV-13 with LC3-GFP plasmid and monitoring formation of autophagosomes via confocal microscopy. Autophagosome formation was monitored by observing ubiquitously expressed LC3-I move to punctate structures after dsRNA stimulation (Figure 2.30). Both cell lines significantly increased their autophagic response following stimulation.

As responsive cell lines do not induce autophagy but do activate a caspase response (opposite to the non-responsive cell lines), we tested if autophagy could be induced if caspase activation was blocked. Cells were pre-treated with pan-caspase inhibitor for 6 hours and then stimulated with pI:pC. LC3 lipidation was monitored via Western blot (Figure 2.31). The responsive cell lines did not increase LC3 lipidation following treatment, suggesting caspase activation and autophagy are not linked in this system.

To determine the mechanism of the autophagic response, both ER stress and the classical autophagic pathways were investigated. Phosphorylation of eIF2-α is indicative of ER stress and is commonly used as an indicator. However, the activated dsRNA receptor, PKR, phosphorylates eIF2-α eliminating this protein as a viable ER stress marker in our system. When stimulated with pI:pC, all four cell lines increased phosphorylation of
eIF2-α (Figure 2.32). A better indicator of ER stress in our system is an increase in BiP or GRP78. A hallmark of ER stress is an accumulation of unfolded proteins in the endoplasmic reticulum (108). BiP, a molecular chaperone, increases when the ER is overwhelmed with unfolded proteins (109). BiP levels did not increase in any of the cell lines suggesting that ER stress is not responsible for the increase in autophagy (Figure 2.33). The classical autophagic pathway was investigated by probing for increased mTOR phosphorylation (S2448) and beclin levels via Western blot. Phosphorylation of mTOR (Figure 2.34) and expression of beclin (Figure 2.35) did not increase following stimulation with pI:pC. These data suggest that like the ER stress pathway, the classical pathway did not appear to be activated.

To probe the importance of autophagy in non-responsive cell line survival, autophagy was blocked via knock down of a key protein ATG5. ATG5 forms a complex with ATG12 localizing to the isolation membrane, and is essential for its elongation into the autophagosome (110). Knockdown of mRNA and protein were successful with a 70% decrease in mRNA (Figure 2.36 A). In the DOV-13 ATG5 knockdown, these cells do not succumb to dsRNA-induced cell death (Figure 2.36 B). In addition, DOV-13 and SKOV-3 cell lines were treated with 3-methyladenine (3-MA), a PI3K inhibitor that blocks classical autophagy. Confocal microscopy revealed that 3-MA blocked the increase in autophagosome formation following stimulation in DOV-13 cells but did not affect basal levels of autophagy (Figure 2.37 A). However, 3-MA treated cells did not undergo increased apoptosis when treated with pI:pC (Figure 2.37 B). 3-MA does affect the basal level of apoptosis in DOV-13 but not SKOV-3 (Figure 2.37 C), indicating that DOV-13...
may be dependent upon autophagy for cell survival. In some cases, autophagy is known to eventually switch from a cell survival to cell death mechanism (107). We exposed DOV-13 to continuous pI:pC stimulation for 9 weeks and assessed apoptotic levels via Hoechst / PI staining every two days. This long-term pI:pC exposure did not alter cell survival (Figure 2.38).

Overall, this data shows that non-responsive cell lines increase autophagy in response to pI:pC treatment. However, when this autophagic response is blocked, dsRNA stimulation does not alter cell survival.
Figure 2.29. LC3 is lipidated in non-responsive cell lines following dsRNA stimulation. Cells were (un)treated with 50 µg/mL pI:pC for 0, 2, 4, 6, 18, or 24 hours and whole cell lysates were collected. Expression of LC3-I and LC3-II were determined by Western blotting. β–tubulin was used as a loading control. * indicates a non-specific band.
Figure 2.30. Non-responsive cell lines increase autophagy in response to dsRNA. DOV-13 and SKOV-3 cell lines were plated on coverslips and transfected with LC3-GFP plasmid for 24 hours. Cells were given fresh media and allowed to rest for 24 hours and were then (un)treated with 50 µg/mL pI:pC for 24 hours. The coverslips were mounted on glass slides and viewed via confocal microscopy. The mean ± SD of 3 independent experiments is shown. Student’s t test was used to determine statistical significance, * indicates significant difference (p≤0.05). Representative pictures of non-autophagic (DOV-13 - pI:pC) and autophagic counts (DOV-13 + pI:pC) cells are shown above.
Figure 2.31. Responsive cell lines do not increase autophagy when caspase activation is inhibited. Ovarian cancer cell lines were (un)treated with 25 µM Z-VAD-FMK pan-caspase inhibitor for 6 hours followed by treatment with 50 µg/mL pl:pC for 24 hours. Whole cell lysates were extracted and expression of LC3-I and II were determined by Western blot. β–tubulin was used as a loading control.
Figure 2.32. Levels of phosphorylated eIF2-α increase in ovarian cancer cell lines following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 0, 2, 4, 6, 18, and 24 hours and whole cell lysates were collected. Expression of phosphorylated eIF2-α and total eIF2-α were determined by Western blotting.
Figure 2.33. BiP levels do not increase following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 0, 2, 4, 6, 18 and 24 hours and whole cell lysates were collected. Expression of BiP was determined by Western blotting. Actin was used as a loading control.
**Figure 2.34. MTOR is not phosphorylated in response to dsRNA stimulation.** Ovarian cancer cell lines were (un)treated with 50 µg/mL pI:pC for 24 hours and whole cell lysates were collected. Expression of phosphorylated mTOR and total mTOR were determined by Western blotting. Actin was used as a loading control.
Figure 2.35. Beclin levels do not increase following dsRNA stimulation. Ovarian cancer cell lines were (un)treated with 50 µg/mL pl:pC for 0, 18, or 24 hours and whole cell lysates were collected. Expression of beclin was determined by Western blotting. Actin was used as a loading control.
Figure 2.36. Knockdown of ATG5 does not cause non-responders to undergo cell death following dsRNA stimulation. A. DOV-13 cells were transfected with ATG5 or scrambled control siRNA for 24 hours. RNA and cell lysates were harvested to determine mRNA levels via qPCR and protein levels via Western blot of ATG5. Actin was used as a loading control. Densitometry shows relative protein induction (bottom graph). B. Apoptosis was measured via Hoechst / PI staining following siRNA nucleofection and treatment with 50 µg/mL pl:pC for 24 and 48 hours. The mean ± SD of 3 independent experiments is shown.
Figure 2.37. Inhibition of the classical autophagic pathway does not sensitize non-responsive cell lines to dsRNA-induced cell death. A. DOV-13 cells were treated with 10nM 3MA for 4 hours followed by treatment with 50 µg/mL pl:pC for 24 hours. Autophagy was assessed via confocal microscopy. The mean ± SD of 3 independent experiments is shown. B. Apoptosis was also monitored in DOV-13 and C. SKOV-3 following 3MA pretreatment and 50 µg/mL pl:pC treatment for 24 and 48 hours via Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. Student’s t test was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 2.38. Long-term exposure to dsRNA does not induce cell death in non-responsive cell line. DOV-13 cells were (un)treated with 50 µg/mL pI:pC. Some cells were passaged and some were assessed for cell death (every other day) via Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. Cells were passaged a total of 9 weeks either untreated or in the presence of pI:pC continuously. Biweekly counts are shown.
2.3.7 DsRNA receptor contribution to dsRNA-induced cell death.

Previous studies have implicated different dsRNA receptors as essential for dsRNA responsiveness (53, 54). Many groups have used siRNA via lipid-based transfection to knock down the receptors one by one to determine importance. We found that when lipid-based reagents are used on the responsive cell lines, both the lipid-based reagent and lipid-based reagent followed by dsRNA treatment increased dsRNA receptor levels significantly. In order to determine receptor contribution without these off target effects, receptor specific agonist or antagonist and introduction of shRNA via lentiviral infection were utilized.

To determine the contribution of TLR3 to dsRNA-induced cell death, the responsive cell line, OVCAR-3, was treated with 5 μg/mL pl:pC or pA:pU (selective for TLR3 at this concentration, delivered directly into the media (111), for 24, 48, and 72 hours (Figure 2.40). Concentration dependent effect of pl:pC and pA:pU were determined via titration (Figure 2.39 A and B). The cell death induced by pl:pC increased over 72 hours, however, the death induced by pA:pU increased at 24 hours but then remained constant. Therefore, TLR3 induced cell death via this ligand, but RIG-I and MDA5 may provide the second wave of apoptotic death seen in the pl:pC samples at 48 and 72 hours but lacking in the pA:pU treatment.

PKR’s contribution to the dsRNA-induced cell death response was analyzed via its selective antagonist, 2-aminopurine (2-AP). CAOV-3 cells were treated with 5 mM 2-AP in the presence of pl:pC at 5 μg/mL for 24 hours. Ability of 2-AP to inhibit eIF2-α phosphorylation was confirmed via Western blot (Figure 2.41 A). The cell death response
was unaffected by inhibition of PKR, and was therefore determined to be non-essential for the dsRNA-induced cell death response (Figure 2.41 B).

