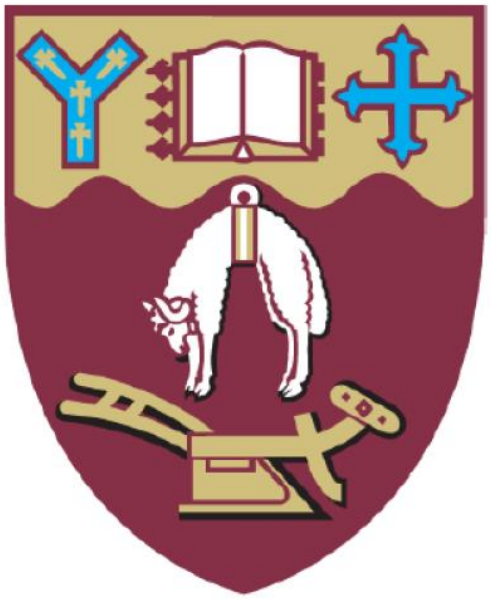
Anti-Tumour Effects of Resveratrol and Pterostilbene on Ovarian Cancer



A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Science in Cellular and Molecular Biology

in the

School of Biological Sciences

By Janelle Hosking

University of Canterbury

2016

Table of Contents

i. Contents……2
ii. Acknowledgments……5
iii. Abstract……6
iv. Abbreviations……7
v. List of Figures……9
vi. List of tables……10
1. Chapter 1 Introduction……11
1.1. Overview……11
1.2. Cancer……11
1.2.1. Ovarian Cancer and the Female Reproductive System……13
1.3. Cell Cycle and Proliferation……18
1.4. Metastasis ……21
1.5. Treatment Options for Ovarian Cancer……24
1.5.1. Chemotherapy……24
1.5.2. Surgery……25
1.5.3. Radiotherapy……27
1.6. Natural Substances, Polyphenols and Cancer……28
1.6.1. Resveratrol……29
1.6.2. Pterostilbene……31
1.7. Two Dimensional and Three Dimensional Cell Cultures……32
1.8. Aims and Hypotheses ……33
1.8.1. Aims……33
1.8.2. Hypotheses……33
1.8.3. Model Cell Lines……33
2. Chapter 2 Materials and Methods……34
2.1. Materials……34
2.1.1. General Materials……34
2.1.1.1. PBS……34
2.1.1.2. Trypsin-EDTA……34
2.1.1.3. DMEM-F12……34
2.1.1.4. Poly-HEMA……34
2.1.1.5. Crystal Violet……35
2.1.2. Anti-Cancerous Drugs……35
2.1.3. ELISA: Buffers and Solutions……35
2.1.4. Western Blot Buffers and Solutions……35
2.1.4.1. 2M Tris-HCl……35

2.1.4.2. 1M Tris-HCL……35
2.1.4.3. Running Buffer……35
2.1.4.4. Tris Buffer Saline (TBS)……36
2.1.4.5. 5X Sample Buffer……36
2.1.4.6. Transfer Buffer……36
2.1.4.7. Blocking Solutions……36
2.1.4.8. Antibody Solutions……36
2.2. Methods……36
2.2.1. Two Dimensional Cell Culture……36
2.2.2. Three Dimensional Cell Culture……37
2.2.3. Drug Treatment……38
2.2.4. Cell Morphology……38
2.2.5. Alamar Blue Assay……38
2.2.6. Crystal Violet Assay……39
2.2.7. VEGF ELISA……39
2.2.8. BCA Protein Assay……39
2.2.9. Western Blotting……40
2.2.10. Statistical Analysis……42
3. Chapter 3 Effects of Polyphenols on Cellular Metabolism and Growth of Ovarian Cancer Cells……43
3.1. Introduction……43
3.1.1. The Cell Cycle and Ovarian Cancer……43
3.1.2. Cellular Metabolism and Cancer……44
3.1.3. Aims of Chapter 3……44
3.2. Methods……45
3.3. Results……45
3.3.1. Effect of Resveratrol on Cell Morphology……45
3.3.2. Effect of Pterostilbene on Cell Morphology……45
3.3.3. Effect of Resveratrol and Pterostilbene Metabolic Activity……51
3.3.4. Effect of Resveratrol and Pterostilbene on Cell Number……51
3.4. Discussion……56
4. Chapter 4 Effects of Polyphenols on the Vascular Endothelial Growth Factor……60
4.1. Introduction……60
4.1.1. Vascular Endothelial Growth Factor……60
4.1.2. Aims of Chapter 4……61
4.2. Methods……62
4.3. Results……62
4.3.1. Effect of Resveratrol and Pterostilbene on VEGF Production……62
4.4. Discussion……64
5. Chapter 5 Effects of Polyphenols on the Expression of Proteins ……66
5.1. Introduction……66

5.1.1. Epidermal Growth Factor Receptor Family……..66
5.1.1.1. The Epidermal Growth Factor Receptor/ErbB1……..……66
5.1.1.2. Human Epidermal Growth Factor Receptor (Her2/Neu Protein)..69
5.1.1.3. Epidermal Growth Factor Receptor/ErbB1 and Neu/Neu Co-expression……..69
5.1.2. Current Drug Targets……70
5.1.3. The Phosphatidylinositol (3) Kinase Protein and Signalling Pathway……71
5.1.3.1. AKT Protein and Role in Signalling……74
5.1.4. Proliferating Cell Nuclear Antigen……75
5.1.5. Cyclin D and Cancer……..77
5.1.6. Aims of Chapter 5……77
5.2. Methods……78
5.3. Results……78
5.3.1. AKT/pAKT Expression after Resveratrol and Pterostilbene Treatment……78
5.3.2. Cyclin D2 Expression after Resveratrol and Pterostilbene Treatment……79
5.3.3. EGFR/pEGFR Expression after Resveratrol and Pterostilbene Treatment.79
5.3.4. Expression of pHER2/HER2 and PCNA after Resveratrol and Pterostilbene Treatment……80
5.4. Discussion……87
…
6. Conclusions……92
7. Limitations and Future Directions……93

References……95

ii. Acknowledgements

Firstly I thank my supervisor Dr. Ashley Garrill for all the support, academic guidance and constant belief in me throughout my thesis.

I thank my fellow students, especially Wafaa Hassan for teaching me the lab techniques required and always being there for support or advice, and Hayley Schoch for making the lab environment an enjoyable place to work.

Lastly, I'm thankful to my family, especially my parents for their endless support and for always picking me up in times of stress. Without them it would have been difficult to make it through my thesis year.

iii. Abstract

Ovarian cancer is the most lethal gynaecologic malignancy, with a high mortality rate that is associated with the difficulty of treating the disease. This is due to the typical onset of symptoms when the disease is at a fairly advanced stage. Improved treatment strategies are required to improve longevity in patients. Recently polyphenols, secondary metabolites in plants, have aroused interest with respect to the treatment of cancer. The purpose of this study was to investigate the effect of two polyphenols, resveratrol and its derivative pterostilbene, on cell metabolism and proliferation in two ovarian cancer cell lines, OVCAR-5 and SKOV-3 that were grown in a three dimensional culture. The proteins VEGF, AKT, EGFR, HER2, cyclin D2 and PCNA were investigated to identify any possible changes to signalling pathways following exposure to the polyphenols. Both resveratrol and pterostilbene affected spheroid metabolism and proliferation in a dose- and cell line-dependant manner. Vascular endothelial growth factor and expression of the various proteins were affected following treatment. This study demonstrates that resveratrol and pterostilbene are capable of down regulating the cell metabolism and inhibiting proliferation in the ovarian cancer spheroids, potentially through several different pathways.

iv. Abbreviations

2D	Two Dimensional
3D	Three Dimensional
ANOVA	Analysis of variance
BAD	bcl-2-associated death promoter
BCA	Bicinchonicic acid assay
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
Crystal Violet	(tris(4-(dimethylamino)phenyl)methylium) chloride
ECM	Extracellular cell matrix
EGFR	Epidermal growth factor receptor
DMEM-F12	Dulbecco's Modified Eagle Medium (Nutrient Mixture F12)
DMSO	Dimethyl sulfoxide
E2F	E2 transcription factor
ECM	Extracellular membrane
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immnosorbent assay
EMT	Epithelial to mesenchymal transformation
FBS	Foetal bovine serum
FOXO	Forkhead box class O
HER2	Human epidermal growth factor receptor
IMRT	Intensity modulated radiotherapy
mAbs	Monoclonal antibodies

MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial (re) transition
MMP	Matrix metalloprotease
OD	Optical density
OVCAR-5	Human ovarian adenocarcinoma cell line 5
pAKT	phosphorylated AKT
PBS	Phosphate buffered solution
PCNA	Proliferating cell nuclear antigen
pEGFR	Phosphorylated EGFR
PH	Plectstrin-homology
PI(3)K	Phosphatidylinositol 3 kinase
PIP2	Phosphatidylinositol-4,5-bis-phosphate
PolyHEMA	Poly-hydroxyethylmethacrylate
Pterostilbene	trans-3,5-dimethoxy-4-hydroxystilbene
PVDF	Polyvinyl difluoride
Resveratrol	3,4,5-trihydroxy-trans-stilbene
rpm	revs per minute
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SKOV-3	Human ovarian serous carcinoma cell line 3
TBS-T	Tris buffered saline and Twee20
TKI	Tyrosine kinase inhibitor
Trypsin-EDTA	Trypsin-Ethylenediamine tetraaccetic acid

VEGF	Vascular Endothelial Growth Factor
WAR	Whole abdominal radiotherapy

v. List of Figures

1.1 The female reproductive system……14
1.2 Cancer cell locations in the ovary……16
1.3 The cell cycle……20
1.4 Proposed model of metastasis……23
1.5 Chemical structure of resveratrol……30
1.6 Chemical structure of pterostilbene……31
3.1 Cell morphology of OVCAR-5 with resveratrol……46
3.2 Cell morphology of SKOV-3 with resveratrol……47
3.3 Cell morphology of OVCAR-5 with pterostilbene……48
3.4 Cell morphology of SKOV-3 with pterostilbene……49
3.5 Spheroid size of OVCAR-5 following resveratrol and pterostilbene……50
3.6 Cell metabolism of OVCAR-5 after resveratrol and pterostilbene……53
3.7 Cell metabolism of SKOV-3 after resveratrol and pterostilbene……53
3.8 Cell number of OVCAR-5 after resveratrol and pterostilbene……54
3.9 Cell number of SKOV-3 after resveratrol and pterostilbene……54
4.1 VEGF secretion by OVCAR-5 cells after resveratrol and pterostilbene……63
4.2. VEGF secretion by SKOV-3 spheroids after resveratrol and pterostilbene…… 63
5.1. EGFR and downstream signalling……68
5.2. PI(3)K/AKT signalling pathway……73
5.3. AKT/pAKT, cyclin D and GAPDH expression in OVCAR-5 spheroids after resveratrol ..81
5.4. EGFR, pHER2 and GAPDH expression in OVCAR-5 spheroids after resveratrol……..82
5.5. AKT/pAKT, cyclin D2 expression in SKOV-3 spheroids after resveratrol……..83
5.6. AKT/pAKT, cyclin D2 and HER2 expression in OVCAR-5 spheroids after pterostilbene..84
5.7. AKT/pAKT, cyclin D2 and GAPDH expression in SKOV-3 spheroids after pterostilbene..85
5.8. EGFR, PCNA and GAPDH expression in SKOV-3 after pterostilbene……86

vi. List of Tables

2.1. Working concentration of western blot antibodies……41
3.1. Cellular metabolism of control after resveratrol and pterostilbene……..55
3.2. Cellular metabolism of control with DMSO after resveratrol and pterostilbene……55

Chapter 1: Introduction

1.1. Overview

Ovarian cancer is the most lethal gynaecologic malignancy and most commonly originates at the surface epithelium of the ovary. Globally, this is the 7th most common diagnosed cancer with 6.3 per 100,000 women being diagnosed. This statistic increases in more resource rich countries to being the 5th most diagnosed cancer with 9.3 cases per 100,000 women (Prat 2014). Overall, ovarian cancer results in approximately 225,000 women being diagnosed and approximately 140,000 deaths globally per year (Banerjee and Kaye 2013).

Current treatment options include surgery and chemotherapy. These can be combined as a reduction in the tumour size can potentially increase the effectiveness and diffusion capabilities of chemotherapeutic drugs, in addition to decreasing the chance of drug resistance by the cells (van der Burg, van Lent et al. 1995). Treatment with chemotherapy has improved throughout the years with attempts to decrease drug toxicity while increasing effectiveness (Agarwal and Kaye 2003). The use of surgery and chemotherapy has now surpassed radiotherapy as the common treatment option due to the limitations which occurred with radiotherapy. However, due to increasing resistance by the cells, these treatment options are limited and therefore further therapies need to be determined.

The use of natural products for the treatment of cancer has been of interest for many decades, however of more recent interest are polyphenols due to their ability to inhibit cell proliferation (Manach, Scalbert et al. 2004, Han, Shen et al. 2007). Of particular interest are two stilbenes, resveratrol and pterostilbene. These have previously been shown to affect various cancers types, but there are limited studies focussing on their effects on ovarian cancer (McCormack and McFadden 2012, Stakleff, Sloan et al. 2012)

1.2. Cancer

The written history of cancer is thought to date back as early as 3000BC, where the first known surviving manuscript described the origin and potential treatment of this disease. Since then there have been hundreds of distinct types of cancer and cancerous subgroups described, and cancer is the second largest cause of death globally (Aggarwal, Danda et al. 2009). In 2012, 14 million people were diagnosed and 8.2 million people died from some form of cancer (World

Health Organisation). These statistics have been relatively static throughout the years, however it is predicted this will increase to 17 million deaths and 27 million diagnoses globally by the year 2030 due to the growing population and increasing longevity. (Aggarwal, Danda et al. 2009).

Five to ten percent of cancer cases are caused directly by genetic factors such as inherited genes containing the cancerous defects or elevated levels of hormone production. The remaining 90-95% are influenced by diet and lifestyle choices including smoking, alcohol consumption or environmental pollutants which lead to cancerous genetic mutations. Statistics indicate that between 30-35% of cancer is associated with diet (Anand, Kunnumakara et al. 2008).

Cancer forms due to uncontrollable cell growth caused by a loss of the balance between cell proliferation and cell death via apoptosis or autophagocytosis. The transition from normal cells to cancerous cells occurs in a two-step process: (1) initiation; during which the DNA becomes damaged, creating a pre-neoplastic cell and (2) promotion; during which the pre-neoplastic cell is promoted by growth factors or hormones which allow tumour growth (Barnard 2004). Abnormal cell growth is enabled by shutting off the cells regulation, either by the alteration of proteins or deregulation of signalling pathways. Proteins and pathways affected vary depending on the type of cancer.

Six important changes are thought to be involved in the majority of cancer types which override the cells defence mechanisms. (1) The ability to be self-sufficient for growth signals. Proliferation in normal cells is controlled by mitogenic growth signals. In many cancers these growth signals are mimicked, either by the alteration of extracellular growth signals, transcellular transducers or cellular circuits that are responsible for translating growth signals. Overall this allows the cancer to be independent from its environment for proliferation. (2) The cancer must become insensitive to the antigrowth signals that are produced by normal cells to block cellular proliferation. Cells achieve this by altering the pathways responsible for producing anti-proliferative signals. (3) Cancer cells must acquire the ability to evade apoptosis, which is typically used to remove abnormal cells. This occurs via a mutation in a signalling pathway responsible for the transmission of anti-apoptotic signals. In many cases the p53 tumour suppressor protein is mutated. (4) The ability to have endless replication of the cells. The hallmarks allowing proliferation to occur and form cancerous cells is limited by

the amount of replications that can occur by the cells. By overcoming this limitation, the cells are capable of continuously replicating, resulting in larger tumours and the potential to spread further throughout the body. (5) Angiogenesis, or the ability to form new blood vessels. In normal cells this is controlled by inducers and inhibitors, however in cancer this control can be lost and the balance must favour the inducers, a process achieved by alterations in gene transcription. (6) To enable relocation of the cancer cells to a nutrient rich environment they must acquire the ability to invade other locations in the body via detachment from initial tumour, and then attachment at the new location (Hanahan and Weinberg 2000).

1.2.1. The Female Reproductive System and Ovarian Cancer

The female reproductive system is comprised of two ovaries, two fallopian tubes, a uterus and a vagina (Figure 1.1). The ovaries are responsible for producing many of the female hormones and the production of eggs. The eggs travel via the fallopian tubes to the uterus where they become fertilised.

The ovaries, which are composed of germ cells and somatic cells, are located near the lateral wall of the pelvic cavity and are responsible for controlling ovulation and the maturation of eggs through the regulation of hormone levels. Early in the menstrual cycle, follicle growth is increased to allow the release of hormones that are important for the continuation of the menstrual cycle. At the start of the cycle, the gonadotropin-releasing hormone level pulsates at a low frequency, stimulating an increase in the amount of the follicle-stimulating hormone being secreted from the anterior pituitary gland, and thus an increase in follicle growth. Halfway through the menstrual cycle the level of pulsing of the gonadotropin-releasing hormone increases in frequency, shutting off follicle growth and increasing the amount of luteinizing hormone that is released from the anterior pituitary gland, resulting in ovulation and maturation of the eggs. When the control of this cycle is lost, various types of cancer can form including cervical, ovarian and endometrial cancers (Richards and Pangas 2010).

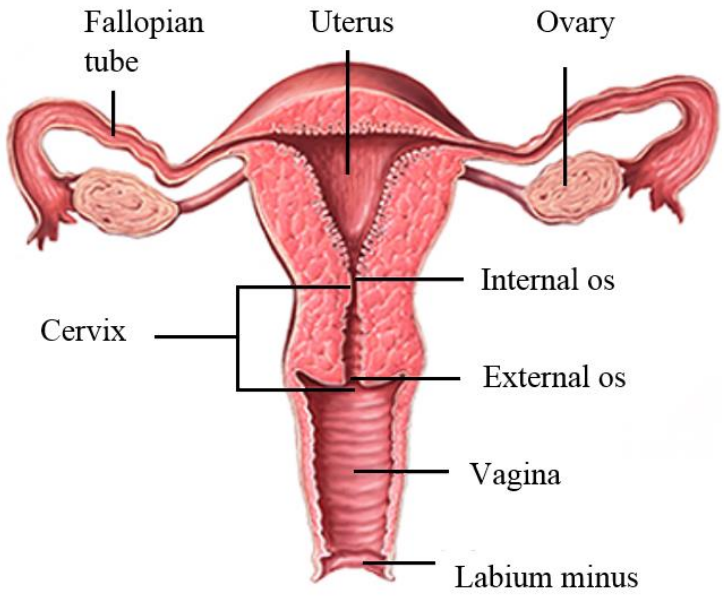


Figure 1.1. An overview of the female reproductive system. The ovaries are two small, oval shape organs which are responsible for the production of female hormones and reproductive eggs. The ovaries are connected to the uterus by the fallopian tube.

Image adapted from
https://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/19263.jpg

A lack of early symptoms and markers make ovarian cancer difficult to detect. 70% of diagnoses occur at an advanced stage and in only 30% of these cases will the patient survive 5 years, a factor that is associated with the high mortality rates for this disease (Cho and Shih 2009, Weiderpass and Labrèche 2012).

There are three classifications of ovarian cancer: (1) epithelial tumours: these initially form on the surface of the ovary, (2) stromal tumours: these originate in the interior of the ovary and (3) germ cell tumours: these form via embryological differentiation (Figure 1.2) (Ang, Chan et al. 2011). The majority (90%) of ovarian cancers are epithelial. The epithelial ovarian cancers are further subdivided into; mucinous, endometroid, clear cell and the most common, serous carcinoma, on the basis of their histology and morphology. Cancers that originate in the stromal and the germ cell account for 5% and <5% of cases respectively (Cho and Shih 2009, Weiderpass and Labrèche 2012). Tumour growth is classified into grades, depending on the amount of growth that has occurred. Low grade tumours, classified as either grade 1 or 2, have at most 50% solid growth. In contrast, a high grade tumour (classified as grades 3 and 4) has at least 50% solid growth (Cho and Shih 2009).

Furthermore, ovarian tumours can be further categorised by type, based on their histology. Type I include low grade serous carcinoma, endometroid, mucinous and clear cell carcinomas. Typically, these are slower growing and confined to either one or both ovaries when diagnosed. Low grade tumours occur via somatic mutations in several genes and are relatively stable. Type II tumours are highly aggressive with rapid growth. Typically at diagnosis these tumours will have extended out of the ovaries and have become advanced and unstable. These include high grade serous carcinomas, high grade endometroid and clear cell carcinomas. Somatic mutations are rarely associated with Type II tumours, instead a mutation in the cell growth control gene, is *TP53*, is believed to be largely responsible (Kurman, Visvanathan et al. 2008, Cho and Shih 2009).

