Exploring Novel Drug Treatments for Chemotherapy Resistance In Human Epithelial Ovarian Cancer (EOC)

by

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Abstract

Chemotherapy resistance in human epithelial ovarian cancer (EOC) is a significant reason for the high rate of death among patients. We hypothesized that chemotherapy-resistant EOC cells will be killed by novel drug treatments in non-adherent culture conditions. The objective of this study was to test the efficacy of novel drugs to affect platinum resistant EOC cell viability. To achieve this, the cell killing efficacy of several drugs were tested on drug-resistant EOCs growing in non-adherent cultures. Both EOC cell lines and primary EOC cells isolated from patient ascites were used for these studies. Two different classes of drugs were tested including multikinase inhibitors (dorsomorphin and LDN-193189), and an understudied class of novel chemotherapeutic agents called glycosylated antitumor ether lipids (GAELs). EOC cells were treated with the drugs at different doses alone or in combination with cisplatin. Because GAELs exhibited promising results in resistant EOC cells, the mechanism of GAEL-induced cell-death was evaluated.

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List of Abbreviations and Symbols

- Aldehyde dehydrogenase (ALDH)
- Alkyllysophospholipids (ALPs)
- Alkylphosphocholines (APCs)
- AMP kinase (AMPK)
- Antitumor ether lipids (AELs)
- Antioxidant 1, a copper chaperone protein (ATOX1)
- AT Rich Interactive Domain 1A (ARID1A)
- B-cell lymphoma 2 (Bcl-2)
- B-cell lymphoma-extra large (Bcl-XL)
- BCL2-Associated X Protein (Bax)
- B-cell lymphoma-extra short (Bcl-Xs)
- Bone morphogenetic protein (BMP)
- B-RAF proto-oncogene (BRAF)
- Breast Cancer Genes 1 and 2 (*BRCA1* & *BRCA2*)
- Copper transporting receptor 1 (CTR1)
- Dimethyl sulfoxide (DMSO)
- Deoxyribonucleic acid (DNA)
- Dorsomorphin (DM)
- Efflux transporters ATPase7A and ATPase7B (ATP7A & ATP7B)
- Epithelial Ovarian Cancers (EOC)
- Fetal bovine serum (FBS)
- Fibroblast growth factor-2 (FGF-2)
- Glycosylated antitumor ether lipids (GAELs)
- Ham's F12 nutrient mixture (DMEM/F12)
- High-grade serous ovarian cancer (HGSOC)
- Hepatocyte growth factor (HGF)

- Interstrand crosslink repair (ICLs)
- Kirsten rat sarcoma viral oncogene homolog (KRAS)
- LDN-193189 (LDN)
- Low-grade serous ovarian cancer (LGSOC)
- Mitogen-activated protein kinase 14 (MAPK14) (p38)
- Mitogen-activated protein kinase 8 (MAPK8) (JNK)
- Non-essential amino acids (1X NEAA)
- Nuclear antigen (Ki-67)
- Nucleotide excision repair (NER)
- Pan caspase inhibitor (QVD-OPh)
- Phosphatase and tensin homolog gene (PTEN)
- Protein kinase B (AKT)
- Serous tubal intraepithelial carcinoma (STIC)
- Serous tubal intraepithelial lesions (STILs)
- Small interfering ribonucleic acid (siRNA)
- Tumor protein 53 gene (TP53)
- Tubal intraepithelial carcinoma (TIC)
- Transforming growth factor (TGF)-β
- The three-dimensional system (non-adherent)

Chapter I: Introduction

1.1. Epithelial Ovarian Cancers (EOC)

EOCs are the most common type of ovarian cancer. There are five major histological subtypes of EOC: high grade serous ovarian cancer (HGSOC), low grade serous ovarian cancer (LGSOC), clear cell, endometrioid, and mucinous. Recently, differences in EOC prognosis have been reported between two subgroups: Type 1 that includes mucinous, LGSOC, low/moderate grade endometrioid, and clear cell, and Type 2 encompassing moderate/high grade serous, high grade endometrioid, undifferentiated, and malignant mixed mesodermal tumours (Lim et al., 2015). HGSOC is the most common subtype accounting for 70% of EOC, followed by 15, 10, 3% for clear cell, endometrioid, and mucinous, respectively (Anglesio et al., 2013; Köbel et al., 2014). LGSOCs are rare. HGSOC accounts for the most EOC mortality primarily because of the late stage at which it is diagnosed, and the common development of drug resistance after initial treatment with chemotherapy (Buys et al., 2011; McGuire et al., 1996). This thesis will evaluate novel drug treatments using different models of chemotherapy resistant EOC cells.

1.2. Stages of EOC

EOC is typically staged using the four FIGO (International Federation of Gynecology and Obstetrics) stages (I-IV) (Pereira et al., 2015). EOC in Stage I occurs only in one or both ovaries. Stage I has three grades (A-C) that classify the disease according to the location of the cancer whether in one ovary or both, inside the ovary or outside. Stage II is characterized by cancer growth extending to one or both ovaries

and involving the pelvic area. It includes three grades as well: A) cancer spread to the fallopian tubes or the uterus, B) cancer involving the bladder, rectum or other tissues in the pelvis, C) cancer invading pelvic tissues and creating fluid inside the abdomen (ascites). When cancer spreads outside the pelvis to the abdominal cavity, surrounding tissues where visual tumor growths can been seen, and to lymph nodes in the upper abdomen, that mean a patient reaches Stage III. Stage IV is the most advanced stage of EOC and can involve tissues and organs outside the peritoneal cavity, such as the lungs. Cancer cells can detach from the primary tumor and move within the peritoneal fluid or migrate via the lymphatic or vascular route to form metastases (Pereira et al., 2015).

1.3. Histological Site of Origin

Until recently it had been thought that all the subtypes of EOC arose from the ovary. There is strong evidence that most EOC subtypes develop from a different non-ovarian site suggesting that each subtype should be considered as a distinct disease, with specific morphology, markers and features that may require different treatment from other subtypes. Unfortunately, all EOCs are treated with the same drug cocktails. Thus, "epithelial ovarian cancer" is an inaccurate term describing these different diseases. Specific histologic strategies have been developed to identify each subtype for accurate diagnoses (Köbel et al., 2014).

1.3.1. Mucinous. Some investigators suggest that mucinous cancer cells spread from gastrointestinal or cervical adenocarcinomas to the ovary as a target site for metastatic

spread (Furuya, 2012; Young, 2007). However, the tissue of origin for this subtype is still unclear. Compared with other subtypes, a high percentage of mucinous EOCs have mutations in kirsten rat sarcoma viral oncogene homolog (KRAS), an essential gene in tissue signaling, and so can be used as a molecular biomarker for mucinous tumours (Enomoto, Weghorst, Inoue, Tanizawa, & Rice, 1991; Furuya, 2012; Gemignani et al., 2003; Ichikawa et al., 1994).

1.3.2. Endometrioid and clear cell. There is excellent correlative evidence that endometrioid and clear cell EOC arise from cells associated with endometriosis, a disorder allowing endometrial tissues to grow outside the uterus (Debra a Bell, 2005; R. Kurman & Shih, 2010). Endometrioid and clear cell EOC commonly have mutations in AT Rich Interactive Domain 1A (ARID1A), a regulator of chromatin structure, at 30% and 57%, respectively (Jones et al., 2010; Wiegand et al., 2010). Additionally, half of endometrioid cancers contain mutations in the phosphatase and tensin homolog (PTEN) gene (Catasús et al., 2004; Obata et al., 1998). PTEN functions as tumor suppressor gene by down regulating specific intracellular pathways, most notably for EOCs the PI3K/AKT pathway (Catasús et al., 2004; Obata et al., 1998).

1.3.3. LGSOC and HGSOC. Within the past 10 years the epithelial lining of the fimbrial portion of the fallopian tube has been identified as a major source of HGSOC (Domcke, Sinha, Levine, Sander, & Schultz, 2013; R. J. Kurman, 2013; Reade et al., 2014). Cellular changes and mutations in LGSOC differ than those in HGSOC,

suggesting that LGSOC has a different cellular origin, possibly the epithelial lining on the surface of the ovary (Sato et al., 2000). Generally, LGSOC accounts for only 10% of serous carcinoma and account for a small percentage of the mortality rate (R. J. Kurman, 2013). Mutations in KRAS and the B-RAF proto-oncogene (BRAF) are commonly found in LGSOC, but not in HGSOC (Singer et al., 2003; Vang et al., 2015). By contrast, mutations in tumor protein 53 (TP53) are a particular marker for HGSOC. 96% of HGSOC contains mutations in *TP53* (The Cancer Genome Atlas; (D. Bell et al., 2011). Most of the literature has focused on HGSOC, therefore, I will discuss this subtype more extensively.

Answering why and how HGSOC arises in fallopian tube, but appears on or in the ovary, thus the assumption that these tumours were "ovarian" cancers, is a controversial question with many viewpoints, including:

1) Because the fallopian tube fimbriae is very close to the ovarian surface, premalignant cells or EOC cells are thought to easily shed and transfer from the epithelium surface of the fimbriae to implant on the epithelium surface of ovary. Such implantations cause malignant cysts on the surface, which then can invade into the ovary for further tumor development. Alternatively, premalignant cells may be altered specifically because of the high level of ovarian hormones, which facilitate metaplastic changes (Dubeau & Drapkin, 2013; Furuya, 2012). In cases where there is no ovarian involvement, EOC cells may detach from the fimbriae into the peritoneum resulting in peritoneal metastases.

2) Another perspective says that the ovaries are the best environment for HGSOC, as well as other subtypes, because some cells in the ovarian stroma are very sensitive to molecules secreted by tumor cells originating in the fimbria, thus promoting stromal cells to surround EOC cells to stimulate implantation and proliferation (Kuhn et al., 2012; R. J. Kurman, 2013). Other stromal cells become active after implantation and secrete ovarian hormones, e.g. steroid hormones, which further enhance EOC cell proliferation. (Kuhn et al., 2012; R. J. Kurman, 2013).

1.4. Main Genetic Changes in HGSOC

1.4.1. TP53 mutations

Mutations in TP53 are an important feature in HGSOC because almost all (96%) contain mutations in the *TP53* gene (The Cancer Genome Atlas; (Bell et al., 2011). *TP53* is a tumor suppressor gene that encodes p53 protein. p53 regulates specific cellular events such as cell cycle, deoxyribonucleic acid (DNA) repair, and apoptosis (Vousden & Lu, 2002). p53 has DNA surveillance activity that examines whether the damaged DNA can be repaired or if the cell will undergo cell death by apoptosis. Therefore, controlling these important functions prevents cells from developing cancer. Alterations in *TP53* gene contribute widely to various human cancers including inherited cancers (Vousden & Lu, 2002).

Although we know that almost all HGSOC contains *TP53* mutations (Bell et al., 2011), only a small number of the mutations have been characterized. Some investigators suggest that *TP53* mutations should be classified according to their

function in the EOC cells as wild type, partial loss and loss of wild type function (Brachova, Thiel, & Leslie, 2013). However, there is no specific mechanism illustrating how p53 mutations contribute to HGSOC.

1.4.2. BRCA1 and BRCA2 mutations

The Breast Cancer Genes 1 and 2 (*BRCA1* & *BRCA2*) produce proteins used by cells for DNA repair (Roy, Chun, & Powell, 2011). During DNA repair, these two proteins are involved in the homologous recombination process; they use an un-damaged copy of the gene to repair DNA with double-strand breaks. The process is important for maintaining cell integrity during proliferation. Mutations in BRCA1 and BRCA2 increase the risk of developing diseases such as ovarian and breast cancer (Miki et al., 1994; Wooster et al., 1995). Individuals with inherited *BRCA1* and *BRCA2* mutations have an increased lifetime risk of developing ovarian cancer by 30-50%, and 50-80% for developing breast cancer (Foulkes, 2008; Roy et al., 2011).

While a wide variety of additional genes, putative oncogenes and tumour suppressor genes, have been examined, none have been shown to contribute substantially to the development of familial or sporadic HGSOC.

1.5. HGSOC Tumor Progression

A potential mechanism that describes the serial events of tumor progression suggests that p53 loss of function is the first initiator of HGSOC, followed by BRCA inactivation (Bowtell, 2010). As mentioned, BRCA proteins are critical for DNA damage and repair, thus BRCA inactivation influences genomic integrity, resulting in

chromosomal instability such as DNA copy number changes, which may cause changes in gene expression that promote tumor growth (Bowtell, 2010).

1.5.1. HGSOC Tumor Initiation

By sectioning fallopian tube specimens of women carrying BRCA mutations, researchers detected some early changes in HGSOC progression (Medeiros et al., 2006; Reade et al., 2014). In the distal fimbrial portion of the fallopian tubes, they found lesions with cellular dysplasia and hyperplasia that resembled HGSOC. An abnormal cellular event called "secretory cell outgrowths", consisting exclusively in fallopian tube secretory cells, present with a normal appearance and have low expression of PTEN and a low Ki-67 index with no *TP53* mutations (E. Y. Chen et al., 2010; Koshiyama, Matsumura, & Konishi, 2014; Roh et al., 2010). However, these cells can transform from benign to malignant with *TP53* mutations as a next step, identified as "serous tubal intraepithelial lesions (STILs)" (E. Y. Chen et al., 2010; Koshiyama et al., 2014). These lesions in the tubal epithelium, termed "tubal intraepithelial carcinoma (TIC)" or "serous tubal intraepithelial carcinoma (STIC)," or "serous intraepithelial carcinoma," positively express p53 and nuclear antigen (Ki-67) in a pattern resembling HGSOC (Medeiros et al., 2006).

1.5.2. Environmental Changes in HGSOC

Invasive EOC cells usually alter the surrounding environment in order to adapt, proliferate, invade and survive. Malignant cells communicate with stroma through a complicated network of cytokine activities, including hepatocyte growth factor (HGF),

fibroblast growth factor-2 (FGF-2), and transforming growth factor (TGF)-β. These factors may activate cancer-associated fibroblasts by specific intracellular signals and thus promote invasion and proliferation (Furuya, 2012; Xouri & Christian, 2010). Stromal changes help EOC cells to evade anti-tumor actions or treatments (Furuya, 2012; Vaughan et al., 2011).

1.6. EOC Chemotherapy Treatment

Patients with EOC are treated with 6 to 9 cycles of a platinum agent (cisplatin or carboplatin) and a taxane (paclitaxel or docetaxel) and/or surgery to remove tumor(s). Epithelial ovarian cancer is sensitive to platinum chemotherapy in early stages, where 85% of EOCs patients experience positive results; however, up to 75% of the same patients relapse. With their relapse comes chemoresistant disease forcing them to undergo secondary treatments (McGuire et al., 1996; Sherman-Baust, Becker, Wood III, Zhang, & Morin, 2011). EOC patients are classified into three groups according to their response to re-treatment:

1) Platinum resistant group: EOC patients who relapse within 6 months after completing initial treatment, wherein 80% do not respond to the second treatment.

2) Platinum sensitive group: those who relapse after 12 months of completing initial treatment, wherein 40% do not respond to re-treatment.

3) Platinum partly sensitive group: patients who relapse between 6 and 12 months, wherein 73% do not respond to the second use of chemotherapy (Bookman, 1999; Cooke & Brenton, 2011).

1.6.1 Cisplatin Mechanism of Action

The anti-tumor action of platinum agents, e.g. cisplatin, is generally a result of the molecule forming crosslinks or adducts in DNA (Gonzalez, Fuertes, Alonso, & Perez, 2001), and thus interfering with DNA replication mechanisms and triggering cell death (Fuertes, Alonso, & Pérez, 2003; Jamieson & Lippard, 1999). These drugs retain their original structures outside the cells because of the high level of chloride, which blocks the formation of mono- and diaquo cis-Pt(II) species. In the low chloride environment inside the cells, platinum agents start to form mono- and diaquo cis-Pt(II) structures, by which one or two chloride groups will be replaced with water. The final structure is $[Pt(H2O)2(NH3)2]^{2+}$ cation, which can easily react with different sites on DNA causing intrastrand and interstrand cross-links (Fichtinger-Schepman, van der Veer, den Hartog, Lohman, & Reedijk, 1985; Kelland, 2007; Miller & House, 1991). Crosslinks can lead to DNA breaks. Failure to repair these cross-links and breaks leads cells to undergo cell death via apoptosis (Barry, Behnke, & Eastman, 1990).

1.7. Chemotherapy Resistance

Chemotherapy resistance reduces the survival rate of EOC patients (Buys et al., 2011). Acquisition of chemotherapy resistance is a result of one of several mechanisms to avoid cell death. Some of these mechanisms include an increase in drug efflux or a decrease in drug influx transporters, enhanced DNA repair activity, and changes in proteins involved in the apoptotic pathways (Howell, Safaei, Larson, &

Sailor, 2010; Köberle, Tomicic, Usanova, & Kaina, 2010; Palm et al., 2011). Each of these mechanisms will be discussed briefly.

1.7.1. Mechanisms of Developing Chemotherapy Resistance in EOCs

1.7.1.1. Influx Transporters

Platinum agents are transferred into cells via influx transporters such as copper transporting receptor 1 (CTR1). CTR1 is a copper transporter localized in the plasma membrane and works as a homotrimer; each molecule has three transmembrane domains forming a pore, which is the channel for copper molecules (Howell et al., 2010). CTR1 has been shown to transport platinum drugs into the cells via methionine residues located in the transmembrane domain of CTR1. Methionine binds to the metal-sulfur bonds of platinum agents (Howell et al., 2010).

A decrease in cisplatin concentration inside cancer cells by 20-70% contributes to chemotherapy resistance of a variety of cancer including EOC (Holzer, Manorek, & Howell, 2006; Siddik, 2003; Song et al., 2004). This is a result of a decrease in influx transporter factors such as CTR1. Platinum resistance may also arise from a low amount of drug uptake because of the degradation of CTR1 by micropinocytosis, mislocalization of influx transporters (Howell et al., 2010), or low expression of CTR1 (Shen, Pouliot, Hall, & Gottesman, 2012). While the overexpression of CTR1 can increase sensitivity to platinum drugs, the complete down regulation of CTR1 significantly increased resistance with no response to the chemotherapy (Larson, Blair, Safaei, & Howell, 2009). Mouse embryonic fibroblasts that are CTR1-/- are resistant

to platinum chemotherapy by 3.2 fold compared to CTR1+/+ cells. CTR1-/- cells show resistant to carboplatin by 2 fold compared with CTR1+/+ cells (Holzer et al., 2006).

Antioxidant 1 (ATOX1), a copper chaperone protein, also contributes to drug resistance indirectly (Howell et al., 2010). ATOX1 is important for the transferring of CTR1 towards the plasma surface, in addition to participating in CTR1 degradation. ATOX1 decreases the level of platinum agents inside the cells via two different ways: 1) polyubiquination and downregulating of CTR1; 2) binding to drugs once they enter the cells and transferring them to the efflux transporters ATPase7A and ATPase7B (ATP7A & ATP7B) (Howell et al., 2010). Cells from ATOX1-/- mice have shown low concentrations of cisplatin inside the cells compared to cells from ATOX1+/+ mice; however, ATOX1-/- cells did not affect the resistance ability significantly (Safaei, Maktabi, Blair, Larson, & Howell, 2009). ATOX1-/- cells were not able to downregulate or ubiquinate CTR1, suggesting that platinum drug transport is associated with ATOX1 activity (Howell et al., 2010).

1.7.1.2. Efflux Transporters

ATP7A & ATP7B are efflux transporters that can pump platinum drugs from inside to outside cells. The main function of ATP7A & ATP7B is to transfer copper between the Golgi apparatus and the plasma membrane. Overexpression of ATP7A & ATP7B is associated with developing chemoresistance (Palm et al., 2011). Patients with tumors that express high ATP7B progress poorly compared to those with low

expression (Nakayama, Miyazaki, Kanzaki, Fukumoto, & Takebayashi, 2001). Moreover, patients with EOC that expressed high levels of ATP7A have a low survival rate compared to EOC patients with low expression level of ATP7A (Katano et al., 2002).

ATP7A has been measured at the protein level in the A2780-cp and 2008/C13 cisplatin-resistant EOC cell lines and was found to be overexpressed. Note that these cell lines have been treated gradually with different graded doses of cisplatin to become resistant. Therefore, these cells may use more than one mechanism to acquire resistance (Katano et al., 2002).

1.7.2 DNA Repair Mechanisms Contribute to Chemotherapy Resistance

1.7.2.1. Excision lesion of DNA

Chemo-resistant cells may use different DNA repair mechanisms in order to fix damage caused by treatment. One mechanism is by removing the lesions in the DNA. Cisplatin-resistant cell lines have higher capacity for removing damaged DNA lesions and DNA repair mechanisms compared to cisplatin-sensitive cell lines. In the A2780 EOC cell line, increased resistance to cisplatin was found to be a result of increased removal of damaged DNA lesions (Johnson et al., 1994; Parker, Eastman, Bostick-Bruton, & Reed, 1991). Thus, removing damaged DNA induced by cisplatin appears one mechanism to enhance EOC chemotherapy resistance.

1.7.2.2. Nucleotide excision repair (NER)

Damaged DNA lesions are removed by nucleotide excision repair (NER), which is the main pathway that fixes damage caused by chemotherapy drugs (Batty & Wood, 2000; Gillet & Schärer, 2006). NER can recognize the damage on DNA and cut the lesion from both sides, followed by DNA synthesis (Aboussekhra et al., 1995). Cisplatin resistant A2780-cp cells had a higher level of NER higher than cisplatin sensitive A2780-s cells, suggesting that NER plays a role in resistance (Ferry, Hamilton, & Johnson, 2000). Cell lines exposed to different doses of cisplatin gradually in order to acquire resistance show higher levels of resistance due to multiple changes, i.e. genetic mutations, that occur during exposure, so the resistance phenotype is not related to one change or one factor only, such as increased expression of NER (Köberle et al., 2010). Thus, this may be different from cisplatin-resistant disease identified in patients.

1.7.2.3. Interstrand Crosslink Repair

In addition to NER, resistant cells use interstrand crosslink repair (ICLs) to fix damage caused by cisplatin (McHugh, Spanswick, & Hartley, 2001), and thus enhance the resistance phenotype. The mechanism of how ICL damage is removed is not well understood (Köberle et al., 2010). An increase in ICL repair activity was observed in cisplatin-resistant EOC cell lines (Zhen et al., 1992). Moreover, EOC tissues have been obtained from patients before chemotherapy treatment and from patients who relapse after completing platinum treatment, and were found to have an increase in

ICL repair activity in tissues from resistant patients compared to sensitive patients (Wynne et al., 2007). This suggests that increased ICLs repair is associated with chemoresistance in EOC (Wynne et al., 2007).

1.7.2.4. Anti-apoptotic Proteins in EOC

Anti-apoptotic proteins were shown to be involved in developing chemotherapy resistance to cisplatin in EOC cell lines (X. Yang et al., 2004). The expression of proteins associated with the apoptotic process (e.g. caspase-3 activity) have been measured in cisplatin-resistant and cisplatin-sensitive A2780 cell lines. They found that the expression of anti-apoptotic proteins, B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-XL), were very high in cisplatin-resistant A2780-cp cells compared to cisplatin-sensitive A2780-s cells. However, the pro-apoptotic proteins, BCL2-Associated X Protein (Bax) and B-cell lymphoma-extra short (Bcl-Xs), showed no difference between cisplatin-resistance and cisplatin-sensitive A2780 cells. This data suggests that the overexpression of Bcl-2 and Bcl-XL, the anti-apoptotic proteins, may contribute to chemotherapy resistance in EOC (X. Yang et al., 2004).

1.7.3. Stem Cell Theory

One aspect of research into cancer cell chemoresistance is the correlation between chemoresistance and cancer stem cells. These cells have been shown to initiate or sustain tumorigenesis, and are resistant to conventional therapies (Kreso & Dick, 2014). Putative EOC stem cells that are present in EOC patient samples and cell lines, can be isolated using different stem cell markers, have the ability to produce new

tumours, and typically exhibit chemotherapy-resistance (Ayub et al., 2015; Kreso & Dick, 2014; Kryczek et al., 2012; Stewart et al., 2011). Aldehyde dehydrogenase (ALDH) is a stem cell marker consistently used to isolate populations of EOC stem cells (Ayub et al., 2015). Studies have demonstrated that ALDH expressing EOC cells are more resistant to chemotherapy; however, the mechanism by which these cells are resistant is not well understood (Guddati, 2012; Meng et al., 2014). In this project, the ALDH marker was used to sort and isolate cells to examine distinct populations within the human EOC A2780 cell lines.