TLR3’s importance to the dsRNA-induced response was also analyzed via shRNA knockdown delivered via lentiviral technology. Analysis of TLR3 protein via Western blot and mRNA using Taqman qPCR revealed an 85% knockdown when compared to uninfected, stimulated control cells (Figure 2.42 A and B). When the TLR3 knockdown cells were treated with pI:pC, they underwent significantly less cell death than cells expressing control shRNA (Figure 2.43). However, the cell death was not at basal levels indicating that other dsRNA receptors may also contribute to the dsRNA-induced cell death response, consistent with the experiments testing pA:pU. When pI:pC is delivered via PEI, the dsRNA-induced apoptotic response is restored in CAOV-3-TLR3 knockdown cells (Figure 2.44). These data show that TLR3 is needed for dsRNA-induced apoptosis if pI:pC is delivered without a vehicle. In addition to CAOV-3, the receptors TLR3 and RIG-I were knocked down in OVCAR-3. The knockdown of each receptor was assessed via Western blot (Figure 2.45 A) and qPCR (Figure 2.45 B). The level of each receptor was successfully reduced, however, additional experiments must be completed to determine how this dual receptor knockdown will effect the dsRNA response.

To determine if an increase in dsRNA receptor expression could cause the non-responsive cell lines to become sensitized to dsRNA treatment, the cells were transfected with dsRNA receptor plasmid. Appropriate DNA amounts were determined via titration (Figure 2.46). When the dsRNA receptors are overexpressed in SKOV-3 cells and stimulated with pI:pC for 24, 48, and 72 hours, cell death increases significantly up to
approximately 20-30% (Figure 2.47). This level is not as high as a responsive cell line, however, the transfection efficiency was only ~35% (Figure 2.48). Western blot analysis confirmed that the overexpression of the dsRNA receptors in non-responsive cell lines was equivalent to responder dsRNA receptor protein expression levels, ± dsRNA stimulation (Figure 2.49). A Western blot analysis verified that non-responsive cell lines, when over expressing the dsRNA receptors, and stimulated with dsRNA, cleaved caspase 3 (Figure 2.50), which may explain the cell death observed in Figure 2.43.

Overall, these data suggest that TLR3, RIG-I and MDA5 coordinate the dsRNA response. When these receptors are overexpressed in non-responsive cell lines, they are able to resensitize these cells to dsRNA stimulation, including caspase 3 cleavage.
Figure 2.39. Titrations of pI:pC and pA:pU. A. OVCAR-3 cells were treated with pI:pC and B. pA:pU at increasing concentrations for 24 hours. Cells were stained with Hoechst / PI to measure apoptosis. The mean ± SD of 3 independent experiments is shown.
Figure 2.40. TLR3 specific ligand induces cell death in a responsive cell line. OVCAR-3 cells were (un)treated with 5 µg/mL pl:pC or pA:pU for 24, 48, or 72 hours. Hoechst / PI staining was used to measure apoptosis. The mean ± SD of 3 independent experiments is shown.
Figure 2.41. PKR is not necessary for dsRNA-induced cell death in ovarian cancer cells. A. OVCAR3 cells were (un)treated with 2-aminopurine (2-AP) vehicle (PBS:acetic acid at 200:1 ratio), 5 µg/mL pI:pC, and either 1, 5, or 10 mM 2-AP for 24 hours. Cells were lysed and phosphorylated eIF2-α (Ser51) and total eIF2-α were analyzed via Western blot. β-tubulin was used as a loading control. B. OVCAR-3 cells were treated with 2-AP vehicle, 5 µg/mL pI:pC, 5 mM 2-AP, or both for 24 hours. Hoechst / PI staining was used to measure apoptosis. The mean ± SD of 3 independent experiments is shown.
Figure 2.42. TLR3 knockdown in CAOV-3 cells via shRNA. A. Stable CAOV-3 scrambled or TLR3 shRNA knockdown cells were (un)treated with 50 µg/mL pl:pC for 24 hours and whole cell lysates were extracted. Expression of TLR3 was assessed via Western blot. Actin was used as a loading control. * indicates a non-specific band. B. Stable CAOV-3 scrambled or TLR3 shRNA knockdown cells were (un)treated with 50 µg/mL pl:pC for 24 hours and RNA was extracted. Levels of dsRNA receptor mRNA were quantified via TaqMan qPCR. Expression is reported as relative induction normalized to GAPDH. The mean ± SD of 2 independent experiments is shown.
Figure 2.43. TLR3 contributes to dsRNA-induced cell death. Stable CAOV-3 scrambled or TLR3 shRNA knockdown cells were established and then were (un)treated with 50 µg/mL pl:pC for 24 hours. Cells were stained with Hoechst / PI to measure apoptosis at 24, 48 and 72 hour time points. The mean ± SD of 3 independent experiments is shown.
Figure 2.44. Cell death via PEI delivery of dsRNA does not require TLR3. CAOV-3 cells or CAOV-3 cells expressing stable TLR3 shRNA were (un)treated with pl:pC alone at 0.2 μg/mL or with PEI delivery at 0.02 μg/mL. Cell death was assessed after 48 hours with Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown.
Figure 2.45. **TLR3 and RIG-I knockdown in OVCAR-3 cells via shRNA.** OVCAR-3 cells were infected with lentiviral shRNA specific to TLR3 or RIG-I. Stable cell lines were established and then were (un)treated with 50 μg/mL pl:pC for 24 hours and whole cell lysates and RNA were extracted. A. Expression of TLR3 and RIG-I were assessed via Western blot. Actin was used as a loading control. * indicates a non-specific band. B. Levels of dsRNA receptor mRNA were quantified via TaqMan qPCR. Expression is reported as relative induction normalized to GAPDH. The mean ± SD of 2 independent experiments is shown.
Experiment by Charlotte Roberts.

**Figure 2.46. Titration of dsRNA receptor DNA.** SKOV3 cells were (un)transfected with 5, 50, or 500 ng of receptor MDA5 or 500 ng of control DNA. 24 hours post-transfection, cells were (un)treated with 50 µg/ml pl:pC for 24, 48, and 72 hours. Hoechst / PI staining was used to measure apoptosis. The mean ± SD of 3 independent experiments is shown.
Figure 2.47. DsRNA receptor over expression in a non-responsive cell line leads to cell death. SKOV-3 cells were transfected with 50 ng of receptor DNA or empty vector (ctrl). The cells were treated with 50 µg/mL pl:pC 24, 48, and 72 hours post-transfection. Hoechst / PI staining was used to measure apoptosis. The mean ± SD of 3 independent experiments is shown.
Experiment by Charlotte Roberts.

Figure 2.48. DsRNA receptor over expression transfection efficiency. SKOV3 cells were (un)transfected with 5, 50, or 500 ng of GFP plasmid, or 500 ng of control DNA. 24 hours later, transfection efficiency was assessed via quantification of GFP fluorescence. The mean ± SD of 3 independent experiments is shown.
Experiment by Charlotte Roberts.

Figure 2.49. Over expression of MDA5 in a non-responsive cell line shows expression levels comparable to responsive cell lines. Ovarian cancer cell lines were (un)transfected with 50 ng (L) or 500 ng (H) MDA5 DNA. Cells were then (un)treated with 50 µg/mL pi:pC for 24 hours. MDA5 expression was observed via Western blot analysis. Actin was used as a loading control.
Figure 2.50. Over expression of dsRNA receptors leads to caspase 3 cleavage in a non-responsive cell line. DOV-13 cells were transfected with 50 ng of control (ctrl) or dsRNA receptor DNA. 24 hours later, the cells were (un)treated with 50 µg/mL pl:pC for 24 hours. Cell lysates were collected and probed for full length (35 kDa) and cleaved (19 and 17 kDa) caspase 3. Actin was used as a loading control.
2.4 DISCUSSION

DsRNA has been shown to induce cell death in multiple cancer cell types including breast (53), prostate (56), melanoma (54, 84), and hepatoma (85). We have found that dsRNA stimulation can induce cell death in ovarian cancer cell lines and ascites-derived patient samples. In addition to the responsive cell lines and patient samples, we have also identified a sub-group that does not respond to dsRNA treatment regardless of dsRNA delivery method. The action of dsRNA in cancer has been attributed to an activation of various pathway components including type I interferons (53), autophagy (54), PKC-α (56), and caspases (53). Each cancer cell type utilized a unique set of pathways and dsRNA receptors to trigger cell death. Thus, predictive biomarkers for dsRNA-responsive tumors have yet to be identified. These current studies in ovarian cancer cell lines provide detailed study of required receptors and pathways to aid in identifying biomarkers for a dsRNA responsive patient population.

Responsiveness to dsRNA requires expression of a cognizant dsRNA receptor. In responsive cell lines and patient samples, dsRNA stimulation leads to an increase in expression of dsRNA receptor mRNA and protein. This response has been found in other cancer cell types as well (84), but we have found that non-responsive cell lines do not induce dsRNA receptor expression to an equivalent level (Figure 2.6). In addition, we are the first group to examine the expression levels of all four dsRNA receptors following stimulation. These data suggest that an increase in dsRNA receptor levels following stimulation could serve as a biomarker for dsRNA-responsive patients. Future studies could examine via immunohistochemistry, the dsRNA receptor expression in tumor
samples.

Given the segregation between responsive and non-responsive cell lines in terms of their dsRNA receptor expression, we probed for global differences in responsive vs. non-responsive cell lines via microarray analysis. Without dsRNA stimulation, responsive and non-responsive cell lines clustered separately, confirming two distinct subgroups (Figure 2.4).