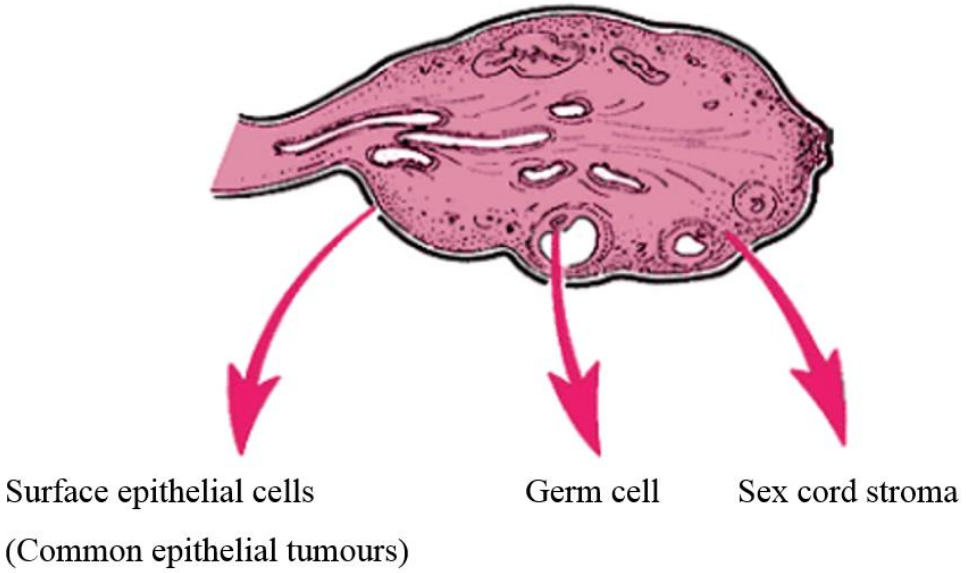


Figure 1.2. Illustration of different locations of cancer cells within the ovary. The surface epithelial cells occur on the edge of the ovary while the germ cells and sex-cord-stroma cells are within the ovary. The germ cell cancers form through embryological differentiation while the sex-cord-stromal cancers form from the interior of the ovary

Image adapted from http://d3jonline.tripod.com/24-Reproduction/pathology_of_ovary_2.gif

Only five to ten percent of ovarian cancer cases are associated with genetic inheritance. Of these, mutations in two tumour suppressing genes, *BRCA1* and *BRCA2*, are responsible for the majority of familial early onset cases and the inheritance of genes causing a genetic predisposition to this disease (Ludwig, Chapman et al. 1997, Kurman, Visvanathan et al. 2008). Mutations affecting the *BRCA1* gene results in a dysfunctional DNA damage repair pathway, ultimately causing an effect on the cell cycles regulation and thus effects proliferation. This subsequently can result in the transfer of the mutated genes to subsequent generations (Chen, Silver et al. 1998, Hilton, Geisler et al. 2002). The mutated *BRCA1* and *BRCA2* genes both result in truncated protein products in germline and somatic mutations. Those occurring in the germline were found to have a much larger occurrence percentage (38%) in women with a family history of either *BRCA1* or *BRCA2* than those without, and somatic mutations have a larger percentage of women without family history being diagnosed (76%), suggesting some of these genes are hereditary (Hilton, Geisler et al. 2002).

Several additional genes have been associated with ovarian cancer, specifically *BRAF*, *KRAS* and *TP53*. If either *BRAF* or *KRAS* are mutated in Type I carcinomas the other will function normally, suggesting both are involved in the same pathway. The associated RAS-RAF-MEK-ERK pathway is responsible for regulating cell growth and proliferation through a RAS-activated protein kinase cascade. The mutation of either *BRAF* or *KRAS* activates the mitogen-activated protein kinase/signal-regulated protein kinase (MAPK/ERK) which is downstream of these genes, resulting in the activation of cell growth, proliferation, and ovarian tumour formation. Both *KRAS* and *BRAF* are observed in more than half of serous carcinomas. P53 is a tumour suppressor protein responsible for regulating the cell cycle and monitoring cell stability. If DNA damage is present, it prevents the cell cycle continuing. Therefore a mutation in the gene for P53, *TP53*, would cause this control to be lost, resulting in an increase in proliferative rates and the formation of tumours. This is one of the most commonly mutated genes in cancer, including ovarian cancer, yet it rarely occurs in Type I tumours (Greenblatt, Bennett et al. 1994, Cho and Shih 2009)

1.3. Cell Cycle and Proliferation

Regulation of the cell cycle is essential in all parts of the body to ensure cells divide at the correct time. Additionally this regulation allows the repair of DNA before completion of the cycle. It has been suggested that the risk of developing ovarian cancer increases when the ovulation cycle is not inhibited, as there is a rupture in the epithelial cells during each ovulation. This damage then requires several rounds of cell division to repair the wound, increasing the possibility of incorrect cell division and growth. To ensure these cells are repaired correctly after damage, a cell cycle repair mechanism controlled by cell cycle checkpoints is required and completed. Initiation of checkpoints later in the cycle is dependent on completion of prior checkpoints, to ensure there is no DNA damage. During cancer formation, these checkpoints are disrupted so recognition of damaged DNA cannot occur, resulting in proliferation of abnormal cells (Godwin, Testa et al. 1992).

The cell cycle is comprised of 5 different phases; the growth phases G_0, G_1, and G_2 which are additionally responsible for checking the completion of the DNA replication prior to the cell finalising replication. The active phases are the DNA synthesis phase (S-phase), which is responsible for duplicating the genome and DNA synthesis, and the mitotic phase (M-phase) which is where mitosis occurs. Each phase is regulated with different control points. Checkpoints between the different phases are responsible for allowing the continuation of the cycle into the next phase. The cycle starts at the G_0 stage, which is quiescent and relies on signals from external stimuli, such as growth signals or mitogens to move it past the G_1 checkpoint and thus begin the cell cycle. The G_1 stage of the cycle is the gap phase between the M-phase (which typically takes 1-2 hours to complete) and the S-phase (which typically takes 2-4 hours to complete). Replication of the cytoplasmic organelles and cell growth occurs in the G_1 phase, which typically takes 12-96 hours to complete. The shift to the S-phase is controlled at the G_1/S checkpoint. Following the 2-4 hours in the G_2-phase, the final checkpoint occurs, allowing transition from G_2-phase to the M-phase, where the cell cycle is completed (Figure 1.3) (Israels and Israels 2000). The control of these mechanisms in mammalian cells is through the association of serine/threonine kinases, known as cyclin dependant kinases (CDKs), which act as a catalytic partner to the regulatory unit cyclin. The interaction between these different cyclins (specifically D and E) and kinases (CDK4, CDK6, and CDK2) result in phosphorylation of the retinoblastoma tumour suppressor protein, and

are required for movement through the cell cycle. The loss of cell regulation and changes in the expression levels of these cyclins or CDKs contribute to cancer pathogenesis and proliferation (D'Andrilli, Kumar et al. 2004, Collins and Garrett 2005).

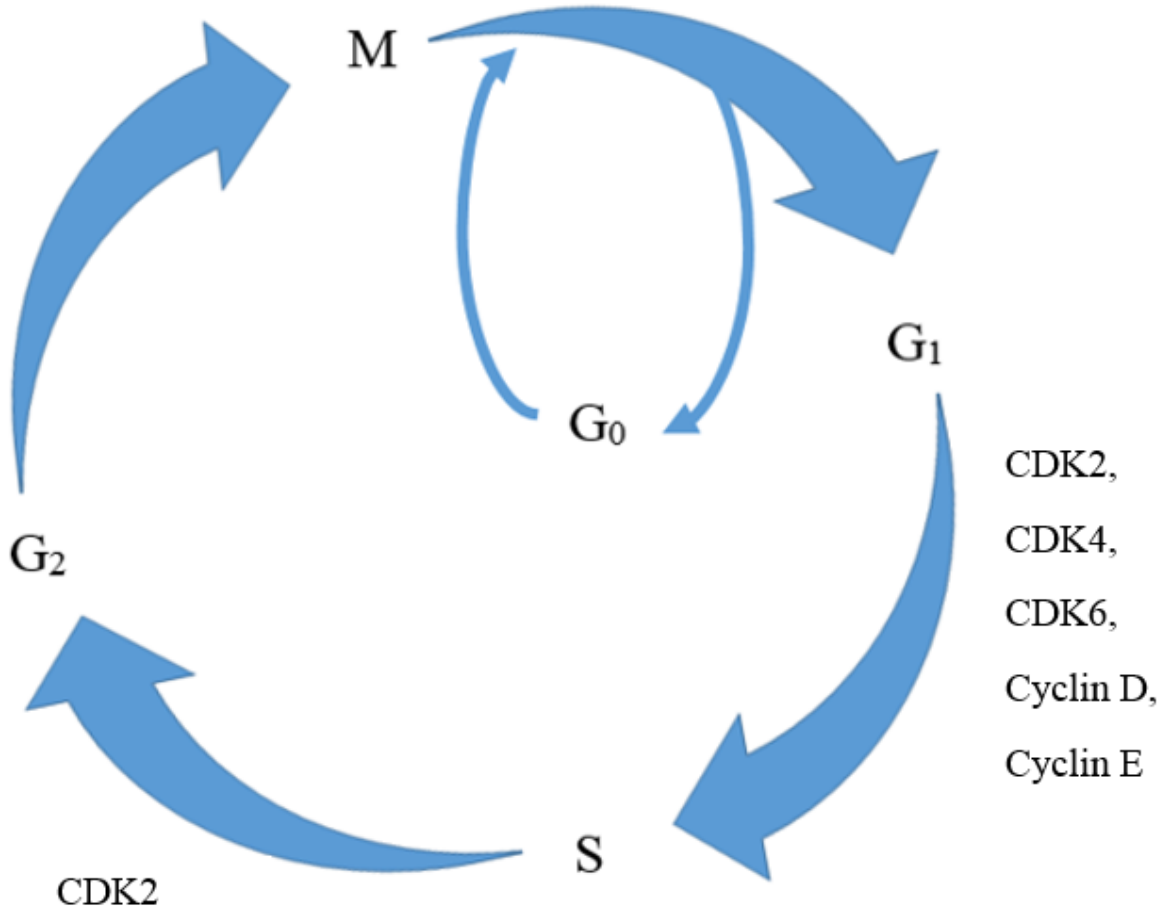


Figure 1.3. The cell cycle. This cycle consists of several checkpoints at different stages of the cycle, responsible for checking for, and maintaining the correct division of cells. G_0 is the start of the cell cycle. After completing the M-phase the cycle is complete. Cyclins and cyclin dependant kinases (CDKs) interactions control these mechanisms and differ at the various stages in the cycle

1.4. Metastasis

Ovarian cancer can spread, either by moving directly to a nearby organ such as the colon or the bladder, or by detaching from the tumour and moving via intraperitoneal dissemination. Most commonly, intraperitoneal dissemination occurs, which allows part of the primary tumour to dissociate and move through the peritoneum via the ascetic fluid until it arrives at a new location, which is generally in the abdominal cavity. This usually occurs early in ovarian cancer and is probably the reason few cases are diagnosed when the cancer is restricted to the ovaries (Lengyel 2010, Nakayama, Nakayama et al. 2012).

The epithelial cells which form the outer layer of the spheroid and which interact with the basement membrane undergo an epithelial-to-mesenchymal (EMT) transformation that causes the inter-cellular adhesions between the cancer cells to loosen, resulting in detachment from the primary tumour. The mesenchymal cells are essential for metastasis because they have an increased migratory ability, are more resistant to apoptosis compared to epithelial cells and can increase the production of extracellular matrix (ECM) components (Kalluri and Weinberg 2009). During the transition, the E-cadherin holding the cells together is lost which allows the cancer cells to become motile and acquire mesenchymal properties (Lengyel 2010). When the cells dissociate from the primary ECM, individual cells and/or cell clusters (spheroids) must overcome apoptotic control, which they do by overexpressing RAB25, which is resistant to apoptosis, enabling movement to the peritoneum or omentum via the peritoneal fluid (Figure 1.4) (Shield, Ackland et al. 2009, Nakayama, Nakayama et al. 2012).

When the cancerous cells come into contact with the peritoneum, an interaction occurs between the cancer cells and the mesothelial cells though cadherin binding and integrins. A β1 integrin which is responsible for the binding to ECM molecules of the mesothelial linings, additionally collagen and laminin are involved in binding (Ahmed, Riley et al. 2005). Following attachment and the upregulation of β1 integrin, the EMT process must be reversed; this mesenchymal-epithelial (re-)transition (MET) is required because the properties of EMT prevent growth and resocialization of the spheroids (Brabletz 2012). Following this process, a matrix metalloprotease (MMP) is activated enabling degradation of the matrix in the mesothelial lining. This is a zinc-dependant peptidase which can degrade the ECM, acting

specifically to break collagen and fibronectin into smaller fragments. This provides a stronger attachment for the newly localised cells. (Ahmed, Riley et al. 2005). At any point throughout this process, where the cells require new blood vessels this is initiated by the Vascular Endothelial Growth Factor (VEGF). Specifically once cells are in the secondary site, the VEGF levels are raised and this helps facilitate new growth (Figure 1.4) (Shield, Ackland et al. 2009, Lengyel 2010).

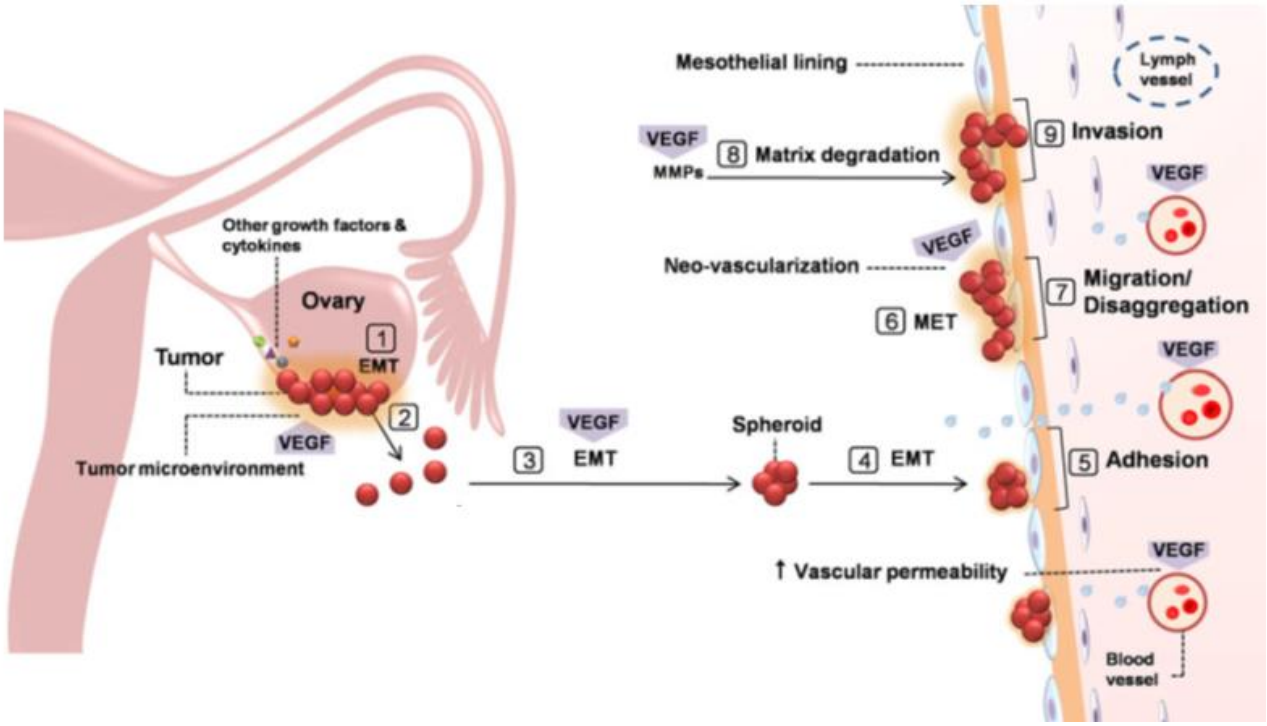


Figure 1.4. Ovarian cancer metastasis. (1) Cells undergo EMT in the ovary to loosen individual cells from the tumour. (2) Individual malignant cells are released from the ovaries ECM into the peritoneum. (3) During transport to new location, malignant cells often reform spheroids to assist their survival by creating an optimum environment. (4) EMT changes occur to further increase the spheroids survival. (5) The transported spheroids attach to the mesothelial lining of the peritoneum through interactions with cadherins and integrins. (6) MET occurs to reverse the initial EMT, resulting in the ability of the metastasised cells to grow. (7) Cells migrate further. (8) MMPs act to break down the mesothelial lining. (9) The cells enter the peritoneum.

At most stages of intraperitoneal dissemination, VEGF is present, to allow the cells to grow by inducing new blood vessel formation. A process which is vital to ensure the growth of the tumour at a new location.

Image altered from (Brabletz 2012).

1.5 Treatment Options for Ovarian Cancer

1.5.1. Chemotherapy

The preferred treatment for ovarian cancer has changed over the years from alkylating agents to platinum treatments, and more recently to combination therapies containing both platinum and taxanes. These all have the potential to kill cancer cells (McGuire and Markman 2003). In the 1960's and 1970's, the treatment of choice was a monotherapy of alkylating agents, such as melphalan. Rosenburg et al (1965) subsequently reported that platinum compounds can affect cancer cell growth and this led to the introduction of cisplatin (cis-diamminedichloroplatinum (II)) as an anticancer drug. Cisplatin causes a chemical reaction by replacing either one or two chloride ions from the cisplatin with water molecules. This ultimately leads to apoptosis via different mechanisms: (1) Once the reaction has taken place, it affects the cells pathways responsible for recognising DNA repair mechanisms, which interferes with the DNA synthesis for further cell development. (2) The reaction causes covalent binding to purine bases, specifically at guanine bases (Reedijk 2003, Kelland 2007). These findings resulted in the main treatment option changing from the alkylating agents to the platinum treatment.

Cisplatin is highly toxic so the dosage cannot be increased following acquired resistance by the cells. To overcome these limitations further research was required to identify a drug that was just as successful but with less toxicity (Reedijk 2003). Carboplatin (cis-diammine-[1,1-cyclobutanedicarboxylato]platinum(II)), a cisplatin analogue, was determined to be suitable for replacing cisplatin, with many studies demonstrating similar effectiveness to cisplatin but with significantly less toxicity. Carboplatin was introduced as an anti-cancer drug in 1985 (Adams, Kerby et al. 1989, Christian and Thomas 2001). In contrast to cisplatin, carboplatin contains a cyclobutane ring which is more stable than the chlorides present in cisplatin. The increased stability and slower reaction time is believed to be the cause of the lower toxicity level (Lebwohl and Canetta 1998).

Although these monotherapy treatments gave improved results against cancer cells, their effectiveness was compromised due to increasing resistance of the cells to them. This led to the development of combination therapies, using a platinum-based treatment combined with one other treatment with the goal of these working synergistically. Taxanes were selected

due to their capability of interfering with the functioning of the mitotic spindle through the alteration of spindle stability. The taxanes prevent microtubule depolymerisation, resulting in the inability to complete mitosis, and thus cause a halt at the M/G2 phase of the cell cycle (Agarwal and Kaye 2003).

The predominant taxane used in drug therapy is paclitaxel. This originates in the tree bark of *Taxus brevifolia* and was discovered as an anticancer treatment in 1971 (Rowinsky 1997, McGuire and Markman 2003). This drug is capable of affecting the mitotic spindle by binding to 31 amino acids at the N-terminal of β-tubulin, one half of the tubulin heterodimer. Additionally paclitaxel affects downstream signalling pathways such as the mitogen activated protein kinase (MAPK), which leads to the phosphorylation of the apoptotic protein, BAD, and thus stimulates apoptosis (Agarwal and Kaye 2003). When used in combination therapy with a platinum based compound, specifically cisplatin, patients responded positively, with an increased longevity. This was limited by an increase in toxicity, however this can be somewhat countered by treatment with the platinum based drug first, or by replacing the cisplatin with carboplatin (Rowinsky 1997, Christian and Thomas 2001)

Since these drug discoveries, further anticancer drugs are being discovered which improve on the efficiency of these previous treatments. Such drugs include docetaxel, a taxane derived from needles in the *Taxus baccata* plant, which in past studies have shown to be as effective as paclitaxel but with less toxic effects (McGuire and Markman 2003).

Although current treatments are giving increased longevity for patients, the mortality rate from this disease remains high, so further treatments or drugs need to be determined.

1.5.2. Surgery

In addition to the combination therapy of platinum and taxane based chemotherapy, debulking, or cytoreductive surgery, is now a common form of treatment for ovarian cancer (Ang, Chan et al. 2011). For chemotherapy to be effective, the cytotoxins must be able to access the tumour, generally via diffusion. If the tumour is large, the drugs may not be able to diffuse to the majority of the tumour due to inefficient blood supply and are therefore ineffective. Additionally, larger tumours have an increased chance of acquiring resistance to chemotherapeutic drugs (van der Burg, van Lent et al. 1995). Griffiths et al (1975) suggested that when a tumours size had been surgically decreased, an increase in the survival rate for

patients occurred. Overall, the amount of residual tumour remaining is the best prognostic indicator. If the residual tumour is ≤1cm in diameter post-cytoreductive surgery, it is considered optimally reduced and potentially increases the survival chance for the patient. In contrast, survival rates with tumours larger than 2cm in diameter do not significantly differ to those who did not undergo surgery (Ang, Chan et al. 2011).