1.8. The Model System

Although, the majority of the literature has used the two-dimensional monolayer system as a model for testing anti-cancer therapies, the success of treatment in clinical trials is about 5% only (Ashworth et al., 2008). Using the monolayer system often shows vast promising results *in vitro*; however, these activities fail *in vivo*, confirming that the two-dimensional system does not reflect the actual environment in a patient. Cells grown as monolayers respond differently to chemotherapy compared to how cells respond in the *in vivo* environment, and activate different signaling pathways that allow the cells to be sensitive to anti-cancer agents during treatment (Ma et al., 2011; Smalley et al., 2006). In the EOC patient's peritoneal cavity, cancer cells can disseminate from the primary tumor, and flow freely in peritoneal fluid as aggregates or spheroid-like structures (Allen, Porter, Gamarra, Piver, & Johnson, 1987; Ernst Lengyel, 2010; Naora & Montell, 2005). These cells can travel and invade distant

organs as well as peritoneal mesothelial cells, which then form secondary tumor growths (H. A. Kenny, Nieman, Mitra, & Lengyel, 2011; E Lengyel et al., 2014). Thus, the two-dimensional system is a very simple model that does not mimic the complexity of the *in-vivo* microenvironment, and is not a strong model for drug discovery and development.

On the other hand, the three-dimensional (non-adherent) system is a better model that more closely reflects the 3-dimensional *in vivo* environment (Ghosh et al., 2005; Gómez-Lechón et al., 1998; P. A. Kenny et al., 2007; E Lengyel et al., 2014). Testing drug treatments using this system may decrease the gap between laboratory research and clinical trials, and improve the outcomes of EOC patients. Recently, several studies used the non-adherent system as an experimental model to study various aspects of EOC, such as the EOC microenvironment, stem cell markers, and evaluating drug resistance and developing anti-cancer agents. Importantly, cells in non-adherent cultures exhibited frequently higher levels of chemoresistance compared to adherent conditions (Junsong Chen et al., 2013; Dong et al., 2010; Lee et al., 2013; Loessner et al., 2010; Z. Yang & Zhao, 2011). Pictures of some EOC cell lines and primary cells growing in adherent and non-adherent conditions are included (Figure 1-1, 1-2 & 1-3). Thus, the non-adherent system was selected as the main condition used for the majority of the experiments in this project by growing EOC cells in low-attachment plates. Adherent cultures were used when needed for comparison purposes.

1.8.1. EOC cell lines and primaries and their chemotherapy resistance status

One of the most popular cell-based models of EOC drug-resistant available for research is the EOC endometrioid subtype A2780-s (sensitive) and A2780-cp (cisplatin resistant) isogenic cell lines (Louie et al., 1985). A2780-cp cells were developed by exposure of A2780-s cells to increasing doses of cisplatin. Cisplatin sensitivity was validated for all cells lines, and are shown in this Introduction as a validation of the model system used in this thesis. A2780s cells were effectively killed by platinum chemotherapy (cisplatin), whether in adherent or non-adherent conditions (Figure 1-4). Cisplatin treatment decreased cell viability of A2780cp effectively when growing in adherent cultures, but not in non-adherent cultures, where A2780cp cells exhibited high level of resistance (Figure 1-4). A2780cp cells grown in non-adherent conditions were killed by increasing the doses of cisplatin up to 90 μM (Figure 1-5). A second cell line used for these studies was the COV362 cell line, which was derived from a HGSOC. By contrast to A2780 cells, the cisplatin sensitivity profile of COV362 cells showed the same trend whether cells were in adherent or non-adherent conditions (Figure 1-6 & 1-7). Surprisingly there was no complete cell death induced by cisplatin for doses up to 90 μM, suggesting that they are even more platinum resistant than A2780-cp cells.

In addition, several primary EOC cells were isolated from ascites (abdominal fluid containing cancer cells) isolated from chemoresistant patients with different subtypes (Shepherd, Thériault, Campbell, & Nachtigal, 2006). The **EOC140** sample was

obtained from a patient with HGSOC, who received several cycles of paclitaxel in addition to carboplatin treatment. She had recurrent disease and received second-line of chemotherapy, and there was no indication of drug resistance clinically. Cisplatin-treated EOC140 cells were sensitive to the low doses of cisplatin, with high effect starts at 10 μM to 90 μM (Figure 1-8). **EOC126** cells were isolated from a patient with clear cell EOC who had received paclitaxel and carboplatin treatment. Her chemoresistance status was not noted in the clinical chart; however, EOC126 cells exhibited resistance to all doses of cisplatin treatment under non-adherent condition *in vitro*, and partially sensitive using high doses with adherent cells (Figure 1-8). **EOC146** and **EOC103E** EOC samples were isolated from patients with HGSOC. Their platinum chemotherapy status was not determined. A summary of EOC cell lines and primary EOC cells in this project is included (Figure 1-9).

1.9. Thesis Objectives and Hypothesis

Given the high rate of chemotherapy resistance in the EOC patient population, and the lack of drugs to effectively clinically manage the recurrent, resistant disease, the goal of this thesis was to provide preclinical data on the use of novel drugs that might be used as chemotherapies for drug resistant EOC. My hypothesis is that the novel drugs will effectively kill drug-resistant EOC cells, including putative EOC stem cells. In particular, the kinase inhibitors dorsomorphin and LDN-193189, and two glycosylated antitumour ether lipids (GAELS) – GLN and MO-101 – were tested in this thesis. Each of these drugs will be introduced further in the respective chapters

where the experiments are described. Chemo-sensitive and –resistant EOC cells grown as non-adherent cultures were used to test this hypothesis.

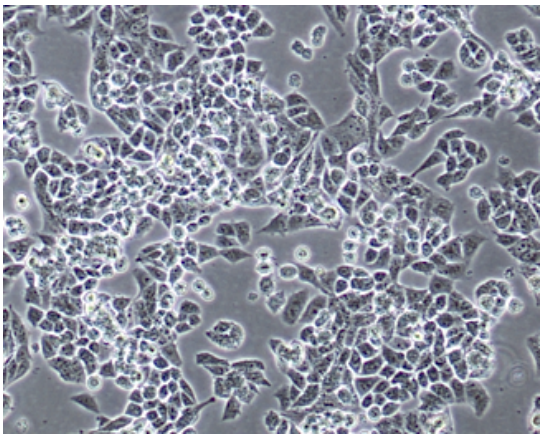
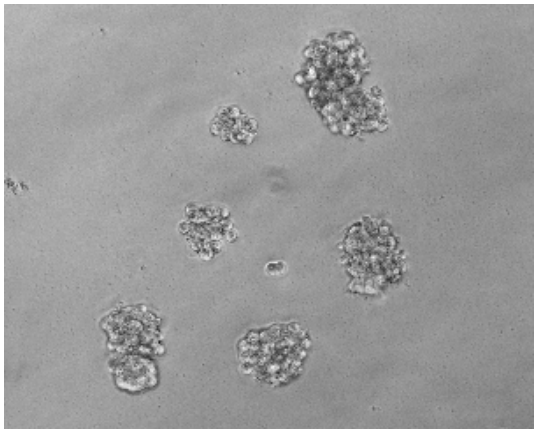
A.  B. 

Figure 1-1. **A.** Adherent and **B.** non-adherent pictures of A2780s cells

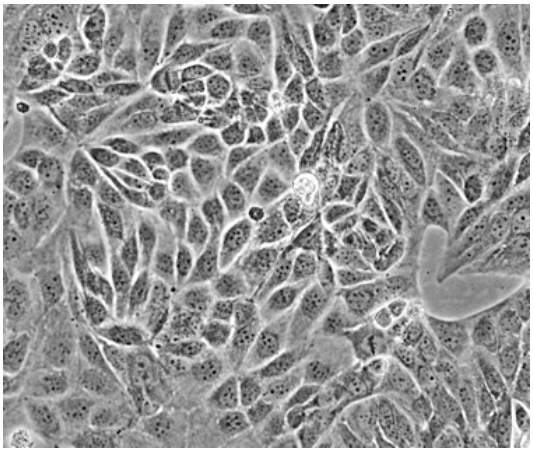
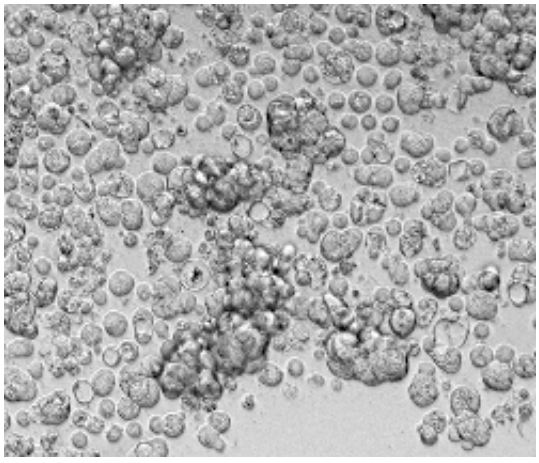
A.  B. 

Figure 1-2. **A.** Adherent and **B.** non-adherent pictures COV362 cells

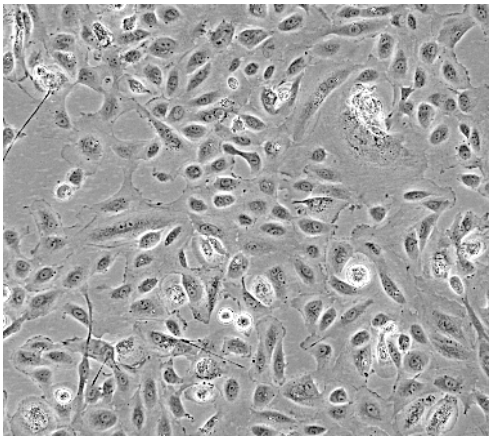
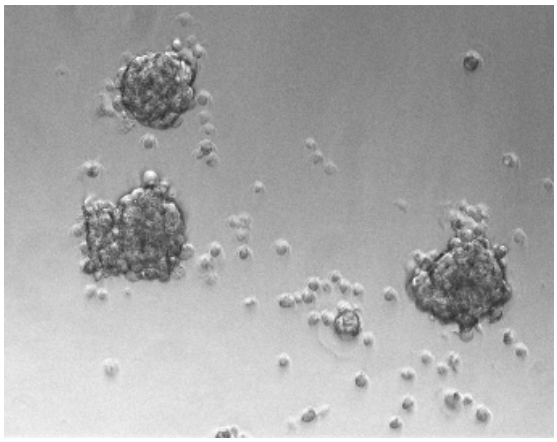
A.  B. 

Figure 1-3. **A.** Adherent and **B.** non-adherent pictures EOC126 cells

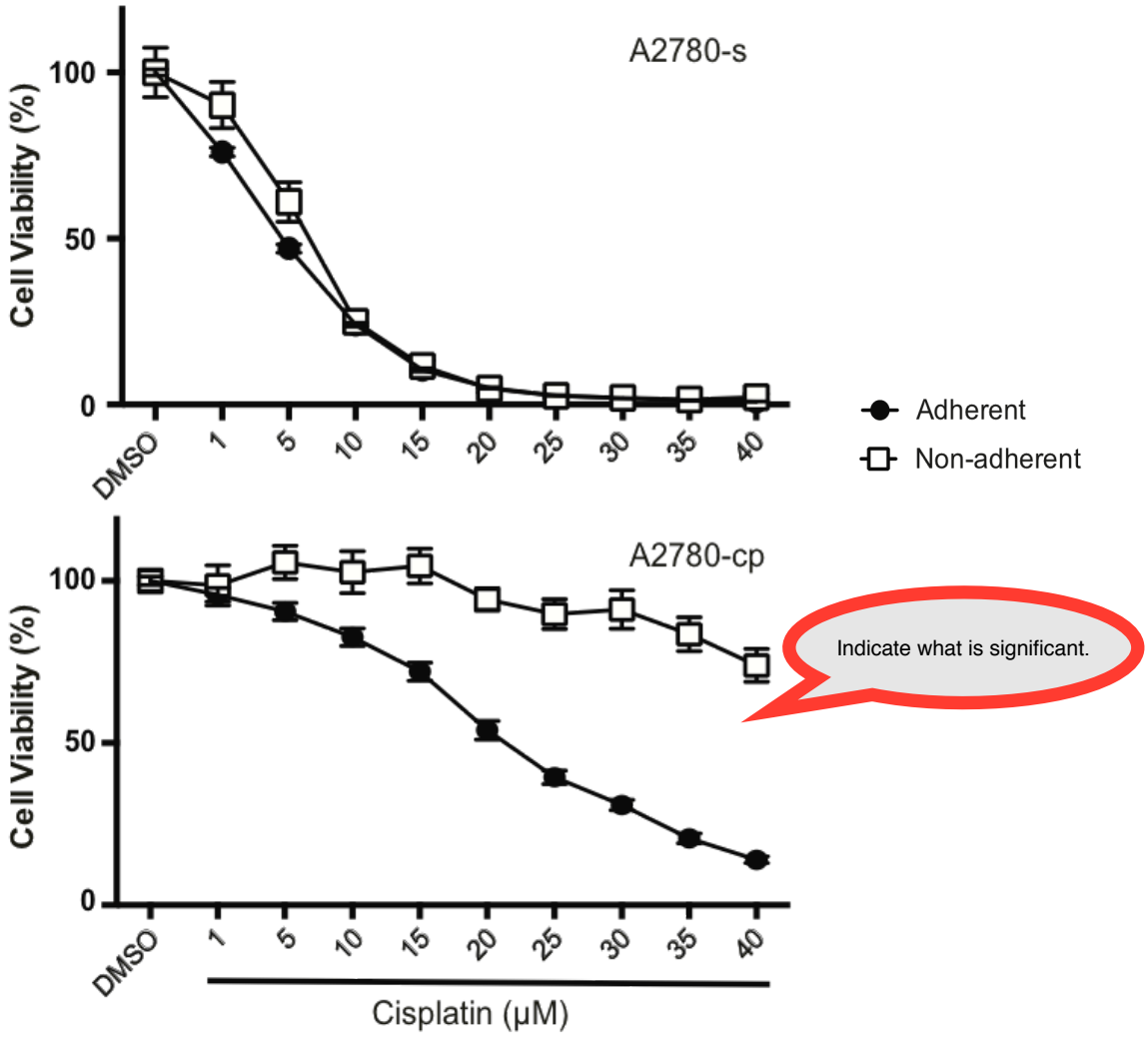


Figure 1-4. Adherent/Non-adherent A2780s/A2780cp cells treated with different doses of cisplatin for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

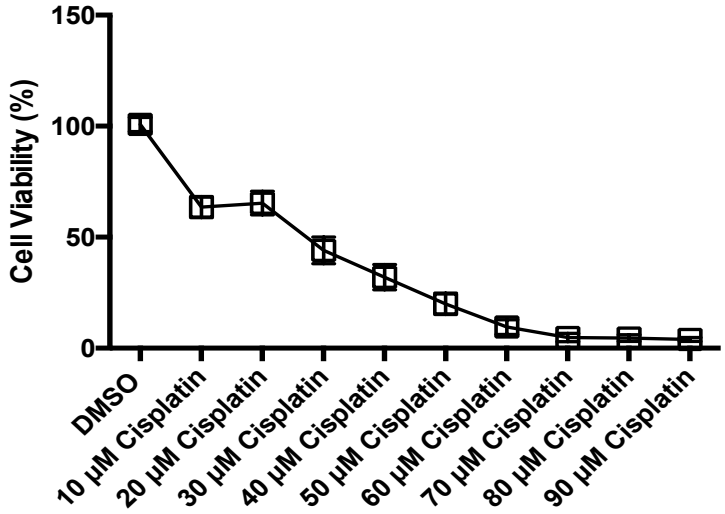


Figure 1-5. Non-adherent A2780cp cells treated with different doses of cisplatin up to 90 µM for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

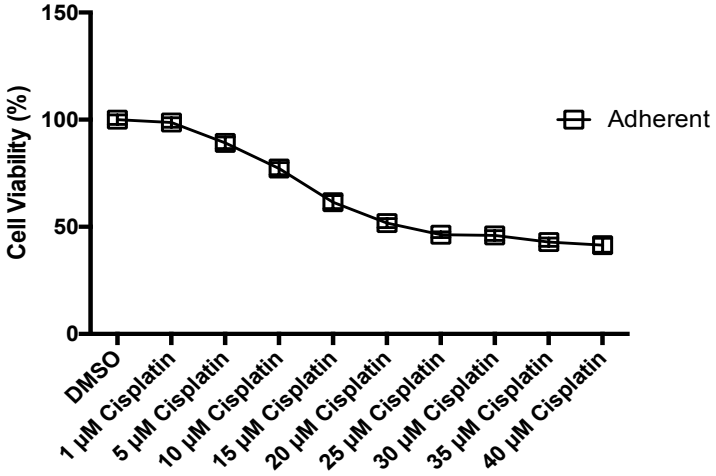


Figure 1-6. Adherent COV362 cells treated with different doses of cisplatin for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

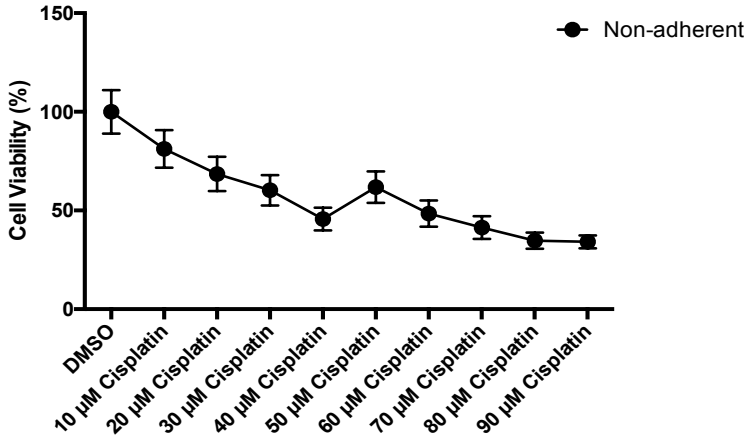


Figure 1-7. Non-adherent COV362 cells treated with different doses of cisplatin for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

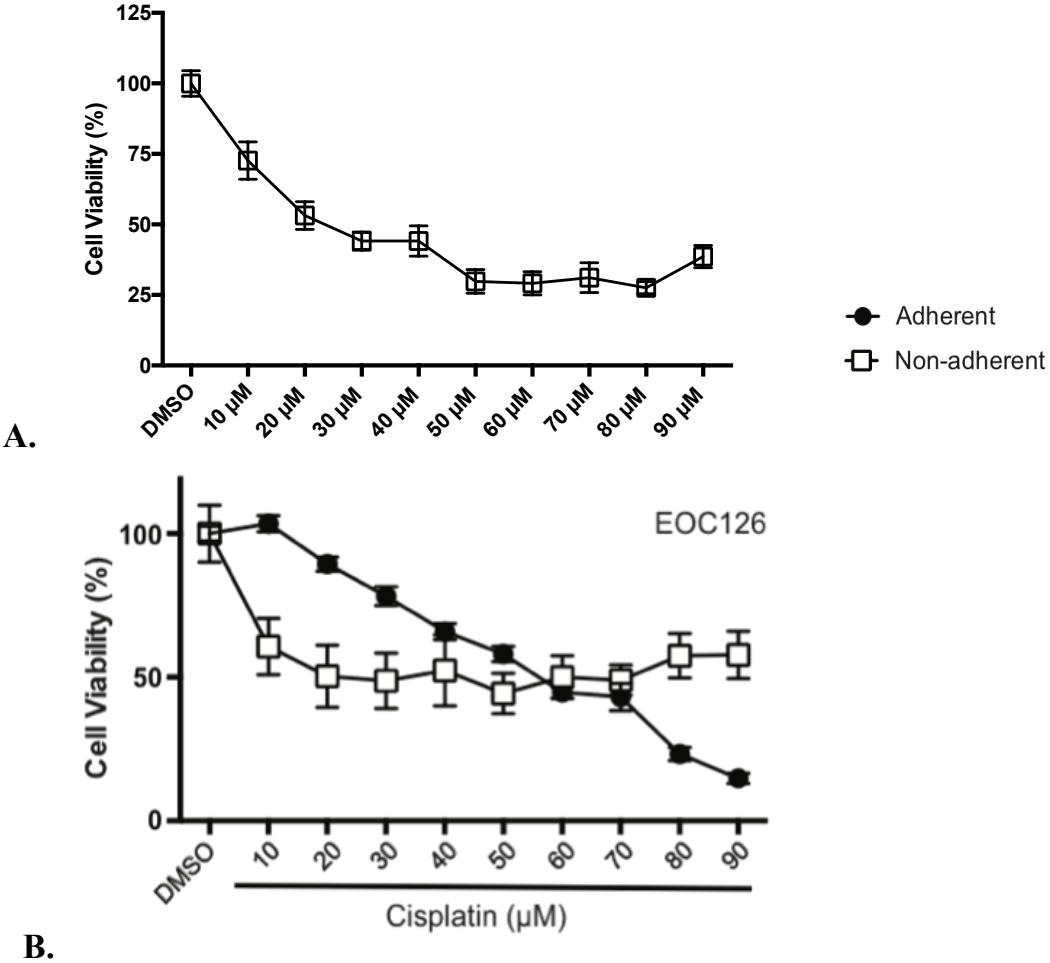


Figure 1-8. A. Non-adherent EOC140 and **B.** Adherent/Non-adherent EOC126 cells treated with different doses of cisplatin for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

EOC Cell lines + Patient Samples	**EOC subtype**	**Genetic Mutations**	**Chemoresistance Status**
A2780	Endometrioid	PIK3CA, PTEN, BRAF, CTNNB1 & ARID1A *	A2780-s: cisplatin sensitive. A2780-cp: cisplatin resistant
COV362	HGSOC *	TP53 & BRCA1 *	Cisplatin resistant
EOC 126	Adenocarcinoma	_	Not determined
EOC 146	HGSOC	_	Not determined
EOC 140	HGSOC	_	Not determined
EOC 103E	HGSOC	_	Not determined

Figure 1-9. Summary of EOC cell lines and primary EOC cells
* (Domcke, Sinha, Levine, Sander, & Schultz, 2013)

Chapter II: Materials and Methods

2. Materials and Methods

2.1. Cell Culture.

2.1.1 Cell Lines. Human endometrioid EOC cells comprised of cisplatin-sensitive A2780s and cisplatin-resistant A2780cp lines were obtained from Dr. B. Tsang (University of Ottawa). Cells were maintained in serum containing medium unless otherwise stated: Dulbecco's modified Eagles medium, Ham's F12 nutrient mixture (DMEM/F12) in addition to 10% fetal bovine serum (FBS) and antibiotics (1x penicillin/streptomycin). A2780 cells between passage 16 to 26 were used to perform experiments. The high-grade serous EOC cell line COV362 was obtained from the European Collection of Cell Culture, distributed by Sigma-Aldrich. COV362 were maintained in serum containing medium unless otherwise stated: DMEM/High Glucose supplemented with non-essential amino acids (1X NEAA), 10% FBS and antibiotics (1x penicillin/streptomycin). Experiments with COV362 cells were performed between passages 41 and 48. All cells were maintained at 37 °C, 5%CO2/95% air, 100% humidity.

The latest authentication of these cell lines was conducted in June 2016 using short tandem repeat profiling on the Promega PowerPlex system.

2.1.2 Patient Samples. Before conducting experiments with primary human EOC cells, institutional approval for research with human materials was received (University of Manitoba, #HS12920), and samples were used after receiving Informed Consent from patients. Primary human EOC cells were isolated from ascites fluid

obtained from patients with stage III or IV high grade serous adenocarcinoma, and grown in serum containing medium: DMEM/F12, 10% FBS and antibiotics (1x penicillin/streptomycin) as previously described (Shepherd et al., 2006). Experiments with human EOC cells were performed between passages 1 and 6. EOC cells were maintained at 37 °C, 5%CO2/95% air, 100% humidity.