DsRNA receptor signaling pathways have been extensively studied in the context of the anti-viral response. Proinflammatory cytokines, type I interferons, and the induction of apoptosis result from dsRNA ligation. We examined the role of each of these pathways in the dsRNA-induced apoptosis observed in the responsive cell lines CAOV-3 and OVCAR-3. We analyzed activation of NF-κB p65 through the phosphorylation of the p65 subunit. Ovarian cancer cell lines phosphorylated NF-κB p65 following stimulation and increased expression of NF-κB-inducible genes (Figure 2.10). These results suggested that in responsive cell lines, NF-κB may contribute to dsRNA-induced apoptosis. In non-responsive cell lines, the NF-κB activation indicated that even the low level of dsRNA receptor expression is capable of responding to ligand and initiating NF-κB activation. Alternatively, dsRNA may activate an, as yet, uncharacterized signaling pathway that activates NF-κB or creates a stress response leading to p65 phosphorylation.

Some groups have shown that the type I interferon pathway is necessary to induce dsRNA dependent cancer cell death (53), while others have found that it is non-essential (54). In ovarian cancer cell lines that are responsive to dsRNA, we have shown that the
The type I interferon pathway is activated following treatment, however, when this pathway is blocked, the cell death does decrease but not to basal level (Figures 2.15 – 2.17). This pathway is contributing to dsRNA-induced cell death, but one or more other pathways are also required for the dsRNA-induced apoptosis. Non-responsive cell lines are not able to activate the type I interferon response due to multiple blockades within this pathway. The cell lines are not able to produce IFN-β mRNA or protein suggesting that either the promoter/gene or a component upstream of the IFN-β promoter is non-functional. In addition, if IFN-β is given exogenously, the cells remain unresponsive even though their JAK/STAT pathways are functional (Figure 2.16). This indicates that the IFNα/βR is not functional. Whether this is due to mutation or an altered expression or trafficking has not been determined.

The responsive cell lines are also able to activate caspases, specifically caspases 8 and 9 that subsequently cleave 3 (Figure 2.21 and Figure 2.22). Non-responders are unable to activate caspases, possibly due to the ubiquitination of RIP1 kinase by c-IAP2 (Figure 2.26 and Figure 2.27). The fact that cell death increases in SKOV-3 when c-IAP2 is inhibited, showed that SKOV-3 is partially dependent on this anti-apoptotic protein for survival (Figure 2.28). DOV-13, however, does not depend on c-IAP2.

In responsive melanoma cells, autophagy has been shown to result in cell death following dsRNA stimulation (54). Both non-responsive cell lines increase autophagy following stimulation with dsRNA, but the increase and basal level in DOV-13 is much more pronounced. Autophagy was not detected in responsive cell lines. In the DOV-13 ovarian cancer cell line, the autophagy does not lead to cell death, even after a long-term (9
week) exposure (Figure 2.38). What is most surprising about the dsRNA-induced autophagy is that it occurs in the absence/low levels of dsRNA receptor. We are unable to attribute the increased autophagy to a stress response as we do not observe an increase in BiP (Figure 2.33). When pretreated with 3MA (a classical autophagy pathway inhibitor of PI3K), we see a return to basal level autophagy, which in the case of DOV-13 is still 30% of the cell population (Figure 2.37). Further studies are necessary to identify the role of autophagy in non-responsive cell lines.

In addition to evaluating the contribution of components downstream of the dsRNA receptors, the importance of the receptors themselves was investigated. Previous studies in multiple cancer cell types have shown differing receptor contribution. When cells were stimulated with pA:pU, a ligand specific for TLR3, cells did undergo apoptosis after 24 hours but the level of cell death did not increase at later time points as is seen with pI:pC (Figure 2.40). This could be due to the fact that activation of the TLR3 pathway and subsequent activation of the type I interferon response can lead to an increase in other dsRNA receptors like RIG-I and MDA-5 (66). As RIG-I and MDA5 do not recognize the pA:pU at this concentration, apoptosis does not increase. TLR3-specific shRNA knockdown via lentiviral infection reduced the levels of TLR3 in CAOV-3 cells. This reduction led to a significant decrease in dsRNA-induced cell death showing that when pI:pC is delivered directly into the media, TLR3 contributes to cell death (Figure 2.43). However, the decrease in cell death did not return to basal levels, indicating that other dsRNA receptors may be activated. This must be the helicases RIG-I and MDA5 due to the exclusion of PKR as an early contributor to dsRNA-induced cell death (Figure 2.41).
Interestingly, when pI:pC is delivered via PEI, loss of TLR3 does not affect the dsRNA-induced level of apoptosis. Further shRNA studies to knockdown RIG-I and MDA5 are needed to assess their role in dsRNA-induced apoptosis.

When the dsRNA receptor expression was restored in non-responsive cell lines, dsRNA-induced, cell death increased in a dsRNA-specific manner. This data shows that receptor expression is key to the dsRNA sensitivity. In addition, when the receptors were overexpressed in these cells, caspase 3 cleavage was restored following dsRNA stimulation. Cleaved caspase 3 expression is highest in cells over expressing TLR3. This could be due to the pI:pC delivery method in which TLR3 would be the first receptor to encounter the pI:pC. If pI:pC was delivered, other dsRNA receptors could also induce increased caspase 3 cleavage. To test this hypothesis, caspase 3 cleavage should be assessed in CAOV-3 TLR3 knockdown cells following PEI-pI:pC stimulation or time points beyond 24 hours with “naked” pI:pC.

Overall, we have identified subsets of ovarian cancer cell lines that are either responsive or non-responsive to dsRNA-induced apoptosis. We have examined dsRNA receptor expression in patient samples and ovarian cancer cells and found increased dsRNA receptor expression following dsRNA treatment in responsive samples. Responsive cell lines undergo a dsRNA-induced, caspase and type I interferon-dependent apoptosis. Non-responsiveness is primarily due to loss of dsRNA-induced expression of dsRNA receptors. Interestingly, these levels are sufficient to activate the NF-κB response. In non-responsive cell lines dsRNA does cause an increase in autophagy, and SKOV-3 increases RIP1 ubiquitination, but the reason why remains unknown.
CHAPTER 3

NOVEL COMBINATION CHEMOTHERAPY IN OVARIAN CANCER

3.1 INTRODUCTION

Customary treatment for ovarian cancer involves surgical debulking of the tumor followed by administration of chemotherapeutic drugs. In most cases, the front line therapy includes a combination of platinum-based drugs and taxane compounds such as carboplatin and paclitaxel (20). This regimen generally elicits a positive response, however, 70-90% of patients relapse and require second-line chemotherapy (7). Cancer cells have mutations that block signaling pathways or have aberrant over expression of proteins that activate pathways in the absence of stimuli. Tumor mutations can desensitize the patient to chemotherapeutic agents leaving them resistant or non-responsive to certain drugs. Drug combinations that target multiple pathways for activation or blockade, are less likely to encounter tumor mutations that overcome the chemotherapeutic signal.

Here we combine the innate immune ligand dsRNA with a variety of chemotherapeutics with distinct cytotoxic mechanisms. These drugs included paclitaxel and carboplatin, which are the current standard of care drug combination in ovarian cancer (20). In addition, we tested dsRNA in combination with vorinostat, a histone deacetylase inhibitor (HDACi) and sorafenib, a tyrosine kinase inhibitor. Vorinostat was tested due to the HDACi’s ability to reverse histone modifications that are known to contribute to ovarian cancer drug resistance and progression (112). Sorafenib, a tyrosine kinase
inhibitor, potentially blocks tumor angiogenesis by inhibiting vascular endothelial growth factor receptor (VEGFR) (113). In ovarian cancer, anti-angiogenesis chemotherapeutics (monoclonal antibodies and tyrosine kinase inhibitors) have met with some success in Phase II clinical trials (114). We included Sorafenib as a representative of this class of chemotherapeutic agents as it has previously been combined with carboplatin and vorinostat (115, 116). The cell lines tested have either mutant or are null for the tumor suppressor p53. This is similar to recently a published status of ovarian cancer tumors, in which 96% of tumor samples (303/316) were p53 mutants (31). In addition to reducing the likelihood of resistance and non-responsiveness, drug combinations may also allow a lower effective dose to be administered while maintaining the same drug efficacy (117). When more than one drug is administered, the drugs may interact in potentially three ways: not interactive; the effect of the drugs will be additive; antagonistic, the two drugs counteract or inhibit the effect of one another; synergistic, the combined effect of the two drugs is greater than the sum or their individual effects (118). This data will show how the drugs interact and if the combinations will be beneficial.
3.2 MATERIALS AND METHODS

3.2.1 Reagents
Vorinostat and Sorafenib were gifts from Dr. Paul Dent’s laboratory. Carboplatin, Paclitaxel and pI:pC were purchased from Sigma. Human-IFN-β was a gift from Dr. Andrew Larner’s laboratory. Anti-caspase-3 antibody was purchased from Cell Signaling Technology, Inc. Anti-LC3 antibody was purchased from Novus Biologicals.