Cytoreductive surgery strategies are based on the type of tumour and how advanced the growth is. Standard cytoreductive surgery specifically targets the middle abdomen and pelvic regions, and often results in the removal of the uterus, cervix, both fallopian tubes and the omemtum, the lining of the peritoneal cavity. Patients with more advanced tumours, specifically advanced epithelial ovarian cancer, often require more extensive surgery which is referred to as radical surgery. This is more invasive and can include the removal of the spleen (splenectomy), surgical resection of the pancreas (distal pancreatectomy), resection of the liver (hepatic resection), partial removal of the lining of the abdominal cavity (peritonectomy) and the removal of the gallbladder (cholecystectomy), (Ang, Chan et al. 2011, Ren, Jiang et al. 2015). The surgery type is typically dependant on the type of tumour. Young women, with low malignant tumours such as stage I epithelial cancer or germ cell tumours may only require the removal of the affected ovary, which would allow them to remain fertile, in contrast to the more invasive surgeries which can lead to loss of fertility (Berman 2003).

The capability of the surgeon can limit the effectiveness of surgery, by altering what is considered optimal cytoreduction by the surgeon based on their abilities, and also their capabilities or preferred techniques may vary from patient to patient (Nguyen, Averette et al. 1993, Aletti, Dowdy et al. 2006). The aggressiveness of the tumour can also influence the overall result despite a tumour being optimally reduced. If the tumour has metastasized, surgery may not be a viable treatment option. (Aletti, Dowdy et al. 2006).

As mentioned above, the mortality rate from ovarian cancer is still very high, despite the fact that treatments using surgery are increasing longevity, further research is required to increase survival statistics further.

1.5.3. Radiotherapy

The use of radiotherapy as a treatment for ovarian carcinomas dates back to the 1950's, and was commonly used as a subsequent treatment to surgery. Initially there were two treatment methods: (1) intraperitoneal radiotherapy, where a radioactive liquid, typically 32P, was put directly into the abdomen via a tube, or (2) external whole abdominal radiotherapy (WAR), the process of directing an external beam of radiation to the abdomen and pelvic region (Cardenes and Randall 2000, Rochet, Sterzing et al. 2010). For advanced ovarian cancer, migration to peritoneal surfaces usually occurs and thus WAR became the preferred radiotherapy option due to the ability to treat the pelvic region and peritoneal surfaces more successfully and with a homogenous distribution (Cardenes and Randall 2000).

Limitations to the use of WAR included a high acute toxicity level, and a high level of late toxicity affecting the bowel resulting in bleeding, perforation and obstruction which is typically difficult to treat with surgery. Nearby organs were also a limiting factor. The kidneys, liver and small bowel required protection from the radiation which affected the homogeneity of this radiation beam and thus resulted in insufficient radiation treatment to these areas. Additionally, WAR was only effective on tumours <2cm in diameter. Subsequently, a different treatment approach was required and with the improved success rates from the advancing chemotherapy regimens, the use of radiotherapy for the treatment of ovarian cancer significantly decreased (Rochet, Sterzing et al. 2010). However, research is ongoing to determine how to alter the original WAR technique to improve on these limitations. Early phase trials have focussed on intensity modulated radiotherapy (IMRT), where the nearby tissues and organs at risk are protected whilst still giving a strong and homogenous dose of radiation to the peritoneal region. However, further research is required to determine the level of toxicity (Rochet, Sterzing et al. 2010).

The use of radiotherapy for the treatment of ovarian cancer is a controversial topic, and many studies suggest different uses for radiation. Specifically the argument that radiotherapy is useful when used with chemotherapy on microscopic tumours. In contrast, some studies suggest radiation becomes ineffective due to long rounds of chemotherapy, resulting in chemoresistant cells (Cardenes and Randall 2000). In contrast, another study suggested that radiotherapy was the best treatment option for chemoresistant cells, and rather than being hindered by the resistance, was an effective treatment and gave a better long term prognosis

(Machida, Takei et al. 2014). Two studies have suggested the use of radiotherapy as a palliative treatment rather than to actually treat the tumour. (Einhorn, Trope et al. 2003, Machida, Takei et al. 2014). Additionally, it has been suggested that radiation can be used after the completion of treatment to target any residual cancer cells left in the body. This was also where IMRT is suggested to be most useful (Rochet, Sterzing et al. 2010). Due to such controversy around this treatment option and the differing results from many studies, further research is required to understand how best this can be used as a treatment.

1.6. Natural Substances, Polyphenols and Cancer

Diet has long been associated with the treatment and/or prevention of various diseases, including cancer. Dating back to the 1940's, natural treatments such as medicinal herbs and plant materials have been used as a form of treatment for cancer (Huang, Cai et al. 2009). This focus has narrowed over the last 20 years towards polyphenols, secondary metabolites present in plants that are used as a defence mechanism against disease from pathogens or ultra violet radiation (Manach, Scalbert et al. 2004). Structurally these are composed of at least one aromatic ring with at least one hydroxyl group attached and occur in many fruit, vegetables and herbs. Polyphenols are natural antioxidants, and therefore may have anti-tumour properties of varying levels depending on the plant source. These properties include the inhibition of angiogenesis (the formation of blood vessels), cell proliferation and ultimately the cell cycle (Manach, Scalbert et al. 2004, Han, Shen et al. 2007). During the development of cancer, changes occur in the cells which can be the result of oxidative stress and the production of free radicals. The antioxidant properties of polyphenols enable the scavenging of these free radicals through the donation of a H-atom from its hydroxyl group (Duthie, Duthie et al. 2000). An examination of 112 medicinal plants focussing on the effects of phenolic compounds has determined that the antioxidant activity was related to total phenolic content in the herbs, and confirmed that the herbs with higher levels correlated with a decreased cancer incidence. In contrast, dietary fruit and vegetables which contain no polyphenols had much lower antioxidant activity, with the exclusion of foods with other antioxidants present. The location of the plant tested appears to be important, as stems and leaves differed in their antioxidant levels (Cai, Luo et al. 2004).

Polyphenols are subdivided as phenolic acids, flavonoids, stilbenes and lignins. These are categorised on the basis of the number of phenolic rings they are comprised of, and the

overall structure of the compound. This research focuses specifically on stilbenes, which are comprised of a 1,2 diphenylethylene nucleus with hydroxyls alongside the aromatic rings and occur as either monomers or oligomers (Manach, Scalbert et al. 2004, Han, Shen et al. 2007).

1.6.1. Resveratrol

Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a stilbene polyphenol with two aromatic rings (Figure 1.5) (Huang, Cai et al. 2009). This compound is present in grapes, red wine and peanuts and acts against tumour growth by inducing apoptosis, preventing angiogenesis through the alteration of important pathways for growth and the inhibition of cell proliferation. Resveratrol has been trialled in the treatment of cardiovascular disease, type II diabetes and autoimmune diseases, however current interest has moved to determining its effects on cancer. Many tumours including colon, breast, prostate and liver cancers have been reduced when treated with resveratrol, however limited research has been done on the effects on ovarian cancer (Marques, Markus et al. 2009, Stakleff, Sloan et al. 2012). Resveratrol has been shown to induce apoptosis by stabilising the p53 tumour suppressor protein. In ovarian cancers where p53 is inactive or apoptotic inhibitors are deactivated, cell death can still occur by autophagocytosis, a type II programmed cell death (Opipari, Tan et al. 2004). An alternative mechanism may be the down-regulation of glycolysis, an essential process in the metabolism of glucose. As autophagocytosis is associated with nutrient deprivation, a decrease in glucose leads to cell death via this pathway, supporting the findings of the previous study (Kueck, Opipari et al. 2007).

Most studies have determined differences in the effects resveratrol has *in vitro* and *in vivo* although continue *to* use *in vitro* experiments. These differences may be due to a lack of *in vivo* bioavailability, the amount of unchanged drug that reaches the systemic circulation. It is only the bioavailable parts that are accessible to the cancer cells. Using urinary excretion data, Walle et al (2004) determined the absorption of resveratrol was at least 70%, however bioavailability via oral exposure to resveratrol through ingestion resulted in a value close to zero, due to most of the resveratrol being moved through the body and excreted in the urine. Small reductions in tumour cells after resveratrol treatment have been observed, specifically in the oral cavity where resveratrol may have collected in the epithelial cells (Walle, Hsieh et al. 2004)

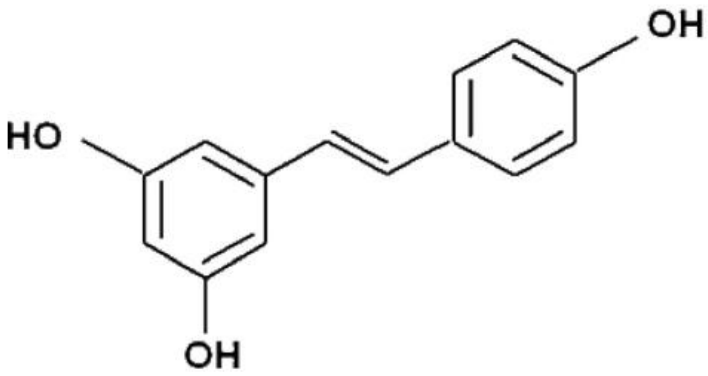


Figure 1.5. The chemical structure of Resveratrol

1.6.2. Pterostilbene

The stilbene pterostilbene (*trans*-3,5-dimethoxy-4-hydroxystilbene) is a dimethylether analogue of resveratrol, commonly found in blueberries. Structurally, the two compounds are similar, differing only in one functional group, the A-ring; resveratrol contains a 3,5-dihydroxy motif compared to pterostilbene which contains a 3,5-dimethyoxy motif (Figure 1.6) (Pan, Chang et al. 2007). Pterostilbene has been shown to be more potent than resveratrol, and appears capable of inhibiting the cancer growth in both *in vitro* and *in vivo* experiments. This is likely due to the greater *in vivo* bioavailability, where 20% is bioavailable, and as such it has aroused interest with respect to clinical applications for cancer treatment (Wang, Ding et al. 2012).

Similarly to resveratrol, pterostilbene is also believed to affect cellular functions through the induction of apoptosis. This occurs through the upregulation of the p53 tumour suppressor protein and various other proteins associated with the cell cycle and proliferation, specifically cyclins, to prevent cell growth completing a full cell cycle (Pan, Chang et al. 2007, Nutakul, Sobers et al. 2011). Further research is required to determine how pterostilbene effects tumour growth in ovarian cancer, and whether or not it affects the pathways in similar ways to other cancers previously studied.

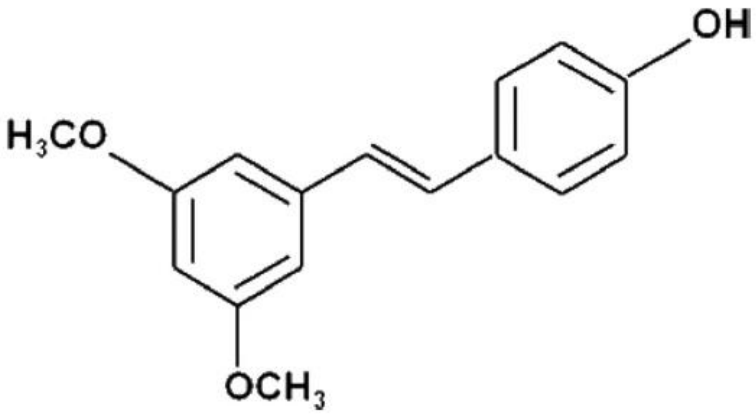


Figure 1.6. The chemical structure of pterostilbene

1.7. Two Dimensional and Three Dimensional Cell Cultures

Two dimensional (2D) monolayer cell cultures have been the most commonly used method for determining the effects of chemicals on cellular proliferation of cancer cells *in vitro*. This is due to the simple preparation and the long term viability of cells when cultured on a flat layer of plastic. However, the optimal functioning of cells *in vivo* requires signalling; cell-cell signalling and signalling with the ECM. This provides the cells with the correct cues for proliferation and differentiation. 2D monolayer cultures lack the three dimensional (3D) environment experienced by cells *in vivo* and therefore also lack 3D signalling systems, which can result in a different morphology, receptor expression and interactions with the ECM than those grown *in vivo* (Breslin and O'Driscoll 2013).

To counter these limitations, a three dimensional culture model has been used by some researchers. There are many different methods for 3D culturing, including: matrices and scaffolds, agitation based approaches such as spinning or rotation, the hanging drop method and the forced floating method, which is the most commonly used method and the one that is used in this research. The forced floating method forces the cells away from the culture flask or well plates surface to bring the cells together to form spheroids (Breslin and O'Driscoll 2013). This enables the cluster of cells to have similar morphology to spheroids *in vivo*, as the cultured spheroids contains cells which are exposed to the external surface and cells that are not. These different environments lead to different cell behaviours, for example, some cells will proliferate while others will be non-proliferating, as observed *in vivo*. (Yamada and Cukierman 2007). Additionally, due to the structure of the spheroid, the cells are more likely to be able to maintain cell-cell and cell-ECM interactions and signalling patterns, and thus are more likely to behave similarly to *in vivo* spheroids (Grun, Benjamin et al. 2009).

For drug discovery and development, 3D cultures have been shown to be better for examining the effects of drugs. As the effects of drugs on growth and cellular pathways are thought to reflect spheroids *in vivo*, this technique is beneficial for researching and testing chemotherapeutic drugs (Ivascu and Kubbies 2006, Breslin and O'Driscoll 2013).

1.8. Aims and Hypotheses

1.8.1. Aims

Given that there have been many studies done on the effects of resveratrol on cancer cells, but few which have focussed on the effects in ovarian cancer, the aim of this research is to investigate the effects of resveratrol and its anologue, pterostilbene on two ovarian cancer cell lines, OVCAR-5 and SKOV-3. This study aims to determine the effect of these drugs on: cellular growth and metabolism, the vascular endothelial growth factor and the protein levels of EGFR, pEGFR, HER2, pHER2, AKT, pAKT, cyclin D2 and PCNA in order to investigate how various protein pathways are affected by these drugs. The effects of the drugs will be tested at various concentrations.

1.8.2. Hypotheses

I hypothesise that resveratrol and pterostilbene will exhibit anti-cancerous effects on both the OVCAR-5 and SKOV-3 cell lines. Based on previous findings stating pterostilbene is more potent, I hypothesise that pterostilbene will be more anti-cancerous than resveratrol, causing significant changes to proliferation and metabolism. Lastly I hypothesise that both these drugs will interfere with pathways required for tumour growth, such as the production of VEGF, and the proteins: EGFR, pEGFR, HER2, pHER2, AKT, pAKT, cyclin D2 and PCNA.

1.8.3. Model Cell Lines

The two ovarian cancer cell lines that were used in this research were SKOV-3 and OVCAR-5. SKOV-3 is the conventional serous carcinoma cell line (Singer, Oldt et al. 2003). SKOV-3 has 4 gene mutations, these occur in: the cyclin dependant kinase inhibitor 2A (*CDKN2A*) which is associated with p16, the mismatch repair gene *MLH1*, *PIK3CA* and in the tumour suppressor gene *TP53*. Both *BRCA1* and *BRCA2* remain normal in these cells. SKOV-3 cells are resistant to tumour necrosis factor and to various cytotoxic drugs (American Type Culture Collection). OVCAR-5 cells have a substitution mutation in the *KRAS* gene and a normal *BRAF* gene. Both *BRCA* genes are normal. Similar to SKOV-3, both the *TP53* tumour suppressor gene and *CDKN2A* genes are also mutated (The Roche Cancer Genome Database 2.0). Both cell lines have proven useful in studies of ovarian cancer and the effects of anti-cancer treatments.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. General Materials

2.1.1.1. 10X PBS (500mL)

For the stock PBS buffer 7.1g Na_2HPO_4 was dissolved in 500mL of MilliQ H_2O, and NaH_2PO_4 was added until the pH was 7.1. NaCl and KCl were then added to adjust the pH to 6.7-6.8. The 10X PBS was further diluted to 1X using MilliQ H_2O when required.

2.1.1.2. 10X Trypsin-Ethylenediamine Tetraaccetic Acid (200mL) (Trypsin-EDTA)

Trypsin EDTA solution was made up using the following: 5g Trypsin powder and 1.5g EDTA were mixed in 200mL 1X PBS and adjusted to pH 8 using NaOH pellets.

2.1.1.3. Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM-F12)

For the Dulbecco's modified Eagle media 1L of 10X base media was made up directly following the instructions on the packet under sterile conditions. 1 packet of alpha medium and 2.438g $NaHCO_3$ were dissolved in MilliQ H_20 and the pH was adjusted to 7.1-7.3. The base media was then sterilised by filtration.

The working media was made up under sterile conditions using 20mL FBS (5%), 4mL 1X PenStrep, 4mL 1X Glutamax and 400µL fungizone, all of which were added to 400mL of base media in a sterile bottle. This media from here on is referred to as working media.

2.1.1.4. Poly-hydroxyethylmethacrylate (Poly-HEMA)

For the Poly-HEMA solution, for a final concentration of 12mg/mL, 1.2g Poly-HEMA powder was dissolved in 100mL of 95% ethanol and heated to 70°C to completely dissolve the PolyHEMA. This was stored under sterile conditions until required for plating. After storage, the Poly-HEMA was reheated to 70°C to ensure it was completely dissolved prior to plating.

2.1.1.5. Crystal Violet Solutions

For the Tris(4-(dimethylamino)phenyl)methylium chloride (crystal violet dye) 0.2% crystal violet powder was mixed with 100mL 2% ethanol.

For the Sodium dodecyl sulphate (SDS), this was diluted to 2% using MilliQ H_2O

2.1.2. Anti-Cancerous Drugs

Resveratrol was dissolved in 100% PBS pH 7.4.

Pterostilbene was dissolved in 100% DMSO.

2.1.3. ELISA: Buffers and Solutions

The washing buffer contained 0.05% Tween20 in 1x PBS pH 7.4.

The reagent diluent contained 2% BSA in 1X PBS pH 7.4.

2.1.4. Western Blot: Buffers and Solutions

2.1.4.1. 2M Tris-HCl (pH 7.5)

2M Tris-HCl was made up using the following: 24.2g Tris base, concentrated HCL to make pH 7.5, then MilliQ H_2O to make up to 100mL.

2.1.4.2. 1M Tris-HCl (pH 6.8)

1M Tris-HCl was made up using the following; 12.1g Tris base, concentrated HCl to make the pH 6.8, then MilliQ H_2O to make up to 100mL.

2.1.4.3. Running Buffer

The electrophoresis buffer contained 50ml Bolt™ 1X MES SDS running buffer and was made up to 2 litres using MilliQ H_2O

2.1.4.4. Tris Buffer Saline (TBS)

TBS was made up containing; 10mL 2M Tris-HCl (pH 7.5), 8.18g NaCl and MilliQ H_2O to make it up to 1L.

TBS-T was made up by adding 1mL Tween-20 to TBS.

2.1.4.5. 5x Sample Buffer

10mL Sample Buffer was made up containing; 0.6mL 1M Tris-HCl (pH 6.8), 5mL 50% glycerol, 2mL 10% SDS, 2mL 1% bromophenol and 0.8mL MilliQ H_2O

2.1.4.6. Transfer Buffer

Transfer buffer was made up containing; 1.93g Tris, 9g glycine in 1L MilliQ H_2O

2.1.4.7. Blocking Solutions

Blocking solutions were made up using either 5% (w/v) low-fat skim milk or 1% (w/v) BSA in TBS-T

2.1.4.8. Antibody Solutions

Antibody solutions were made up using solutions were made up using either 5% (w/v) low-fat skim milk in TBS-T or 1% (w/v) BSA in TBS-T in a 1:1 ratio

2.2. Methods

2.2.1. Two Dimensional Cell Culture

The two cell lines, OVCAR-5 and SKOV-3, were obtained from Dr Kenny Chitcholtan (Department of Obstetrics and Gynaecology, University of Otago, Christchurch), and maintained in a 50mL culture flask with DMEM-F12 working media, at 37°C in a humidified 5% CO_2 incubator. The media was replaced every 2 days, until the cells reached near confluence, as determined using a light microscope. Once about 90% confluent, the cells were subcultured. In a cell culture laminar flow cabinet, the used media was discarded and the culture flask was rinsed once with sterile 1X PBS before incubating with sterile 1X Trypsin-

EDTA for 20-30 minutes to detach the cells from the culture flask surface. Cells were then transferred to a 50mL tube and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded. The cells were resuspended in 7mL of fresh working media and a sample of this suspension was transferred to a new culture flask and mixed with fresh working media to make up to 6ml total volume.