2.2. Drugs.

2.2.1 Cisplatin, dorsomorphin (DM), LDN-193189 (LDN). CP and DM were obtained from Tocris Bioscience, and LDN from Selleck Chemical. These drugs were reconstituted in 100% dimethyl sulfoxide (DMSO), which was used as the vehicle control.

2.2.2 Glycosylated antitumor ether lipids (GAELs)

GAELs were synthesized in and obtained from Dr. Frank Schweizer's laboratory (Department of Chemistry, University of Manitoba): compound A 1-*O*-hexadecyl-2-*O*-methyl-3-*O*-(2'-deoxy-2'-amino-β-D-glucopyransoyl)-sn-glycerol **(β-D-GLN)**; compound B 1-*O*-hexadecyl-2-*O*-methyl-3-*O*-(2'-amino-2'-deoxy-α-D-glucopyranosy)-sn glycerol **(α D-GLN)**; compound C 1-*O*-Hexadecyloxy-2R-(–α-L-rhamnopyranosyl)-3-amino glycerol **(Rham-GAEL)**; compound D 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2',6'-diamino-2',6'-dideoxy–α-D-glucopyranosyl)-sn-glycerol **(MO-101)**; and compound E 1-*O*-Hexadecyloxy-2R-*O*-methyl(2'amino-2'deoxy–α-L-

glucopyranosyl)-3-amino glycerol **(L-GLN)**. All were dissolved in 95% Ethanol, which used as a vehicle control.

2.3. Dose-Response Assays

2.3.1 Adherent Cultures. EOC cell lines or primary EOC cells were passaged in T75 flasks. For experiments, cells were plated at 4000 cells per well of a 96-well plate and grown for 24 hours before adding increasing doses of the drugs. Cells were treated with drugs for the time pints shown in the figures.

2.3.2 Non-Adherent Cultures. Cells were plated at 10000 cells per well of a 96-well ultra-low attachment plate (Cellstar, 96 well, cell-repellent surface) and grown for 48 hours to allow time to form cell aggregates, reminiscent of how the cells appear in ascites. Cells were treated with drugs for the time points shown in the figures.

2.3.3 Cell viability measurement. Cell viability was measured using a modified tetrazolium assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega Corporation). Twenty microliters of the Cell Titer 96 Aqueous One Solution was added into each well, incubated for 1 hour for adherent plates, or for 2-3.5 hours in case of non-adherent plates at 37°C, 5%CO2/95% air, 100% humidity. A Spectra Max M2e (Molecular Devices) was used to read absorbance at 490 nm. The number of viable cells was measured based on the quantity of formazan production.

2.4. Cell Sorting based on Aldehyde Dehydrogenase (ALDH) activity.

Cell sorting was conducted based on Aldehyde Dehydrogenase (ALDH) activity using the Aldefour kit (Stem Cell Technologies). Prior to sorting, cells were

trypsinized and counted. Since this is a fluorescent assay, all subsequent steps were conducted in low light. 30 x 10^6 cells were resuspended in 30 mL of Aldefluor buffer. Cell "staining" was produced by adding 5 µL of ALDH reagent (BODIPY-aminoacetaldehyde, BAAA) for every 1 mL of cells (according to manufacturer's protocol). One control contained unstained cells. A second control contained stained cells in the presence of the 4-Diethylaminobenzaldehyde (DEAB) inhibitor. All three tubes were incubated in 37 °C water bath for 40 minutes. Cells were centrifuged, re-suspended with ALDH buffer, and dispersed into single cells by using needles (B-D 21 G 1). All tubes were placed on ice in preparation for cell sorting. Cell sorting operated by Monroe Chan, flow operator and manager at Regenerative Medicine Program. Flow cytometry-based sorting simply identifies stem cells or cancer stem cells based on their ability to metabolize a synthetic ALDH substrate to produce a fluorescent signal. The reaction inhibit by diethylamino-benzaldehyde, which is exploited as a negative control. Distinct ALDH populations were then collected, ALDH ^{Low}, ALDH ^{High}, and ALDH$^-$. ALDH ^{Low} cells constituted low percentage of ALDH activity, while ALDH ^{High} cells constituted the highest ALDH activity of the total cell fraction. ALDH$^-$ population has no ALDH activity.

2.5. Annexin V in Non-adherent Cultures

A2780cp ALDH ^{High} and ALDH ^{low} cells were plated into 24 well ultra low attachment plates and allowed to grow for 48 hours to develop cell aggregates prior to

drug addition. Wells were treated with 4 different concentrations of MO-101 (5, 7.5, 12.5, 15 μM) and 4 different concentrations of GLN (10, 15, 20, 25 μM) for 48 hours at 37°C prior to cells being harvested for flow cytometry. Cells were pelleted and treated with Accutase (Accutase ® solution from Sigma Life Science) to allow cell aggregates to dissociate into single cells. Cells were resuspended in PBS and counted. 200,000 cells per condition were aliquoted into Eppendorf tubes and resuspended into Annexin V binding buffer. Cells were then stained with APC Annexin V stain and 7AAD for 15 minutes at room temperature in the dark. Each tube was topped up with 400 μL of Annexin V binding buffer and analyzed by flow cytometry. Jennifer Ali conduced all experiment of Annexin V in non-adherent Cultures in addition to Ludivine Morrison, who is a research technician for Dr. Tamra Ogilvie at Biochemistry and Medical Genetics performed the flow cytometry on a GalliosTM flow cytometer, and analyzed samples using Beckman Coulter Gallios flow cytometer (8 color/2 lasers-561 ready).

2.6. Use of CaspGlow to measure caspase activity

Cells were seeded into 24 well ultra-low attachment plates, 40.000 cells/well, for 48 hours at 37°C. Drugs were added on the second day after seeding and incubated for 24 hours. Cells were prepared for flow cytometry using the CaspGlow Fluorescein Active Caspase Staining Kit (BioVision Incorporated) dissociating aggregates by with Accutase and using 200,000 cells resuspended in 300μL medium per condition. 1 μL

of CaspGlow reagent was added into each tubes and incubated for one hour at 37°C. Unstained cells served as a negative control. After an hour of incubation, cells were pelleted and resuspended in 500µL Caspglow washing buffer. Washing was repeated prior to resuspending cells in 300µL of Caspglow washing buffer, and transfer to flow cytometry tubes. Ludivine Morrison preformed the flow cytometry on a GalliosTM flow cytometer.

2.7. Cell viability measurements using Annexin V staining

A2780-cp ALDH $^{High/low}$ cell viability was assessed using an APC Annexin V staining kit (BD Pharmigen, 559763). Cells were dissociated and a single cell suspension at a density of $3x10^6$ cells/mL was stained simultaneously with Annexin V-APC and 7-aminoactinomycin D (7-AAD) using concentrations of 7.5 µM/100,000 cells, 12.5 µM/100,000 cells and 15 µM/100,000 cells. Samples were incubated for 15 minutes and then resuspended in 400 mL of 13 Binding Buffer. J. Ali conduced the experiments and L. Morrison preformed the flow cytometry on a GalliosTM flow cytometer and analyzed using Kaluza analysis software (Beckman Coulter).

2.8. Cell cycle analyses by BrdU incorporation

A2780cp ALDH ^{High} and ALDH ^{low} cells were plated into 24 well ultralow attachment plates and allowed to grow for 48 hours prior to drug addition. Wells were treated with 4 different concentrations of MO-101 (5, 7.5, 12.5 and 15 µM) and 2 different concentrations of GLN (10, 25 µM) for 48 hours at 37°C prior to cells being harvested for flow cytometry. Prior to harvesting, cells were pulsed for 3 hours at

37°C with 1mM BrdU. Cells were pelleted and then treated with Accutase to allow for aggregates to dissociate. Cells were then resuspended in PBS, counted and aliquoted into microcentrifuge tubes at 400,000 cells per condition. Cells were fixed using BD Cytofix/Cytoperm buffer for 20 minutes at room temperature. Cells were washed with BD Perm/Wash Buffer, centrifuged and then permeabilized with BD Cytoperm Permeabilization buffer plus for 10 minutes on ice. Washed again with BD Perm/Wash Buffer and then centrifuged. Cells were then fixed again with BD Cytofix/Cytoperm buffer for 5 minutes at room temperature. Washed with BD Perm/Wash buffer, centrifuged and then treated with 300 μg/mL in PBS for 1 hour in 37°C. Cells were then washed with wash buffer, centrifuged and stained with anti-BrdU antibody (1:50) for 20 minutes at room temperature in the dark. Cells were washed with wash buffer, centrifuged, resuspended in 300 μL of PBS and stained with 7AAD. Cells were then passed through cell strainers into flow cytometry tubes. J. Ali conduced the experiments, and the cells were analyzed by M. Chan by using the flow cytometry machine at 400 events/second.

2.9. Caspase Dependent Cell Death Assays

Cells were plated in either 96 well or 24 well ultralow attachment plates and cells were allowed to grow for 48 hours prior to drug treatment. Cells were pre-treated for 4 hours with the pan caspase inhibitor QVD-OPh and then different concentrations of MO-101 and GLN were added to alone or in combination with inhibitor for 48 hours.

Cells were then analyzed for viability using CellTiter or measuring Annexin V binding by flow cytometry.

2.10. Statistical Analysis

Cell viability was measured by using Prism 6 program. One way ANOVA with Dunnett's multiple comparison test were conducted with p values <0.05, and the comparison based on the standard error of the mean. Multiple t test were used where needed with p values <0.05.

Chapter III: Testing the efficacy of dorsomorphin (DM) and LDN-193189 (LDN) on drug-resistant EOCs growing in non-adherent cultures

2.1. Introduction

Chemoresistance of EOC cells is a major contributor to reducing the survival rate among EOC patients (Buys et al., 2011). Chemoresistance is defined clinically by disease recurrence in less than six months after initial treatment. Currently, recurrent disease is not treatable. However, some drugs, such as gemcitabine, liposomal doxorubicin or topotecan, increased disease-free survival by 10-30% in platinum-resistant EOC (Coleman, Monk, Sood, & Herzog, 2013a; Hiss, 2012). Thus, evaluating novel drugs becomes critical for outcome improvement. This chapter focuses on dorsomorphin (DM) and the chemically related compound, LDN-193189 (LDN) as treatment agents for chemoresistant EOCs.

The EOC microenvironment contains several factors that may be tested as target for treatment. This includes autocrine factors, which affect EOC cell activities such as maintenance of cellular morphology, migration and invasion. The bone morphogenetic protein (BMP) family of secreted cytokines are EOC autocrine factors. Studies showed that autocrine factors are capable of altering EOC cell morphology, migration and invasion (Ali et al., 2015; Moll et al., 2006; Thériault, Shepherd, Mujoomdar, & Nachtigal, 2007). Prior to 2008, there were no selective small molecule inhibitors targeting BMP signaling; however, a chemical screening study showed that compound C enhanced dorsalization in zebrafish because of a block in BMP signaling. Thus, compound C was renamed dorsomorphin (DM) (Yu et al., 2008). Compound C was originally identified as an AMP kinase (AMPK) inhibitor

(Zhou et al., 2001), but it also has the ability to inhibit BMP type I serine/threonine kinase receptors (Boergermann, Kopf, Yu, & Knaus, 2010; Yu et al., 2008). LDN was developed by alterations of the DM chemical backbone (Cuny et al., 2008), and it also inhibits BMP type I receptors, but has greater potency.

Pharmacologic studies *in vitro* showed that DM and LDN inhibit a wide range of kinase targets with different specificity, suggesting that DM and LDN can induce a variety of cellular responses (Vogt, Traynor, & Sapkota, 2011). Diverse studies showed that DM and LDN are multikinase inhibitors, which inhibit a wide variety of receptor and intracellular kinases including BMP type I serine/threonine kinase receptors, mitogen-activated protein kinase 14 (MAPK14) (p38), mitogen-activated protein kinase 8 (MAPK8) (JNK), and Protein kinase B (AKT) (Boergermann et al., 2010; Pachori et al., 2010; Vucicevic et al., 2011). Several multikinase inhibitors have been used in studies for the treatment of EOC. For example, dasatinib is a multikinase inhibitor that targets BCR/ABL and Src tyrosine kinases, and thus affects EOC invasion and apoptosis (Konecny et al., 2009). Moreover, small interfering ribonucleic acid (siRNA) knockdown of multiple kinases in EOC induces microtubule stability and cytotoxicity (Ahmed et al., 2011). Therefore, kinases inhibitors are considered as viable targets to develop treatments for EOC.

DM and LDN are capable of killing cancer cells (Cuny et al., 2008; Hao et al., 2010; Yu et al., 2008). Previously, the Nachtigal laboratory had examined the effects of DM and LDN on the cell viability of EOC cells in monolayer culture (Ali et al.,

2015). We observed that DM and LDN effectively killed EOC cells, including platinum-resistant cells (e.g. A2780-cp), by a caspase-independent cell death process. DM and LDN induced EOC cell death by autophagy or induction of reactive oxygen species, respectively (Ali et al., 2015). The first goal from this aim was to test the efficacy of DM or LDN in combination with cisplatin using non-adherent cultures. There was significant cell death in A2780-s with single drug treatment and combination treatment. By contrast to the results observed in monolayer, DM was not effective on killing cisplatin-resistant A2780-cp cells using single drug or combination treatments when cells were grown as non-adherent cultures. LDN effectively kill A2780-cp cells at the highest dose.

3.2. Results

3.2.1 DM and LDN affect cell viability and EOC aggregate formation in non-adherent cultures

DM and LDN induced a dose-dependent decrease in the viability of EOC cell lines growing in non-adherent conditions (Figure 3-1, 3-2, & 3-5). All cell lines were treated with increased doses of DM or LDN, from 0.1 μM to 20 μM. Cell viability was reduced significantly at the highest doses of treatment in, 15 μM and 20 μM, except A2780cp treated with DM. In COV362 cells, LDN exhibited a greater effect on cell viability at lower doses starting at 7.5 μM; this was not observed in A2780-s and A2780-cp cells. A2780-cp cells were resistant to DM treatment compared to the

other cell lines; however, LDN was able to kill A2780-cp cells completely at 15 μM and 20 μM. In addition, DM/LDN are observed to prevent EOC aggregate formation at high doses of the drugs (Figure 3-3).

3.2.2 DM and LDN treatment in cell lines growing adherent vs. non-adherent conditions

For comparison purposes, COV362 cells were treated with increased doses of DM/LDN, while growing in adherent and non-adherent cultures (Figure 3-4 & 3-5). Cells exhibited significant reduction in cell viability at high doses of drugs in both conditions similarly, with LDN having a greater cell killing.

3.2.3 DM/LDN co-treatment with cisplatin in cells growing in non-adherent cultures

A2780s cells were treated with different doses of DM/LDN, from 0.1 μM to 10 μM, and co-treated with 5 μM cisplatin (Figure 3-6, 3-7). Similarly, A2780-cp cells were treated with different doses of DM/LDN, from 0.1 μM to 10 μM, and co-treated with 25 μM cisplatin (Figure 3-8 & 3-9). Different doses of cisplatin were used for co-treatment because of the A2780-cp chemoresistance phenotype. In both cell lines, LDN + cisplatin were more effective on killing cells at the high doses compared to DM + cisplatin. DM + cisplatin induced partial cell death in A2780-s cells, but there was no reduction in cell viability in EOC resistant cell line, A2780-cp. DM/LDN treatment with cisplatin showed no synergistic effect. Moreover, the *in vivo* combination therapy experiments using Rag2 mice bearing tumors with A2780s cells

strongly suggest that DM or LDN are unlikely to be effective as adjuvant chemotherapy agents in EOC (Figure 3-10).

3.3. Discussion

Primary or acquired chemotherapy resistance contributes to the high EOC mortality rate. Thus investigations for novel drugs become important to increase EOC survival rate (Buys et al., 2011; Coleman, Monk, Sood, & Herzog, 2013b). Previous studies showed that DM and LDN are multikinase inhibitors that can inhibit a wide variety of receptor and intracellular kinases (Boergermann et al., 2010; Pachori et al., 2010; Vucicevic et al., 2011). DM and LDN are also capable of affecting EOC proliferation in adherent conditions (Ali et al., 2015). Similarly LDN effectively killed resistant EOC cell lines growing in non-adherent cultures, where cell viability reduced significantly at the highest doses of treatment. DM induced significant cell death in COV362: however, was not able to reduce resistant EOC cell viability (A2780cp), even at the high doses. In addition, testing DM/LDN as adjuvant chemotherapy agents with cisplatin showed no synergistic reduction in resistant EOC cell viability in our non-adherent conditions. These results suggest that EOC cells growing as non-adherent cultures more accurately reflect the drug-resistant profile exhibited by EOC patients. The *in vivo* combination therapy experiments conducted in the lab also strongly suggest that DM or LDN are unlikely to be effective as adjuvant chemotherapy agents in EOC. Thus, this line of investigation was terminated.

Figures

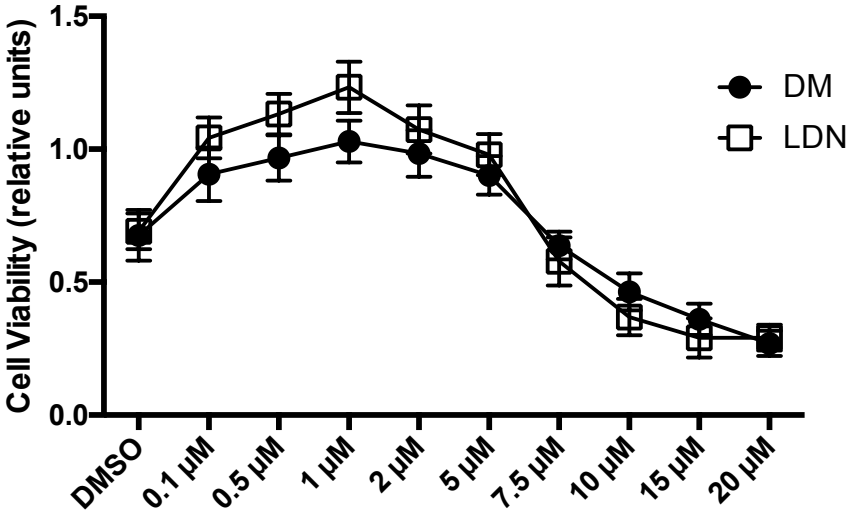


Figure 3-1. Non-adherent A2780s cells treated with different doses of DM/LDN for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

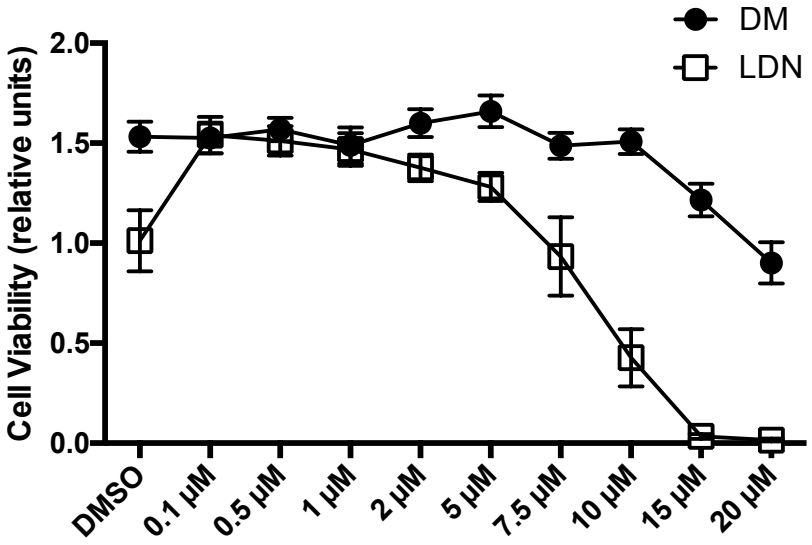


Figure 3-2. Non-adherent A2780cp cells treated with different doses of DM/LDN for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

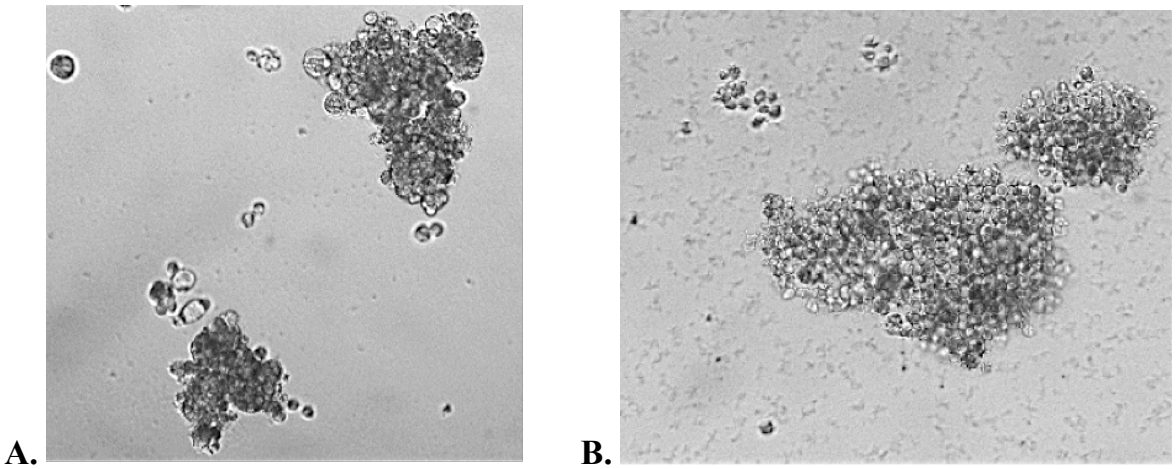


Figure 3-3. COV362 cells grow in non-adherent conditions. **A.** Non-treated COV362 cells forming aggregates. **B.** COV362 cells treated with 15 μM LDN.

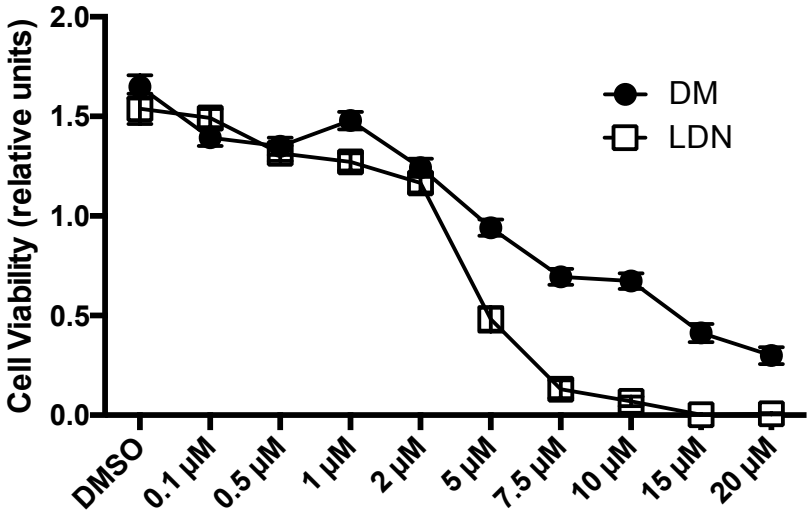


Figure 3-4. Adherent COV362 cells treated with different doses of DM/LDN for three days. N = 4, n = 24/condition. Analyzed by One-way ANOVA multiple comparisons.

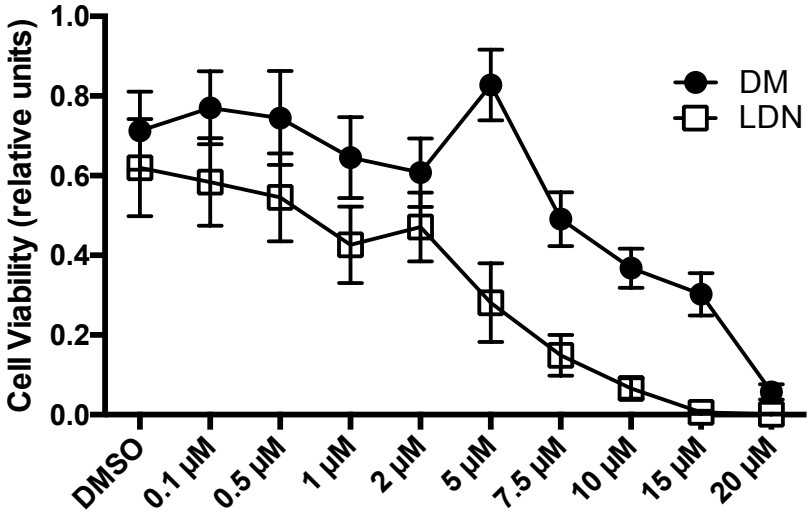


Figure 3-5. Non-adherent COV362 cells treated with different doses of DM/LDN for three days. N = 2, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons.