3.2.2 Cell culture
Ovarian cancer cell lines OVCAR-3, CAOV-3, and SKOV-3 were purchased from ATCC. The ovarian cancer cell line DOV-13 was a gift from Dr. Xianjun Fang’s laboratory. Cells were cultured in RPMI 1640 medium supplemented with 10% low endotoxin FBS, 20 mM L-glutamine, 100 mM HEPES, 10 mM sodium pyruvate and 1X non-essential amino acids solution at 37°C in 5% CO₂. Immortalized non-tumorigenic ovarian epithelial cell lines IOSE 385 and IOSE 386 were a gift from the Canadian Ovarian Tissue Bank. Cells were cultured in 50% MCDB 105 (Sigma) / 50% M199 (Invitrogen) medium pH 7.2, 5% low endotoxin FBS, 4 mM L-glutamine, and 50 µg/mL gentamycin.

3.2.3 Drug stimulation
Cells were plated and allowed to adhere overnight. Drugs (Human-IFN-β, pI:pC, Sorafenib, Vorinostat, Carboplatin, and Paclitaxel), and a vehicle control (DMSO) were diluted in media and added to wells at specified concentrations and time points. Human-IFN-β was only used at 1000 units/µL.

3.2.4 Quantitation of cell death
Cells were (un)stimulated with 5 or 50 µg/ml pI:pC and monitored for apoptotic / other cell death at 24, 48 and 72 hour time points by Hoechst / propidium iodide (PI) staining of nuclear DNA. DNA was visualized under a Nikon TE300 Eclipse microscope equipped with a Hg-lamp and blue excitation fluorescence filter (excitation 330-380 nM / emission 420 long pass). Percentage of cell death was determined by counting normal (diffuse nuclear DNA) versus apoptotic or other cell death in three fields per well of >100 cells per field. Each experiment contained three wells per condition and the experiment was repeated three times.

3.2.5 MTT assay

Cells were plated in 96-well plates and allowed to adhere overnight. Cells were stimulated with drugs at specified concentrations. After 48 hours post-drug treatment, 10 µL of MTT solution was added to each well and allowed to incubate for 4 hours, according to the manufacturer’s protocol (Vybrant MTT Cell Proliferation Kit V-12154 Invitrogen). To avoid interference by phenol red, cells were placed in phenol red free media before addition of the MTT. The media used was RPMI medium 1640 (+ L-glutamine, - phenol red) supplemented with 10% low endotoxin FBS, 100 mM HEPES, 10 mM sodium pyruvate and 1X non-essential amino acids solution. DMSO was used to solubilize the formazan crystals, and absorbance was recorded at 540 nM after a 10 minute incubation. Experiments were performed in triplicate and the data were expressed as a relative percentage compared to untreated cells. Drug combination experiments were set up according to methods proposed by Chou (119). Cells were treated with increasing levels of single agents to determine their ED50 value, the concentration at which cell viability is
decreased by 50%. The data were fit to a 4-parameter logistic curve using SigmaPlot software (Systat Software, Inc.) to derive ED50 values. All $R^2$ values were $\geq 0.95$. Drug interactions were determined by isobologram analysis via CalcuSyn software (Biosoft, Cambridge, UK).

3.2.6 Western blotting

Cells, plated at $1\times10^6$ cells per 100x20 mm tissue culture dish, were harvested and lysed (100 $\mu$L of 0.02 M Tris; pH 8, 0.15 M NaCl, 1 mM DTT, 1% NP-40, 1 X protease inhibitor cocktail (Roche complete EDTA-free), 25 mM NaF, 10 X PhosSTOP (Roche)) at specified time points following stimulation. Lysates were separated by SDS-PAGE (20-50 $\mu$g/lane, Bio-Rad assay), transferred to nitrocellulose and immunoblotted with specified antibodies. For RIG-I an 8% Tris-Glycine gel was used, for LC-3, an 18.5 X 20 cm 15% Tris-Glycine gel was used, and for caspase 3, a 10% Tris-Glycine gel was used. All other Western blots were performed using 10% gels. Western blots were developed with chemiluminescent reagents from Thermo Scientific (Supersignal West Dura) or GE Healthcare (ECL Plus).

3.2.7 Statistical analysis

One-Way ANOVA or Student’s t test was used for statistical analyses using JMP 8.0 software (SAS Institute Inc.). A P-value $\leq 0.05$ was considered significant.
3.3 RESULTS

3.3.1 Overview of drug studies.

Preliminary experiments were performed on cell lines that are non-responsive to dsRNA to determine if, and at what level, other established chemotherapeutics led to cell death. These chemotherapeutics included paclitaxel, carboplatin, vorinostat, and sorafenib. The initial drug studies tested individual and combination therapies at concentrations found in the literature using an apoptosis assay. To determine optimal drug concentrations for dual drug studies, MTT assays were completed using the method established by Chou (119). Following the determination of the ED50 values for each individual drug, the ability of p1:pC to enhance the action of these drugs was determined via MTT assay. In responsive cell lines, the MTT assay was used to determine the ED50 value for individual drugs. Subsequently, drug combination studies via MTT assay were used to determine drug interactions (synergistic, additive, or antagonistic). For the drug combination experiments, SKOV-3 and OVCAR-3 cell lines were used due to their ability to form tumors in mice (120). This will allow any successful drug combinations to be further studied in an animal model.

3.3.2 Paclitaxel.

Paclitaxel is a mitotic inhibitor that was isolated from the Pacific yew tree, *Taxus brevifolia* (121). The drug stabilizes microtubules not allowing them to disassemble, a process necessary for proper cell division. The important action of these drugs is the suppression of spindle-microtubule dynamics, which results in the slowing or blocking of
mitosis at the metaphase–anaphase transition and induction of apoptotic cell death (122).

Paclitaxel is well known for its efficacy in ovarian cancer treatment as part of a combination regimen including the platinum-based drug, carboplatin. In addition to an abundance of studies and clinical trials targeting ovarian cancer, paclitaxel has been shown to be effective against breast (123), lung (124), and head and neck cancers (125).

The taxane-sensitivity of our panel of ovarian cancer cell lines was determined by a cell death assay testing increasing concentrations of paclitaxel. All cell lines undergo apoptosis in response to paclitaxel treatment, however, the drug concentration used proved too high, with the percentage of cell death reaching close to 100% at the lowest concentration and time point (Figures 3.1 and 3.2). To determine the optimal drug concentration for future experiments, an MTT assay was performed to determine the ED50 value. ED50 for OVCAR3 is 9.4 ng/mL and for SKOV-3 is 1.2 ng/mL (Figure 3.3).
Figure 3.1. Paclitaxel titration in ovarian cancer cell lines CAOV-3 and OVCAR-3. Responsive cell lines CAOV-3 (top panel) and OVCAR-3 (bottom panel), were left untreated or treated with paclitaxel at 0, 0.025, 0.25, 2.5 or 25 µg/mL for 24, 48, and 72 hours. A vehicle control is included. The mean ± SD of 3 independent experiments is shown. Cell death was measured via Hoechst / PI staining.
Figure 3.2. Paclitaxel titration in ovarian cancer cell lines DOV-13 and SKOV-3. Non-responsive cell lines DOV-13 (top panel) and SKOV-3 (bottom panel), were left untreated or treated with paclitaxel at 0, 0.025, 0.25, 2.5 or 25 µg/mL for 24, 48, and 72 hours. A vehicle control is included. The mean ± SD of 3 independent experiments is shown. Cell death was measured via Hoechst / PI staining.
Figure 3.3. Determination of the ED50 value for paclitaxel. OVCAR-3 and SKOV-3 cell lines were (un)treated with increasing concentrations of paclitaxel for 48 hours. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. Data were fit to a four-parameter logistic curve to derive the ED50.
3.3.3 Carboplatin

In combination with paclitaxel, the platinum-based drug carboplatin has been established as a first line chemotherapeutic regimen for ovarian cancer patients. Carboplatin was designed specifically to reduce side effects of cisplatin, another platinum-based compound from which it was derived (126). These drugs act by creating intrastrand cross-links between two adjacent guanine residues on the same strand of DNA (127). This would disrupt DNA replication and require a functional DNA repair response to repair these cross-linked strands. In addition to application in ovarian cancer, the use of this drug has expanded greatly in recent years, with clinical trials performed in melanoma (128), as well as lung (129), testicular (130), and cervical cancers (131). Although some trials use carboplatin as an individual agent, most use carboplatin in combination with one or more other chemotherapeutic agents. Despite the initial positive response to carboplatin/paclitaxel treatment in ovarian cancer, more than 70-90% of patients relapse and require second-line chemotherapy (7). If the relapse is within 1 month the patient is considered refractory to platinum treatment, within 6 months the patient is platinum-resistant, and after 6 months the patient is platinum sensitive (23). Patients become platinum resistant due to the presence of resistant subclones present in the tumor at the time of drug presentation or the occurrence of resistance mutations following chemotherapy (132). Including carboplatin in novel drug combinations with distinct mechanisms of action could aid in overcoming platinum resistance.