2.2.2. Three Dimensional Cell Culture

For the generation of multicellular spheroids in a 3D culture, 12-well plates were pre-coated with 12mg/ml Poly-HEMA and incubated at 37°C on an orbital shaker set at 50rpm for 24 hours, to prevent cell adhesion to the culture wells. Before use, the 12-well plates were washed once with 1X PBS. Cells were subcultured from 2D monolayer following the above methods. Once the cells were resuspended in fresh working media, 100µL of resuspended cells and 100µL 1X PBS were transferred into an eppendorf tube and cells were counted using a haemocytometer to determine the approximate cell number in the cell suspension. Cells were then plated at approximately 200,000 cells per well on a 12-well plate. Media was changed every 2 days for 6 days by tilting the plate for about 5 minutes to allow cells to accumulate at the bottom of the well before aspirating off the media. The media was replaced with 1mL fresh working media.

2.2.3. Drug Treatment

On the sixth day of 3D culturing, the media was removed by tilting the 12-well plate for approximately 5 minutes to allow the cells to accumulate in the bottom of the well. The media was then aspirated off and 1ml of fresh working media containing the specific drug concentration of resveratrol or pterostilbene treatment (10µM, 30µM and 50µM) was added. For pterostilbene experiments, control cells were incubated with the highest concentration of solvent (DMSO) used for dissolving pterostilbene in. For resveratrol, the control consisted of working media. Treatments and samples were made in triplicate.

2.2.4. Cell Morphology

Following 6 days of drug treatment, images were collected using a Leica DFC290 camera attached to an inverted light microscope with a 20X/NA=0.4 objective lens for spheroid morphology. Spheroid size was measured using a micrometre slide.

2.2.5. Alamar Blue Assay

Alamar Blue (resazurin) is a metabolism-sensitive dye used to determine the viability, and the migratory and invasive characteristics of cells. When the dye is added to a sample it has a blue colour and is non-fluorescent, but when reduced by components of metabolism, for example mitochondrial enzymes or cytochromes, it becomes pink and fluorescent (Vega-Avila and Pugsley 2011). After 11 days of 3D cell culturing, the 12-well plate was tilted to collect the cellular spheroids in the bottom of the well and 0.5mL of culture media was aspirated off. 50µL Alamar Blue dye was added to each of the wells and incubated at 37°C for 4 hours. Following incubation, 200µL from each sample was loaded onto a 96-well plate. The optical density was the difference between values determined using 570nm (OD_{570}) and 600nm (OD_{600}) on a microplate reader. Measurements for each sample were made in triplicate.

2.2.6. Crystal Violet Assay

Crystal violet assays are used to assess cell viability and quantify cell number through staining and determination of optical density. The violet dye stains the cellular DNA, therefore the more intense the staining, the more cells are present and viable (Vega-Avila and Pugsley 2011). Following the Alamar Blue assay, the remaining Alamar blue dye was aspirated off and the 12 wells were rinsed with 1X PBS. Cells were then collected in 4mL round bottom eppendorf tubes (1 tube for each well) and centrifuged for 5 minutes at 2500rpm. The supernatant was discarded and the pellet was mixed with 1X PBS and centrifuged at 2500rpm. The supernatant was again discarded and 300µL 1X PBS and 700µL 10X trypsin-EDTA was added to the eppendorf tube to break up the spheroids. After 30 minutes of incubation at 37°C with mixing every 10 minutes, the eppendorf tubes were centrifuged at 2500rpm for 5 minutes, and the supernatant was again discarded. 200µL of crystal violet dye was added to each tube and left at room temperature for 30 minutes. The cells were then washed with MilliQ H_20 and centrifuged, discarding the supernatant each time. This washing was repeated until all excess dye was removed and the supernatant was clear. The cells were then lysed

with 2% (w/v) SDS and left in room temperature for 10 minutes. 200µL from each sample was then added to a 96-well plate. The optical density was determined at 560nm (OD_{560}) on a microplate reader. Measurements for each sample were made in triplicate.

2.2.7. Vascular Endothelial Growth Factor ELISA

The secretion of VEGF produced by the OVCAR-5 and SKOV-3 spheroids was measured using the DuoSet Human VEGF ELISA kit (R&D systems, New Zealand). Following 6 days of drug treatment the cultured spheroids in growth media were centrifuged at 2000rpm for 5 minutes and the supernatant was aspirated off and frozen at -80^{o}C. To start the ELISA, a 96 well plate was coated with the capture antibody diluted in 1X PBS pH 7.4 and incubated at room temperature overnight. The next day the wells were washed four times using the wash buffer and then blocked using reagent diluent and incubated for 1 hour. The wells were again washed with the wash buffer 4 times before adding the cell media samples and VEGF standards (2ng/mL, 1ng/mL, 0.5nm/mL, 0.25ng/mL, 0.125ng/mL, 0.06ng/mL and 0.03ng/mL of VEGF standard diluted in reagent diluent) to the designated wells and incubated for 2 hours. Wells were washed 4 more times and the detection antibody diluted in reagent diluent was added to the wells and incubated at room temperature for a further 2 hours before being washed with washing buffer 4 more times. Following washing, streptavidin-HRP diluted in reagent diluent was added to each well and incubated in the dark for 20 minutes. The wells were then washed with washing buffer 4 times before adding TMB-substrate and incubating for 20 minutes at room temperature. After 5 minutes $2NH_2SO_4$ was added to stop the reaction and the optical density was then read at 450nm (OD_{450}) using a microplate reader. The samples were then plotted against a graph of the standards to determine the concentration of VEGF in each sample. Each treatment was tested in triplicate.

2.2.8. BCA Protein Assay

A protein assay was used to determine the concentration of protein present in the cell pellets to normalise the amount of protein that was run for the western blot. A BCA™ Protein Assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) was used to determine protein levels in each sample by comparing samples to a standard curve from known concentrations of BSA. Cells were centrifuged at 2000rpm for 5 minutes and the media was aspirated off. Cell pellets were lysed by adding 200µL of 0.1% SDS to each cell sample, and 12.5µL of each sample and

standards were loaded into a 96 well plate. 200µL of BSA working reagent was added to each well. Cells were shaken for 30 seconds then incubated at 37°C for 30 minutes. Absorbance was read at 562nm using a microplate reader, and protein concentration determined to give 10µg protein per sample for loading in the gel.

2.2.9. Western Blotting

Following the BCA protein assay, each cell lysate was prepared for gel loading by mixing them with 20µL 5x sample buffer. Samples were then vortexed and heated at 100°C for 10 minutes then cooled on ice for 10 minutes and centrifuged at 10,000rpm for 10 minutes. Samples were loaded into Bolt™ 4-12% Bis-Tris Plus gels at the volume required to give 10µg protein. MagicMark™ Western Standard (Invitrogen, New Zealand) and Precision Plus Protein™ Duel Colour Standards (BIO-RAD, New Zealand) were used as gel markers. Gels were run in Bolt™ MES SDS Running Buffer (1X) for approximately 120 minutes at 120 Volts. Polyvinyl difluoride (PVDF) membranes (BIO-RAD, Hercules, USA) were soaked in methanol for 5 minutes and then in cold transfer buffer for 15 minutes and then the separated proteins were transferred to the membranes using a Trans-Blot® SD cell (BIO-RAD, New Zealand) for 60 minutes at 15 Volts. Following transfer, gels membranes were blocked with blocking solution specific to each protein for 60 minutes (Table 2.1). Blocking buffer was then discarded and membranes soaked in the diluted primary antibody solution at 4°C overnight (Table 2.1). Primary antibody solution was then discarded and membranes washed four times in fresh TBS-T for 10 minutes on an orbital shaker at 20rpm. Membranes were then incubated in the appropriate secondary antibody solution for 90 minutes at room temperature on an orbital shaker at 10rpm (table 2.1). Membranes were then washed as before with fresh TBS-T. The developer (Clarity™ Western ECL Substrate, BIO-RAD, USA) was used to image protein bands and densitometry was then performed.

Primary Antibody	Company, (Clone): Catalogue number, Lot number	Species Antibodies Raised	Concentration of Primary Antibody	Blocking Solution	Concentration of secondary Antibody
Anti-EGFR	Santa Cruz Biotechnology, (F-2): sc-514995, H1115	Mouse	1/500	1% (w/v) BSA	1/10000
Anti-pEGFR	Santa Cruz Biotechnology, (F-3): sc-377547, G1515	Mouse	1/500	1% (w/v) BSA	1/10000
Anti-HER2/neu	Santa Cruz Biotechnology, (A-2): sc-393712, B1315	Mouse	1/1000	5% (w/v) low-fat skim milk	1/5000
Anti-pHER2/neu	Santa Cruz Biotechnology, (Tyr 1248)-R: sc-12352-R, J0314	Rabbit	1/500	1% (w/v) BSA	1/10000
Anti-AKT	Santa Cruz Biotechnology, (F-7): sc-5270, G0213	Rabbit	1/500	1% (w/v) BSA	1/10000
Anti-pAKT	ThermoFisher, (14-6): 44-621G, QG217213	Rabbit	1/500	1% (w/v) BSA	1/10000
Anti-Cyclin D2	ThermoFisher, (DCS-3.1): AHF0112	Mouse	1/500	1% (w/v) BSA	1/10000
Anti-GAPDH	Santa Cruz Biotechnology, (FL-335): sc-25778, c2415	Rabbit	1/2000	5% Skim milk	1/5000
Anti-PCNA	ThermoFisher, (-): PA5-27214, RB2156511	Mouse	1/500	5% Skim milk	1/1000

Table 2.1. Working concentrations of antibodies used for Western Blots. Both secondary antibodies obtained from ThermoFisher Scientific and were raised in goat.

2.2.10. Statistical Analysis

Statistical analysis was performed using GraphPad Prism® software (La Jolla, CA, USA). Experimental results were assessed for significance using a one way ANOVA followed by a Tukey test. $P<0.05$ was the measure of statistical significance.

Chapter 3: Effects of Polyphenols on Cellular Metabolism and Growth of Ovarian Cancer Cells

3.1. Introduction

3.1.1. The Cell Cycle and Ovarian Cancer

The cell cycle and its importance was introduced in section 1.3. Different regulators are required at each of the cell cycle checkpoints. For G_1/S phase regulation, cyclin D1, CDK4 and p16 have significant roles (Figure 1.3). The gene which encodes the 36kDa cyclin D1 protein, (*CCND1*), is usually regulated by mitogens, which induce the cell cycle. However, during tumourigenesis, this gene can be altered, resulting in gene deregulation and an overexpression of cyclin D1. When cyclin D1 is present in the nucleus, the cell cycle will progress beyond the G_1 checkpoint. In ovarian cancer, when there is overexpression of D1 in the nucleus, it will bind with CDK4 or CDK6, which enables the cell cycle to continue (Dhar, Branigan et al. 1999). The overexpression of CDK4 has been observed in up to 15% of large ovarian tumours (D'Andrilli, Kumar et al. 2004). A tumour suppressor protein, p16, binds to the CDKs in normal cells to prevent association with the cyclins and thus these function in tumour suppression. This role is important, as it prevents phosphorylation and activation of the retinoblastoma protein, a tumour suppressor protein, and thus is responsible for stopping the cell cycle if there is damage. This process can become interrupted allowing the formation of ovarian carcinomas by preventing the cell regulation system functioning (Fang, Jin et al. 1998, Bali, O'Brien et al. 2004). Fang, Jin et al. (1998) found a deletion in p16 occurred in 50% of the ovarian cancer cell lines that they tested.

A second pathway for regulating the G_1/S transition checkpoint involves cyclin E, CDK2 and inhibitors of Cip/Kip. Similar to cyclin D, cyclin E is overexpressed in ovarian tumours. The overexpression has been found to occur in up to 45% of ovarian cancers and the level of overexpression appears to be associated with the malignancy of the tumour; benign tumours have little overexpression, in contrast to malignant tumours have greater overexpression. Thus, the overexpression of cyclin E affects both development and malignancy. This overexpression is matched by CDK2, which is associated with cyclin E, and similarly there is more overexpression of this protein in malignant tumours. Two Cip/Kip CDK inhibitors, p27 and p21 also affect this pathway. In normal cells, p21 is present in the cell nucleus, which

enables it to bind the CDKs and prevent cyclin binding, which subsequently prevents the transition to the S phase of the cell cycle. In cancer cells p21 can become overexpressed, resulting in its presence in the cytoplasm, where it becomes inactive. In contrast, p27 is down regulated in ovarian cancer cells. Similarly to p16, due to the decreased amount of p27, the cyclin-CDK complex can form even under conditions where there is damage, further enabling cellular proliferation. (Kim, Shim et al. 2003).

3.1.2. Cellular Metabolism and Cancer

Metabolic adjustment is an important process for continued proliferation in cancer cells. With each cell division, the production of proteins, nucleic acids and lipids is required for both daughter cells, thus the metabolic process must change to facilitate increased production of these (DeBerardinis, Sayed et al. 2008). An increase in the activity of the glycolytic pathway leads to the increased production of ATP and other metabolites that are required for growth. Although there is an increase in glucose consumption in proliferating cancer cells, most of this is converted to lactate and little is used for cellular respiration; this has been referred to as the Warburg effect (Munoz-Pinedo, El Mjiyad et al. 2012). This is advantageous for cancer cells, because it allows them to grow in hypoxic conditions, where they wouldn't be able to survive if they relied simply on aerobic respiration. Additionally, by producing lactate as an end product, it acts to provide a favourable condition for further cell growth by acting against immune responses that could potentially target the abnormal cells (Kroemer and Pouyssegur 2008). Proliferating cells use glucose uptake to activate the pentose phosphate pathway, leading to the production of nucleic acids. Due to the importance of cellular metabolism in proliferation and subsequently cell migration, metabolic processes are a desirable target for drug treatments as a slowed down metabolism, or one which cannot adjust could have significant effects in treatment.

3.1.3. Aims of Chapter 3

The aim of this chapter is to investigate the effects of resveratrol and pterostilbene on cell morphology, metabolism and cell numbers in OVCAR-5 and SKOV-3 spheroids.

3.2. Methods

3D Cell culture – See chapter 2 for details.

Alamar Blue Assay – See chapter 2 for details.

Crystal Violet Assay – See chapter 2 for details.

3.3. Results

3.3.1. Effect of Resveratrol on Spheroid Morphology

Resveratrol treatment had no obvious effect on spheroid morphology at 10µM, relative to the control, while a decrease in spheroid number was observed after 30µM and 50 µM treatments (Figure 3.1). The average size of the OVCAR-5 control spheroids was 49.14µm. No significant decrease in spheroid size occurred at any resveratrol concentration tested (Figure 3.5a).

Resveratrol treatment on SKOV-3 cells had no obvious effect on spheroid morphology at any of the concentrations tested, and spheroids did not start to break apart. Spheroids appeared to be highly layered and thus measuring individual spheroid size was not viable due to lack of clear outlines or separate spheroid clusters (Figure 3.2).

3.3.2. Effect of Pterostilbene on Spheroid Morphology

Treatment of OVCAR-5 spheroids with pterostilbene had no obvious effect on morphology at 10µM relative to the control, however there was a decrease in spheroid number at concentrations of 30µM and 50µM (figure 3.5). Exposure to 50µM pterostilbene resulted in a significant decrease in average spheroid size relative to the other treatment concentrations tested ($P \leq 0.0001$, ANOVA, n=29) (Figure 3.5b).

The effect of pterostilbene treatment to SKOV-3 spheroids was a decreasing trend in overall spheroid size relative to the control for each concentration tested. Following 50µM pterostilbene, the spheroids were broken up into smaller clusters in addition to the decrease in overall size. The SKOV-3 cell line has a high level of cell layering and thus actual spheroid size could not be measured (Figure 3.6).

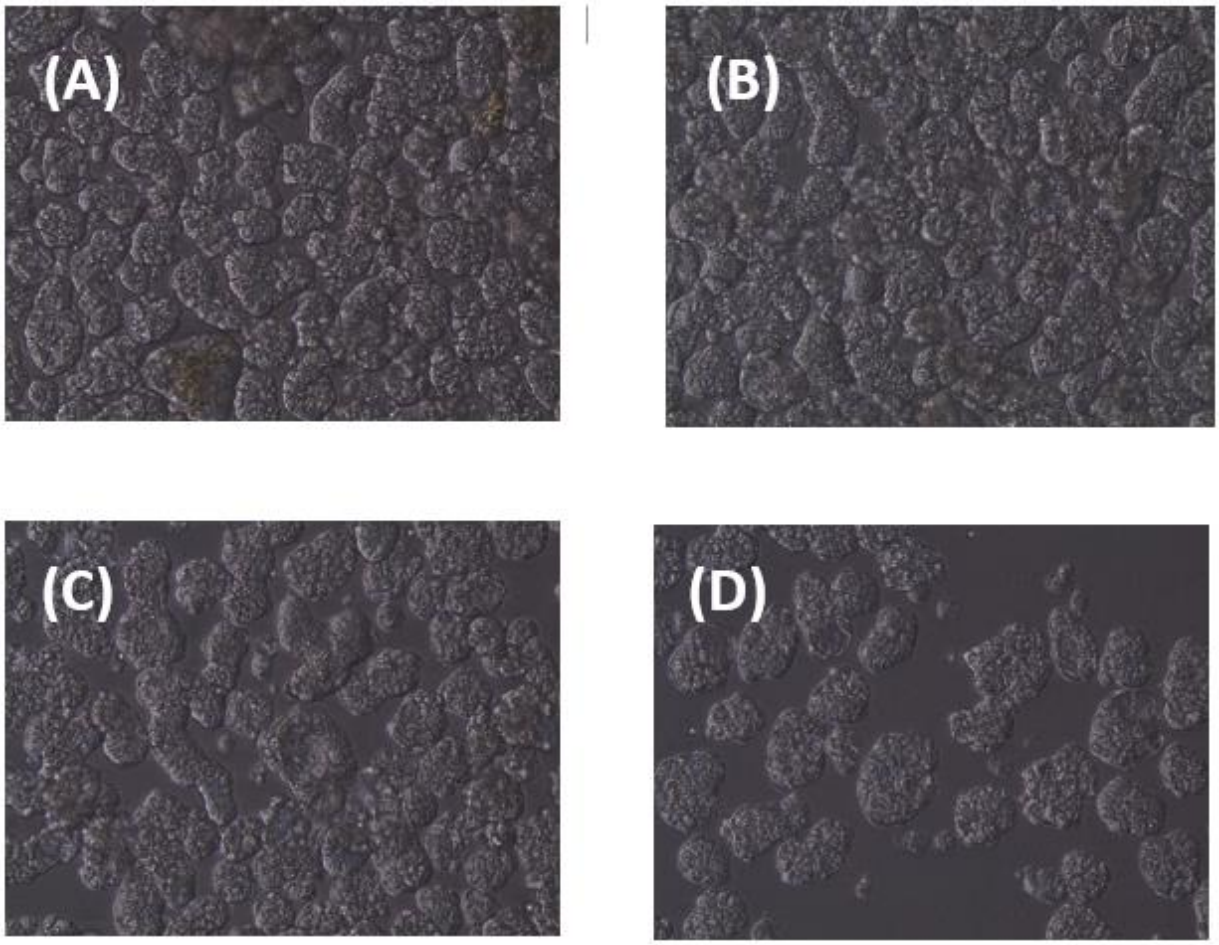


Figure 3.1. OVCAR-5 spheroids after treatment with resveratrol. The images show the effects of (A) no resveratrol (control); (B) 10µM resveratrol; (C) 30µM resveratrol; and (D) 50µM resveratrol. Differences in the number of spheroids present occurred with the 30µM treatment and a significant difference of cluster number occurred after the 50µM treatment.