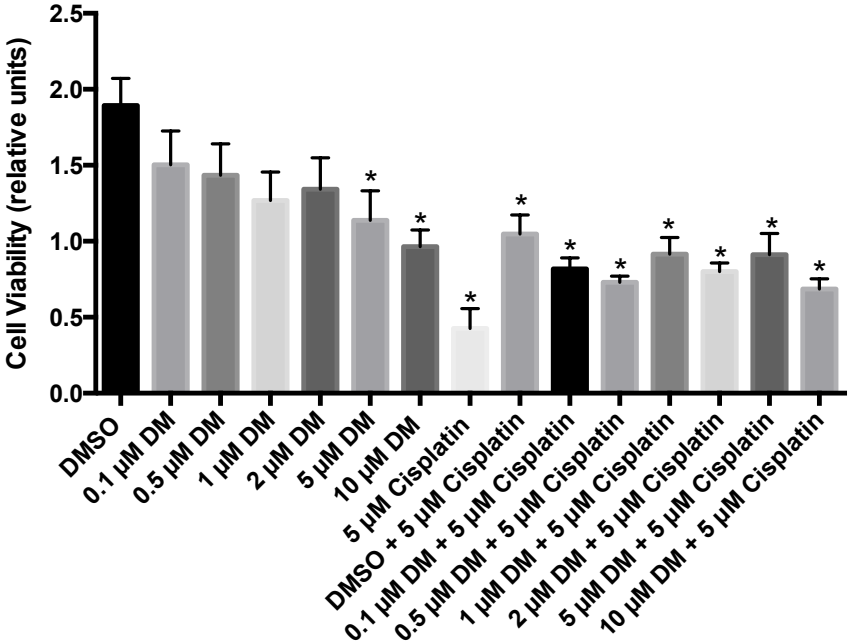


Figure 3-6. Non-adherent A2780s cells treated with different doses of DM + CP for three days. N = 2, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons.

* Compared to DMSO, + compared to the dose control, ^ compared to Cisplatin

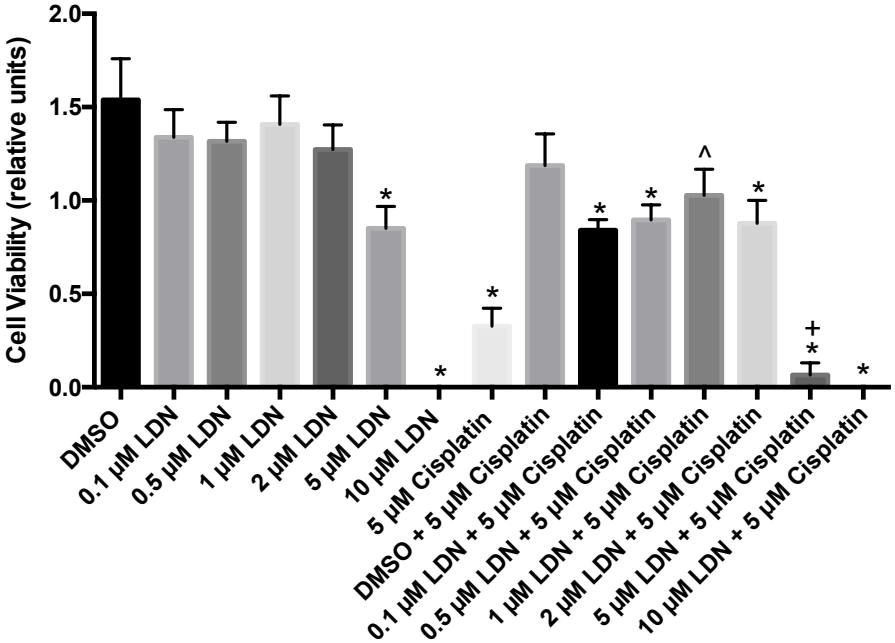


Figure 3-7. Non-adherent A2780s cells treated with different doses of LDN + CP for three days. N = 2, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons.

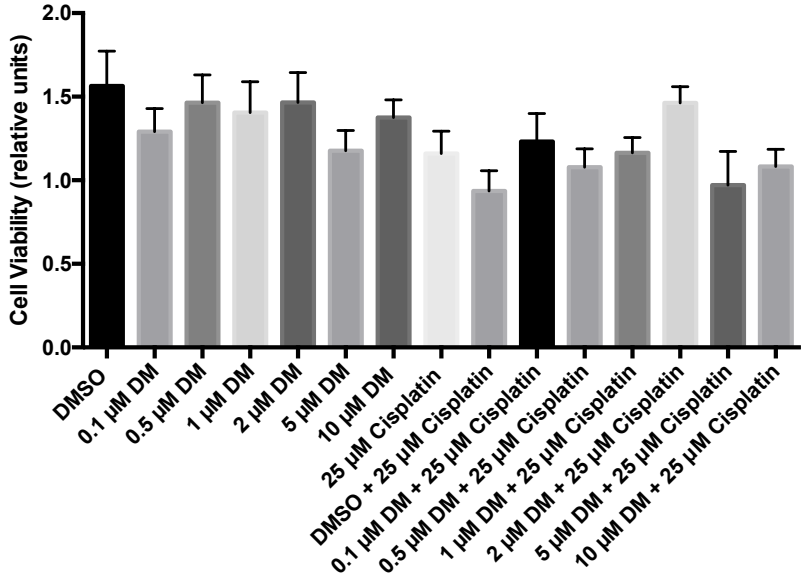


Figure 3-8. Non-adherent A2780cp cells treated with different doses of DM + CP for three days. N = 2, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons.

* Compared to DMSO, + compared to the dose control, ^ compared to Cisplatin

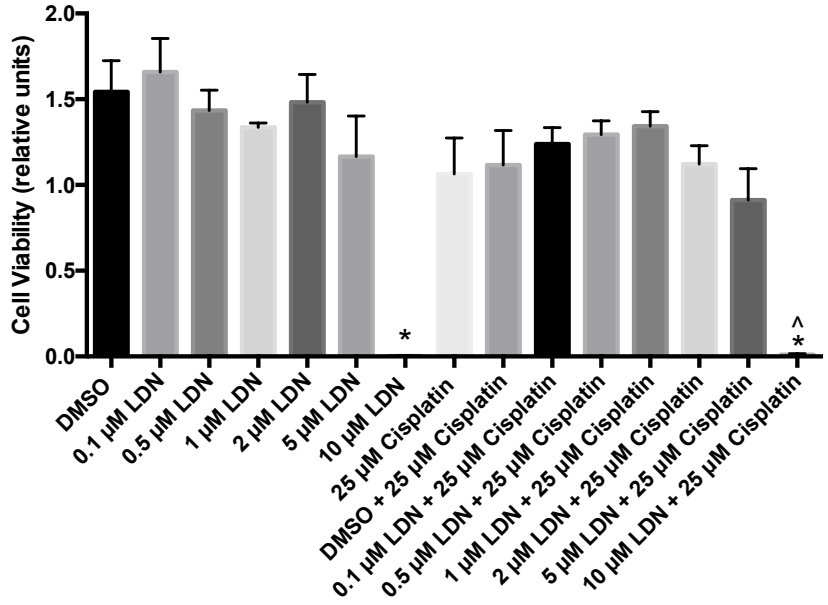


Figure 3-9. Non-adherent A2780cp cells treated with different doses of LDN + CP for three days. N = 2, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons.

* Compared to DMSO, + compared to the dose control, ^ compared to Cisplatin

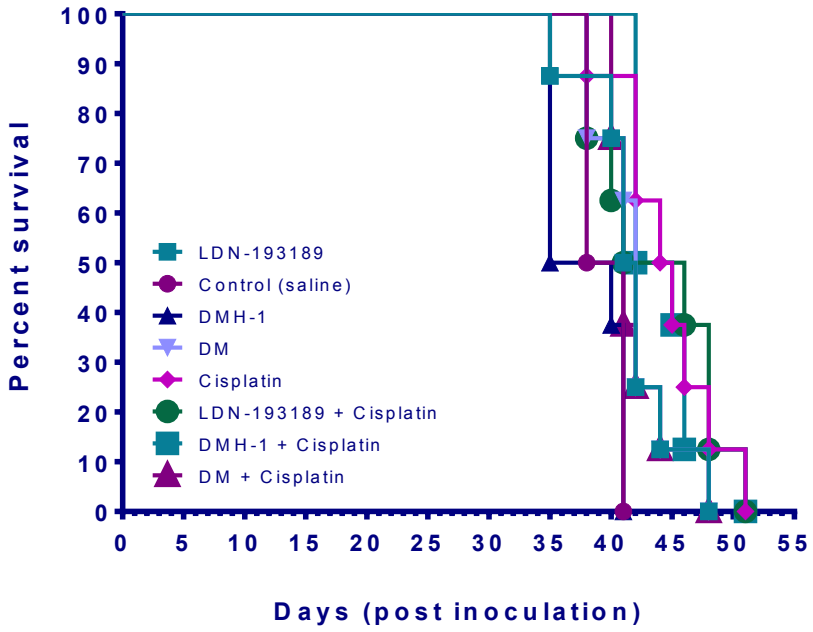


Figure 3-10. Kaplan Meier Curves (% Survival) for Test Articles administered to Rag2 mice bearing A2780-s ovarian cancer.

Chapter IV: Test efficacy of novel anti-tumour lipid compounds (GAELs; glycosylated antitumor ether lipids) -/+ cisplatin using EOC cell lines and primary EOC cells isolated from patients in non-adherent cultures

4.1. Introduction

The mortality rate of EOC has not changed significantly over many decades since the introduction of platinum-based and taxane chemotherapeutics (Buys et al., 2011; McGuire et al., 1996; Siegel, Miller, & Jemal, 2015; Who, 2011). EOC is diagnosed mostly at advanced stages, where there is limited effective treatment currently (Buys et al., 2011; McGuire et al., 1996; Siegel et al., 2015; Who, 2011). Despite the heterogeneity of EOC, all EOC histotypes are treated equally by a combination of surgery and chemotherapy (Coleman et al., 2013b; Goh et al., 2015). Killing EOC cancer cells is mostly based on damaging the DNA, preventing DNA synthesis, or targeting the cell cycle to stop cell proliferation (MacFarlane, 2009). Targeting these biological events activates apoptotic pathways in order to induce cell death (MacFarlane, 2009). However, EOC cells are either inherently resistant or capable of developing resistance during chemotherapy treatment by various pathways to evade apoptosis (Howell et al., 2010; Köberle et al., 2010; Palm et al., 2011). Thus, discovering new agents that can induce non-apoptotic cell death become critical to obtain effective treatment and improve overall survival. An understudied class of novel chemotherapeutic agents is called glycosylated antitumor ether lipids (GAELs). Previous work showed that GAELs induced cell death in a variety of human cancer cell lines, including effectively killing breast cancer stem cells (Arthur & Bittman, 2014; Samadder, Xu, Schweizer, & Arthur, 2014). Importantly, GAELs produced cell death via a non-apoptotic mechanism in the cells tested for these experiments (Arthur

& Bittman, 2014; Samadder et al., 2014). The aim of this part of the thesis was to test the efficacy of GAELs to produce cell death in EOC cell lines, and primary EOC cells isolated from patient samples.

GAELs are the third subclass of antitumor ether lipids (AELs), along with the other two subclasses, alkyllysophospholipids (ALPs) and alkylphosphocholines (APCs) (Brachwitz & Vollgraf, 1995). AELs are synthetic phospholipids that were determined to have antitumor activity, thus initiating a new area for cancer therapeutic research (Brachwitz & Vollgraf, 1995; Samadder et al., 2014). Several studies were conducted using ALPs and APCs subclasses showing that they kill cancer cells by inducing apoptosis (Gajate, Santos-Beneit, Modolell, & Mollinedo, 1998; Ruiter, Zerp, Bartelink, Van Blitterswijk, & Verheij, 1999). However, GAELs exhibited a distinct mechanism of action from other AELs and current anti-cancer agents, by which GAELs enter cancer cells via the endocytosis pathway, then generate large acidic vacuoles that lead to release of acid hydrolases, including cathepsin D, that induce a caspase-independent form of cell death (Arthur & Bittman, 2014; Samadder et al., 2014). Few studies have been conducted using GAELs as anti-cancer agents; therefore, much more research is needed to discover the cellular and molecular events behind the specific mechanism of action of GAELs (Arthur & Bittman, 2014; Samadder et al., 2014).

GAELs are derived from AELs by adding a sugar molecule at position (*sn-3*) on the AEL backbone, *e.g.* 1-*O*-octadecyl-2- *O*-methyl-glycerophosphocholine

(Edelfosine) (ET-18-OCH3, Figure 4-1) (Arthur & Bittman, 2014). GAELs include a number of compounds that differ in their sugar type (glucose, galactose, or mannose), number, and position of NH_2 group (Arthur & Bittman, 2014; Samadder et al., 2014). In collaboration with Dr. Gilbert Arthur (Biochemistry & Medical Genetics), who co-invented GAELs and holds the patent for them, and Dr. Frank Schweizer (Chemistry), we obtained five different GAEL compounds: compound A, 1-*O*-hexadecyl-2-*O*-methyl-3-*O*-(2'-deoxy-2'-amino-β-D-glucopyransoyl)-sn-glycerol **(β-D-GLN)**; compound B 1-*O*-hexadecyl-2-*O*-methyl-3-*O*-(2'-amino-2'-deoxy-α-D-glucopyranosy)-sn glycerol **(α D-GLN)**; compound C 1-*O*-Hexadecyloxy-2R-(–α-L-rhamnopyranosyl)-3-amino glycerol **(Rham-GAEL)**; compound D 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2',6'-diamino-2',6'-dideoxy–α-D-glucopyranosyl)-sn-glycerol **(MO-101)**; and compound E 1-*O*-Hexadecyloxy-2R-*O*-methyl(2'amino-2'deoxy–α-L-glucopyranosyl)-3-amino glycerol **(L-GLN)** (Figure 4-2). As part of this thesis all five compounds were tested on EOC cells; however, this thesis will focus on the parental compound β-D-GLN and its modified analog MO-101.

4.2. Results

4.2.1. EOC Cell Lines.

4.2.1 1. A2780s. A2780s cells were treated with increasing doses of all GAEL compounds, 2.5, 5, 7.5, 10, 12.5, 15, 20, 25, and 30 μM, while growing in non-adherent conditions (Figures 4-3 & 4-4). All GAEL compounds showed the same

trend of inducing a reduction in cell viability, with almost complete cell killing occurs at 25 and 30 μM. These results confirm that GAEL compounds are effective at killing this platinum-sensitive EOC cell line.

4.2.1. 2. A2780cp. A2780cp cells were treated with the same increasing doses of GAEL compounds, which were determined to effectively kill A2780cp (Figure 4-5, & 4-6). However, compared to other GAEL compounds, MO-101 exhibited a greater reduction on cell viability, starting at with less than 50% viable cells at 10 μM. Thus, MO-101 and its parent compound GLN became the focus of this thesis.

4.2.1 3. COV362. Similar to A2780cp, GAEL compounds significantly killed COV362 cells growing in non-adherent conditions, with almost a complete cell death at 30 μM (Figure 4-7). MO-101 again showed the greater effect at low doses of treatment in comparison to β-D-GLN.

GAEL compounds effectively showed their ability to induce significant cell death in platinum-sensitive and more importantly platinum-resistant EOC cell lines. Having these positive results, primary EOC cells were tested to further evaluate the effect of GAELs on EOC cell viability.

4.2.2 Primary EOC Cells.

4.2.2. 1. EOC126. EOC126 cells were treated with increased doses of β-D-GLN or MO-101, range between 1 μM to 10 μM, in adherent/non-adherent cultures (Figure 4-8, & 4-9). Generally, all primaries EOC cell samples required lower doses of GAELs compared to EOC cell lines. β-D-GLN decreased EOC126 cell viability significantly

in adherent condition as well as non-adherent culture. MO-101 killed EOC126 cells at very low doses under adherent system, starting at 2 μM. Under non-adherent condition, EOC126 cells were able to slightly resist MO-101 treatment, with no complete cell death (%100). Other GAEL compounds, Rham-GAEL and L-GLN, affected EOC126 cell viability only at high doses, but αD-GLN induced significant reduction at 6 μM to 10 μM (Figure 4-10).

4.2.2 2. EOC146, EOC103E & EOC140. EOC146 cells were treated with β-D-GLN or MO-101 under non-adherent conditions only (Figure 4-11). The results showed that MO-101, but not β-D-GLN, induced cell death significantly. EOC103E cells were treated with MO-101 only, and it induced a significant reduction in cell viability at 10 μM (Figure 4-12). EOC140 cells were very sensitive to MO-101 treatment, showing significant cell death starting at low doses, 2-10 μM.

These results from primary EOC cells confirm that GAEL compounds, especially MO-101, have high anti-cancer activity on platinum-sensitive and -resistant EOC cells growing in both adherent and non-adherent systems. Knowing that combination chemotherapy can produce effective cell killing, co-treatment experiments using GAEL compounds, β-D-GLN/MO-101, in combination with platinum agents, cisplatin, were conducted.

4.2.3 Combination treatment of β-D-GLN/MO-101 with cisplatin in EOC cell lines

A2780s cells were treated with increasing doses of β-D-GLN/MO-101 alone and in combination with a single dose of cisplatin, 5 μM (Figures 4-13 & 4-14). Similarly, A2780cp cells were treated with increasing doses of β-D-GLN/MO-101 alone and in combination with 35 μM cisplatin (Figure 4-15 & 4-16). Cisplatin doses were selected based on the sensitivity profile from the dose response assays. The results showed that there is no synergistic effect using combination treatment of GAEL compounds and cisplatin. Highest doses of treatments exhibited significant reduction in EOC cell lines, but this seems to be due to the GAEL compounds alone, and not due to additive effects. Evaluating the GAEL mechanism of action will be described in chapter 5.

4.3. Discussion

Despite the diverse research studies on EOC, the survival rate is low among EOC patients (Buys et al., 2011; McGuire et al., 1996; Siegel et al., 2015; Who, 2011). Inherent or acquired chemoresistance is one of the major reasons contributing to the high mortality rate of EOC (Howell et al., 2010; Köberle et al., 2010; Palm et al., 2011). The majority of different chemotherapeutic agents that have been used clinically produce cell death by inducing apoptosis (Howell et al., 2010; Köberle et al., 2010; MacFarlane, 2009; Palm et al., 2011; Vaughan et al., 2011). EOC cancer cells are highly capable of acquiring chemotherapy resistance and evade apoptotic pathways

(Howell et al., 2010; Köberle et al., 2010; MacFarlane, 2009; Palm et al., 2011; Vaughan et al., 2011). There is still a huge demand for more effective agents that target EOC cell death. GAEL compounds are novel agents proven to have effective anti-cancer activity on several types of cancers via non-apoptotic mechanisms. Studies showed that GAELs induce cell death through releasing lysosomal cathepsin D, thus generating large acidic vacuoles that leads to cell death (Arthur & Bittman, 2014; Samadder et al., 2014).

GAELs were tested in three EOC cell lines, two of which are platinum chemoresistant (A2780cp and COV362), in addition to several primary EOC cells isolated from platinum sensitive- and resistant-EOC patients. Experiments were conducted using non-adherent culture conditions for two important reasons: 1) non-adherent culture conditions more closely resemble how cells grow *in vivo* (Ghosh et al., 2005; Gómez-Lechón et al., 1998; P. A. Kenny et al., 2007; E Lengyel et al., 2014), and 2) cells in non-adherent cultures show a higher level of drug-resistance than monolayer cultures, again reflecting the *in vivo* conditions (Junsong Chen et al., 2013; Dong et al., 2010; Lee et al., 2013; Loessner et al., 2010; Z. Yang & Zhao, 2011).

Overall, the results showed that GAELs, especially MO-101, can reduce cell viability in resistant-EOC cancer cells. Generally, resistant-EOC cell lines required higher doses of GAELs, up to 30 μM, compared to primary EOC cell samples, 10 μM. A2780cp cells have been generated using graded doses of cisplatin to become resistant

(Louie et al., 1985). Therefore, these cells may use more than one mechanism to acquire resistance (Katano et al., 2002). This may explain the reason behind the high doses required to induce cell death. Importantly, all GAEL compounds were able to kill A2780 cells (<90%) at 30 μM in adherent/non-adherent conditions. The cisplatin-resistant COV362 cells showed similar responses under non-adherent conditions.

By contrast, there were variable responses to the different GAELs based on which primary EOC cells were used. For example, in EOC126 cells, β-D-GLN, Rham-GAEL and E L-GLN could not cause significant reduction in cells viability (<50%) at the high dose of treatment, 10 μM, in non-adherent cultures comparing to MO-101. EOC146 exhibited resistance to β-D-GLN treatment, but not MO-101. These results may reflect the inherent differences in biology between the different EOC histotypes.

Using low doses of chemotherapy treatment is recommended and preferable to limit drug toxicity (Ozguroglu, Sari, & Turna, 2006; Stampler, Holtz, & Dunton, 2011). Current chemotherapy treatments of EOC have various side-effects with different severity, such as loss of hearing and acute renal failure (Ozguroglu et al., 2006). However, unpublished work from Dr. G. Arthur's laboratory testing the toxicity profile of GAELs (Rham-GAEL) in Rag2 female mice indicated that they had no toxicity up to 300 mg/kg for oral administration and 50 mg/kg for intravenous administration. One solution to reduce the side-effects of current treatments are to use different agents as adjuvant treatment along with platinum chemotherapy. Therefore, GAEL activity in combination with cisplatin was tested on platinum-sensitive and –

resistant A2780 cells growing in non-adherent conditions. Results showed no additive effects, providing a negative answer to the hypothesis. However, synergistic affects might occur in a dose dependent manner, by which using higher and different doses of cisplatin or the GAELs may have shown synergistic effects. Thus, using GAELs as adjuvant agents requires further experimentation.

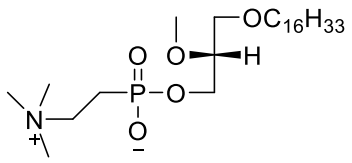


Figure 4-1. Edelfosine: AEL compound.

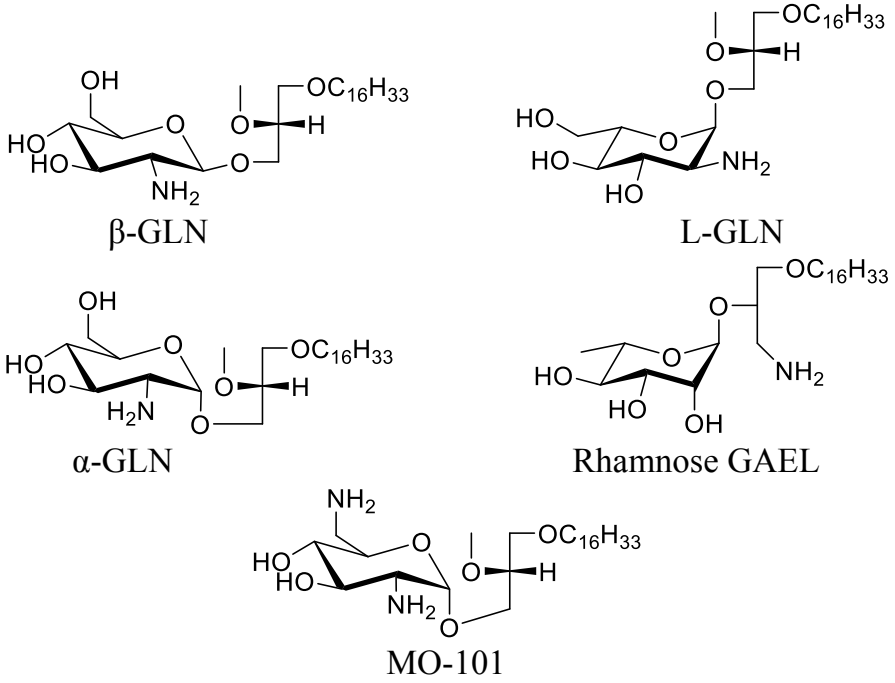


Figure 4-2. GAEL compound structures.