The platinum-sensitivity of our panel of ovarian cancer cell lines was determined by a cell death assay testing increasing concentrations of carboplatin. All cell lines undergo
apoptosis in response to carboplatin treatment, however, dsRNA-responsive cell lines CAOV-3 and OVCAR-3 (Figure 3.4) are more sensitive to carboplatin than DOV-13 and SKOV-3 (Figure 3.5). To determine the optimal drug concentration for future experiments, an MTT assay was performed to determine the ED50 value. The ED50 for OVCAR3 is 46.3 µg/mL and for SKOV-3 is 6.8 µg/mL (Figure 3.6).
Figure 3.4. Carboplatin titration in ovarian cancer cell lines CAOV-3 and OVCAR-3. Responsive cell lines CAOV-3 (top panel) and OVCAR-3 (bottom panel), were left untreated or treated with carboplatin at 0, 0.5, 5, 50 or 500 µg/mL for 24, 48, and 72 hours. Cell death was measured via Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown.
Figure 3.5. Carboplatin titration in ovarian cancer cell lines DOV-13 and SKOV-3. Non-responsive cell lines DOV-13 (top panel) and SKOV-3 (bottom panel), were left untreated or treated with carboplatin at 0, 0.5, 5, 50 or 500 µg/mL for 24, 48, and 72 hours. Cell death was measured via Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown.
Figure 3.6. Determination of the ED50 value for carboplatin. OVCAR-3 and SKOV-3 cell lines were (un)treated with increasing concentrations of carboplatin for 48 hours. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. Data were fit a four-parameter logistic curve to derive the ED50.
3.3.4 Vorinostat

Vorinostat, a histone deacetylase inhibitor (HDACi), belongs to an emerging class of anticancer agents targeting gene expression, an approach that has been termed epigenetic therapy. Post-translational modifications of histone tails such as acetylation and methylation affect chromatin structure and gene expression, and are one component of epigenetic regulation in mammalian cells (133). Transcription is favored when histone acetylases (HATs) add acetyl moieties to histones leading to an open chromatin structure. Repression of gene transcription due to condensed chromatin results from histone deacetylases (HDACs) removing the acetyl moiety from histones and transcription factors. Blocking the activity of HDACs with HDACi leads to relaxing of the chromatin and transcription of genes that may have been blocked. This altered cell homeostasis can lead to cell death (134). While induction of apoptosis appears to be the predominant mechanism of HDACi-mediated cell death, alternative mechanisms such as increased autophagy may also result (135). Vorinostat’s ability to induce cancer cell death has led to its application in a variety of cancer types as a single agent (136). Vorinostat selectively alters the transcription of relatively few genes, and normal cells are at least 10-fold more resistant than transformed cells to the drug (137). Combining HDACi with other pro-apoptotic agents can result in synergistic apoptosis and superior anti-tumor activities compared to those observed using single agents (138). Given that HDACi appears to be well tolerated in patients and has the capacity to act synergistically with a diverse range of pharmacological and biological agents, HDACi may be best utilized as part of a combination regimen. Clinical trials are underway pairing vorinostat with radiation, targeted therapies, and
cytotoxics (139). In addition to these promising applications of HDACi, development of novel drug combinations will aid in overcoming patient disease heterogeneity, chemoresistance, and dosage toxicity.

Initially, we targeted cell lines that are not responsive to dsRNA with vorinostat. A titration determined the concentration to be used in preliminary experiments (Figure 3.7). Later, to establish a more accurate concentration for further experiments, the ED50 was determined via MTT assay (Figure 3.8) for SKOV-3 (ED50 = 967 nM) and OVCAR-3 (ED50 = 1057 nM) cell lines. Preliminary studies have been completed in order to determine the mechanism of vorinostat-induced cell death in DOV-13 and SKOV-3.

Vorinostat’s ability to alter gene expression led us to probe for dsRNA-receptor expression. A Western blot for RIG-I showed that vorinostat treatment does not lead to an increase in receptor expression following dsRNA treatment, however, the other receptors have not been tested (Figure 3.9). A similar result was obtained when probing for caspase 3 cleavage, showing no caspase 3 cleavage following vorinostat treatment (Figure 3.10). These studies indicate that vorinostat-induced cell death in these cell lines is not caused by an increase in RIG-I receptor expression or caspase 3 cleavage. When probing for autophagy, we observed a marked increase in LC3 lipidation following vorinostat treatment, especially in SKOV-3 cells (Figure 3.11). However, what role autophagy plays in cell death has not been determined. More mechanistic studies must be completed to determine the complete mechanism of vorinostat-induced cell death.
**Figure 3.7. Vorinostat titration in ovarian cancer cell line DOV-13.** DOV-13 cells were left untreated or treated with vorinostat at 100, 200, 300, 400, 500 or 600 nM for 24, 48, and 72 hours. Cell death was measured via Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown.
Figure 3.8. Determination of the ED50 value for vorinostat. OVCAR-3 and SKOV-3 cell lines were (un)treated with increasing concentrations of vorinostat for 48 hours. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. Data were fit to a four-parameter logistic curve to derive the ED50.
Figure 3.9. Vorinostat stimulation does not lead to an increase in RIG-I expression. Non-responsive cell lines SKOV-3 and DOV-13 were (un)treated with 600 nM vorinostat for 24 hours and whole cell lysates were collected. Expression of RIG-I was determined by Western blotting. Actin was used as a loading control.
Figure 3.10. Vorinostat stimulation does not lead to caspase 3 cleavage in non-responsive cell lines. Non-responsive cell lines SKOV-3 and DOV-13 were (un)treated with 600 nM vorinostat for 24 hours and whole cell lysates were collected. Expression of full length (35 kDa) and cleaved (19 and 17 kDa) caspase 3 was determined by Western blotting. Actin was used as a loading control.
Figure 3.11. Vorinostat stimulation leads to LC3 lipidation in non-responsive cell lines. Cells were (un)treated with 600 nM vorinostat for 0, 2, 4, 6, 18, or 24 hours and whole cell lysates were collected. Expression of LC3-I and LC3-II were determined by Western blotting. β–tubulin was used as a loading control. * indicates a non-specific band.
3.3.5 Drug combinations.

Combining two or more chemotherapeutics with distinct mechanisms of action greatly increases the apoptotic response while decreasing overall dosage to achieve target cell death (140). Preliminary experiments were performed on cell lines that are non-responsive to dsRNA to determine which drugs individually and in combination lead to cell death. In addition, we investigated the ability of dsRNA to enhance the effect of these drugs. The drugs tested included previously discussed pI:pC, carboplatin and vorinostat, as well as IFN-β and Sorafenib. As shown in Figures 2.16 and 2.17, IFN-β can induce apoptosis in responsive cell lines and when the IFNα/βR is blocked, the pI:pC induced apoptosis is blunted. Sorafenib, a tyrosine kinase inhibitor, potentially blocks tumor angiogenesis by inhibiting vascular endothelial growth factor receptor (VEGFR) (113). In ovarian cancer, anti-angiogenesis chemotherapeutics (monoclonal antibodies and tyrosine kinase inhibitors) have met with some success in Phase II clinical trials (114). We included Sorafenib as a representative of this class of chemotherapeutic agents as it has previously been combined with carboplatin and vorinostat (115, 116). Drug concentrations in these experiments were determined from the literature.

To establish the effect of the drugs on non-cancerous ovarian cells, IOSE cell lines 385 and 386 were tested. When the IOSE cell lines 385 and 386 were treated with IFN-β they responded slightly at 72 hours (Figure 3.12). This response increased significantly when carboplatin or vorinostat were combined with IFN-β, but not sorafenib. Carboplatin, at a treatment concentration often used in the literature, caused 100% cell death in the IOSE cell line and thus it was not possible to assess interactions with other drugs.
However, when IFN-β was combined with vorinostat, there was a significant increase in cell death at the latest time point, 72 hours. SKOV-3 and DOV-13 also had a slight response to IFN-β at 72 hours (Figure 3.13). IFN-β did not significantly increase the effect of carboplatin treatment on these cells but did significantly increase the cell death induced by vorinostat at 48 and 72 hours.

When the IOSE cell lines were treated with carboplatin they underwent 100% cell death (Figure 3.14). Due to this effect, no conclusions can be made about the interaction of carboplatin with other chemotherapeutics. In SKOV-3 and DOV-13, carboplatin alone greatly increased cell death at 48 and 72 hours (Figure 3.15). The addition of carboplatin to IFN-β did not produce increased cell death, however, pI:pC did increase cell death significantly at 72 hours. Sorafenib added to carboplatin did not lead to an increase in cell death and vorinostat seemed to produce an additive effect. Overall, in SKOV-3 and DOV-13, only pI:pC enhanced the cell death induced by carboplatin beyond an additive effect.

Sorafenib did induce cell death in the IOSE 385 cell line (~50%) but had less of an effect on the IOSE 386 cell line (Figure 3.16). This effect was enhanced by pI:pC and vorinostat but not IFN-β. SKOV-3 and DOV-13 did not respond to sorafenib alone and only the addition of vorinostat significantly increased cell death (Figure 3.17). Given that the addition of pI:pC to sorafenib did not result in a significant increase in cell death, additional combination studies using this drug were not pursued.

The IOSE cell lines did respond to vorinostat alone, but addition of pI:pC and IFN-β increased it’s response (Figure 3.18). Interestingly, the addition of IFN-β increased cell death much more than pI:pC. SKOV-3 and DOV-13 also responded to vorinostat alone
(Figure 3.19). This effect was enhanced by both pI:pC and IFN-β at roughly equal amounts. Overall, the non-cancerous IOSE cell lines are sensitive to the drugs so proper dosage must be determined to decrease cytotoxic effects on “normal” cells as much as possible.