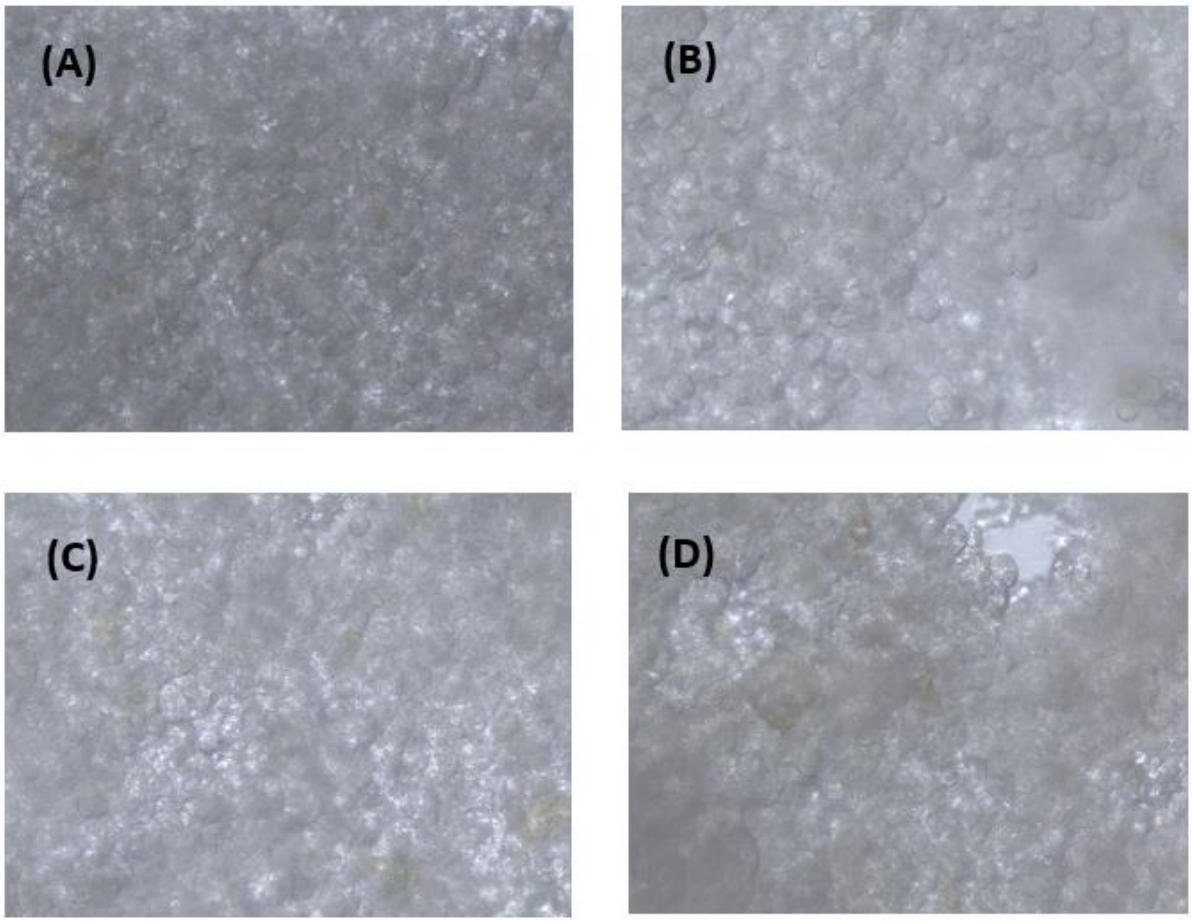


Figure 3.2. SKOV-3 spheroids after treatment with resveratrol. The images show the effects of (A) no resveratrol (control); (B) 10µM resveratrol; (C) 30µM resveratrol; and (D) 50µM resveratrol. Spheroids are highly layered and few morphological changes occur between the treatments.

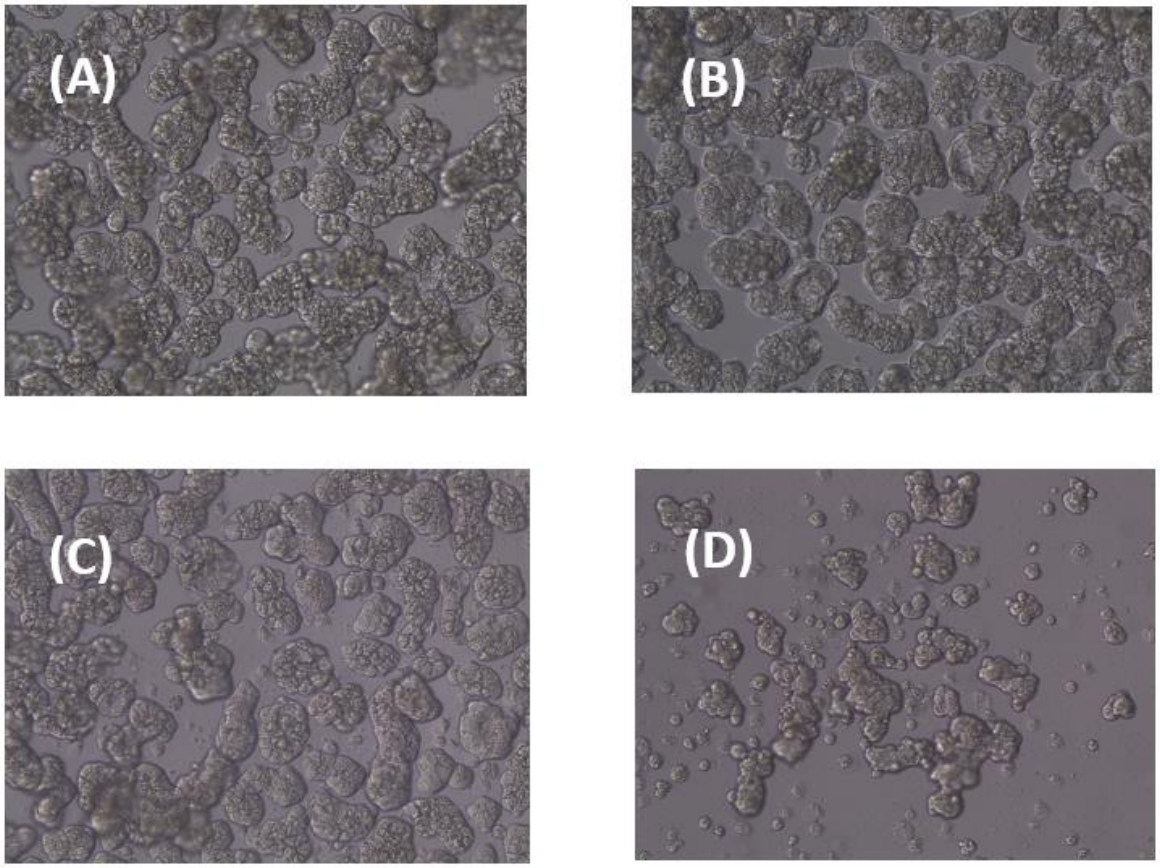


Figure 3.3. OVCAR-5 spheroids after treatment with pterostilbene. The images show the effects of (A) no pterostilbene (control); (B) 10µM pterostilbene; (C) 30µM pterostilbene; and (D) 50µM pterostilbene. A difference in the number of cell clusters occurred following the 50µM treatment.

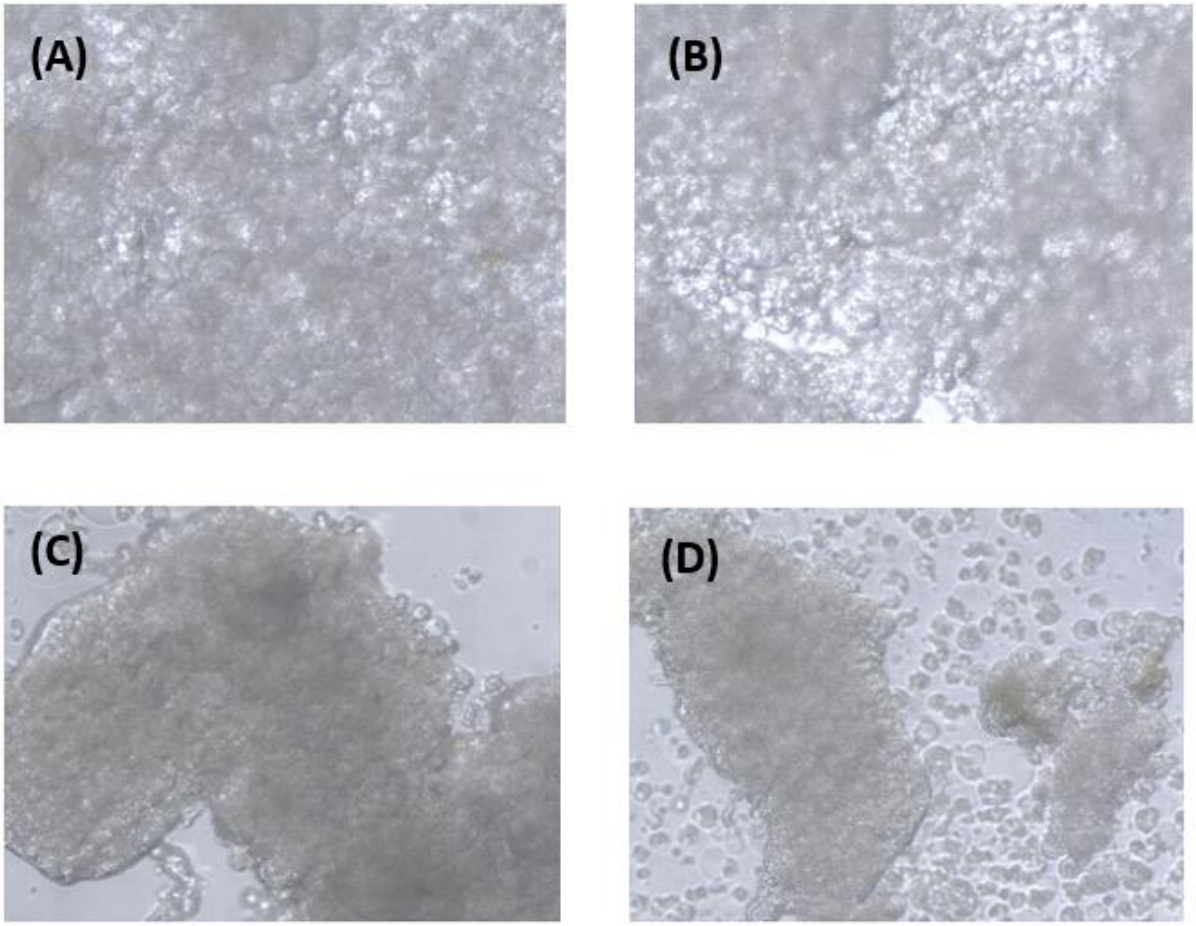


Figure 3.4. SKOV-3 spheroids after treatment with pterostilbene. The images show the effects of (A) no pterostilbene (control); (B) 10µM pterostilbene; (C) 30µM pterostilbene; and (D) 50µM pterostilbene. Spheroid size decreased at the higher concentrations and spheroids appeared to break up into smaller groups of spheroids after treatment with 50µM.

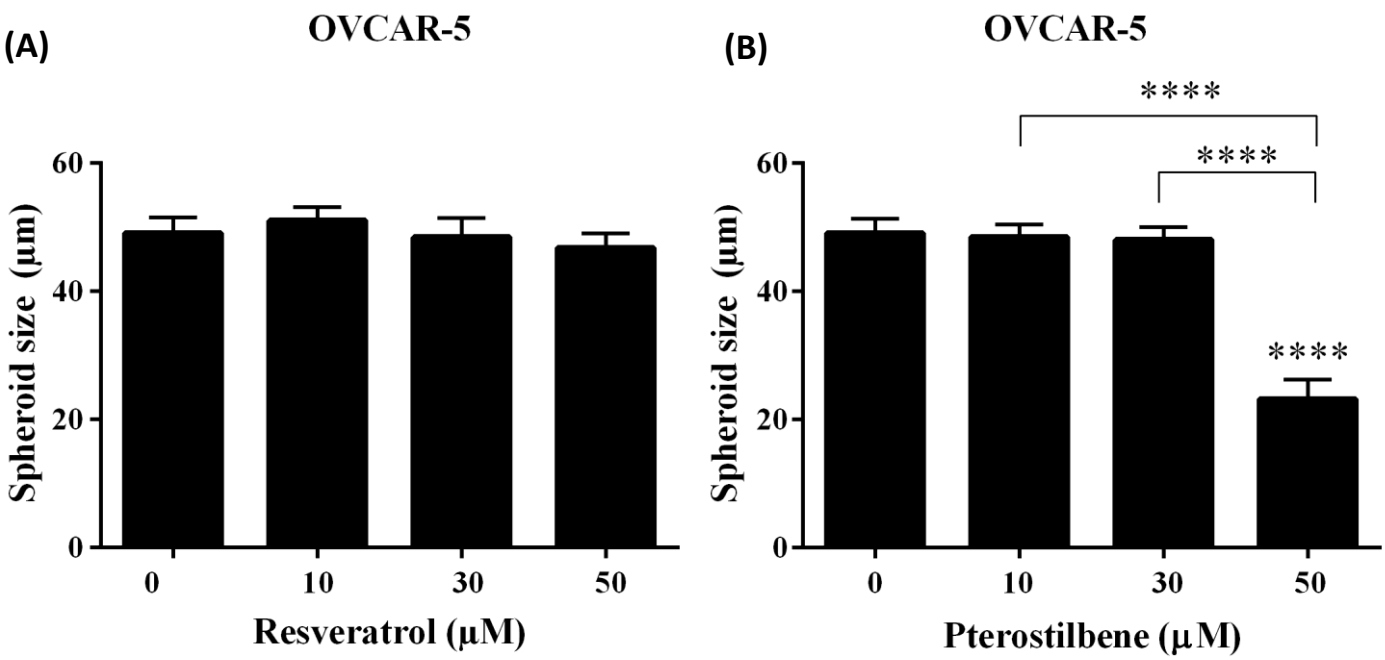


Figure 3.5. OVCAR-5 spheroid size following treatment with (A) resveratrol and (B) pterostilbene at 0µM (control), 10µM, 30µM and 50µM. Measurements were taken from images obtained using light microscope with Leica camera and micrometre slide. **** denotes statistical significance (P≤0.0001, ANOVA, n=29).

3.3.3. Effect of Resveratrol and Pterostilbene on Metabolic Activity of OVCAR-5 and SKOV-3

The metabolic activity of OVCAR-5 spheroids was significantly reduced in the presence of 50μM resveratrol (P≤0.05, ANOVA, n=9) (Figure 3.9). The metabolic activity was also significantly lower in the 50μM treatment relative to the 30μM treatment (P≤0.05, ANOVA, n=9). The lower resveratrol concentrations (10μM and 30μM) did not show any significant difference relative to the control (Figure 3.9a). There was no significant change in OVCAR-5 metabolic activity in response to pterostilbene at any of the tested concentrations relative to the control (P>0.05, ANOVA, n=9) (Figure 3.9b).

The metabolic activity of SKOV-3 spheroids was not significantly reduced at any of the concentrations of resveratrol tested (Figure 3.10a) (P>0.05, ANOVA, n=9). In contrast, there was a statistically significant reduction in metabolism in the presence of 30μM (P≤0.001, ANOVA, n=9) and 50μM (P≤0.0001, ANOVA, n=9) pterostilbene. The 50μM pterostilbene treatment also gave a statistically significant decrease in metabolic activity relative to the 10μM and 30μM treatments (Figure 3.10b).

DMSO at the highest concentration tested had no effect on metabolic activity relative to the DMEM-F12 control (Table 3.1).

3.3.4. Effect of Resveratrol and Pterostilbene on Cellular Quantification of OVCAR-5 and SKOV-3

The number of OVCAR-5 cells was significantly reduced in the presence of 50μM resveratrol (P≤0.05, ANOVA, n=9) (Figure 3.11a). At lower concentrations there was no significant effect on cell number. Pterostilbene, significantly decreased the number of OVCAR-5 cells at concentrations of 30μM (P≤0.01, ANOVA, n=9) and 50μM (P≤0.001, ANOVA, n=9). Additionally, the 50μM treatment significantly decreased cell number relative to the 10μM treatment (Figure 3.11b).

Resveratrol had no significant effect on the number of SKOV-3 cells at any of the concentrations tested (P>0.05, ANOVA, n=9). In contrast, pterostilbene significantly reduced

SKOV-3 cell number at each of the concentrations tested: 10µM ($P \leq 0.001$, ANOVA, n=9), 30µM ($P \leq 0.0001$, ANOVA, n=9) and 50µM ($P \leq 0.001$, ANOVA, n=9) (Figure 3.12b).

Again DMSO controls similar to those used in the pterostilbene samples were found to have no significant effect on cell number relative to the DMEM-F12 controls (Table 3.2).

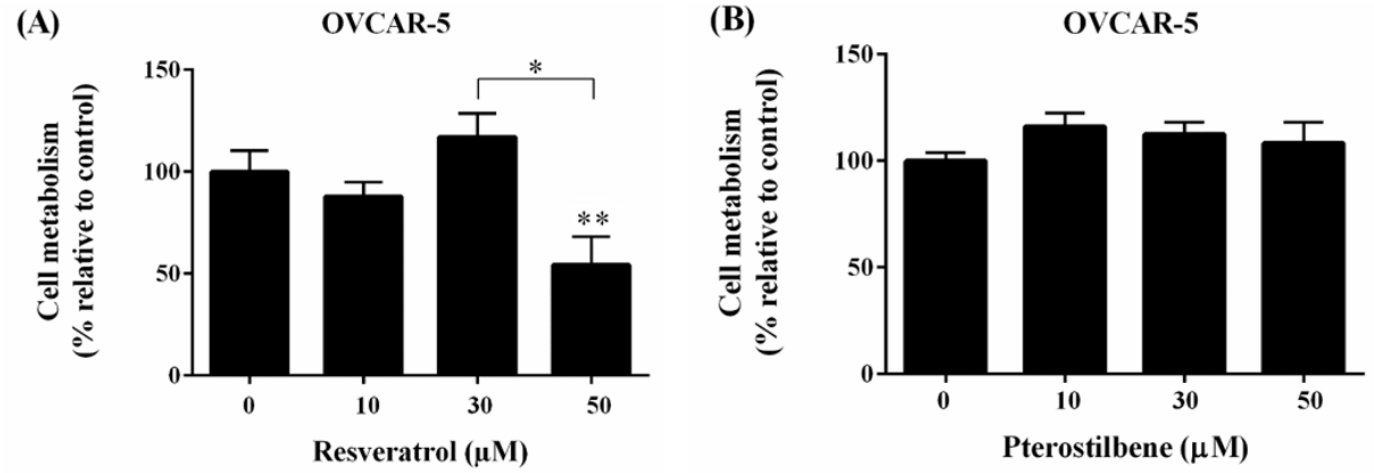


Figure 3.6. Cell metabolism as determined by an Alamar Blue assay of OVCAR-5 cells treated with (A) resveratrol and (B) pterostilbene at 0µM (control), 10µM, 30µM and 50µM. Data was normalised and the control had a mean absorbance value of 0.435nm and 0.212nm for the resveratrol and pterostilbene samples respectively. Data was collected from three individual experiments and carried out in triplicate ± SEM. * denotes statistical significance (P≤0.05, ANOVA, n=9) relative to the control.

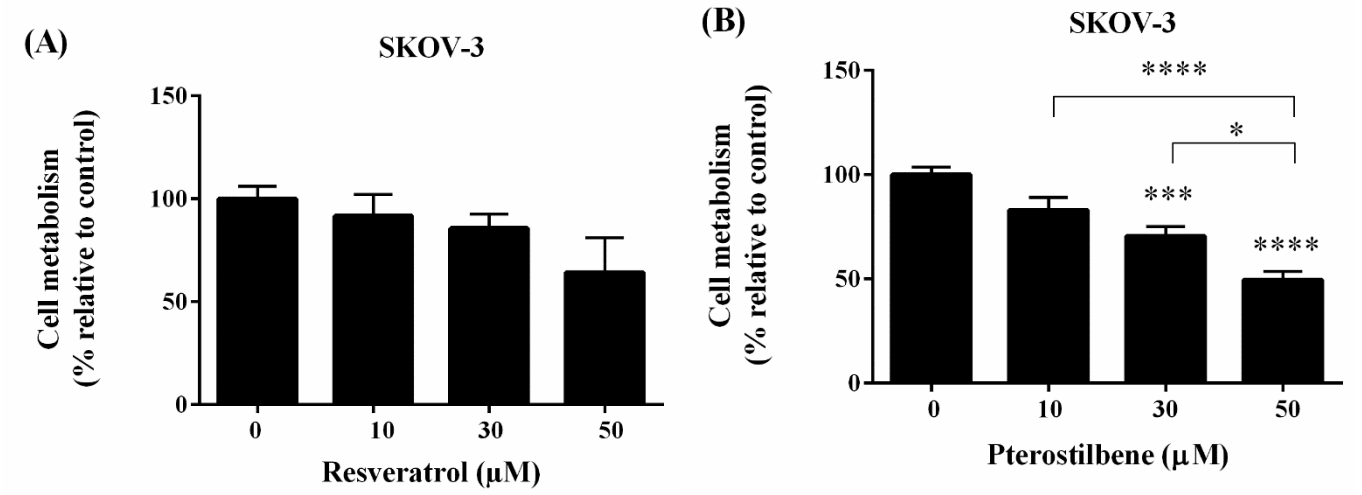


Figure 3.7. Cell metabolism as determined by an Alamar Blue assay of SKOV3 cells treated with (A) resveratrol and (B) pterostilbene at different concentrations (0µM (control), 10µM, 30µM and 50µM). Data was normalised and the control has a mean absorbance value of 0.321nm and 0.372nm for resveratrol and pterostilbene samples respectively. Data is collected from three individual experiments carried out in triplicate ± SEM. * denotes statistical significance (P≤0.05 ANOVA,) relative to the control. *** denotes statistical significance (P≤0.001, ANOVA,) relative to the control. **** denotes statistical significance (P≤0.0001 ANOVA) relative to the control.