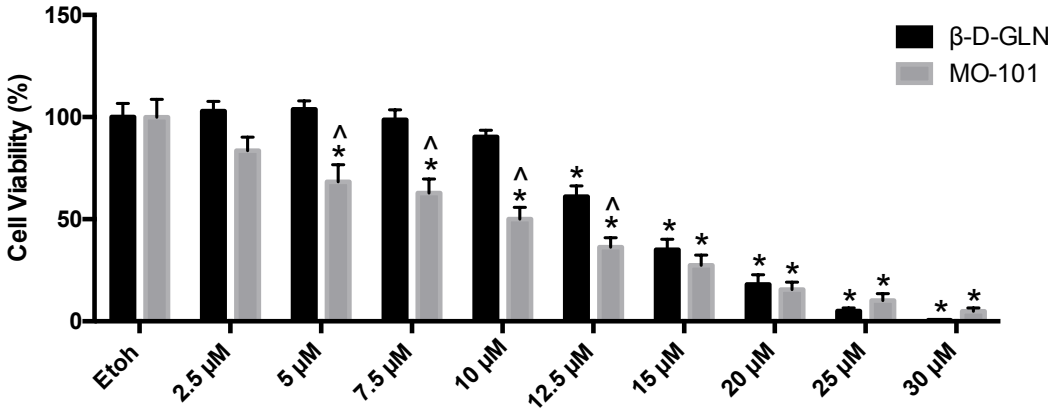


Figure 4-3. Non-adherent A2780s cells treated with different doses of β-D-GLN/MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* Compared to Etoh, ^ compared to β-D-GLN

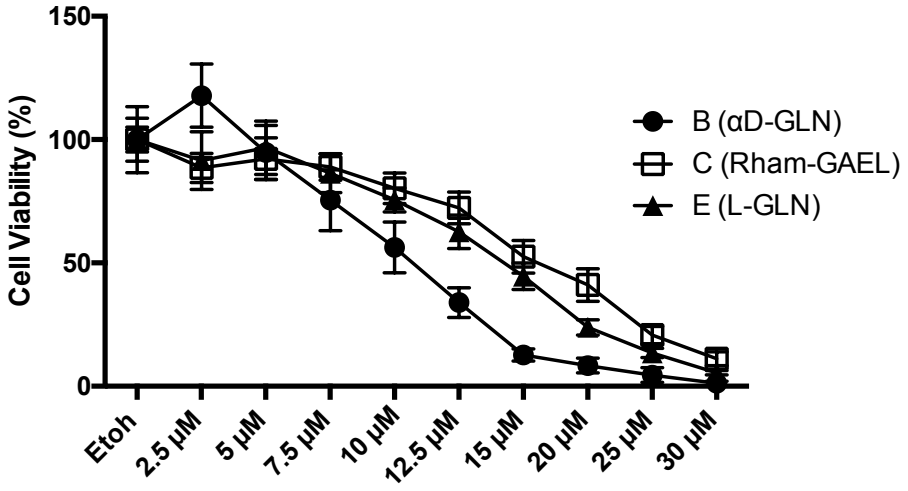


Figure 4-4. Non-adherent A2780s cells treated with different doses of compounds B (αD-GLN), C (Rham-GAEL) & E (L-GLN) for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

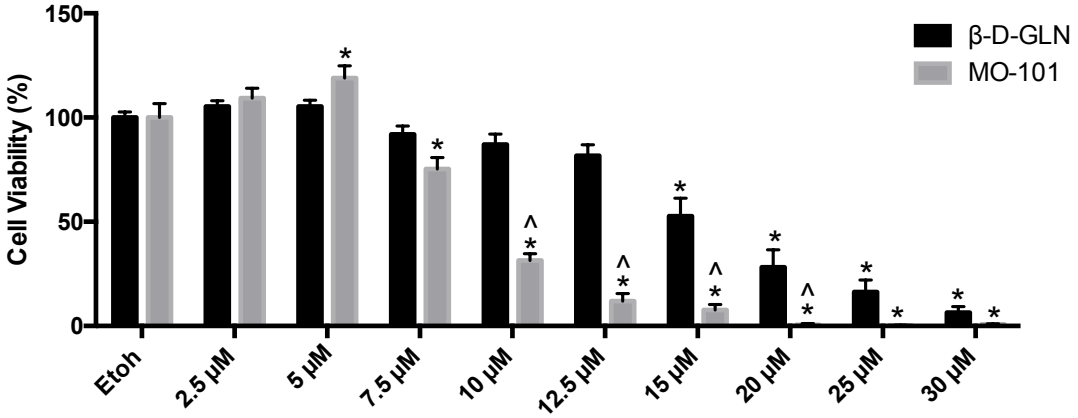


Figure 4-5. Non-adherent A2780cp cells treated with different doses of β-D-GLN/MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* Compared to EtOH, ^ compared to β-D-GLN

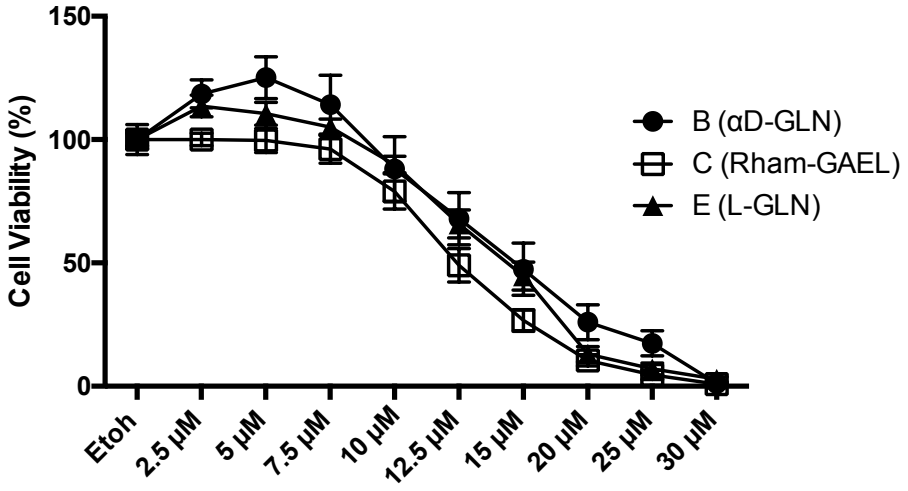


Figure 4-6. Non-adherent A2780cp cells treated with different doses of compounds B (αD-GLN), C (Rham-GAEL) & E (L-GLN) for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

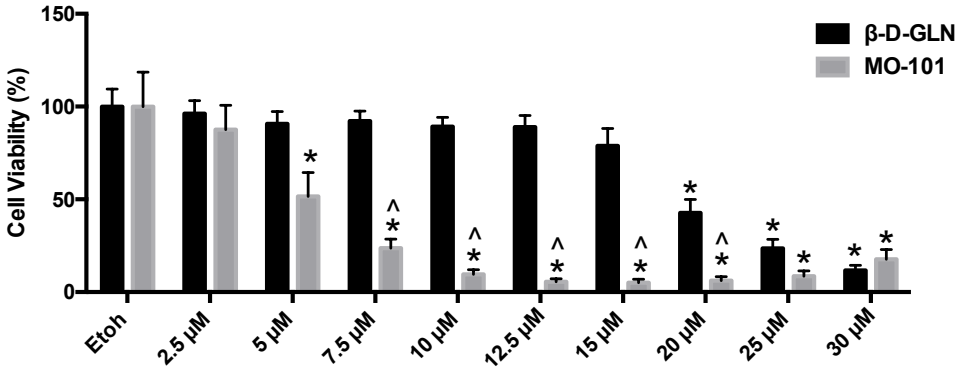


Figure 4-7. Non-adherent COV362 cells treated with different doses of β-D-GLN/MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* Compared to EtOH, ^ compared to β-D-GLN

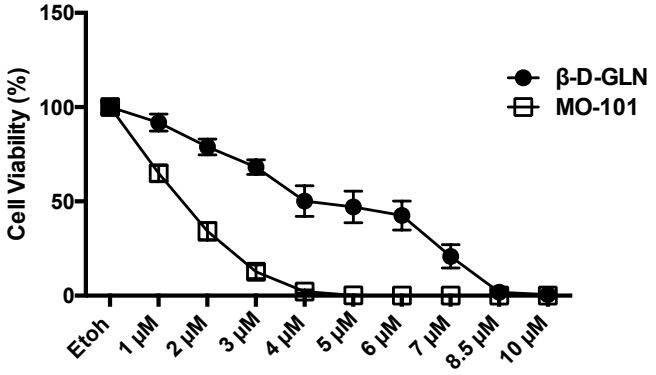


Figure 4-8. Adherent EOC126 cells treated with different doses of β-D-GLN and MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

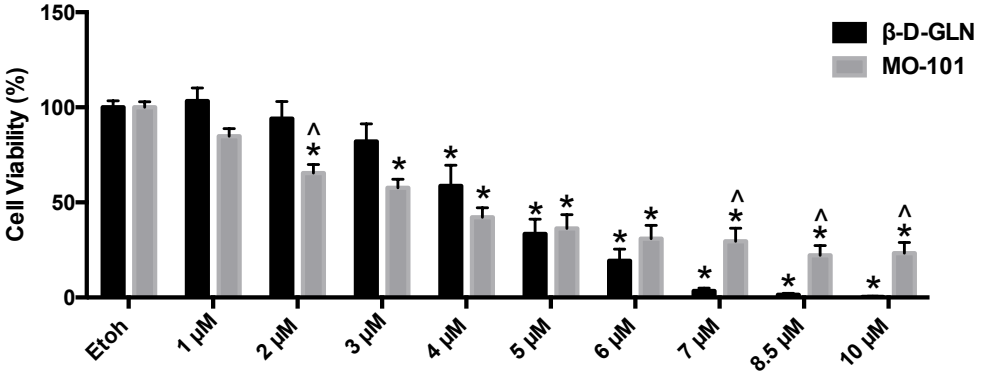


Figure 4-9. Non-adherent EOC126 cells treated with different doses of β-D-GLN and MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* Compared to EtOH, ^ compared to β-D-GLN

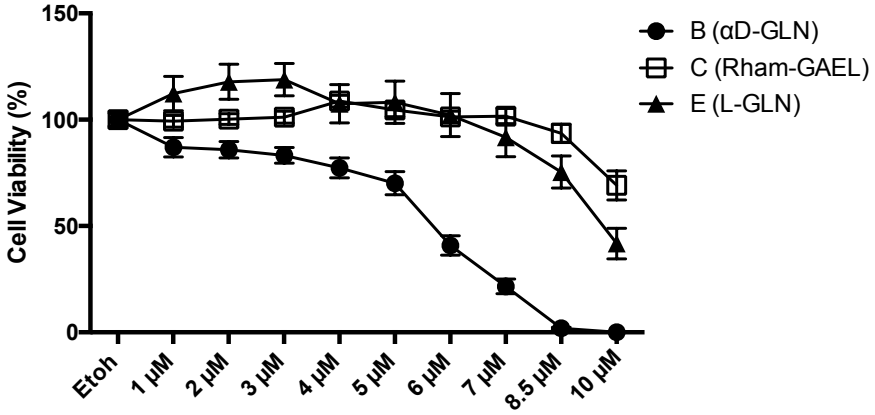


Figure 4-10. Non-adherent EOC126 cells treated with different doses of compounds B (αD-GLN), C (Rham-GAEL) & E (L-GLN) for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

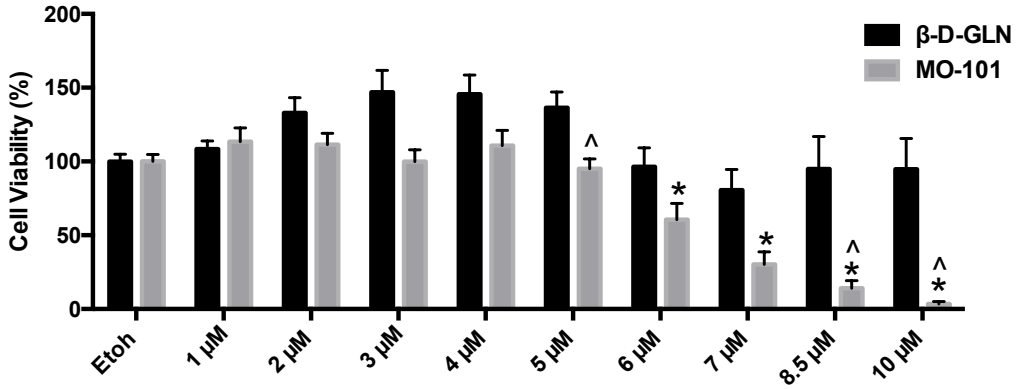
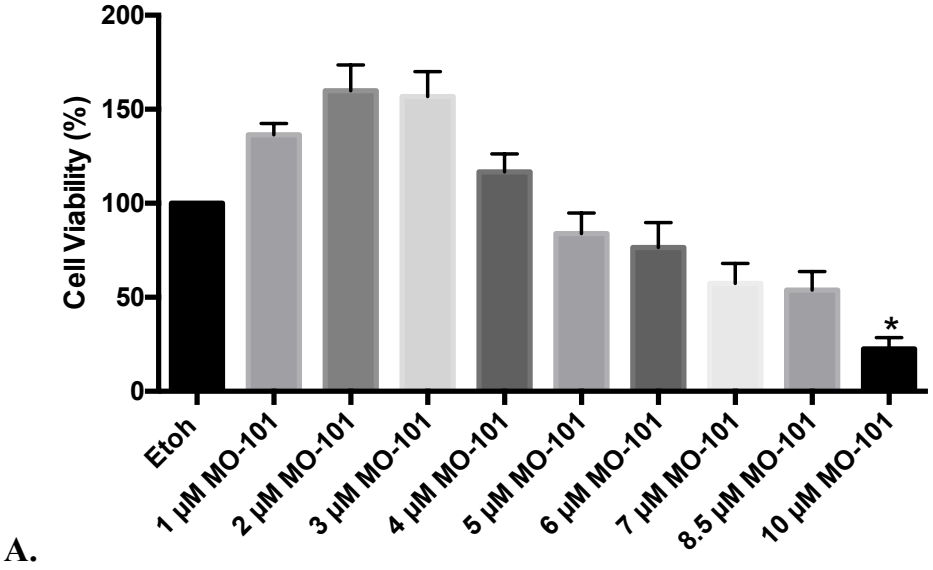
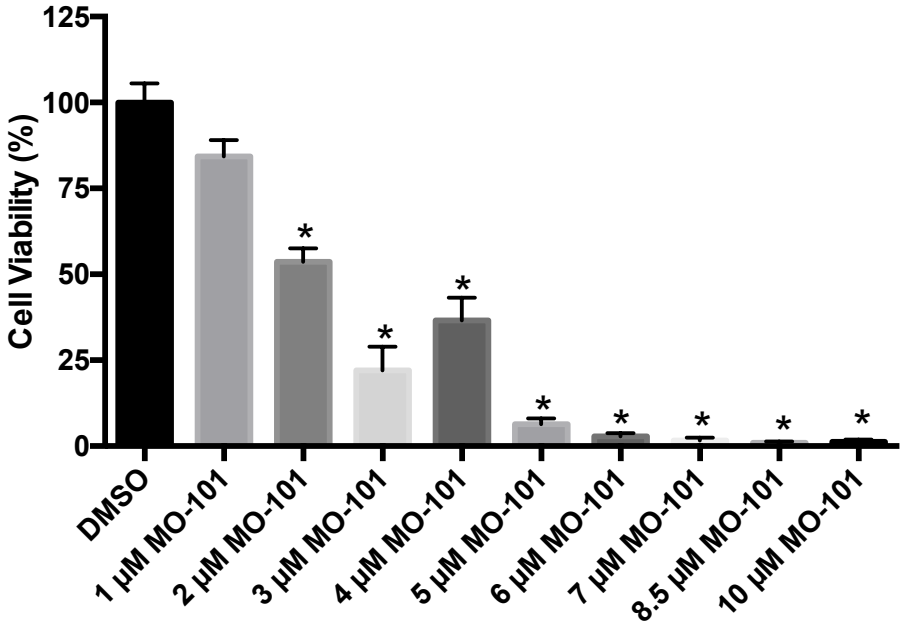


Figure 4-11. Non-adherent EOC146 cells treated with different doses of β-D-GLN and MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* Compared to EtOH, ^ compared to β-D-GLN



A.



B.

Figure 4-12. **A.** Non-adherent EOC103E and **B.** non-adherent EOC140 cells treated with different doses of MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* compared to DMSO.

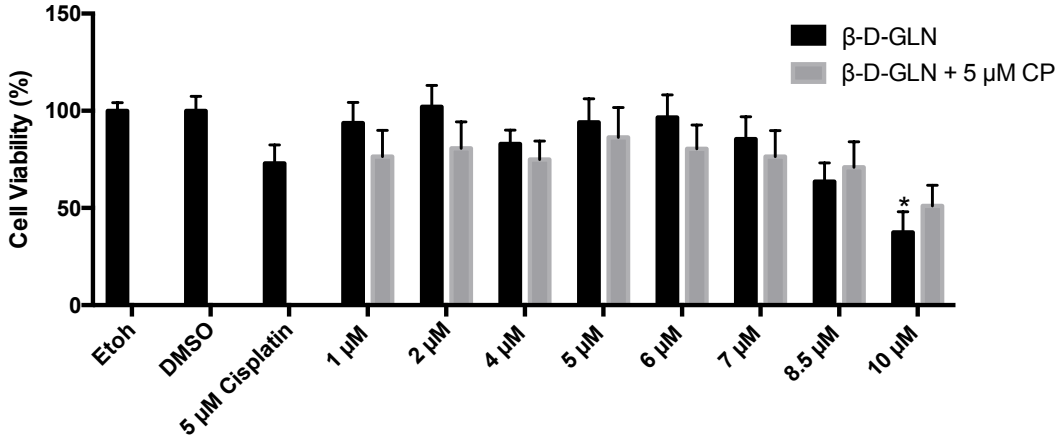


Figure 4-13. Non-adherent A2780s cells treated with different doses of β-D-GLN alone as control, and in combination with 5 μM cisplatin for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* Compared to controls: EtOH, DMSO, & cisplatin, and + compared to MO-101.

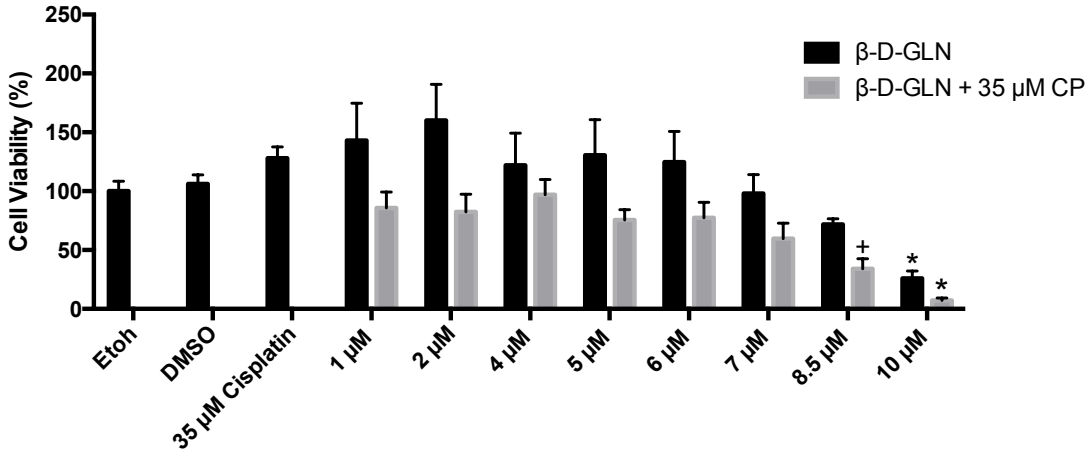


Figure 4-14. Non-adherent A2780cp cells treated with different doses of β-D-GLN alone as control, and in combination with 35 μM cisplatin for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* compared to controls: EtOH, DMSO, & cisplatin, and + compared to MO-101.

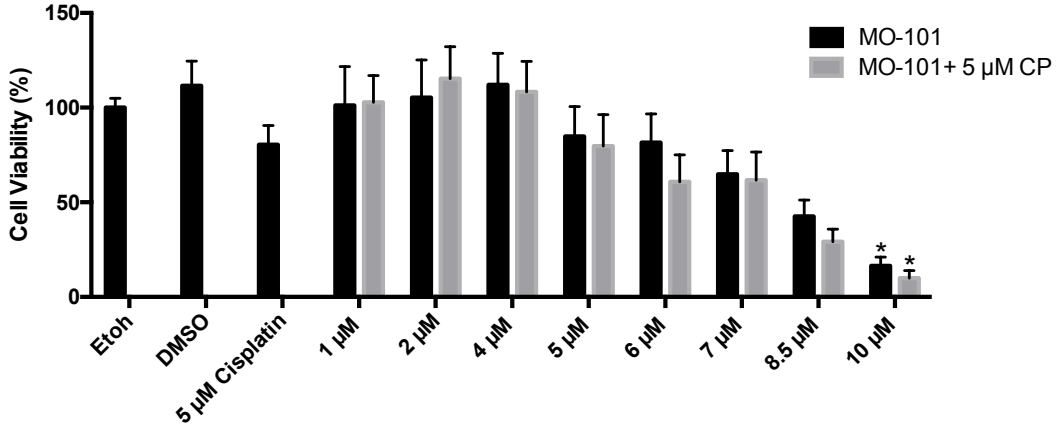


Figure 4-15. Non-adherent A2780s cells treated with different doses of MO-101 alone as control, and in combination with 5 µM cisplatin for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
* compared to controls: EtOH, DMSO, & cisplatin.

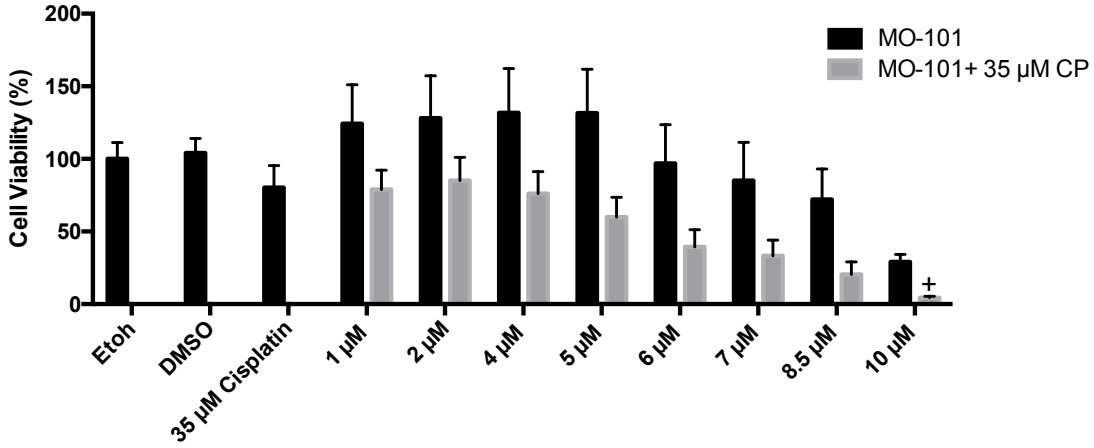


Figure 4-16. Non-adherent A2780cp cells treated with different doses of MO-101 alone as control, and in combination with 35 µM cisplatin for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.
+ compared to MO-101.