The cell death assays show that in SKOV-3 and DOV-13 cell lines, pI:pC has the potential to enhance the action of other chemotherapeutics. As pI:pC has no effect on DOV-13 or SKOV-3 as a single agent, the dual therapy effect cannot be defined as synergistic, but rather pI:pC enhances the action of the other drug (141). As shown in Figure 3.20, SKOV-3 cells are resistant to pI:pC treatment and therefore no ED50 value could be determined. However, the ED50 values were determined for paclitaxel (Figure 3.3), carboplatin (Figure 3.6), and vorinostat (Figure 3.8). With this information, pI:pC was combined over a range of concentrations (0.5 – 1000 µg/mL) with multiple chemotherapeutics held constant at their ED50 value.

When SKOV-3 cells were treated with paclitaxel (ED50 = 1.2 ng/mL), combined with pI:pC (0.5 – 1000 µg/mL), pI:pC enhanced the growth inhibition of paclitaxel in a dose-dependent manner (Figure 3.21). As pI:pC increased up to 500 µg/mL, the percent enhancement also increased. At this concentration, pI:pC enhanced the action of paclitaxel by approximately 30%. When the pI:pC concentration was increased further to 1000 µg/mL, the percent enhancement no longer increased. Similar to paclitaxel, carboplatin (ED50 = 6.8 µg/mL) was also enhanced by pI:pC in a dose-dependent manner (Figure 3.22) up to 500 µg/mL pI:pC in SKOV-3. At this concentration, pI:pC enhanced the action
of carboplatin by approximately 35%. Of the three tested chemotherapeutics, the action of vorinostat (ED50 = 967 nM) was most enhanced by pI:pC (Figure 3.23). At 500 μg/mL pI:pC, enhancement was approximately 50%. As with paclitaxel and carboplatin, enhancement did not increase at higher pI:pC concentrations.

Unlike SKOV-3, OVCAR-3 cells are able to respond to pI:pC individually, which allowed us to determine how pI:pC interacts with chemotherapeutics paclitaxel, carboplatin, and vorinostat. In addition to combining pI:pC with these chemotherapeutics we tested a combination of carboplatin and paclitaxel, the first line ovarian cancer regimen. Also, we combined each of these drugs with the HDACi vorinostat. The determination of ED50 values for each drug (Figures 3.3, 3.6, 3.8, and 3.20) established equipotency. Serial dilutions of individual drugs were generated while maintaining a 1:1 equipotent ratio. Dilutions tested were 0.25, 0.5, 1, 2, and 4 fold the ED50 value as described (119). The effect of each combination on OVCAR-3 was evaluated by MTT assays at 48 hours after treatment. The 48 hour time point was chosen, as it allowed sufficient time for the cells to respond, but not enough time for unresponsive cells to reach confluency. To assess the type of drug interaction, isobologram analysis was performed. This analysis provides a combination index (CI) value, which is a quantitative measure of the degree of drug interaction between two or more agonists (118). A CI<1.0, = 1.0, or >1.0 would indicate synergism, additive and antagonistic effect, respectively. When pI:pC was combined with paclitaxel, the growth inhibition was significantly greater than when either drug was used alone (Figure 3.24 A). The dilutions 1, 2, and 4 X ED50 produced a synergistic effect, while the 0.25 and 0.5 X ED50 dilutions produced an additive effect (Figure 3.24 B).
Combining pI:pC with carboplatin showed greater inhibition than the drugs alone (Figure 3.25 A), while antagonism was seen with the 0.25 and 0.5 X dilutions (Figure 3.25 B). However, as the drug concentrations increased to the ED50 value or greater, the drugs interacted synergistically. Concentrations above the ED50 value began trending up towards additive, establishing the ED50 value as optimal for a synergistic effect. In addition to carboplatin and paclitaxel, pI:pC was combined with the HDACi vorinostat. The combination showed greater inhibition than the drugs individually (Figure 3.26 A). As the drug concentrations increased, the combinations became more synergistic, consistently trending downwards (Figure 3.26 B). These results show that pI:pC has the ability to act synergistically with multiple chemotherapeutics with different mechanisms of action. In addition, these data establish that pI:pC could potentially be successful in combination with chemotherapeutics that are currently used in ovarian cancer treatment.

Considering that carboplatin and paclitaxel are consistently used in combination against ovarian cancer, we tested the combination in OVCAR-3. When these drugs were combined, the drug combination showed greater inhibition than the drugs alone (Figure 3.27 A). However, the difference was not as significant as with the other drug combinations discussed. The range of concentrations showed additive or antagonistic effects (Figure 3.27 B), but not synergism. The 0.5 X ED50 CI is especially high at 4, but this may be an outlier. When these two drugs were combined with vorinostat, the combinations show greater inhibition than the individual drugs (Figures 3.28 A and 3.29 A). The combinations similarly show antagonism at the lower concentrations of 0.25 and 0.5 X ED50, but are synergistic at the ED50 and 2 X ED50 (Figures 3.28 B and 3.29 B).
At 4 X ED50, the trend starts to increase toward additive in both combinations. Overall, carboplatin and paclitaxel do not produce a synergistic effect in these cell lines, but when vorinostat is combined with either drug, synergy is observed.
Figure 3.12. IFN-β in combination with other chemotherapeutics in immortalized ovarian surface epithelium cells. IOSE 385 and 386 cells were (un)treated with vehicle DMSO (D), 1000 units/mL IFN-β (I), IFN-β + 3 µM sorafenib (S), IFN-β + 500 µg/mL carboplatin (C), or IFN-β + 600 nM vorinostat (V) for 24, 48, and 72 hours. Cell death was measured by Hoechst/PI staining. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 3.13. IFN-β in combination with other chemotherapeutics in non-responsive cell lines. SKOV-3 and DOV-13 cells were (un)treated with vehicle DMSO (D), 1000 units / mL IFN-β (I), IFN-β + 3 μM sorafenib (S), IFN-β + 500 μg/mL carboplatin (C), or IFN-β + 600 nM vorinostat (V) for 24, 48, and 72 hours. Cell death was measured by Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 3.14. Carboplatin in combination with other chemotherapeutics in immortalized ovarian surface epithelium cells. IOSE 385 and 386 cells were (un)treated with vehicle DMSO (D), 500 µg/mL carboplatin (C), carboplatin + 50 µg/mL pL:pC (P), carboplatin + 1000 units/mL IFN-β (I), carboplatin + 3 µM sorafenib (S), or carboplatin + 600 nM vorinostat (V) for 24, 48, and 72 hours. Cell death was measured by Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. * Indicates significant difference from individual drugs.
Figure 3.15. Carboplatin in combination with other chemotherapeutics in non-responders. SKOV-3 and DOV-13 cells were (un)treated with vehicle DMSO (D), 500 µg/mL carboplatin (C), carboplatin + 50 µg/mL pI:pC (P), carboplatin + 1000 units/mL IFN-β (I), carboplatin + 3 µM sorafenib (S), or carboplatin + 600 nM vorinostat (V) for 24, 48, and 72 hours. Cell death was measured by Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference (p<0.05).
Figure 3.16. Sorafenib in combination with other chemotherapeutics in immortalized ovarian surface epithelium cells. IOSE 385 and 386 cells were (un)treated with vehicle DMSO (D), 3 µM sorafenib (S), sorafenib + 50 µg/mL pl:pC (P), sorafenib + 1000 units/mL IFN-β (I), sorafenib + 500 µg/mL carboplatin (C), or sorafenib + 600 nM vorinostat (V) for 24, 48, and 72 hours. Cell death was measured by Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 3.17. Sorafenib in combination with other chemotherapeutics in non-responders. SKOV-3 and DOV-13 cells were (un)treated with vehicle DMSO (D), 3 µM sorafenib (S), sorafenib + 50 µg/mL pI:pC (P), sorafenib + 1000 units/mL IFN-β (I), sorafenib + 500 µg/mL carboplatin (C), or sorafenib + 600 nM vorinostat (V) for 24, 48, and 72 hours. Cell death was measured by Hoechst/PI staining. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference (p<0.05).
Figure 3.18. Vorinostat in combination with other chemotherapeutics in immortalized ovarian surface epithelium cells. IOSE 385 and 386 cells were (un)treated with vehicle DMSO (D), 600 nM vorinostat (V), vorinostat + 50 µg/mL pI:pC (P), vorinostat + 1000 units/mL IFN-β (I), vorinostat + 500 µg/mL carboplatin (C), or vorinostat +3 µM sorafenib (S) for 24, 48, and 72 hours. Cell death was measured by Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference (p≤0.05).
Figure 3.19. Vorinostat in combination with other chemotherapeutics in non-responders. SKOV-3 and DOV-13 cells were (un)treated with vehicle DMSO (D), 600 nM vorinostat (V), vorinostat + 50 µg/mL pI:pC (P), vorinostat + 1000 units/mL IFN-β (I), vorinostat + 500 µg/mL carboplatin (C), or vorinostat + 3 µM sorafenib (S) for 24, 48, and 72 hours. Cell death was measured by Hoechst / PI staining. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference (p<0.05).
Figure 3.20. Determination of the ED50 value for pI:pC. OVCAR-3 and SKOV-3 cell lines were (un)treated with increasing concentrations of pI:pC for 48 hours. Cell viability was measured via MTT assay. The mean ± SD of 3 independent experiments is shown. Data were fit to a four-parameter logistic curve to derive the ED50.
Figure 3.21. DsRNA enhances paclitaxel-induced cell death in SKOV-3 cells. SKOV-3 cells were (un)treated with paclitaxel at the ED50 value of 1.2 ng/mL, and increasing concentrations of pI:pC (0.5 – 1000 µg/mL) for 48 hours. Data is represented as % enhancement of growth inhibition relative to cells treated with the ED50 of paclitaxel alone, as determined via MTT assay. The mean ± SD of 3 independent experiments is shown.
Figure 3.22. DsRNA enhances carboplatin-induced cell death in SKOV-3 cells. SKOV-3 cells were (un)treated with carboplatin at the ED50 value of 6.8 µg/mL, and increasing concentrations of pI:pC (0.5 – 1000 µg/mL) for 48 hours. Data is represented as % enhancement of growth inhibition relative to cells treated with the ED50 of carboplatin alone, as determined via MTT assay. The mean ± SD of 3 independent experiments is shown.
Figure 3.23. DsRNA enhances vorinostat-induced cell death in SKOV-3 cells. SKOV-3 cells were (un)treated with vorinostat at the ED50 value of 967 nM, and increasing concentrations of pl:pC (0.5 – 1000 µg/mL) for 48 hours. Data is represented as % enhancement of growth inhibition relative to cells treated with the ED50 of vorinostat alone, as determined via MTT assay. The mean ± SD of 3 independent experiments is shown.
Figure 3.24. DsRNA and paclitaxel act synergistically to induce OVCAR-3 cell death.
A. OVCAR-3 cells were (un)treated with pl:pC and paclitaxel individually and in combination at the indicated concentrations. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference comparing the combination to the individual drugs (p≤0.05).
B. Combination index (CI) values were calculated using the CalcuSyn software. The dotted line represents additivity, where CI = 1.
Figure 3.25. DsRNA and carboplatin act synergistically to induce OVCAR-3 cell death. A. OVCAR-3 cells were (un)treated with pl:pC and carboplatin individually and in combination at the indicated concentrations. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference comparing the combination to the individual drugs (p ≤ 0.05). B. Combination index (CI) values were calculated using the CalcuSyn software. The dotted line represents additivity, where CI = 1.
Figure 3.26. DsRNA and vorinostat act synergistically to induce OVCAR-3 cell death. 