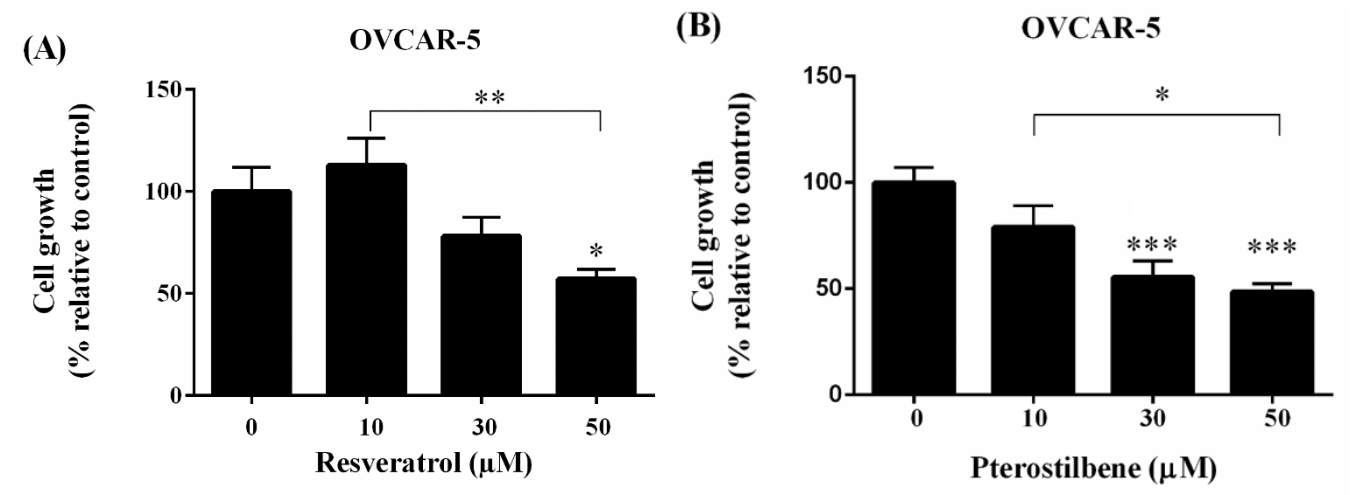


Figure 3.8. Number of OVCAR-5 cells determined by the Crystal Violet assay after treatment with (A) resveratrol and (B) pterostilbene at different concentrations (0µM (control), 10µM, 30µM and 50µM). Data was normalised and the control has a mean absorbance value of 0.757nm and 1.162nm for resveratrol and pterostilbene samples respectively. Data is collected from three individual experiments carried out in triplicate ± SEM. * denotes statistical significance (P≤0.05, ANOVA), ** denotes statistical significance (P≤0.01, ANOVA) and *** denotes statistical significance (P≤0.001, ANOVA) relative to the control.

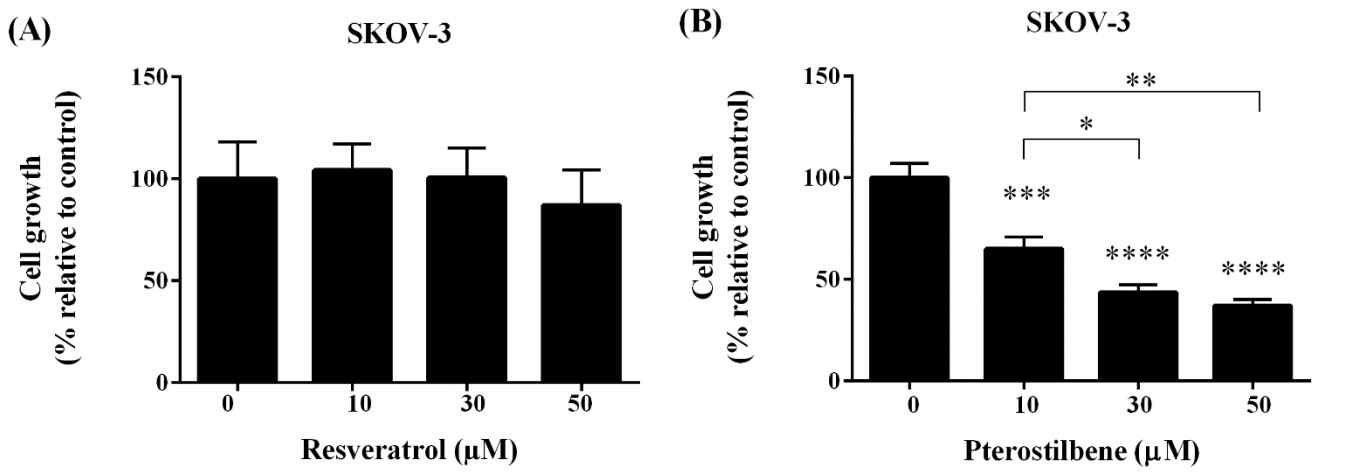


Figure 3.9. Number of SKOV-3 cells determined by Crystal Violet assay after treatment with (A) resveratrol and (B) pterostilbene at different concentrations (0µM (control), 10µM, 30µM and 50µM). Data was normalised and the control has a mean absorbance value of 0.769nm and 1.756nm for resveratrol and pterostilbene samples respectively. Data is collected from three individual experiments carried out in triplicate ± SEM. * denotes statistical significance (P≤0.05, ANOVA), ** denotes statistical significance (P ≤ 0.01, ANOVA), *** denotes statistical significance (ANOVA, P ≤ 0.001) and **** denotes statistical significance (P≤0.0001, ANOVA,) relative to the control.

	Control absorbance (570nm-600nm)	Control with DMSO absorbance (570nm-600nm)	Significance (yes/no)
OVCAR-5	0.354	0.476	no
SKOV-3	0.442	0.365	no

Table 3.1. Comparison between cell metabolism in the control media and control media with added DMSO. There was no significant difference in metabolism and thus DMSO did not affect cell metabolism.

	Control absorbance (560nm)	Control with DMSO absorbance (560nm)	Significance (yes/no)
OVCAR-5	1.016	0.876	no
SKOV-3	0.919	0.627	no

Table 3.2. Comparison between cell number in control media sample and control media with added DMSO. There was no significant different in cell number and thus DMSO did not affect the growth of the cells.

3.4. Discussion

The preventative and/or curative effects against cancer cells of natural products present in the diet is an area that is of current interest given the possible resistance of these cells to chemotherapy and the toxicity of the chemotherapeutic drugs. Two such compounds, the stilbenes resveratrol and pterostilbene were the focus of the current research. Previous research on resveratrol has focussed on colon, breast and prostate cancer and has shown that the compound reduced cell proliferation and growth (Aziz, Kumar et al. 2003). Research on pterostilbene has shown that the compound reduced cell proliferation and growth in a dose-dependent manner in bladder, pancreas, colon, breast, prostate and stomach cancers. However research on the effects of both these stilbenes on ovarian cancer cells has been limited (McCormack and McFadden 2012, Stakleff, Sloan et al. 2012). This chapter focussed on the effects of these two stilbenes on metabolism and cell number of the OVCAR-5 and SKOV-3 ovarian cancer cell lines.

The effect of resveratrol on ovarian cancer was cell line dependant; OVCAR-5 had a significantly decreased cell metabolism and cell number; in contrast resveratrol had no significant effect on the SKOV-3 cells. Furthermore, these decreases in OVCAR-5 were dose dependant; only 50µM resveratrol caused a significant decrease in cell metabolism and number. This is in line with a previous study which tested the effects of resveratrol on 6 different cell lines from different cancers (colon, oesophageal, leukaemia and breast cancer) at various concentrations of resveratrol (0µM, 30µM, 50µM, 100µM, 200µM and 300µM). All cell lines showed a significant reduction in growth after exposure to 100µM resveratrol, and three (oesophageal adenocarcinoma Seg-1, leukaemia HL-60 and breast MCF-7) were more sensitive with a significant reduction after exposure to 50µM resveratrol (Joe, Liu et al. 2002). There are similarities between various breast and ovarian cancers, specifically in the germline mutations *BRCA1* and *BRCA2*, and genetic mutations in *PTEN*, *CDH1*, *PALB2* and *TP53* (National Cancer Institute). Due to the similarities in genetic mutations between breast and ovarian cancer, the similarity in growth inhibition of the breast cancer cell line tested by Joe, Liu et al. (2002) and the OVCAR-5 result from the present study would encourage further concentrations of resveratrol to be tested on ovarian cancer cell lines to determine if a similar pattern would occur.

There are limited studies which have made a comparative examination of the effect of resveratrol on different ovarian cancer cell lines. Lee, Choi et al. (2009) examined three different ovarian cancer cell lines: PA-1, TOV-112D and SKOV-3, in a monolayer culture with increasing levels of resveratrol (0μM, 12.5μM, 25μM, 50μM, 100μM and 200μM), to which the cells were exposed for different lengths of time. Similar to the present study, SKOV-3 growth was not significantly reduced when the resveratrol concentration was less than 50μM. The higher non-physiological levels of resveratrol (≥50μM) produced significant decreases, demonstrating dose dependency, an effect not tested in the present study as concentrations tested did not exceed 50μM. The three cell lines tested by Lee, Choi et al. (2009) differed in their responses to resveratrol, as did the SKOV-3 and OVCAR-5 in the present study. This further suggests that, in addition to different cancer types responding differently to the compound, different cell lines of the same cancer can have a different responses. Thus the different mutations in these cells lines may lead to differing sensitivities to resveratrol.

The OVCAR-5 cell line contains a KRAS:G12V mutation, while SKOV-3 has the wild type gene. This mutation leads to a missense substitution in the *KRAS* gene, which can result in the activation of the MAPK/ERK pathway and subsequently effects cell cycle progression and cell motility (The Roche Cancer Genome Database 2.0). SKOV-3 contains a missense mutation in H1047R, leading to a mutated *PIK3CA* gene, while OVCAR-5 contains the wild type. This could potentially affect the PI(3)K signalling pathway and ultimately increase migration and tumourigenesis. Treatment with resveratrol at high concentrations could affect these proteins differently, to cause a change to the level of proliferation in these cell lines. Further research would be required to determine this.

There are few studies that have investigated the anti-cancerous effect of resveratrol on ovarian cancer 3D spheroids. A recent study focussed on the effects of resveratrol and several of its derivatives on the SKOV-3 cell line and demonstrated significant reduction in cell growth at concentrations of 10 and 50μM resveratrol (Hogg, Chitcholtan et al. 2015). This study used similar techniques to those in the present study, so the difference in the reported results and in the present study are difficult to explain. Different laboratories and different batches of chemicals used may be a factor in why these two studies gave different results. Cells for the current study were donated from the other laboratory, thus the cells may have acquired resistance to resveratrol over time prior to the current study being carried out.

The decrease in cellular metabolism following resveratrol treatment was cell line- and dose-dependent, as resveratrol caused a significant decrease only at 50µM in the OVCAR-5 cell line. A previous study tested the effects of resveratrol on glucose metabolism in several cell lines, including two types of OVCARs and SKOV-3 (Kueck, Opipari et al. 2007). The study revealed that resveratrol acts as a starvation agent, resulting in a decrease in glucose uptake and subsequently glucose metabolism. A greater decrease in glucose uptake was observed in the OVCA432 and OVCA429 cell lines compared to SKOV-3 with 50µM resveratrol (Kueck, Opipari et al. 2007). The DMEM/F12 media used in the current study is high in glucose, which thus suggests that a similar decrease in glucose uptake may be responsible for the results in the current study. This is consistent with the OVCAR-5 spheroids being more sensitive to resveratrol than the SKOV-3 cells.

As hypothesised, pterostilbene had a greater effect than resveratrol; pterostilbene caused significant decreases in cell number at lower concentrations in both OVCAR-5 and SKOV-3 cells and a decrease in cell metabolism in SKOV-3 cells. Cell number was significantly decreased in SKOV-3 spheroids following 10µM, 30µM and 50µM treatment, in contrast to OVCAR-5 which was only significantly decreased at 30µM and 50µM. The level of significance for the decrease in OVCAR-5 cell number following 50µM treatment was in the same range as the significance for the 10µM treatment on SKOV-3, further demonstrating the differences this compound has on the two different cell lines. Pterostilbene produced a dose- and cell line- dependent effect on metabolism. SKOV-3 spheroids resulted in a significant reduction following treatment with 30µM and 50µM pterostilbene. There was no significant decrease in metabolism at any tested concentration of OVCAR-5 or following the 10µM pterostilbene treatment of SKOV-3 cells, even though there was a decrease in cell number. This result is difficult to explain as it would be expected that a decreasing number of viable cells would occur when there was a decrease in cell metabolism. This could be due to the size of the spheroids and the methodology for testing metabolism. Spheroids are quite large, especially the OVCAR-5 spheroids and because the cells were not trypsinized before the addition of the Alamar Blue dye with incubation, the dye may only be interacting with the cells on the outside of the 3D spheroid. Thus as cell number may be decreasing, the size of the spheroid may not be significantly changed, resulting in little difference to the measured metabolic activity.

To the best of my knowledge, the effect of pterostilbene on cell metabolism and cell number of ovarian cancer cells has not been a focus of any previous study. One study has compared the effect of pterostilbene on gastric carcinoma, HL-60 leukaemia and hepatocellular carcinoma cells at a range of concentrations up to 100µM. The pterostilbene had a dose dependant effect, decreasing cell viability in each of the cell types tested. For the HL-60 leukaemia cells there was a significant decrease in cell viability following a 10µM treatment, while Hep G2 cells were first significantly reduced following a 80µM treatment (Pan, Chang et al. 2007). This further demonstrates differences that occur between cell lines following pterostilbene treatment, similar to the results observed in the present study. Alosi, McDonald et al. (2010)) tested the effects of pterostilbene, up to a concentration of 100µM, on two breast cancer cell lines, MCF-7 and MDA-MB-231, grown and treated in a monolayer culture. Similar to the OVCAR-5 results in the current study, treatment with 50µM caused a significant decrease in cell proliferation. Given that monolayer based experiments typically result in a larger statistical decrease, strengthens the idea that SKOV-3 is affected by pterostilbene more than the other cell lines, as cell number was decreased at lower concentrations in a 3D culture compared to OVCAR-5 and previous findings which had used monolayer cultures.

The absorbance values of the control samples following the resveratrol and pterostilbene treatment for both OVCAR-5 and SKOV-3 cell lines varied. A direct experiment comparing these two control types determined that there was no significant difference in the cells metabolic activity or cell number when grown with these environments. This suggests that the varying values are not caused by the treatments but are a result of varying cell growth within the samples.

Chapter 4: Effects of Polyphenols on the Vascular Endothelial Growth Factor

4.1. Introduction

4.1.1. Vascular Endothelial Growth Factor in Cancer

The vascular endothelial growth factor (VEGF) is a 45kDa homodimeric glycoprotein which is secreted by cells to either induce vascular generation and permeability, or to stimulate endothelium mitosis (Ferrara, Gerber et al. 2003, Li, Wang et al. 2004). Additionally, VEGF is responsible for the formation of new blood vessels via angiogenesis in diseases such as cancer. This is critical for tumours larger than 1-2mm in size; without the blood vessels insufficient oxygen and nutrients would diffuse to the tumour cells and this would impact on cell survival and growth (Holmes and Zachary 2005). Tumour metastasis occurs as a result of the formation of new blood vessels, and the expression of VEGF in ascetic fluids in addition to the tumour fluids present. Following relocalisation of the spheroids, VEGF enables growth of the tumour in a new area (Figure 1.4) (Yamamoto, Konishi et al. 1997, McMahon 2000, Ferrara 2004, Brabletz 2012). The control for the VEGF function is known as the angiogenic switch; normal cells have a balance of pro-angiogenic molecules and anti-angiogenic molecules, in contrast to tumours where this is switched off resulting in an increase in the pro-angiogenic molecules, and this can lead to angiogenesis (Carmeliet and Jain 2000). Additionally VEGF can further promote tumour growth by inhibiting dendritic cell maturation or increasing the adhesion between natural killer cells and microvessels to disrupt the immune response, or by upregulating the *bcl-2* gene responsible to interfere with apoptosis (Ferrara 2005).

To function, VEGF (VEGF-A) binds to receptors that are present on the surface of vascular endothelial cells, either VEGFR-1/Flt-2 or VEGFR-2/KDR. Once the growth factor has bound, the receptor dimerizes and becomes phosphorylated, which subsequently allows downstream signalling to occur (Carmeliet 2005). These receptors are believed to lead to different signal transduction pathways; VEGFR-1 does not lead to proliferation but does result in cell migration, in contrast VEGFR-2 activates the MAPK, resulting in cellular proliferation. Therefore, the functions of these receptors are linked as the VEGFR-2 activation benefits VEGFR-1 and the proliferated cells can then migrate. Following VEGFR activation, protease

levels for the first steps in angiogenesis are increased (Neufeld, Cohen et al. 1999). The ability to block these receptors could therefore be vital in cancer treatment.

For intraperitoneal dissemination metastasis, VEGF is essential in that it facilitates the formation of new blood vessels. As discussed, VEGF is required for the initial tumour to proliferate, and once dissociated from the initial tumour, VEGF is required the EMT, MET and migration/disaggregation to occur and as it allows further growth in the new location, similarly to the initial tumour (Figure 1.4) (Brabletz 2012).

It is suggested that VEGF levels that are present in cells are increased in tumours. Yamamoto et al (1997) determined that VEGF was present in 96% of carcinomas that they tested, compared to 33% of benign cells. This suggests that the presence of VEGF is associated with advanced tumours and with more ascites. Overexpression of VEGF may therefore indicate a poor prognosis for patients, due to increased metastasis and proliferation. VEGF can be used as a marker for ovarian tumours due to the increased levels in tumours. Yamamoto et al (1997) further demonstrated the importance of VEGF and its potential use as a marker by comparing the level of the growth factor prior to, and after surgical removal of a tumour, reporting a decrease in VEGF when the tumour was debulked. VEGF is an appealing target for drug therapy because it circulates in the body rather than just being confined to the tumour and as such could be easier to treat. This gives the potential to stop metastasis by preventing angiogenesis (Ferrara 2004). Drugs such as bevacizumab are currently being trialled for ovarian cancer treatment that act against VEGF and these can increase survival times for patients (Banerjee and Kaye 2013). However, these effects follow combinatorial treatments with cisplatin and carboplatin, so may also be limited with respect to toxicity and cell resistance. (Banerjee and Kaye 2013). Further research for other treatments targeting VEGF with a lower toxicity would be beneficial.

4.1.2. Aims of Chapter 4

To determine the effects of resveratrol and pterostilbene on the secretion of vascular endothelial growth factor in OVCAR-5 and SKOV-3 spheroids.

4.2. Methods

3D cell culture – See chapter 2 for details.

VEGF ELISA – See chapter 2 for details.

4.3. Results

4.3.1. Effect of Resveratrol and Pterostilbene on VEGF Production by OVCAR-5 and SKOV-3 Spheroids

The concentrations of VEGF that was secreted into the media of the control samples for the OVCAR-5 spheroids were 1.58ng/mL/mg and 1.32ng/mL/mg for resveratrol and pterostilbene respectively. For the SKOV-3 spheroids the VEGF concentration in control media was 1.83ng/mL/mg and 1.78ng/mL/mg for resveratrol and pterostilbene respectively.

Following the 6 days of treatment with resveratrol, the amount of VEGF secreted by the spheroids did not result in a significant decrease in VEGF production, relative to the control, in either the OVCAR-5 or SKOV-3 cell line at any concentration tested ($P \geq 0.05$, ANOVA, n=9) (Figure 4.1a, 4.1b). The 50µM resveratrol treatment decreased the amount of VEGF secretion in OVCAR-5 spheroids relative to the 10µM treatment ($P<0.05$, ANOVA, n=9) (Figure 4.1a).

In contrast to the effect of resveratrol, SKOV-3 spheroids had a significant reduction in VEGF secretion after treatment with 50µM pterostilbene relative to the control ($P<0.05$, ANOVA, n=9) (Figure 4.2b). The 50µM pterostilbene treatment decreased the amount of VEGF secretion in SKOV-3 spheroids relative to the 10µM treatment ($P< 0.05$, ANOVA, n=9). No other treatments showed any significant reduction.