Chapter V: Isolate putative EOC stem cells from cell lines and test the efficacy of GAELs -/+ cisplatin on cell viability in non-adherent cultures

5.1. Introduction

One important aspect involved in EOC chemoresistance is the link between chemoresistance and EOC stem cells (Steg et al., 2012). Cancer stem cells have the ability to initiate or sustain tumorigenesis, and are resistant to conventional therapies (Kreso & Dick, 2014). Studies have suggested that cancer stem cells are responsible for disease recurrence with chemoresistance (Eramo et al., 2006; Garvalov & Acker, 2011; Hermann et al., 2007; Li et al., 2008). Cancer stem cells may have inherent resistance, or can acquire resistance to apoptotic cell death after exposure to chemotherapeutic agents (Eramo et al., 2006; Hermann et al., 2007; Li et al., 2008). Therefore, disease recurrence is produced with new drug-resistant cancer cells (Jian Chen et al., 2012; Driessens, Beck, Caauwe, Simons, & Blanpain, 2012; Schepers et al., 2012). It is theorized that killing EOC stem cells would eliminate the original source of resistant EOC cells, and more importantly prevent disease recurrence.

Putative EOC stem cells that are present in EOC patient samples and cell lines, can be isolated using different stem cell markers, one of which is aldehyde dehydrogenase (ALDH) (Ayub et al., 2015; Kryczek et al., 2012; Stewart et al., 2011). ALDH is a stem cell marker consistently used to isolate populations of EOC stem cells (Ayub et al., 2015; Kryczek et al., 2012; Meng et al., 2014). ALDH is highly expressed in stem cells and cancer stem cells including breast, colon, brain, live and EOC cells (Vasiliou & Nebert, 2005). It is an enzyme expressed in the cytoplasm and central to the metabolism of retinol to its active state (retinoic acid), which can then

enter the nucleus and regulate transcription via retinoic acid receptors. Studies have demonstrated that ALDH expressing EOC cells are more resistant to chemotherapy; however, the mechanism of resistance is not well understood and may be unrelated to the biological activity of ALDH (Guddati, 2012; Meng et al., 2014). We isolated putative EOC stem cells based on ALDH activity using A2780 and COV362 cell lines. Sorted populations were examined for their responses to cisplatin and GAEL compounds.

5.2. Results

5.2.1 EOC cell sorting

Cells were sorted and isolated by flow cytometry using the Aldefluor kit (StemCell Technologies), which allows isolation of live cells expressing ALDH.

5.2.1. 1. A2780cp. To ensure that the populations of A2780cp cells were cisplatin resistant, A2780cp cells were treated once per week to 10 μM cisplatin. After 3 weeks, resistant A2780cp cells were sorted into ALDH ^{Low} and ALDH ^{High} by Dr. G. Arthur's laboratory using the cell sorting facility. The ALDH ^{High} cells constituted more than 70% of the total cell fraction (Figure 5-1). Evaluation of A2780s cells showed less than 2% ALDH ^{High} cells; therefore, these cells were not isolated. To be sure that the sorted cells were viable as non-adherent cultures, proliferation and cell viability was measured over time (Figure 5-2). After almost eight months of using the cells after sorting, the ALDH status was measured and 64% of the ALDH ^{High} cells retained expression of ALDH (Figure 5-3).

5.2.1. 2. COV362. COV362 ALDH ^{High} cells constituted 79% of the total cell fraction (Figure 5-4). Two populations were collected from COV362, ALDH ^{Low}, and ALDH ^{High}.

5.2.2 Platinum sensitivity of sorted EOC cells

5.2.2. 1. A2780cp ALDH $^{High/Low}$. A2780cp ALDH $^{High/Low}$ cells were treated with increasing doses of cisplatin (10 μM to 90 μM) under non-adherent culture conditions. Overall, A2780cp ALDH $^{High/Low}$ cell viability decreased gradually until a significant reduction (>50%) was reached at 80 μM and higher in A2780cp ALDH ^{Low}, and from 50 μM and higher in A2780cp ALDH ^{High} (Figure 5-5). Contrary to the literature, these results show that A2780cp ALDH ^{High} were more platinum sensitive than A2780cp ALDH ^{Low}. The cisplatin sensitivity of A2780cp ALDH $^{High/Low}$ cells was higher compared to the mixed population of parental A2780cp cells, with A2780cp ALDH ^{Low} being the most resistant population.

5.2.2. 2. COV362 ALDH $^{High/Low}$. COV362 $^{High/Low}$ cells were treated with increasing doses of cisplatin using the non-adherent culture system. COV362 ALDH ^{High} cells were more resistant to cisplatin than COV362 ALDH ^{Low} only at the highest does (90 μM) (Figure 5-6).

5.2.3 GAELs dose-response assays on sorted EOC cell line

A2780cp ALDH $^{High/Low}$ cells were exposed to increasing doses of GAEL compounds (2.5 – 30 μM). Both populations responded similarly to β-D-GLN treatment, with cell viability significantly reduced starting at 20 μM ~~only~~ (Figure 5-7).

MO-101 was more effective at decreasing cell viability at lower doses, starting from 10 μM (Figure 5-8). Moreover, A2780cp ALDH ^{High} cells were affected by MO-101 treatment more than A2780cp ALDH ^{Low} cells.

Additional GAEL compounds were tested on A2780cp ALDH $^{High\ /Low}$ cells, including α-D-GLN, Rham-GAEL, and L-GLN. The response trend was similar between the two populations to each compound, with α-D-GLN reducing cell viability at modestly lower doses (Figure 5-9 & 5-10).

Generally, A2780cp ALDH $^{High/Low}$ cells were slightly more resistant to GAEL treatment compared to parental A2780cp cells, with MO-101 having the greater cell killing effect. Thus, these sorted EOC cells were selected to evaluate GAEL cell death mechanisms. The parental compound β-D-GLN in addition to the modified MO-101, were used for these experiments.

5.2.4 Evaluating the GAEL-induced cell death mechanism of action

5.2.4. 1. EOC cell line

- **Lysosomal cell death**. Previous work showed that GAELs induced cell death in a variety of human cancer cell lines, including breast cancer stem cells, via caspase- and ROS-independent cell death (Arthur & Bittman, 2014; Samadder et al., 2014). Once GAELs enter cancer cells, they produce release of lysosomal cathepsin D to induce cell death (Arthur & Bittman, 2014; Samadder et al., 2014). We evaluated this mechanism by co-treating A2780cp ALDH $^{High/Low}$ cells with pepstatin A, an inhibitor

of aspartyl proteases including cathepsin D, plus β-D-GLN or MO-101 (J. Ali conducted these experiments). The cells were treated with 25 μM and 30 μM of β-D-GLN, and 12.5 μM of MO-101 alone or in combination with different doses of pepstatin A (20, 25 and 30 μg/mL) under non-adherent culture conditions. GAEL-induced cell death was not rescued by pepstatin A (Figure 5-11 & 5-12), suggesting that GAELs may induce cell death via another mechanism in non-adherent conditions. The same experiment was conducted using adherent conditions, but exhibited the same results (Figure 5-13). Therefore, we tested whether GAEL-induced cell death was occurring via a different mechanism.

- **Apoptotic cell death.** To investigate the possibility that GAELs induced cell death via caspase activation, A2780cp ALDH $^{High/Low}$ cells were treated with GAELs and activated caspases were measured using the Casp-GLOW kit. The cells were exposed to 25 μM and 12.5 μM of β-D-GLN or MO-101, respectively, or 20 μM of staurosporine, which was used as a positive control for caspase activation. Activated caspases were detected by flow cytometry (performed by Ludivine Morrison in Dr. Tamra Werbowetski-Ogilvie's laboratory). A2780cp ALDH $^{High\ /Low}$ cells showed increased caspase activation relative to the vehicle control with both GAEL compounds (Figure 5-14). A2780cp ALDH $^{High\ /Low}$ cells were also pre-treated with a pan-caspase inhibitor, QVD-OPH, to further evaluate GAEL-induced cell death.

A2780cp ALDH $^{High/Low}$ cells were treated with 20 μM β-D-GLN or 7.5 μM MO-101 receptively, in addition to 25 μM of QVD-OPH in non-adherent cultures. Cell death was rescued by QVD-OPH in A2780cp ALDH ^{High} cells, but not ALDH ^{Low} cells (Figure 5-15).

To gain further insight into how GAELs may be affecting EOC cell viability, cell cycle analysis by flow cytometry was conducted using BrdU and 7AAD in A2780cp ALDH $^{High /Low}$ cells under non-adherent conditions. No significant changes in any specific stage of the cell cycle in response to GAEL treatment was observed (data not shown). Live/dead cell analysis using Annexin V and 7AAD in A2780cp ALDH $^{High/Low}$ cells was done by J. Ali and flow cytometry performed by L. Morrison (Werbowetski-Ogilvie laboratory). ALDH $^{High /Low}$ cells were treated with three different doses of β-D-GLN or MO-101 (indicated in the figure legend). The most significant cell death in A2780cp ALDH ^{High} cells was exhibited at the highest doses of GAEL treatment, 15 μM MO-101 and 25 μM GLN, at 68.4% and 80.6% respectively (Figure 16). A2780cp ALDH ^{Low} cells showed significant cell death with 15 μM MO-101 (73.1%) and 25 μM GLN (68.4%) treatment (Figure 5-16). To investigate the potential mechanism of enhanced phosphatidylserine flipping to the outer membrane leaflet (detected by Annexin V), Annexin V/7AAD experiments were performed with cells that had been pre-treated with the pan-caspase inhibitor, QVD-OPH. A2780cp ALDH $^{High/Low}$ cells were pre-treated with 25 μM QVD-OPH for four hours, then 12.5 μM MO-101 and 20 μM GLN were added for two days. Cell death was prevented in

both cell populations for each compound, although the effect was greater for GLN, probably due to the higher level of cell death induced by this compound (Figure 5-17, & 5-18). These results strongly suggest that GAEL-induced cell death may be partially induced by an apoptotic mechanism in sorted EOC cell lines.

5.2.4. 2. Primary EOC cells

To further evaluate these results, selected experiments were conducted using primary EOC cell samples. Unsorted primary EOC cells were used because there were no significant differences in the majority of the results from sorted A2780cp cells.

- **Lysosomal cell death.** EOC140 and EOC126 cells were treated with 2, 5 μM of MO-101 alone and in combination with different doses of pepstatin A for two days (Figure 5-19). Unfortunately, MO-101 was not effective at killing the cells at the selected doses, therefore an effect of pepstatin A could not be measured.

- **Apoptotic cell death.** GAEL-induced cell death was measured in the presence or absence of the pan-caspase inhibitor, QVD-OPH. QVD-OPH did not prevent MO-101-induced death in primary EOC cells, EOC126 and EOC146. However, QVD-OPH effectively rescued cisplatin-induced cell death (Figure 5-20 A & C). These results from primary EOC cells strongly suggest that GAEL-induced cell death may be induced by a non-apoptotic mechanism, supporting the previous studies on GAEL mechanism of action (Arthur & Bittman, 2014; Samadder et al., 2014).

5.3. Discussion

Cancer stem cells are thought to be involved into disease recurrence by generating a new generation of cancer cells that are drug-resistant and capable of evading apoptotic pathways (Eramo et al., 2006; Garvalov & Acker, 2011; Hermann et al., 2007; Li et al., 2008). Killing EOC stem cells would eliminate the original source of resistant EOC cells, and more importantly prevent disease recurrence. Thus, we isolated putative EOC stem cells that are present in EOC cell lines as well as patient samples based on ALDH activity. ALDH is a stem cell marker consistently used to isolate populations of EOC stem cells (Ayub et al., 2015; Kryczek et al., 2012; Meng et al., 2014). We then tested their platinum chemotherapy sensitivity and the efficacy of GAELs compounds.

Contrary to published literature where Aldefluor sorted A2780-cp populations grown as monolayers exhibited a higher level of cisplatin resistance in the ALDH ^{High} cell populations (Meng et al., 2014), our ALDH ^{Low} cells showed more cisplatin-resistance than the ALDH ^{High} cells when grown as non-adherent cultures. This may be a reflection of growing as non-adherent cultures. Jennifer Ali tested the responses to cisplatin when cells were grown as monolayers to see if we could recapitulate the published research. ALDH ^{Low} cells were slightly more cisplatin-resistance than the ALDH ^{High} cells when grown as adherent cultures. It should be noted that evaluating ALDH status was then conducted to ensure ALDH stability in those populations, and the results showed that these A2780cp sorted populations maintained their ALDH

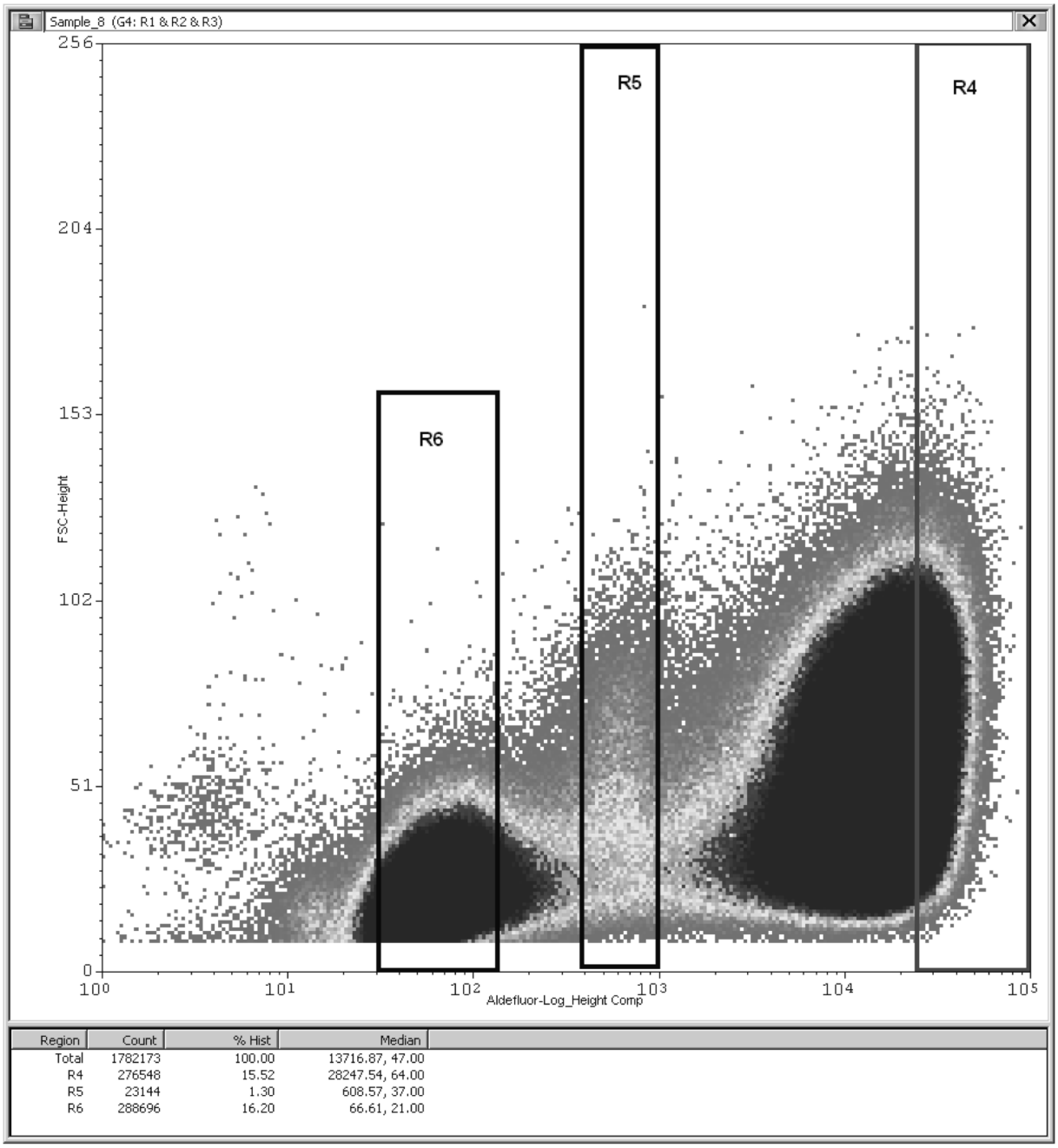
status. These contrary results might be due to our specific population of A2780 cells, and does not invalidate the published literature.

Another EOC cell line, COV362, was also sorted into ALDH $^{High/Low}$ cells. Parental COV362 cells were more sensitive to cisplatin treatment than COV362 ALDH ^{High}. Thus, the utility of using ALDH as a selection tool to isolate putative EOC stem cells and/or drug-resistant EOC cells is limited in our hands.

Despite the variable responses of sorted cells to cisplatin, GAELs efficiently killed all ALDH populations exhibiting chemotherapy resistance. A2780cp ALDH $^{High/Low}$ cells were used to test the efficacy of β-D-GLN and MO-101. The cells were treated with increasing doses of the drugs, ranging between 2.5 μM – 30 μM. β-D-GLN treatment reduced cell viability significantly at 30 μM only (>50%). While MO-101 effectively decreased A2780cp ALDH $^{High/Low}$ cell viability at lower doses, starting from 15 μM (>50%). Similar to cisplatin treatment, A2780cp ALDH^{+} cells were affected by MO-101 treatment more than A2780cp ALDH ^{Low} cells. Generally, A2780cp ALDH $^{High/Low}$ cells were slightly resistant to GAEL treatments compared to parental A2780cp cells, with MO-101 having the greater cell killing. Thus, these sorted EOC cells were selected to evaluate GAEL-induced cell death mechanisms, especially after confirming that GAEL compounds were capable of inducing cell death in EOC cells.

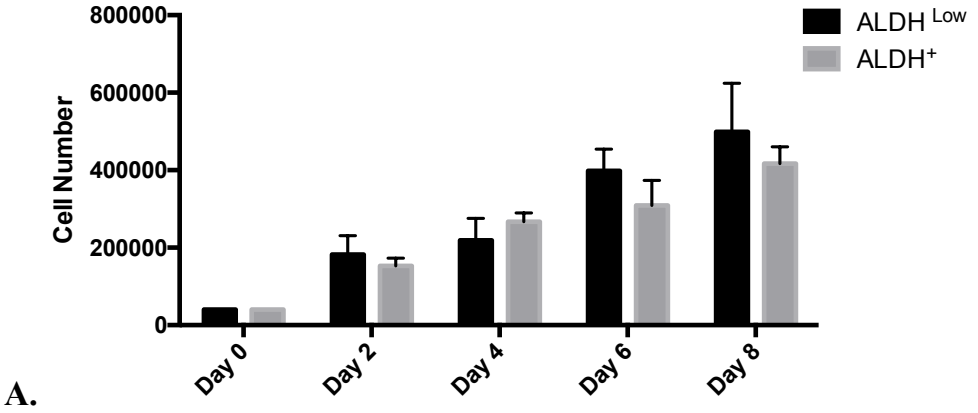
Previous work showed that once GAELs enter cancer cells, they produce release of lysosomal cathepsin D to induce cell death, via a non-apoptotic mechanism (Arthur

& Bittman, 2014; Samadder et al., 2014). We evaluated this mechanism in our EOC sorted cell populations. Inhibiting the lysosomal cathepsin D did not rescue cell death in adherent and non-adherent conditions. The results from A2780cp ALDH $^{High/Low}$ cells strongly suggest that GAELs can induce cell death by apoptotic pathways. Moreover, results from measuring apoptotic pathways by several different techniques showed activation of caspase pathways, indicating that GAELs are capable of inducing cell death by apoptotic pathways in A2780 cells. Surprisingly, results from primary EOC cells exhibited no activity in apoptotic pathways when treated with β-D-GLN and MO-101 in non-adherent cultures. The results from primary EOC cells strongly suggest that cell death may be induced via a caspase-independent mechanism, supporting the previous studies on GAEL mechanism of action (Arthur & Bittman, 2014; Samadder et al., 2014). The different responses between EOC cell lines and primary cells strongly suggests that results from cell lines need to be interpreted with caution as they may not reflect the nature of the disease. In this manner, the use of primary cell samples in the non-adherent culture system provide an improved model to test responses of drug-resistant cells to novel treatments.

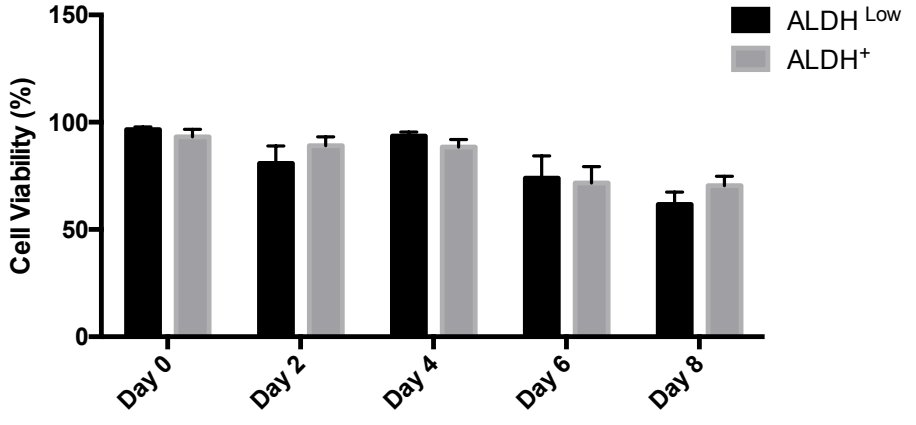


Region	Count	% Hist	Median
Total	1782173	100.00	13716.87, 47.00
R4	276548	15.52	28247.54, 64.00
R5	23144	1.30	608.57, 37.00
R6	288696	16.20	66.61, 21.00

Figure 5-1. Sorting image of A2780 cell lines. R6 is ALDH^{-}, R5 is ALDH ^{low}, and R4 indicate ALDH ^{High} population.

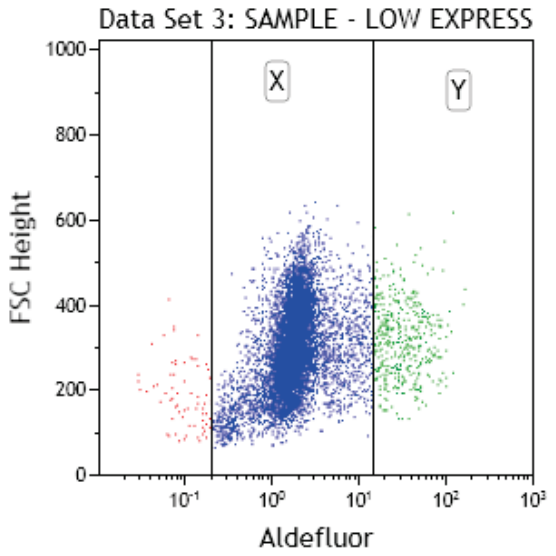


A.

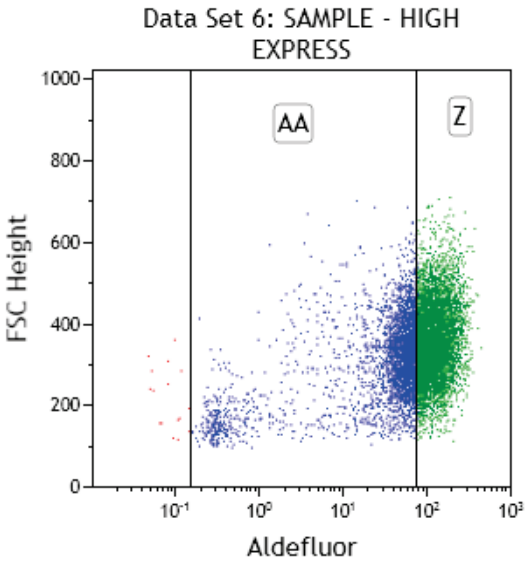


B.

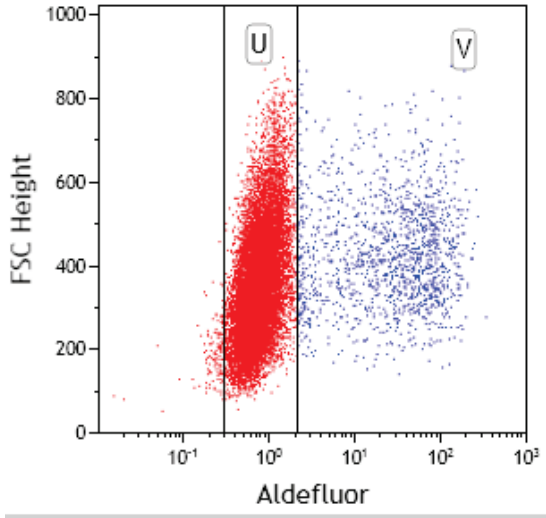
Figure 5-2. A. Cell proliferation of A2780cp ALDH $^{Low/\ High}$ cells during eight days. **B.** Cell viability of A2780cp ALDH $^{Low/\ High}$ cells during eight days.