A. OVCAR-3 cells were (un)treated with pl:pC and vorinostat individually and in combination at the indicated concentrations. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference comparing the combination to the individual drugs (p ≤ 0.05).

B. Combination index (CI) values were calculated using the Calcusyn software. The dotted line represents additivity, where CI = 1.
Figure 3.27. Carboplatin and paclitaxel do not act synergistically to induce OVCAR-3 cell death. A. OVCAR-3 cells were (un)treated with paclitaxel and carboplatin individually and in combination at the indicated concentrations. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference comparing the combination to the individual drugs (p≤0.05). B. Combination index (CI) values were calculated using the CalcuSyn software. The dotted line represents additivity, where CI = 1.
**Figure 3.28. Paclitaxel and vorinostat act synergistically to induce OVCAR-3 cell death.** A. OVCAR-3 cells were (un)treated with paclitaxel and vorinostat individually and in combination at the indicated concentrations. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference comparing the combination to the individual drugs (p ≤ 0.05). B. Combination index (CI) values were calculated using the CalcuSyn software. The dotted line represents additivity, where CI = 1.
Figure 3.29. Carboplatin and vorinostat act synergistically to induce OVCAR-3 cell death. A. OVCAR-3 cells were (un)treated with carboplatin and vorinostat individually and in combination at the indicated concentrations. Cell viability was measured via MTT assay. Values are reported relative to untreated control. The mean ± SD of 3 independent experiments is shown. One-Way ANOVA analysis was used to determine statistical significance, * indicates significant difference comparing the combination to the individual drugs \((p \leq 0.05)\). B. Combination index (CI) values were calculated using the Calcusyn software. The dotted line represents additivity, where CI = 1.
3.4 DISCUSSION

Drug combinations are widely used because multiple drugs affect a variety of targets and cell subpopulations to overcome potentially chemoresistant tumor cells. The primary aim is a mutual enhancement of the therapeutic effects, while other benefits may include decreased dosage that leads to reduced side effects and the delay or prevention of drug resistance (142). In ovarian cancer, patients are often given a combination of paclitaxel and carboplatin (3). This regimen elicits a positive response in 65% of patients but the remaining 35% and those with recurring disease may require alternate treatment if they are considered platinum-resistant (20).

DsRNA has been studied as an individual drug, but has not been investigated as part of a combination regimen. In this work we have examined the effect of individual drugs and combinations of these drugs on dsRNA responsive and non-responsive ovarian cancer cell lines, as well as a “normal” IOSE cell line. Preliminary experiments with cell lines that are non-responsive to dsRNA showed that dsRNA and IFN-β (downstream cytokine produced by dsRNA signaling pathways), both enhance the action of other chemotherapeutics. This led us to determine the ED50 values for multiple chemotherapeutics, and the effect of drug combinations with a range of pI:pC concentrations. DsRNA was able to enhance the action of paclitaxel, carboplatin, and vorinostat. These data show that even in cell lines that do not respond to dsRNA individually, this agent could be used in drug combinations to improve the efficacy of other chemotherapeutics.
When combined, drugs that as single agents elicit a response interact to give a synergistic, additive, or antagonistic effect. The drug interactions between pI:pC and paclitaxel, carboplatin, and vorinostat were examined in the dsRNA responsive cell line, OVCAR-3. DsRNA exhibited a synergistic effect in combination with paclitaxel, carboplatin, and vorinostat. These data showed that dsRNA could act as a potential combination chemotherapeutic with these already established drugs. The combination of carboplatin with paclitaxel did not produce a synergistic effect, but an additive effect and antagonistic effect were observed over the concentrations tested. Instead of equipotent ratios of carboplatin/paclitaxel, unequal ratios could be examined to define the optimal dosage. The combination of the HDACi vorinostat with carboplatin and paclitaxel has been shown effective in non-small cell lung cancer (143). When carboplatin or paclitaxel were combined with vorinostat, synergistic growth inhibition occurred. These data have identified vorinostat as a potential combination chemotherapeutic with drugs that are currently used in first-line ovarian cancer treatment.

These data show the ability of pI:pC to enhance the action of chemotherapeutics in dsRNA non-responsive cell lines, as well as act synergistically with chemotherapeutics in dsRNA responsive cell lines. Further experiments are required to determine the mechanism by which pI:pC both enhances death in non-responsive cell lines, and works synergistically with drugs in responsive cell lines. In addition, the applicability of these drug combinations must be explored in an animal model. These studies will utilize dsRNA responsive cell line OVCAR-3 and the dsRNA non-responsive cell line SKOV-3, due to their ability to form tumors in mice (120). In addition, a syngenic model will be used to
examine how the innate immune agonist in addition to it’s direct effect on tumor cell viability, may elicit an immune response.
CHAPTER 4

GENERAL DISCUSSION AND FUTURE DIRECTIONS

“Ovarian cancer is a disease that is neither common nor rare, has no specific symptoms, is primarily detected at an advanced stage, and often becomes resistant to chemotherapy” (144). Regular pelvic exams miss early stage tumors, and use of clinical imaging for ovarian cancer (such as ultrasonography) is limited to detection of cysts and solid benign lesions (145). In addition to evaluating imaging as a diagnostic tool, the serum biomarker CA-125 is being investigated as a predictive biomarker (146). CA-125 is the only serum biomarker for ovarian cancer, and although CA125 is elevated in 80% of ovarian cancer patients, levels can be elevated due to other conditions such as endometriosis, leiomyomas, congestive heart failure, cirrhosis, and menstruation (12).

Because 90% of stage I patients respond to treatment, predictive biomarker development remains a high priority in the field. Following diagnosis with ovarian cancer, patients are given a combination of platinum and taxol-based chemotherapeutics such as carboplatin and paclitaxel. If the patient is not responsive initially, or has a recurrence within six months of treatment, they are identified as resistant to platinum drugs. In these cases, the patients are treated with one of many possible single agent chemotherapeutics that have shown efficacy, or the patient is recommended for a clinical trial (20). A second priority in the field of ovarian cancer research is the development of novel chemotherapeutic agents to address the chemoresistance (to platinum/taxol-based agents) observed in 35% of initially diagnosed patients, and those who face recurring
chemoresistant tumors. Up to 95% of ovarian cancer cases are sporadic with the remaining ~5% attributed to germ line BRCA1/2 mutations (9). Only 25% of cases are diagnosed in stage I when the tumor is confined to the ovary (19). At this stage, 90% of the patients are “cured.” The remaining 75% of the patient population are diagnosed when the cancer has spread beyond the boundaries of the ovary, owing in part to the location of the ovary in direct contact with the peritoneum. Why is this disease primarily detected only in its later metastatic stages? Regular pelvic exams fail to identify tumors that are still confined to the ovary, and symptoms like abdominal discomfort could be attributed to multiple other gastrointestinal and reproductive diseases (9). At this time the field lacks a specific biomarker for the onset of the disease. The goal of this study was to determine if the innate immune ligand, dsRNA, could be used as an effective treatment in ovarian cancer as a single chemotherapeutic and in combination with other cytotoxic drugs. As a targeted therapy, the components of signaling pathways in multiple cell lines were examined to identify components essential to the dsRNA-induced response that could be used to select dsRNA-responsive patients (Chapter 2).