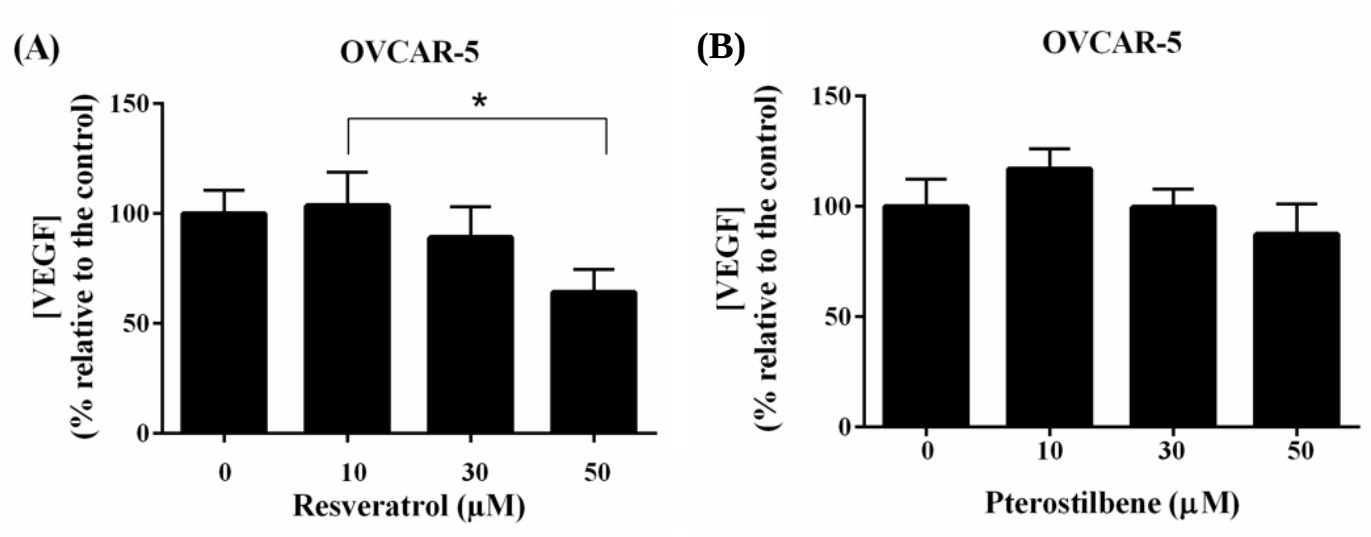


Figure 4.1. VEGF concentration present in media determined by VEGF ELISA following OVCAR-5 cell treatment with (A) resveratrol and (B) pterostilbene at different concentrations (0µM (control), 10µM, 30µM and 50µM). Data was normalised and average control values were 1.575ng/mL/mg and 1.318ng/mL/mg for resveratrol and pterostilbene respectively. Data is collected from three individual experiments carried out in triplicate ± SEM. No statistically significant changes from the control occurred at any drug concentration tested.

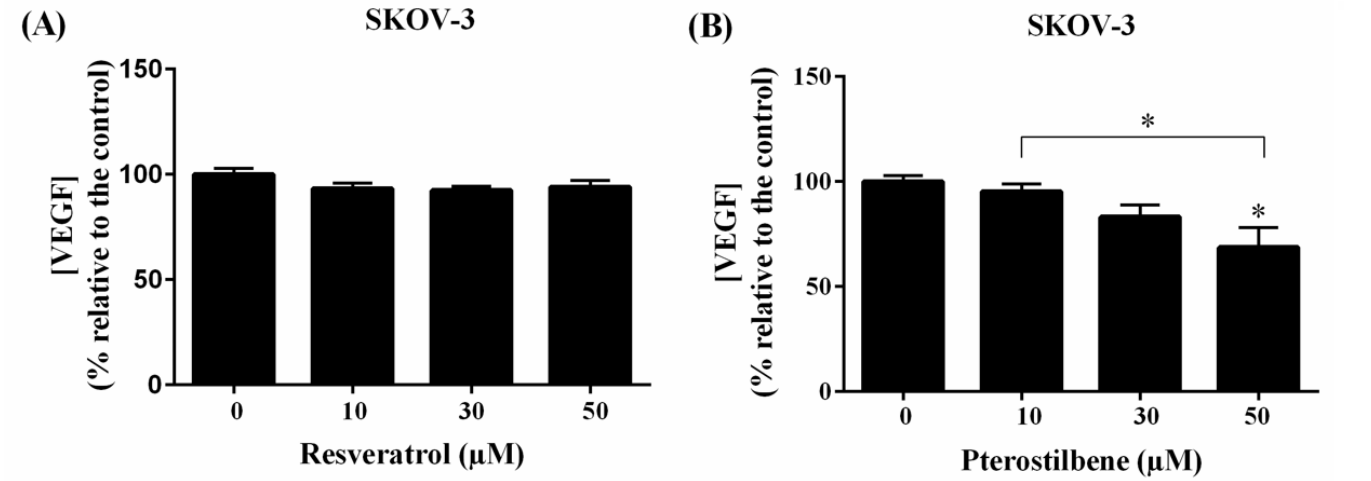


Figure 4.2. VEGF concentration present in media determined by VEGF ELISA following SKOV-3 cell treatment with (A) resveratrol and (B) pterostilbene at different concentrations (0µM (control), 10µM, 30µM and 50µM). Data was normalised and average control values were 1.825ng/mL/mg and 1.779ng/mL/mg for resveratrol and pterostilbene respectively. Data is collected from three individual experiments carried out in triplicate ± SEM. * denotes statistical significance (ANOVA, $P \leq 0.05$).

4.4. Discussion

The increased growth and proliferation following VEGF activation in tumours makes the VEGF an ideal target for drug treatment. Advanced cancers typically produce a higher level of VEGF compared to benign or less advanced tumours. The ability to decrease the level of VEGF could potentially decrease tumour proliferation and metastasis (Yamamoto, Konishi et al. 1997, McMahon 2000).

As discussed in chapter 3, pterostilbene caused a decrease in cell metabolism in the SKOV-3 spheroids after concentrations of 30µM and 50µM, and a decrease in the cell number of OVCAR-5 spheroids after treatment with 50µM resveratrol, and 30µM and 50µM pterostilbene. SKOV-3 cell number was significantly reduced by concentrations of 10µM, 30µM and 50µM pterostilbene. The decrease in VEGF secretion, relative to the control, was only significant after 50µM pterostilbene in the SKOV-3 spheroids. For the remaining treatment samples, VEGF is functioning, and able to assist in proliferation, and therefore these decreasing cell numbers may be influenced by changes to pathways and signalling systems independent from the VEGF signalling pathway. This is consistent with a similar study which obtained significant decreases in the cell growth of SKOV-3 cells after treatment with 10µM, 50µM and 100µM resveratrol but only observed a significant decrease in the VEGF secretion after 50µM and 100µM treatments. In contrast to the current study, there was a significant decrease in VEGF secretion after 50µM resveratrol. (Hogg, Chitcholtan et al. 2015). The current study shows a decreasing trend in VEGF secretion for OVCAR-5 after resveratrol treatment and SKOV-3 after pterostilbene. More replicates may produce a comparable result to that of Hogg, Chitcholtan et al. (2015) as the number of replicates for this study were relatively low.

The present study also contrasts with the study by Cao, Fang et al. (2004) which examined the effects of resveratrol on VEGF secretion in the ovarian cancer cell lines A2780/CP70 and OVCAR-3. VEGF secretion in A2780/CP70 cells was significantly decreased after exposure to 25µM, 50µM and 100µM resveratrol, and in OVCAR-3 cells after exposure to 10µM 25µM, 50µM and 100µM resveratrol. This further demonstrates the differences in the response to resveratrol by different ovarian cancer cell lines and the requirement for further studies to determine why there are differences in response and how to determine which cell line will be

affect more significantly with lower concentrations. Cao, Fang et al. (2004) and Hogg, Chitcholtan et al. (2015) determined that in all cell lines tested, 50µM and 100µM of resveratrol resulted in a significant decrease in VEGF secretion. Therefore the drug concentration range tested in the current study may be too low to elicit a significant decrease. The study by Cao, Fang et al. (2004) used a monolayer culture and thus caution should be exercised when directly comparing this study to the current one. Monolayer cultures do not mimic *in vivo* conditions as the 3D cultures do, and as such may give significant decreases at concentrations where there would not have been one if cultured in 3D.

Differences between cell lines isn't limited to ovarian cancer. A previous study which tested the effect of resveratrol on VEGF secretion by the melanoma cell lines A375 and YLZAZ6 determined that although both had significantly reduced in VEGF secretion relative to the control, the level of reduction was more significant in the A375 spheroids (Trapp, Parmakhtiar et al. 2010). Therefore, the results obtained from the current study are similar to those of other cancers in that there is variability between different cell lines.

To the best of my knowledge there have been limited studies which have tested the effects of pterostilbene on VEGF levels in cancer cells, with none focussing on ovarian cancer. One study which focussed on human hepatocellular carcinoma cells, $HepG_2$, reported a decrease in the enzyme activities in VEGF following treatment with 50mg/kg pterostilbene. The use of different methodologies to assay changes in VEGF levels makes it difficult to directly compare the effect of pterostilbene on VEGF secretion between these two studies. However, VEGF secretion does appear to be following the same pattern as that observed on the SKOV-3 cells after pterostilbene in the present study (Pan, Chiou et al. 2009).

VEGF drug resistance has been previously studied in cancer cells, where an extrinsic resistance occurred; the VEGF is successfully inhibited but the signalling required for angiogenesis and growth by the spheroids is still produced via the upregulation of independent pathways, such as IL-8, to act as a survival mechanism for the cells (Kieran, Kalluri et al. 2012). Intrinsic resistance, where the pathway directly responsible for the VEGF production acquires resistance to the anti-angiogenic drugs, has not been observed in previous studies. Thus, this suggests that the lack of a significant decrease in VEGF secretion in the current study are likely due to the drugs having little to no effect rather than due to acquired resistance in VEGF signalling, although further research would be required to determine this.

Chapter 5: Effects of Polyphenols on the Expression of Proteins

5.1. Introduction

5.1.1. Epidermal Growth Factor Receptor Family

The Erb family consists of four different receptor tyrosine kinases: epidermal growth factor receptor (EGFR)/ErbB1, HER2/ErbB2, Her3/ErbB3 and Her4/ErbB4. These transmembrane proteins are structurally similar and consist of a cytoplasmic region with enzymatic activity and an extracellular ligand-binding domain. Ligand binding is specific to the various Erb proteins and enables specific signalling processes to occur, including signalling from epidermal growth factor molecules (Yarden 2001). When a signalling molecule has bound, Erb proteins will structurally alter to form a homodimer or heterodimer, which subsequently activates downstream signalling for proliferation, survival, adhesion, migration and differentiation (Yarden 2001, Normanno, De Luca et al. 2006).

5.1.1.1. The Epidermal Growth Factor Receptor (EGFR)/ErbB1

The Epidermal Growth Factor Receptor (EGFR) is a 170kD glycoprotein which when activated by ligand binding changes conformation to become a dimer, associating with either another EGFR molecule (becoming a homodimer) or another Erb protein (becoming a heterodimer). Following structural rearrangement, the tail region is phosphorylated on a tyrosine residue to activate the downstream signalling pathways: PI3K, Ras-Raf-MEK, JAK SRC and PLCγ DAG (Figure 5.1) The Ras-Raf-MEK mitogen pathway and the phosphotidylinositol 3 kinase (PI3K) pathway can both control the ability of the cell to grow. The Ras-Raf-MEK pathway affects cell cycle progression and motility while the PI(3)K pathway affects nutrient availability to growing cells, tumourigenesis and also has the ability to inhibit apoptosis, which are all hallmarks of cancer. The STAT and the PLCγ DAG pathways which can affect the cells ability to survive, differentiate and proliferate (Figure 5.1) (Herbst 2004).

Through the regulation of the signalling pathways mentioned above, EGFR has the potential to play a role in the development of cancer by promoting motility and adhesion. A loss of regulation in these pathways can stimulate further cell growth, and thus can influence cancer.

Factors that can affect EGFR and the promotion of cancer include mutations, increased expression of binding ligands and an increase in dimerization levels (Ciardiello and Tortora 2003). Overexpression appears to be the most significant factor due to the intensification of the signals for the pathways, a factor which correlates with a worse prognosis for patients, the formation of advanced tumours and resistance to treatments such as chemotherapy (Ciardiello and Tortora 2003, Normanno, De Luca et al. 2006).

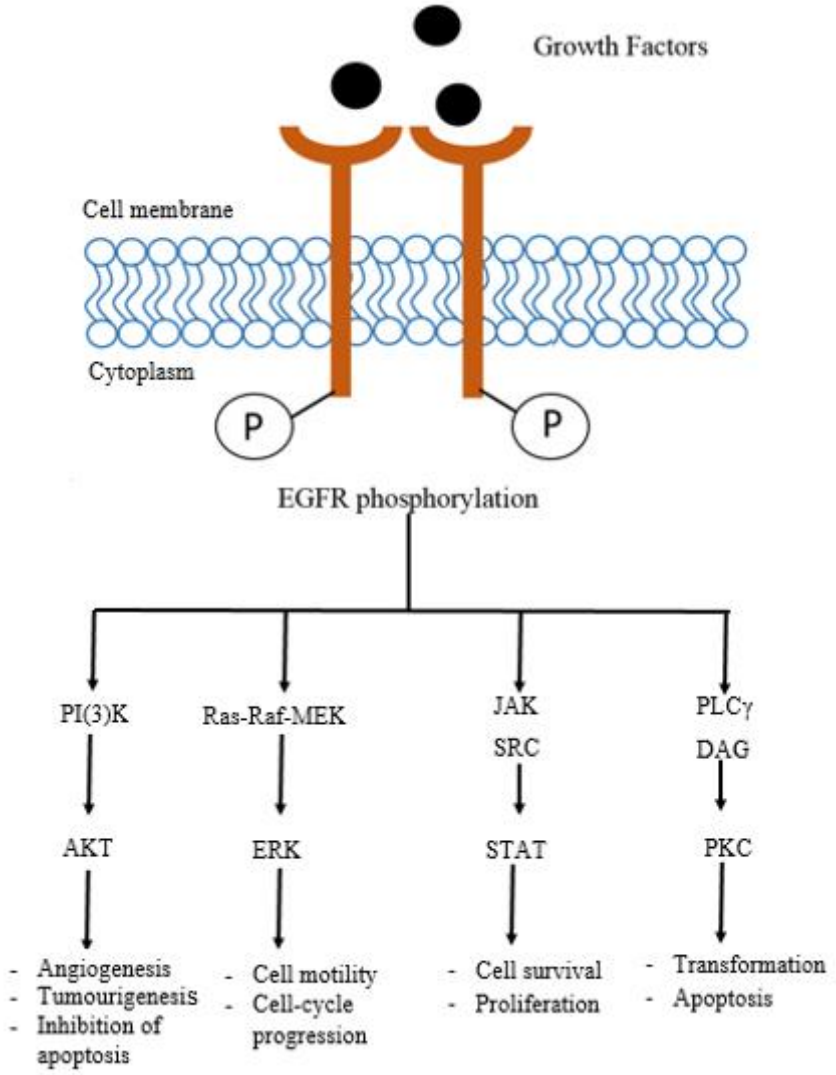


Figure 5.1. EGFR and downstream signalling pathways. Growth factors activate EGFR, resulting in dimerization and the phosphorylation of a tyrosine residue in the tail region. This leads to many downstream signalling pathways that are responsible for cellular survival, progression or growth.

5.1.1.2. Human Epidermal Growth Factor Receptor (HER2/Neu Protein)

HER2/neu is the second member of the Erb family and is responsible for regulating cell proliferation. Although structurally similar to EGFR, this 185kD glycoprotein differs from other family members as no known ligands bind to this protein to activate signalling. Instead, HER2 forms a heterodimer with the other Erb proteins which activates the tyrosine kinases and hence signalling (Serrano-Olvera, Dueñas-González et al. 2006, English, Roque et al. 2013). Similarly to EGFR, overexpression of this protein has been observed in a range of cancers, including ovarian cancer (Verri, Guglielmini et al. 2005). This causes the constitutive activation of tyrosine kinases, loss of regulation for the downstream signalling pathways such as Ras-Raf-MAPK and PI(3)K-AKT, and thus results in increased cell growth (English, Roque et al. 2013). Overall this overexpression is believed to correlate with a worse prognosis, a higher recurrence frequency, decreased sensitivity to platinum chemotherapy and increased tumour aggression. However, there are some conflicting results suggesting this may occur more with certain carcinomas and may be influenced by the location of the tumour in the body; therefore the use of this as a prognostic factor is somewhat limited (Serrano-Olvera, Dueñas-González et al. 2006).

5.1.1.3. Epidermal Growth Factor Receptor/ErbB1 and HER2/Neu Co-Expression

Although EGFR and HER2 can individually have significant effects on tumour formation and growth, when they are coexpressed more aggressive tumours can occur, resulting in a worse prognosis for the patient. (Normanno, De Luca et al. 2006). As EGFR is the main binding partner for HER2, the effect of coexpression is observed in a number of different cancers. The ability of HER2 to slow the dissociation of ligands from EGFR and to remain at the cell surface longer, before being internalised enables this interaction to prolong the signalling for the EGFR downstream pathways (Yarden 2001). The five year survival rate for a patient expressing the EGFR is 51% and decreases to 33% when there is coexpression of the EGFR/ErbB1 and HER2/neu receptors.

5.1.1.4. Current Drug Targets

The increased activation of EGFR and HER2 observed in cancer makes them an appealing target for drug therapy. Currently there are two ways to target EGFR. Firstly, to utilise monoclonal antibodies (mAbs) to target the extracellular region of the receptor. This would increase competitive binding and decrease the level of signalling and activation for the downstream pathways, resulting in inhibition and delayed growth and progression of the tumour (Seshacharyulu, Ponnusamy et al. 2012). Currently there are three anticancer mAbs in current use: cetuximab, pantitumumab and mimituzumab, however studies have suggested the effectiveness of these decrease when used multiple times (Baselga 2002, Reichert and Dhimolea 2012). Secondly, to target EGFR through the use of small molecule tyrosine kinase inhibitors (TKIs), such as ZD1839, which bind to the cytoplasmic side of the receptor in the ATP-binding pocket. This prevents phosphorylation occurring and therefore prevents signalling even after ligand binding (Baselga 2002, Nyati, Morgan et al. 2006).

The ability to decrease dimerization and subsequent functioning of HER2 is a target for cancer research. Two drugs have previously been used to decrease HER2 activation. Trastuzumab, a humanised mAb IgG1 antibody has provided decreased activation when used against breast cancer by preventing dimerization. The similarities between breast and ovarian cancer led to the belief that this drug may produce similar results against ovarian cancer, however the effect in ovarian cancer was limited (Bookman, Darcy et al. 2003). Similarly, pertuzumab was of interest for its ability to interfere with the EGFR dimerization (English, Roque et al. 2013). Recent interest has changed to determine the effects of these drugs when used in combination. Pertuzumab and Trastuzumab were combined in drug therapy with the drug Docetaxel and tested on the HER2 activation and overall survival of patients with metastatic breast cancer. Observations from this study included the increase in median overall survival of up to 15.7 months compared to previous treatments. It is believed this increase is due to these drugs binding to different epitopes on the protein, resulting in increased anti-HER2 activity. In ovarian cancer however, further research is required to find drugs that effectively alter the ability of these proteins to become activated (Swain, Baselga et al. 2015).

5.1.2. The Phosphatidylinositol (3) Kinase Protein and Signalling Pathway

The Phosphatidylinositol 3 kinase (PI(3)K) protein belongs to a family which is divided into three different classes. The kinase that is largely associated with cancer formation is part of the class I family. This is further subdivided into two classes: Class IA, which is activated by receptors with tyrosine kinase activity, and is considered the most important for controlling cellular proliferation in tumour growth, and class IB, which is activated by receptors with G-coupled proteins (Luo, Manning et al. 2003). PI(3)K is a class IA heterodimer comprised of a catalytic subunit, p110, and a regulatory subunit, p85. Of these, several isoforms have been identified, however the catalytic subunit isoform p110α is the subunit of particular interest for its effects in cancer when it is overexpressed (Yuan, Sun et al. 2000).

In cancer PI(3)K can undergo structural rearrangement in both subunits following the binding of a growth factor with tyrosine kinase activity (Luo, Manning et al. 2003). Without stimulation, the p85 subunit is bound to the p110α to prevent signalling. However, when a growth factor is present, the p85 will instead bind to the phosphorylated receptor tyrosine kinase, resulting in the relocation of the protein to the plasma membrane. This causes the activation site of the p110α to open, enabling the phosphorylation of the inositol ring at the 3 position of phosphatidylinositol-4,5-bis-phosphate (PIP2) to create PIP3. PIP3 is capable of interacting with molecules containing a FYVE zinc finger domain (named after the proteins it has been found in such as Fab1, YOTB, Vac1 and EEA1) or plectstrin-homology (PH) domain. Specifically for the PI3K pathway, the serine-threonine kinase AKT/Protein Kinase B is bound, resulting in a conformational change to open the phosphorylation sites for activation. Although phosphorylation at the threonine 308 site partially activates the AKT, it also relies on phosphorylation at Serine 473 to become completely activated at which point it has the ability to control cell proliferation, survival, growth and other processes important in cancer growth, via subsequent downstream signalling (Figure 5.2) (Luo, Manning et al. 2003, Osaki, Oshimura et al. 2004).

Mutation or overexpression of the PI(3)K protein can result in the activation of the signalling cascade leading to tumourigenesis. The *PIK3CA* gene encoding the p110α subunit is amplified in many different cancers, while the gene is mutated in only 4-12% of cases. These mutations

tend to occur in either the catalytic domain or the helical domain, locations believed to be responsible for kinase activation, due to an observed increase in AKT phosphorylation when these mutations are present. Additionally, cell migration has been shown to increase when this mutation is present (Samuels and Ericson 2006). Overall, mutations occurring in this gene alter the functioning of the pathway by controlling the PI(3)K protein and subsequently, its downstream signalling.