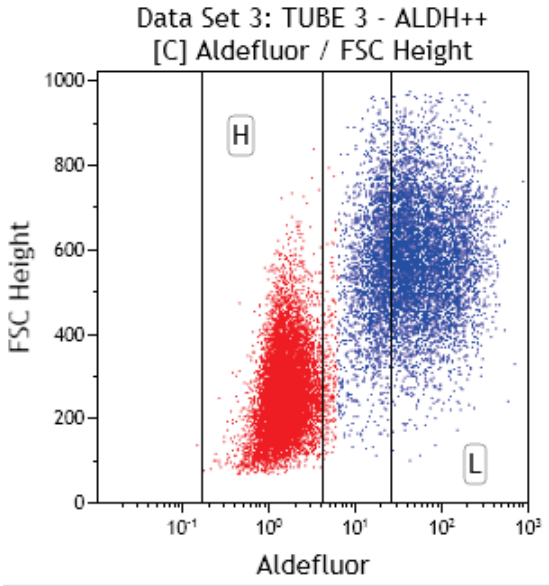
Gate	Number	%Gated
All	10,142	100.00
X	9,661	95.26
Y	412	4.06



Gate	Number	%Gated
All	10,239	100.00
AA	3,609	35.25
Z	6,614	64.60



Gate	Number	%Gated	X-Med
All	20,498	100.00	0.76
U	18,967	92.53	0.73
V	1,416	6.91	31.73



Gate	Number	%Gated	X-Med
All	20,433	100.00	2.26
H	11,898	58.23	1.45
L	5,955	29.14	62.09

Figure 5-3. Results of measuring ALDH status in A2780cp eight months after sorting.

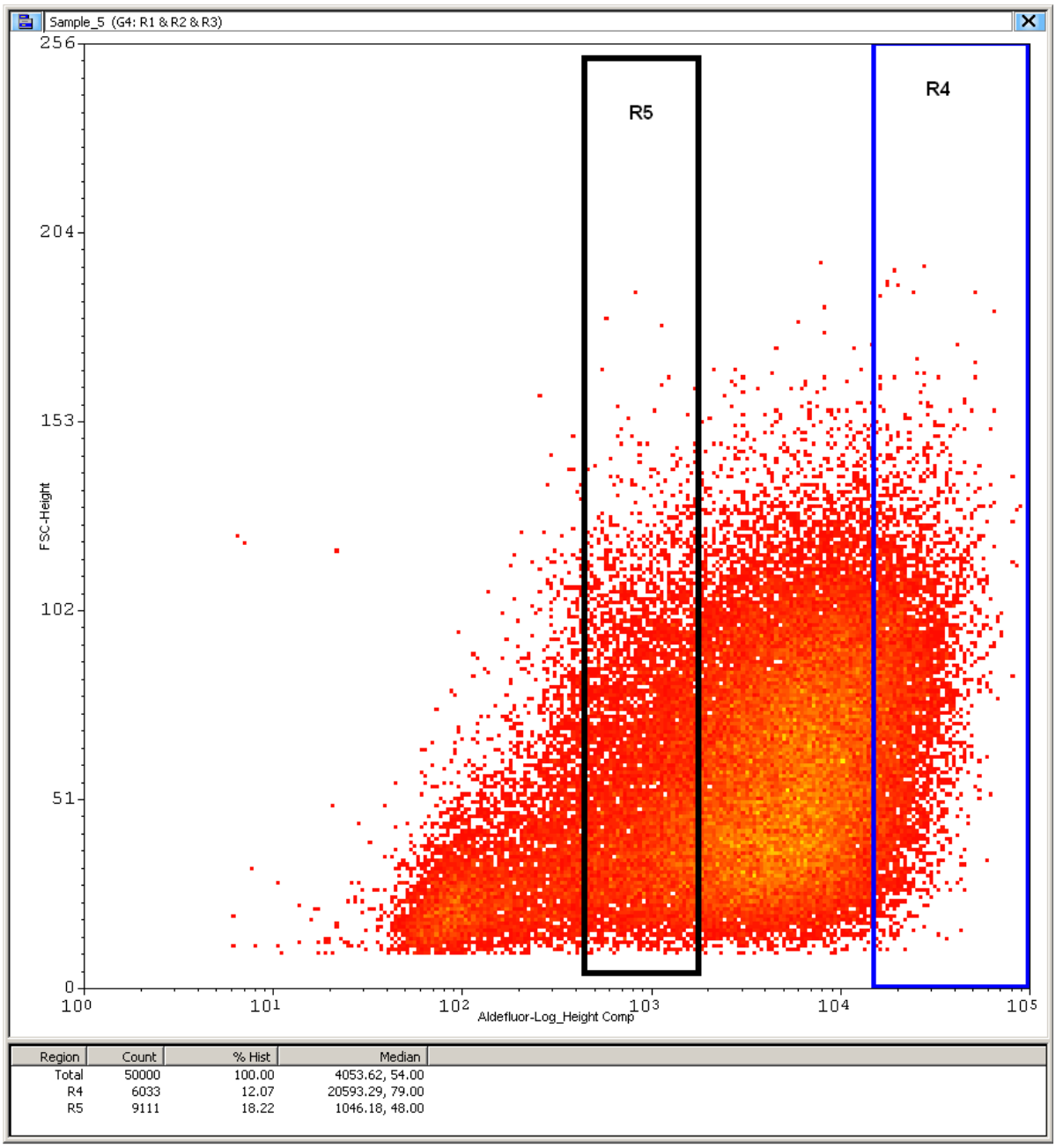


Figure 5-4. Sorting image of COV362 cell lines. R5 is ALDH ^{low}, and R4 indicate ALDH ^{High} population.

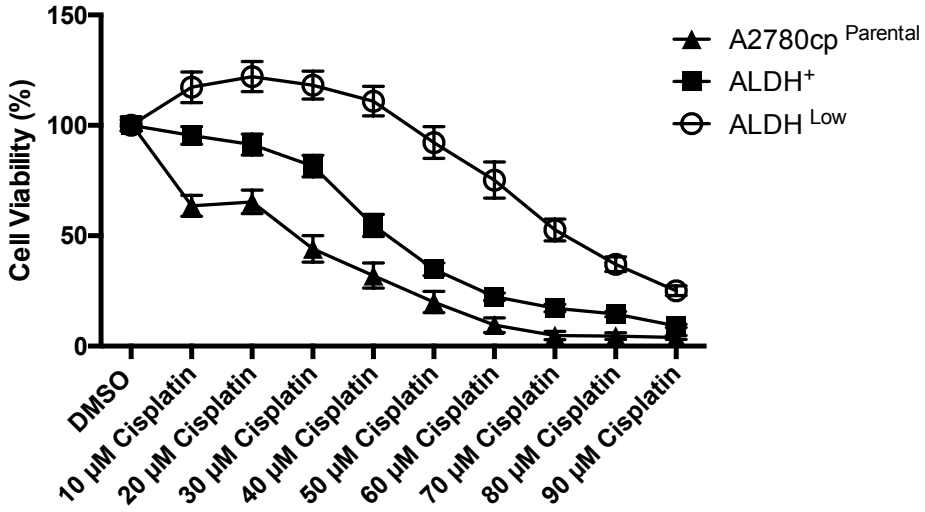


Figure 5-5. Non-adherent A2780cp ^{parental}, A2780cp ALDH ^{High} and A2780cp ALDH ^{Low} cells treated with different doses of cisplatin for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

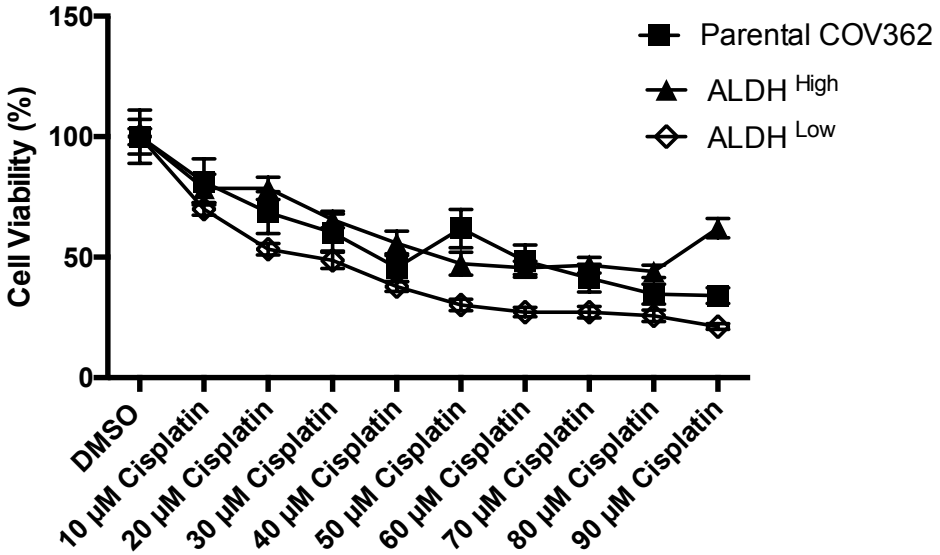


Figure 5-6. Non-adherent COV362 ^{parental}, COV362 ALDH ^{High} and COV362 ALDH ^{Low} cells treated with different doses of cisplatin for three days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

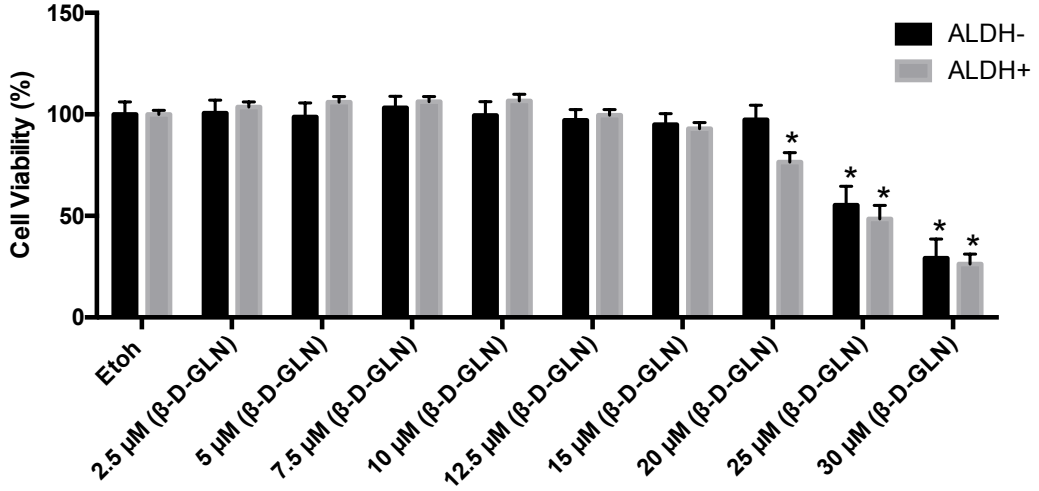


Figure 5-7. Non-adherent A2780cp ALDH $^{Low/High}$ cells treated with different doses of β-D-GLN for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to EtOH, ^ compared to ALDH$^-$

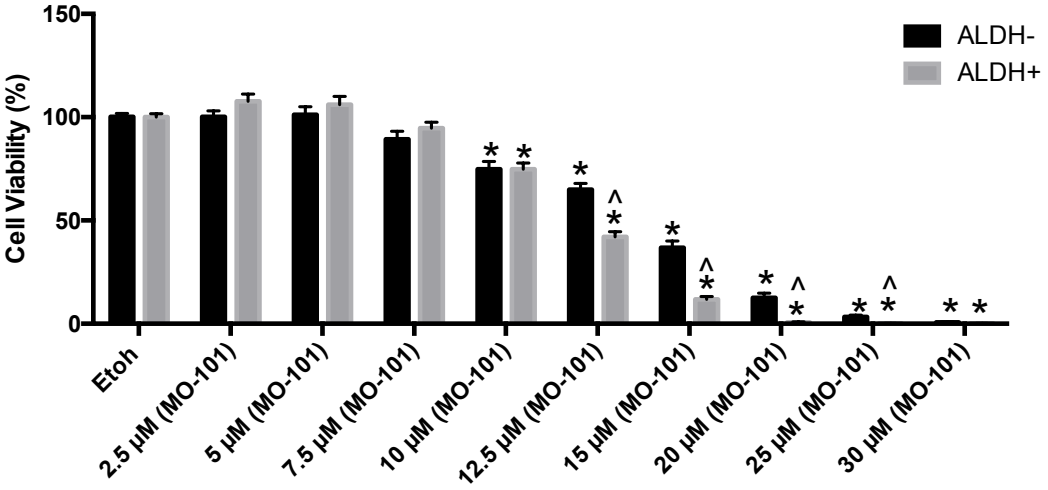


Figure 5-8. Non-adherent A2780cp ALDH $^{Low/High}$ cells treated with different doses of MO-101 for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to EtOH, ^ compared to ALDH$^-$

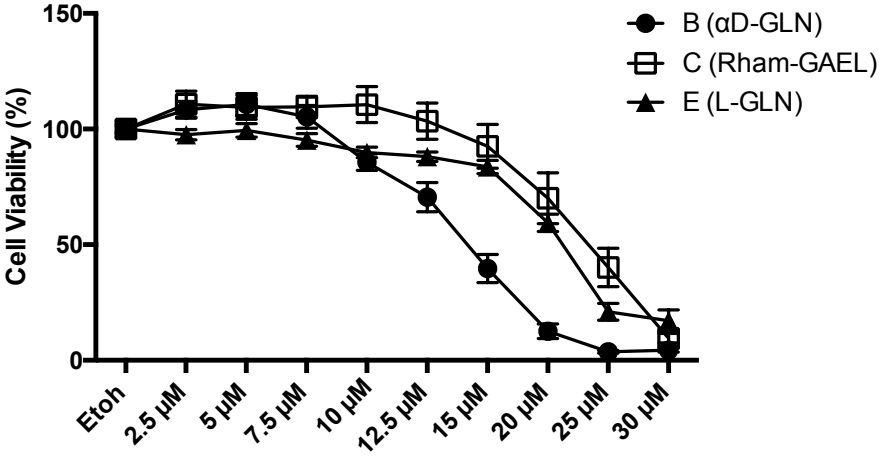


Figure 5-9. Non-adherent A2780cp ALDH ^{Low} cells treated with different doses of compounds B (α-D-GLN), C (Rham-GAEL) & E (L-GLN) for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

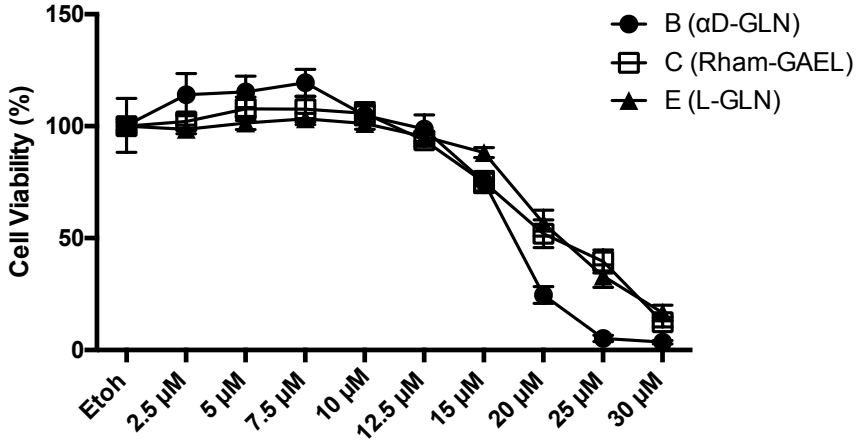


Figure 5-10. Non-adherent A2780cp ALDH ^{High} cells treated with different doses of compounds B (α-D-GLN), C (Rham-GAEL) & E (L-GLN) for two days. N = 3, n = 18/condition. Analyzed by One-way ANOVA multiple comparisons.

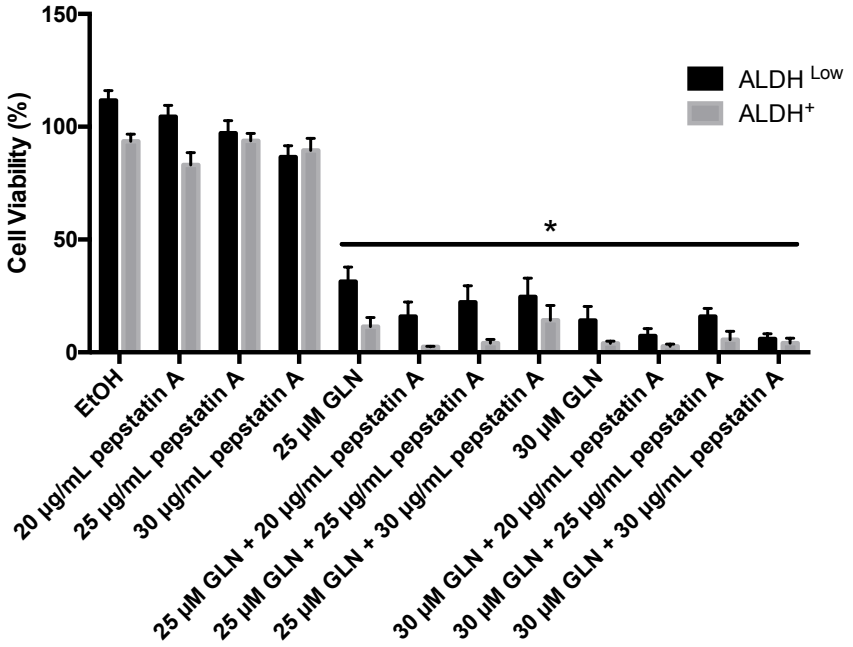


Figure 5-11. Non-adherent A2780cp ALDH $^{Low/\ High}$ cells treated with different doses of β-D-GLN and pepstatin A for two days. N = 3, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to controls

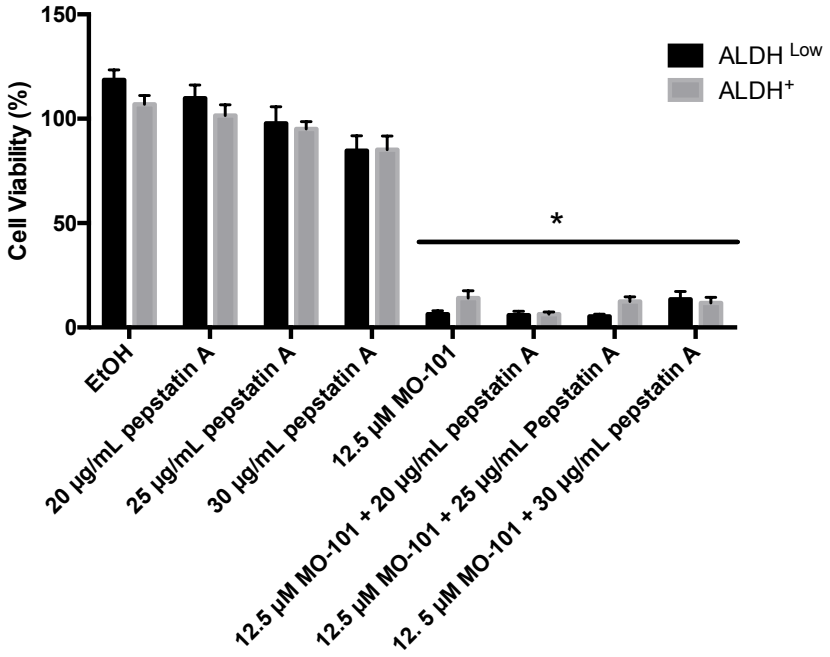
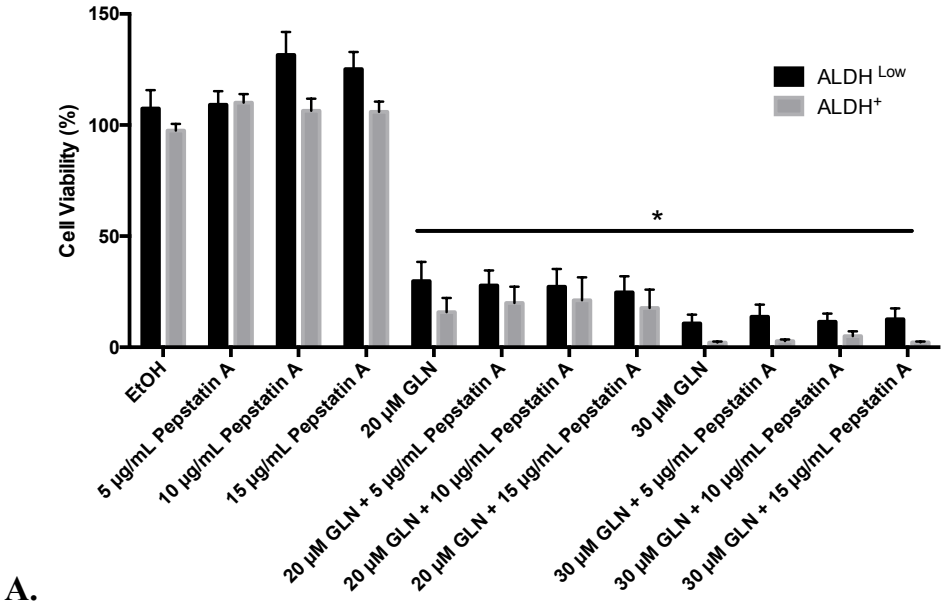
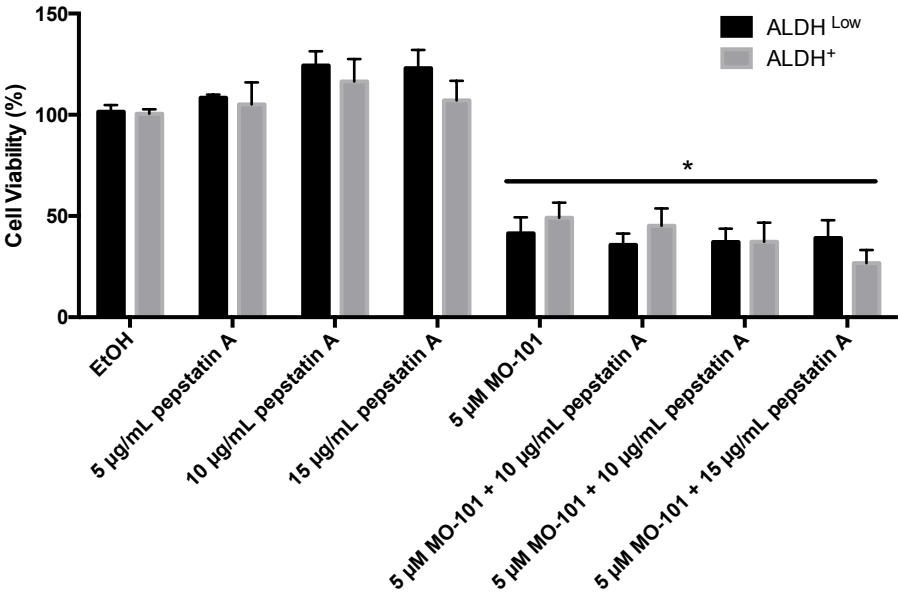


Figure 5-12. Non-adherent A2780cp ALDH $^{Low/\ High}$ cells treated with different doses of β-D-GLN and pepstatin A for two days. N = 3, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to controls



A.



B.

Figure 5-13. **A.** Adherent A2780cp ALDH $^{Low/\ High}$ cells treated with different doses of β-D-GLN and pepstatin A for two days. N = 3, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons. **B.** Adherent A2780cp ALDH $^{Low/\ High}$ cells treated with different doses of MO-101 and pepstatin A for two days. N = 3, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to controls

EOC Cells	25 μM GLN	12.5 μM MO-101	20 μM staurosporine
A2780-cp ALDH^{low}	14.6% +/- 5.1%	9.25% +/- 1.4%	2.8%+/- 1.0%
A2780-cp ALDH^{high}	23.5% +/-12.6%	10.8% +/- 4.9%	4.8% +/- 0.7%

Figure 5-14. Casp-glow experiment on A2780cp ALDH $^{Low/ High}$ cells treated with 25 μM β-D-GLN, 12.5 μM MO-101, and 20 μM staurosporine. The table shows fold increase in activated caspase activity relative to vehicle control.