DsRNA has been shown to induce cell death in a variety of cancer cell types including melanoma (54, 84) breast (53), prostate (56) and hepatoma (85) cell lines. In ovarian cancer cell lines and patient-derived ascites samples, we have found that dsRNA stimulation can induce cell death. We have also identified a sub-group of ovarian cancer cells that does not respond to dsRNA treatment regardless of the dsRNA delivery method. The apoptotic effect of dsRNA on cancer cells has been attributed to dsRNA-induced activation of caspases (53), production of type I interferons (53), increased autophagy (54),
and activation of PKC-α (56). Each study also implicated a different subset of dsRNA receptors as essential for dsRNA responsiveness.

The cell lines that are responsive to dsRNA treatment increase mRNA and protein expression levels of the dsRNA receptors after treatment while the non-responsive cell lines do not detectably increase protein expression, and only increase mRNA at much lower levels. Therefore, an increase in dsRNA receptor levels following dsRNA stimulation could function as a biomarker for dsRNA responsiveness. Expression of the dsRNA receptor TLR3 has previously been proposed as a potential biomarker in breast cancer to identify dsRNA-responsive patients (86). In our evaluation, we only observe this increased mRNA/protein expression of dsRNA receptors after dsRNA treatment in culture. As this represents a more labor-intensive diagnostic tool that requires sufficient viable cells from patient ascites, this evaluation is not ideal for clinical evaluation. We would like to as part of our future studies, evaluate benign vs. malignant patient samples via immunohistochemistry for dsRNA receptor expression and correlate this to dsRNA responsiveness.

A second approach we took to characterize responsive versus non-responsive cell lines was microarray analysis of unstimulated and stimulated samples in collaboration with the Dumur group. The data showed that the responsive cell lines clustered separately from the non-responsive cell lines before and after treatment (Figure 2.8). Given the fact that analysis of the gene set prior to dsRNA stimulation can distinguish responsive from non-responsive cell lines, microarray data could potentially be used to identify dsRNA
responsive patients. As only four cell lines were tested, additional cell lines and patient samples will need to be examined to validate these findings.

To define signaling pathways required for dsRNA responsiveness, we investigated the known signaling pathways for activation status and subsequently inhibited these pathways to confirm contribution to dsRNA-induced apoptosis. We probed for activation of NF-κB via the phosphorylation status of p65. The ability of all the ovarian cancer cell lines to phosphorylate NF-κB p65 following dsRNA stimulation and increase expression of NF-κB-inducible genes, shows that this pathway is functional in all cell lines despite the low dsRNA receptor expression levels in non-responsive cell lines. As all cell lines activate this pathway, NF-κB most likely is not a major contributor to the dsRNA-induced apoptotic response.

In addition to activating NF-κB, previous studies have shown that dsRNA can induce a type I interferon response (147). Microarray after dsRNA treatment in responsive cell lines showed alterations in many IFN-inducible genes. In some cancer cell types, this pathway has been found to be essential for dsRNA-induced cell death, while in others it is not required (53, 54). In ovarian cancer cell lines that are responsive to dsRNA, we have shown that the type I interferon pathway is activated following treatment, however, when this pathway is blocked, the cell death does decrease but not to basal level. This indicates that this pathway is contributing to dsRNA-induced cell death, but that one or more other pathways are also contributing to dsRNA-induced cell death. Non-responsive cell lines are not able to activate the type I interferon response due to multiple inoperative points in the
pathway. The cell lines are not able to produce IFN-β mRNA or protein. In addition, if IFN-β is given exogenously, the cells still cannot respond even though their pathway components are functional. This indicates that the IFNα/βR is not operative, but whether this is due to mutation or altered expression or trafficking is unknown.

DsRNA is known to activate caspases, leading to an apoptotic response (148). This was also the case in the responsive cell lines where caspase 3 cleavage was identified, and the effector caspases 8 and 9 were found to be necessary for dsRNA-induced apoptosis. In the signaling pathway, RIP1 kinase associates with FADD that leads to the activation of caspase 8. When RIP1 kinase is ubiquitinated the RIP1 kinase-FADD complex leads to the activation of NF-κB. Non-responsive cell lines are unable to activate caspases possibly due to ubiquitination of RIP1 kinase by c-IAP2. DsRNA-induced expression of c-IAP2 has been reported for several cancer cell lines including SKOV-3. When pI:pC and a c-IAP2 inhibitor (RMT 5265) were coadministered, SKOV-3 showed increased caspase 8/3/7 activity. The authors concluded that for pI:pC to induce cell death the concomitant inhibition of c-IAP2 was required (149). In our studies, SKOV-3 uses this pathway but must also use additional survival mechanisms.

Previously, pl:pC-induced autophagy had been observed in melanoma that led to increased apoptosis (54). Both non-responsive cell lines increased autophagy after dsRNA stimulation while the responsive cells did not. In responsive melanoma cells, autophagy has been shown to result in cell death following dsRNA stimulation (54). In ovarian cancer cell lines the autophagy does not lead to cell death, even after a long-term (9 week) exposure to pl:pC or blockade of the classical autophagy pathway by 3-MA which
increases autophagy levels. Autophagy could serve as one of many mechanisms the non-responsive cells utilize to survive.

In addition to evaluating the contribution of components downstream of the dsRNA receptors, the importance of the receptors themselves was investigated. Previous studies in multiple cancer cell types have shown differing receptor contribution. Some studies show that the helicases are essential while TLR3 is not required (54, 84). Another identifies TLR3 as essential for dsRNA-induced cell death (53). In the responsive cell lines, PKR was found to be unnecessary for dsRNA-induced cell death. However, TLR3 was essential, with cell death decreasing significantly when TLR3 was knocked down. Although cell death does decrease, apoptosis levels are not basal, indicating involvement of additional receptors. One or both of the helicases RIG-I and MDA5 may contribute. Further shRNA experiments will be necessary to identify how each receptor adds to the dsRNA response.

Knowing that responsive cell lines significantly increase dsRNA receptor levels following stimulation, we overexpressed the receptors in non-responsive cell lines to determine if responsiveness could be restored. Indeed, the non-responsive cell lines did undergo dsRNA-induced cell death with increased receptor, indicating that upregulation of receptors is key to responsiveness and that all required apoptotic signaling pathways are functional.

Following the analysis of the mechanism of dsRNA-induced cell death in ovarian cancer cells, we tested the efficacy of this ligand in combination with other established chemotherapeutics (Chapter 3). We combined the innate immune ligand with a variety of chemotherapeutics with distinct apoptosis-inducing mechanisms. These drugs included
paclitaxel and carboplatin, which have shown great success in triggering cell death in ovarian cancer. In addition, we tested dsRNA in combination with vorinostat, a histone deacetylase inhibitor (HDACi) and sorafenib, a tyrosine kinase inhibitor.

Preliminary experiments with non-responsive cell lines showed that sorafenib did not increase cell death individually or in combination with other drugs, however, the cell lines were responsive to paclitaxel, carboplatin, and vorinostat. In addition to responding to these drugs individually, the addition of pI:pC enhanced the action of these drugs. After determination of the ED50 values for these drugs, drug interaction studies confirmed that pI:pC does in fact enhance the action of paclitaxel, carboplatin, and vorinostat. These data demonstrate that in ovarian cancer cells that are not responsive to dsRNA individually, this drug may be used to enhance the action of established chemotherapeutics.

In dsRNA-responsive cells, drug interactions, including synergy, additive, or antagonistic effects, could be assessed. When pI:pC was added to paclitaxel, carboplatin, and vorinostat, synergistic growth inhibition was observed. Considering the use of carboplatin and paclitaxel in front-line treatment of ovarian cancer, we tested the interaction of this combination but found only an additive effect. When either of these two drugs were combined with vorinostat however, synergy was observed in both cases. These data establish dsRNA as a potential combination chemotherapeutic with three established drug classes. Additionally, vorinostat could be used in combination with established ovarian cancer chemotherapeutics paclitaxel or carboplatin.

The ultimate goal of all novel chemotherapeutics is application in a clinical setting. Part of reaching this goal is determining the drug’s effectiveness in an animal model.
Future studies will include xenograft models using SKOV-3 and OVCAR-3 along with a syngenic model. A syngenetic model will examine how dsRNA in addition to a having direct effect on cell viability, may activate the immune system. Furthermore, understanding the mechanism of action for these combinations will help identify biomarkers that may in/exclude patients that would respond to these treatments. However, these experiments remain for future study. This dissertation adds to the expanding data on the efficacy of dsRNA as chemotherapeutic, both individually and in combination with established chemotherapeutics. In addition, this thesis also contributes to resolving the mechanism of dsRNA-induced apoptosis in ovarian cancer cells and the dsRNA receptors required for this response. This work provides the initial examination of dsRNA as a single or dual therapy agent to address chemoresistance in the silent killer, ovarian cancer.
Literature Cited
Literature Cited

7. www.ovariancancer.org. [cited; Available from:
32. Strausberg RL. Tumor microenvironments, the immune system and cancer survival. Genome Biol 2005; 6: 211.
73. Galabru J, Hovanessian A. Autophosphorylation of the protein kinase dependent on double-stranded RNA. J Biol Chem 1987; 262: 15538-44.


VITA

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