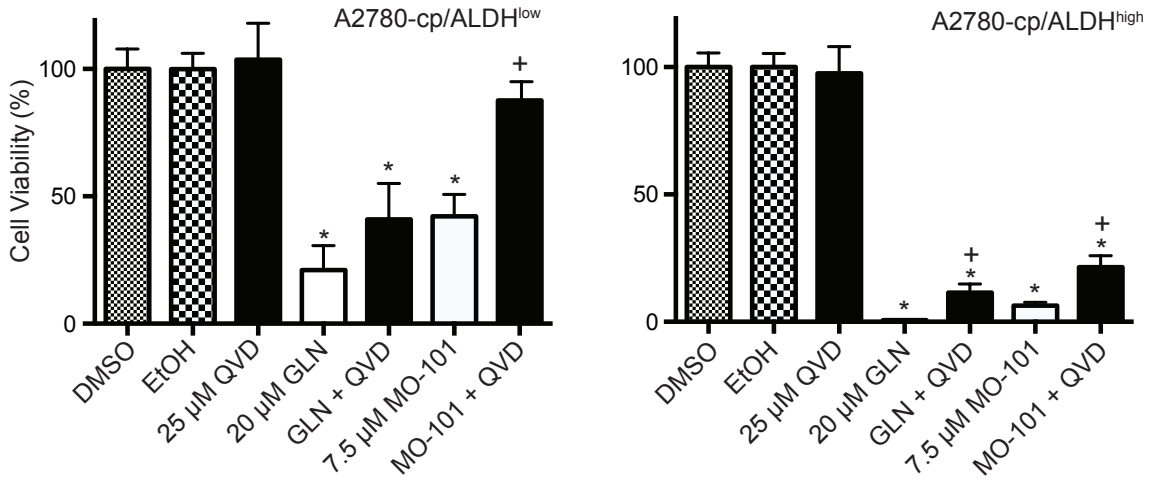


Figure 5-15. Non-adherent A2780cp ALDH $^{Low/ High}$ cells treated with different doses of β-D-GLN/MO-101 and pan-caspase inhibitor for two days. N = 3, n = 9/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to controls: DMSO & EtOH, + compared to MO-101 alone

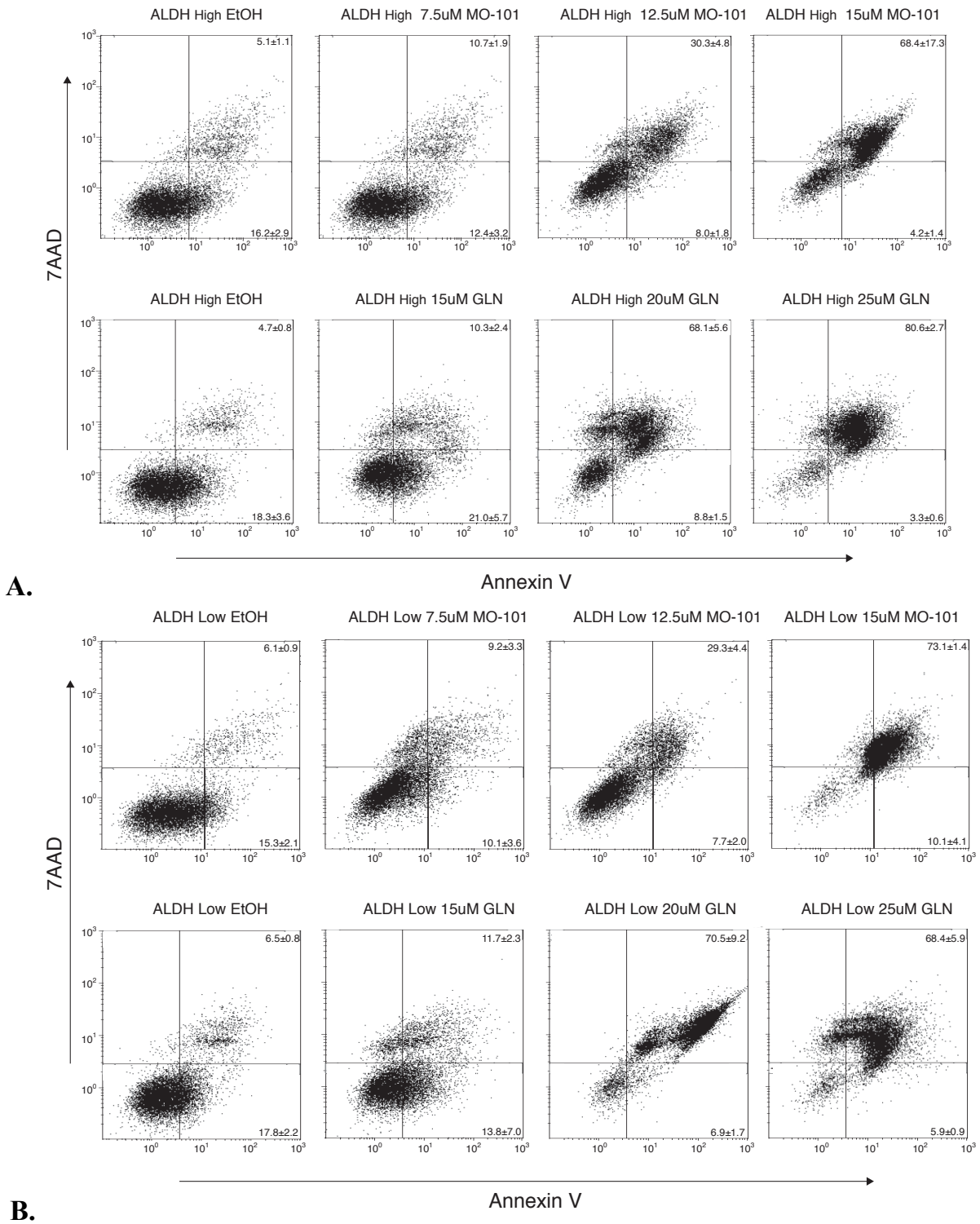


Figure 5-16. Annexin V and 7AAD analysis in **A.** A2780cp ALDH ^{High} and **B.** A2780cp ALDH ^{Low} cells. The cells were treated with 7.5, 12.5, and 15 μM of MO-101, plus 15, 20, and 25 μM of β-D-GLN in non-adherent cultures.

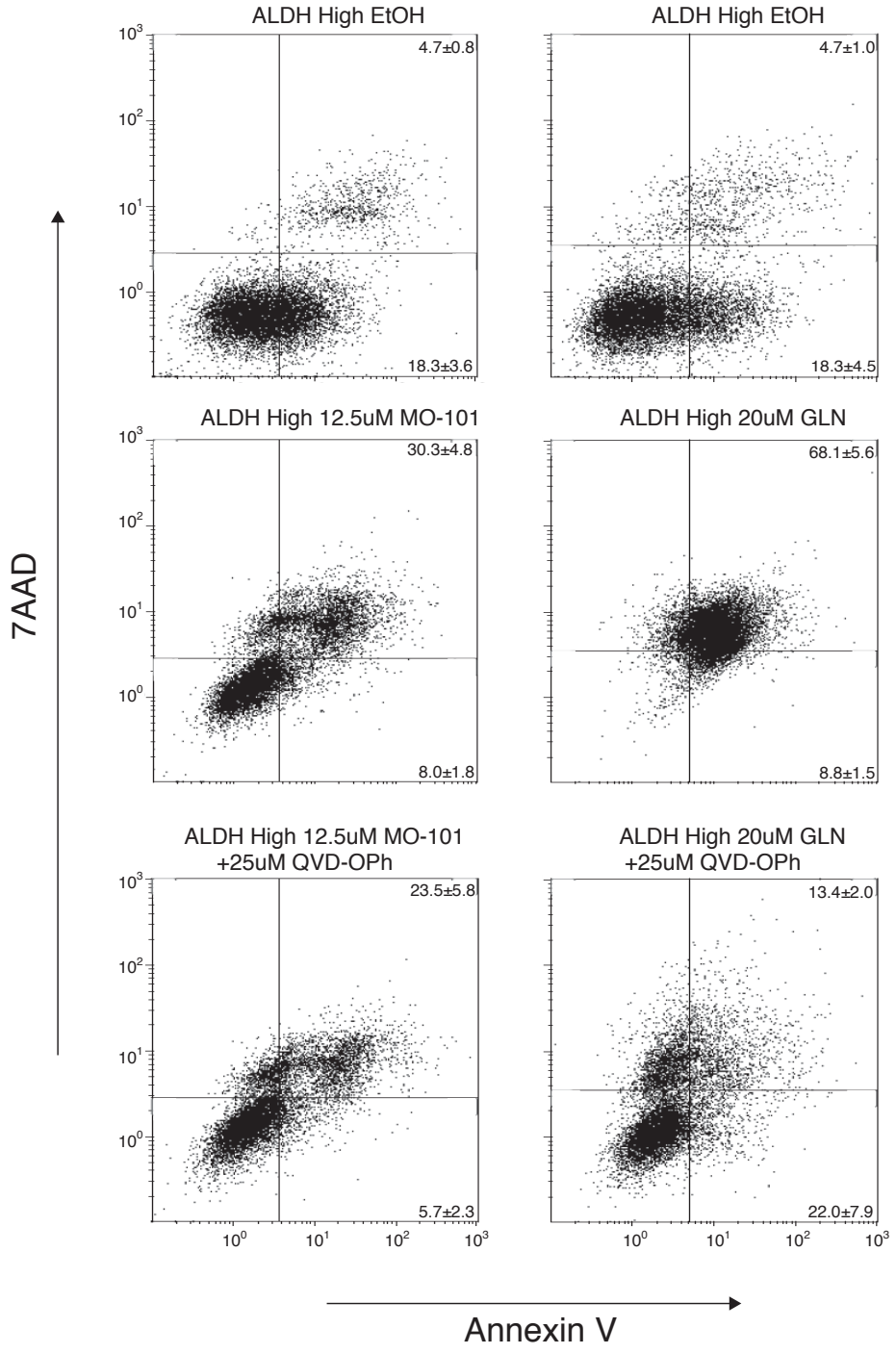


Figure 5-17. Annexin V and 7AAD analysis in A2780cp ALDH ^{High} cells with the pre-treatment of pan-caspase inhibitor, QVD-OPH. The cells were treated with 12.5 μM of MO-101, and 20 μM of β-D-GLN plus 25 μM QVD-OPH, in non-adherent cultures.

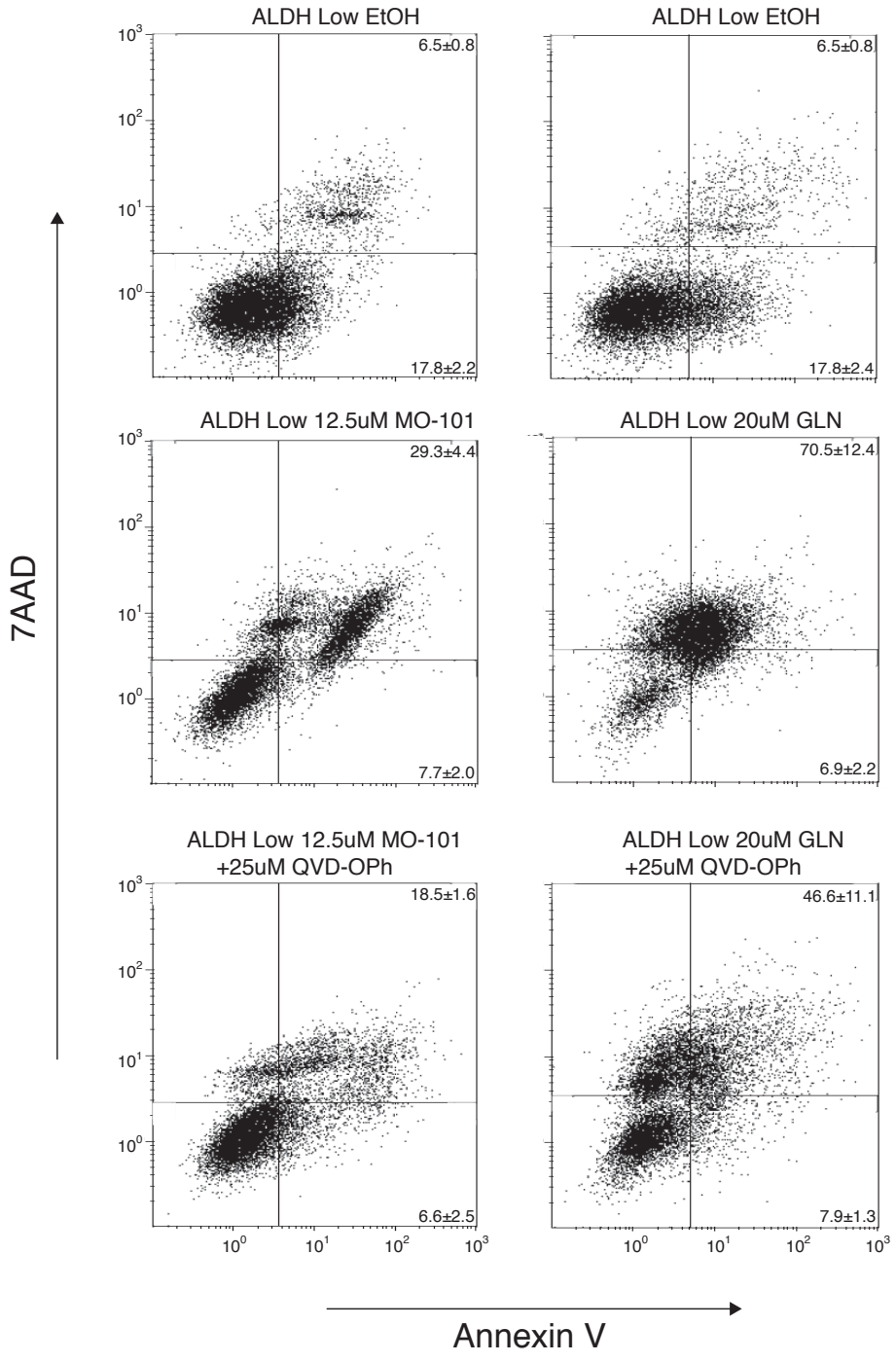


Figure 5-18. Annexin V and 7AAD analysis in A2780cp ALDH ^{Low} cells with the pre-treatment of pan-caspase inhibitor, QVD-OPH. The cells were treated with 12.5 μM of MO-101, and 20 μM of β-D-GLN plus 25 μM QVD-OPH, in non-adherent cultures.

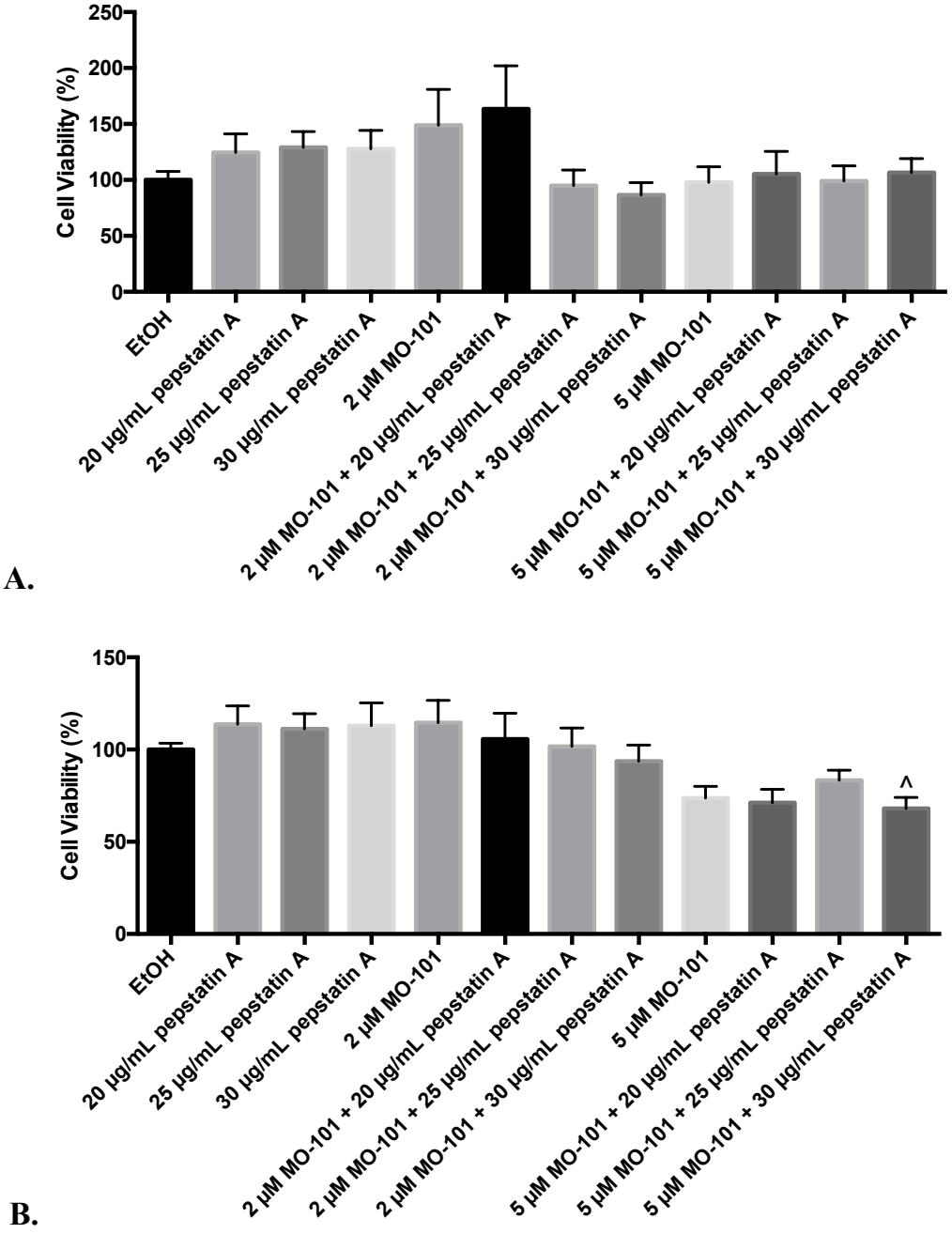


Figure 5-19. A. Non-adherent EOC140 and **B.** EOC126 cells treated with different doses of MO-101 and pepstatin A for two days. N = 3, n = 12/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to EtOH, ^ compared to pepstatin A control.

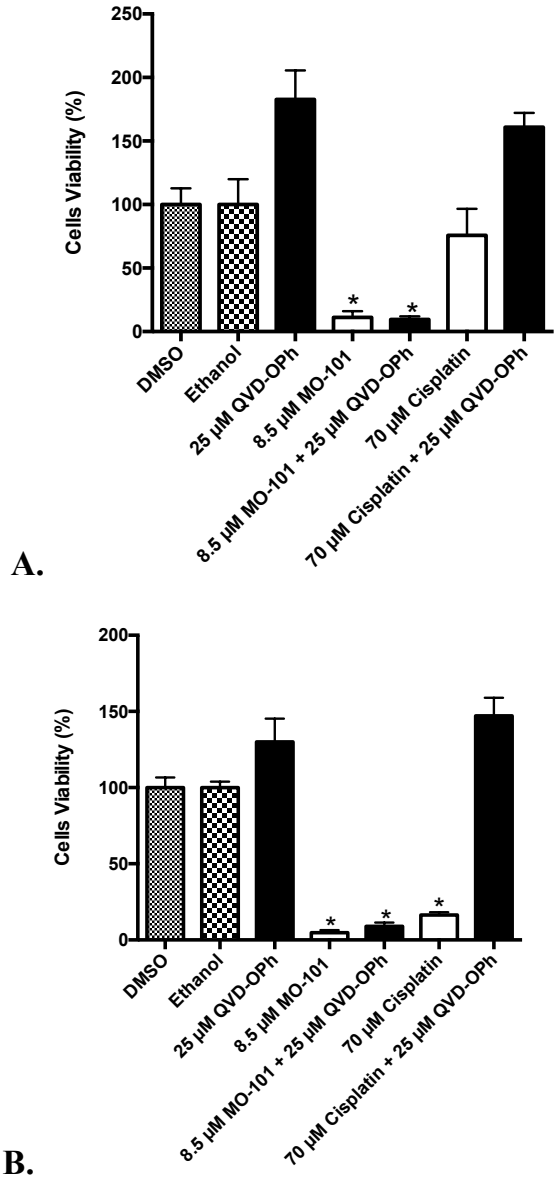


Figure 5-20. **A.** EOC126, and **B.** EOC146 cells treated with different doses of β-D-GLN/MO-101 and pan-caspase inhibitor for two days. N = 3, n = 9/condition. Analyzed by One-way ANOVA multiple comparisons. * Compared to controls: DMSO & EtOH, + compared to QVD.

Chapter VI: Summary & Future Directions

Summary and Future Directions

EOC has a high level of mortality, and chemotherapy resistance in EOC is a significant reason for the high rate of death among patients (Buys et al., 2011) (McGuire et al., 1996). Developing drug resistance is a result of one of several mechanisms to avoid cell death (Köberle et al., 2010). The goal of this thesis project was to evaluate the response of chemotherapy-resistant EOC cells to novel drug treatments. The efficacy of novel drugs to affect platinum resistant EOC cell viability were tested by **1)** evaluating the efficacy of kinase inhibitors (DM, LDN) on drug-resistant EOCs, **2)** evaluating the efficacy of GAELs -/+ cisplatin using EOC cell lines and primary EOC cells, and **3)** isolating putative EOC stem cells from cell lines to test the efficacy of GAELs -/+ cisplatin on cell viability. While monolayer cultures are still being widely used, non-adherent culture conditions were tested herein.

The efficacy of DM and LDN were tested on drug-resistant EOCs growing in non-adherent cultures. Previous research showed that DM and LDN induced EOC cell death by autophagy or induction of reactive oxygen species, respectively (Ali et al., 2015). The first goal from this aim was to test the efficacy of DM or LDN in combination with cisplatin using non-adherent cultures. While the *in vitro* results were promising, the *in vivo* combination therapy experiments strongly suggest that DM or LDN are unlikely to be effective as adjuvant chemotherapy agents in EOC. Therefore, further experiments with DM and LDN were not justified.

GAELs were tested as novel agents that might prove effective against drug-resistant EOC cells. As single treatment agents, GAEL compounds decreased the EOC cell viability in a dose dependent manner. MO-101 was consistently more effective than other GAELs. EOC cell lines required higher doses of GAELs to induce cell death compared to primary EOC cell samples. Interestingly, GAELs effectively killed cisplatin sensitive and resistance EOC cells. However, there was no synergistic effect using combination treatment of GAEL compounds and cisplatin. Synergistic affects might occur in a dose dependent manner, by which using higher and different doses of cisplatin or the GAELs may have shown synergistic effects, but this would require further experiments to delineate a combination index. One limitation of this research was that GAEL compounds were not tested in combination with platinum drugs in primary EOC cultures, which would be a worthwhile experiment to conduct.

The most consistent method emerging in the literature to isolate EOC stem cells is to sort cells based on ALDH activity (Ayub et al., 2015; Kryczek et al., 2012; Stewart et al., 2011). ALDH was used as a marker to sort and isolate putative EOC stem cells. All ALDH populations exhibited a dose-dependent reduction in cell viability in response to GAELs; however, GLN was only effective at the highest doses, whereas MO-101 more frequently produced cell death at lower doses.

The mechanism of GAEL-induced cell-death was evaluated. The results from EOC cell lines suggest that cell death may be induced by an apoptotic mechanism. However, primary EOC results strongly support the previous studies on GAELs

mechanism of action (Arthur & Bittman, 2014; Samadder et al., 2014), which induce cell death by non-apoptotic mechanism.

The significance of this project is to identify novel drugs that may be used as first-line agents or adjuvant chemotherapy to treat recurrent, drug-resistant EOC. Understanding the importance of chemotherapy resistance in human EOC, and the approach to testing novel drugs for re-sensitizing chemotherapy resistant EOC cells will contribute to improve chemotherapy treatment for patients, and reduce the rate of mortality.

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