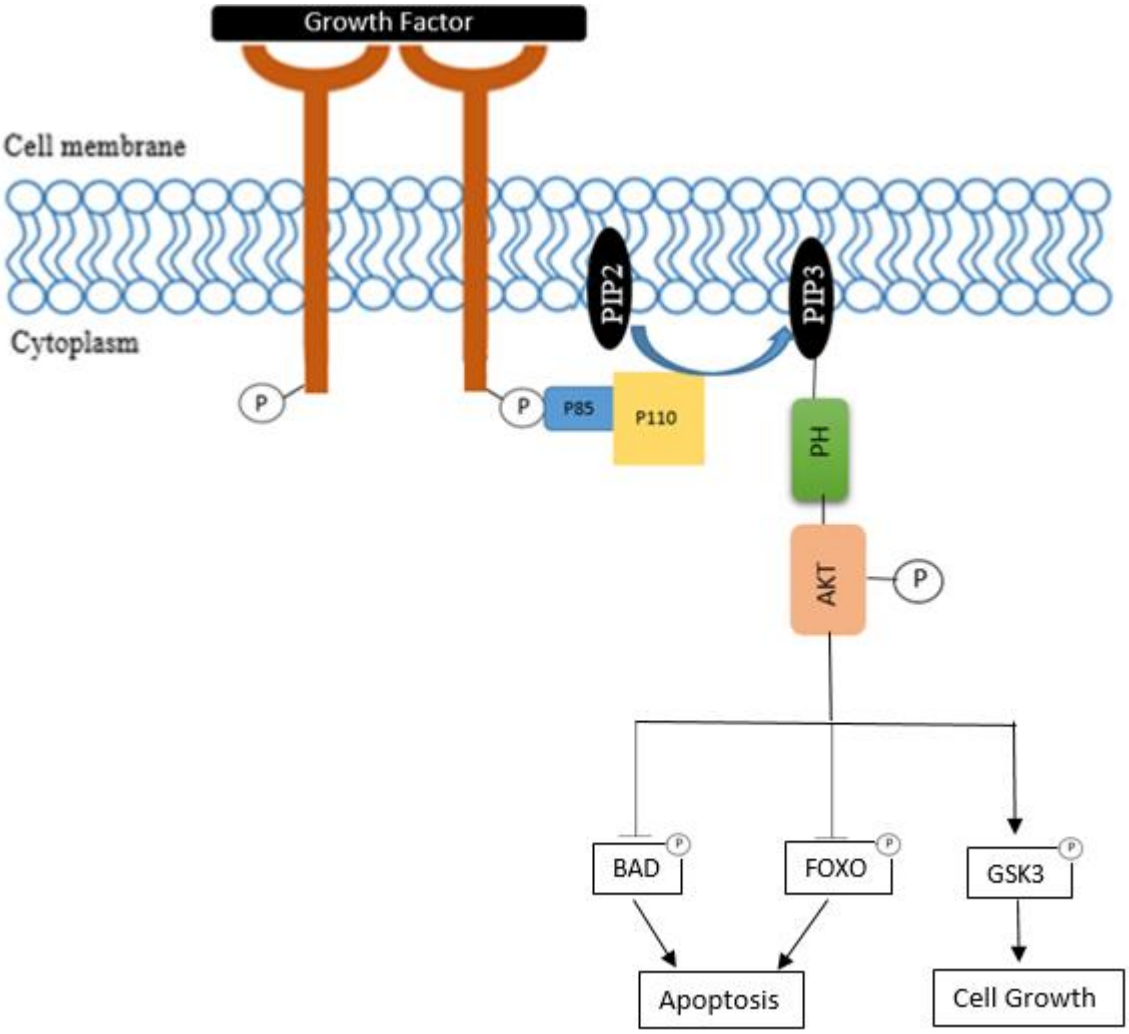


Figure 5.2. PI3K/AKT signalling pathway. Following binding of the growth factor. The heterodimer is activated causing the PI3K p85 to bind the phosphorylated tyrosine kinase and to release p110 to the membrane. This relocation allows p110 to open up and PIP2 to convert to PIP3, capable of interacting with proteins with PH domains, such as those in AKT. AKT becomes phosphorylated and activates downstream signalling leading to proliferation and cell growth through the upregulation and phosphorylation of GSK3 and prevent apoptosis by phosphorylating and downregulating apoptotic proteins BAD and FOXO.

5.1.2.1. AKT Protein and Role in Signalling

The AKT protein which has a molecular weight of 57kD is composed of three parts: an N-terminus containing the plectstrin-homology domain, a central kinase domain and the C-terminal regulatory domain. There are three AKT isoforms which make up the AKT family: AKT1, AKT2 and AKT3, all of which act independently to one another and are encoded by different genes. Homology between these isoforms is high (80%), however differences have been determined specifically at the phosphorylation sites for activation. These differences occur at Threonine309 and Serine474, which are the equivalent to the AKT sites mentioned above. AKT is upstream of many different pathways affecting cell proliferation, specifically those at the transition point from the G1 to the S phase of the cell cycle (Osaki, Oshimura et al. 2004). In normal cells, the glycogen synthase kinase-3 (GSK-3) prevents the cell cycle from progressing by phosphorylating key components such as glycogen synthase or cyclin D, thus causing inactivation. However, in cancer cells, AKT can inhibit this action through the phosphorylation of the serine residue in the N-terminal domain of the GSK-3, the site responsible for the downstream phosphorylation and activation of signalling (Figure 5.2). Specifically, this affects β-catenin, a protein responsible for regulating gene transcription. Due to AKT inhibiting this downstream phosphorylation, β-catenin remains active and present in the nucleus, leading to the transcription of genes for the activation of cyclin D1, therefore resulting in the continuation of the cell cycle and progression (Osaki, Oshimura et al. 2004).

Due to the role of the PI(3)K signalling pathway in cellular proliferation, during tumourigenesis cell cycle regulating components must be deactivated. The CDK inhibitors $P21^{Cip1}$ and $P27^{Cip2}$ are both targeted by the activated AKT protein, both directly and indirectly. $P21^{Cip1}$ is down-regulated, either directly by AKT or by the GSK-3 when this is downregulated by AKT. Both the CDK inhibitors get phosphorylated to prevent their translocation to the nucleus where they can inhibit cell cycle progression (Osaki, Oshimura et al. 2004). The CDK inhibitor is also indirectly affected through phosphorylation of a member of the Forkhead family of transcription factors, the Forkhead box class O (FOXO). When AKT is activated, this phosphorylates the FOXO at serine/threonine residues resulting in it being removed from the nucleus where it would normally function to regulate the $P27^{Kip2}$ (Figure 5.2)(Chang, Lee et al. 2003, Bader, Kang et al. 2005).

In addition to affecting the proteins responsible for controlling the cell cycle, activated AKT can affect apoptosis in two ways, firstly by inhibiting procaspase-9, a significant caspase in the apoptotic pathway. In addition, it can phosphorylate the Bcl-2-associated death promoter (BAD), resulting in inhibition and therefore the lack of control for apoptosis (Osaki, Oshimura et al. 2004).

In contrast to the PI(3)K, no genetic mutations in AKT have been detected, however overexpression/amplification has been reported (Osaki, Oshimura et al. 2004). To the best of my knowledge, no mutations have been detected to date. This overexpression appears to be isoform specific; AKT1 has not been detected to be overexpressed/amplified in any of the ovarian cancer tumour types examined, and AKT2 is overexpressed/amplified in 10-20% of cases (Yuan, Sun et al. 2000, Osaki, Oshimura et al. 2004). Overexpression has been shown to occur late in the development of ovarian cancer, specifically in stages III or IV, suggesting that when this change to protein function is present, the cancer is in an advanced stage and is more aggressive, which signifies a worse prognosis for the patient. Specifically, as the grade of cancer increases, the overexpression and subsequent activation of the AKT protein also increases (Yuan, Sun et al. 2000, Altomare and Testa 2005). For this reason, it is an ideal target for treatment in these later stages and grades of ovarian cancer.

In addition to being a major part of the PI(3)K signalling pathway, AKT also has a significant role in the process of metastasis, where the increased expression of AKT results in a possible increase in metastasis. Studies have shown that the invasion stage of metastasis is increased due to an increase in the amount of β1 integrin present and thus an increase between β1 integrin and collagen on the ECM, and subsequently increased MMP levels (Arboleda, Lyons et al. 2003).

Overall, due to the significant pathways the AKT protein is involved in, this is an ideal protein to target for the prevention of further cell growth and migration.

5.1.3 Proliferating Cell Nuclear Antigen

The proliferating cell nuclear antigen (PCNA) is involved in DNA replication, epigenetic maintenance and DNA repair processes including mismatch repair, base and excision repair and is highly expressed in the G_1-S stage of the cell cycle (Figure 1.3). This 36kDa protein was originally discovered approximately 30 years ago where it was determined to be an antigen

for an autoimmune disease. Subsequent to this another study determined this protein had a role in the cell cycle, and called it cyclin. It was later determined that this was the same protein and was renamed PCNA (Strzalka and Ziemienowicz 2011). Structurally PCNA is composed of 3 monomers, formed by 2 domains that are joined together by a β-sheet in an antiparallel direction, resulting in a ring formation similar to DNA sliding clamps. This structure allows for the PCNA to slide along the DNA polymerase-δ during chromosomal DNA replication. Although overall PNCA is negatively charged, the internal ring is positively charged due the presence of arginines and lysines. The negatively charged DNA double helix can then slide through the internal ring for processing (Naryzhny 2008).

In addition to sliding along DNA, PCNA interacts with proteins involved in the cell cycle, specifically cyclins and cyclin dependant kinases, and thus has an important role in proliferation. Specifically the cell cycles S-phase proteins cyclin A and CDK2 which are drawn to the PCNA to form a complex. Due to PCNA being bound to the DNA as a sliding clamp, the ability to bind other proteins is beneficial to proliferation as it brings the required proteins to the replication site (Maga and Hübscher 2003). However, cyclin A is not the only cyclin associated with PCNA. In contrast, cyclin D acts a regulator, capable of binding to PCNA and preventing association with the replication complex required (Naryzhny 2008). Regulation of PCNA also occurs through the p21 protein. Additional to the capability of p21 to bind CDKs at the N-terminal to prevent CDK-cyclin complexes forming, when present at a high level it can bind to PCNA at the C-terminal to inhibit DNA replication by preventing the interactions required for PCNA to function with the DNA polymerase δ. In cancer, the cells regulation is often shut down so if p21 is not active or overexpressed and therefore relocated in the cell where it is no longer useful, proliferation would be capable of continuing via the interaction PCNA has with the regulatory machinery (Maga and Hübscher 2003, Strzalka and Ziemienowicz 2011).

5.1.4. Cyclin D and Cancer

Cancer is the disease of uncontrolled cell growth, which makes the cell cycle an important aspect of this disease. As mentioned previously, the cell cycle begins in a quiescent state before moving to the G_1 stage of the cycle. The family of cyclin D is responsible for this initial transition into the G_1 following induction by mitogenic cycles, present only when the cell is required to replicate. This cyclin family consists of three different cyclins (D1, D2 and D3) and although there is similarity in the amino acid sequences, they are expressed differently in each cell type and cell line, and not all are present in each cell line (Choi, Li et al. 2012). Following mitogen activation, cyclin D binds to the appropriate CDKs, specifically CDK4 and CDK6, a complex which then relocates to the cell nucleus to become phosphorylated by the CDK-activating kinase (CAK), enabling the subsequent phosphorylation of the retinoblastoma protein. The retinoblastoma protein is essential for the transition from the G_1 phase to the S phase. In the non-phosphorylated state, this protein binds E2F, a family of transcription factors, preventing them from transcribing the genes required at the end of the G_1 phase. Following phosphorylation of the retinoblastoma by the CDK-cyclin D complex, the E2F is released and to undergo transcription (Pines 1995, Giacinti and Giordano 2006). Cyclin D2 is a 34 kDa proto-oncogene which is amplified in some ovarian cancers. In addition to regulating the cell cycle through the retinoblastoma phosphorylation, cyclin D2 is capable of ectopic overexpression, which prevents the cell cycle from continuing, resulting in the cell exiting the cell cycle rather than continuing to undergo division and proliferation. The importance of the cyclin D proteins in the cell cycle therefore make it a desirable target for drug treatment, and this study aims to determine if there are changes in the cyclin D2 protein expression (Pines 1995).

5.1.5. Aims of Chapter 5

To investigate the effects that resveratrol and pterostilbene have on the protein expression of Epidermal Growth Factor Receptor/ErbB1 and HER2/neu in both the phosphorylated and non-phosphorylated forms. Also to determine the level of protein expression of AKT and the level of PCNA.

5.2 Methods

3D cell culture –See chapter 2 for details.

BCA protein Assay – See chapter 2 for details.

Western Blotting – See chapter 2 for details.

5.3 Results

Protein expression was determined using western blot and densitometry, however, due to limited time and resources not all proteins for each cell line were imaged and due to the housekeeping protein, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) only working for half the samples, not all were able to have densitometry carried out and therefore results for those proteins are based on band observation only. Due to the time restraints there was only one repeat (n=1) of each protein sample tested.

5.3.1. AKT/pAKT Expression after Resveratrol and Pterostilbene Treatment

Resveratrol caused an increase in AKT levels in OVCAR-5 spheroids following 10µM, 30µM and 50µM treatments relative to the control. 30µM and 50µM treatments decreased relative to the 10µM (Figure 3.5a). The level of the activated AKT, pAKT, had a large increase after 10µM and 50µM resveratrol, increasing the densitometry ratio by 0.37 (Figure 5.3b). In SKOV-3 spheroids, the AKT level is static in the 10µM and 30µM relative to the control but decreases after 50µM resveratrol. pAKT had no change from the after 10µM and 30µM resveratrol, but increases after 50µM resveratrol treatment (Figure 5.5). In OVCAR-5 spheroids, 50µM pterostilbene caused an increase in protein expression of AKT, while the other treatment concentrations were similar to the control (Figure 5.6). In contrast, pAKT had a decrease in expression following each treatment sample of pterostilbene in OVCAR-5 spheroids (Figure 5.6). Pterostilbene treatment to the SKOV-3 spheroids resulted in an increase in AKT expression following 10µM and 30µM treatment, then decreased at 50µM back to a level similar to the control (Figure 5.7a). Although the densitometry ratio illustrates an increase in pAKT expression, the ration is only increased by 0.000169, suggesting no change in the expression of pAKT (Figure 5.7b).

5.3.2. Cyclin D2 Expression after Resveratrol and Pterostilbene Treatments

Resveratrol caused an increase in the expression of cyclin D2 in OVCAR-5 spheroids after 10µM and 50µM but no change after 30µM (Figure 5.3c). In contrast, pterostilbene treatment resulted in a dose dependant decrease in protein expression in the OVCAR-5 spheroids, where expression was decreased after 30µM and 50µM pterostilbene (Figure 5.4). SKOV-3 spheroids after resveratrol produced similar results to the control (Figure 5.5). Densitometry illustrated an increase in the 10µM and 30µM pterostilbene treatments in SKOV-3 spheroids for cyclin D2 expression, and no obvious change from the control after 50µM treatment. However, the increase in densitometry ratio was only 0.00015, suggesting no change. However, the images obtained from the western blot suggest an increase in expression following 10µM and 30µM (figure 5.7c).

5.3.3. EGFR/pEGFR Expression after Resveratrol and Pterostilbene Treatments

Images for EGFR were limited to resveratrol treatment to OVCAR-5 spheroids and pterostilbene treatment to SKOV-3 spheroids. In the OVCAR-5 sample, a decrease in expression occurred after 30µM and 50µM treatment (Figure 5.4a). In contrast, SKOV-3 after pterostilbene resulted in an increase in the 10µM and 30µM samples (Figure 5.8a).

The western blot for pEGFR in both samples produced a clear ladder marker, suggesting a successful western blot, but limited protein bands, suggesting this protein was not present in the active form. This image was not included.

5.3.4. Expression of pHER2/HER2 and PCNA after Resveratrol and Pterostilbene treatment

Due to limited time and resources the western blots for pHER2/HER2 and PCNA samples did not obtain results from all samples. Using densitometry, pHER2 in OVCAR-5 spheroids after pterostilbene did not change from the control in any treatment concentration tested (10µM, 30µM or 50µM). However, in the image produced from the western blot it there appears to be an increase after the 30µM and 50µM treatment, illustrating the need for replicates. (Figure 5.4b). HER2 in OVCAR-5 spheroids after pterostilbene gave a dose dependant decrease in expression. (Figure 5.6).

In SKOV-3 spheroids, an increase in PCNA expression was observed following 10µM treatment, and a large increase occurred following 50µM pterostilbene, observed by an increase in the densitometry ration of 0.24 (Figure 5.8b).

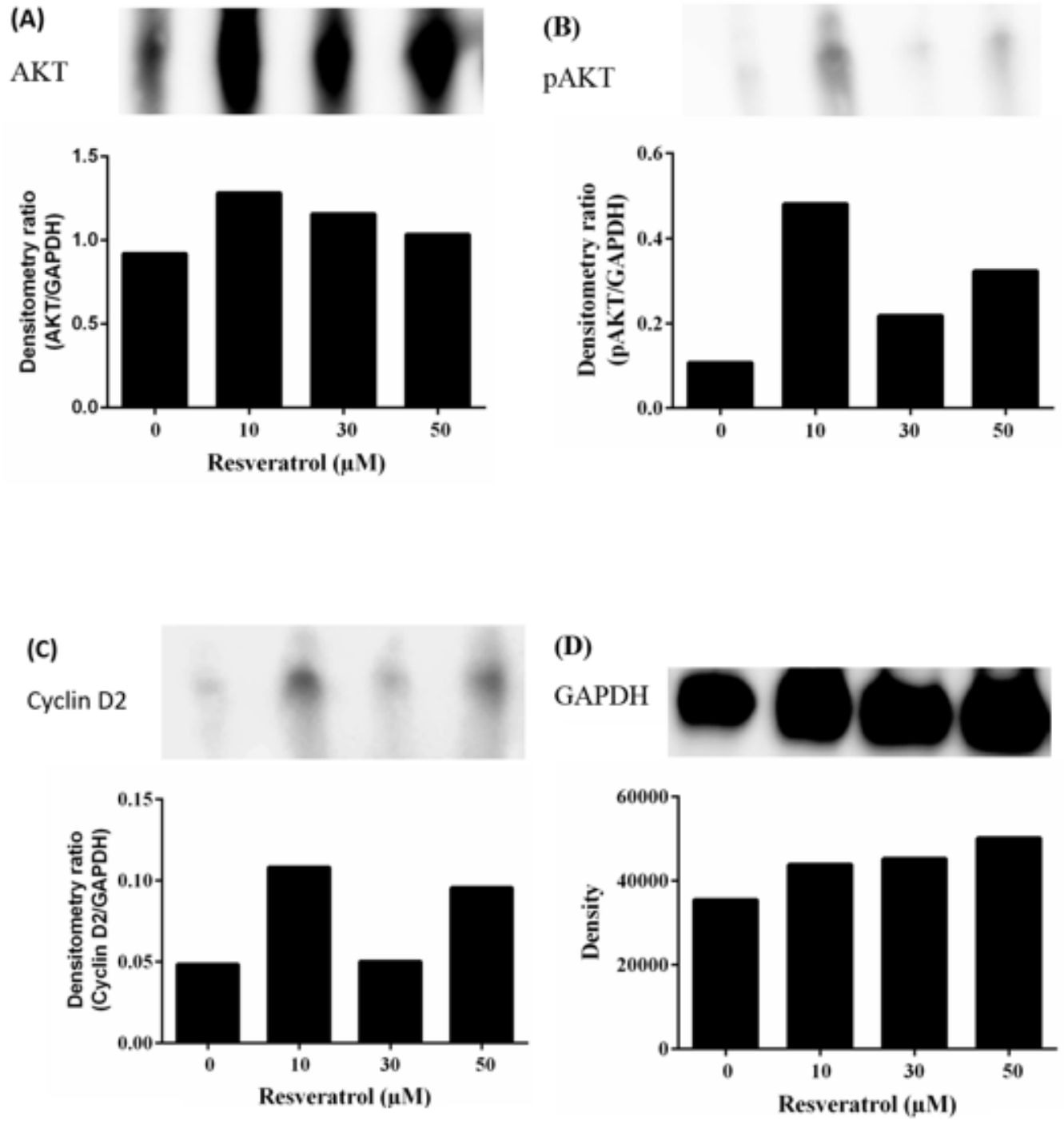


Figure 5.3. Western blot for protein expression of (A) AKT; (B) pAKT; (C) cyclin D2 and (D) GAPDH in OVCAR-5 spheroids after resveratrol treatments at 0μM, 10μM, 30μM and 50μM. 10μg of protein sample was loaded for each sample and compared to a reference protein, glycolytic enzyme GAPDH.

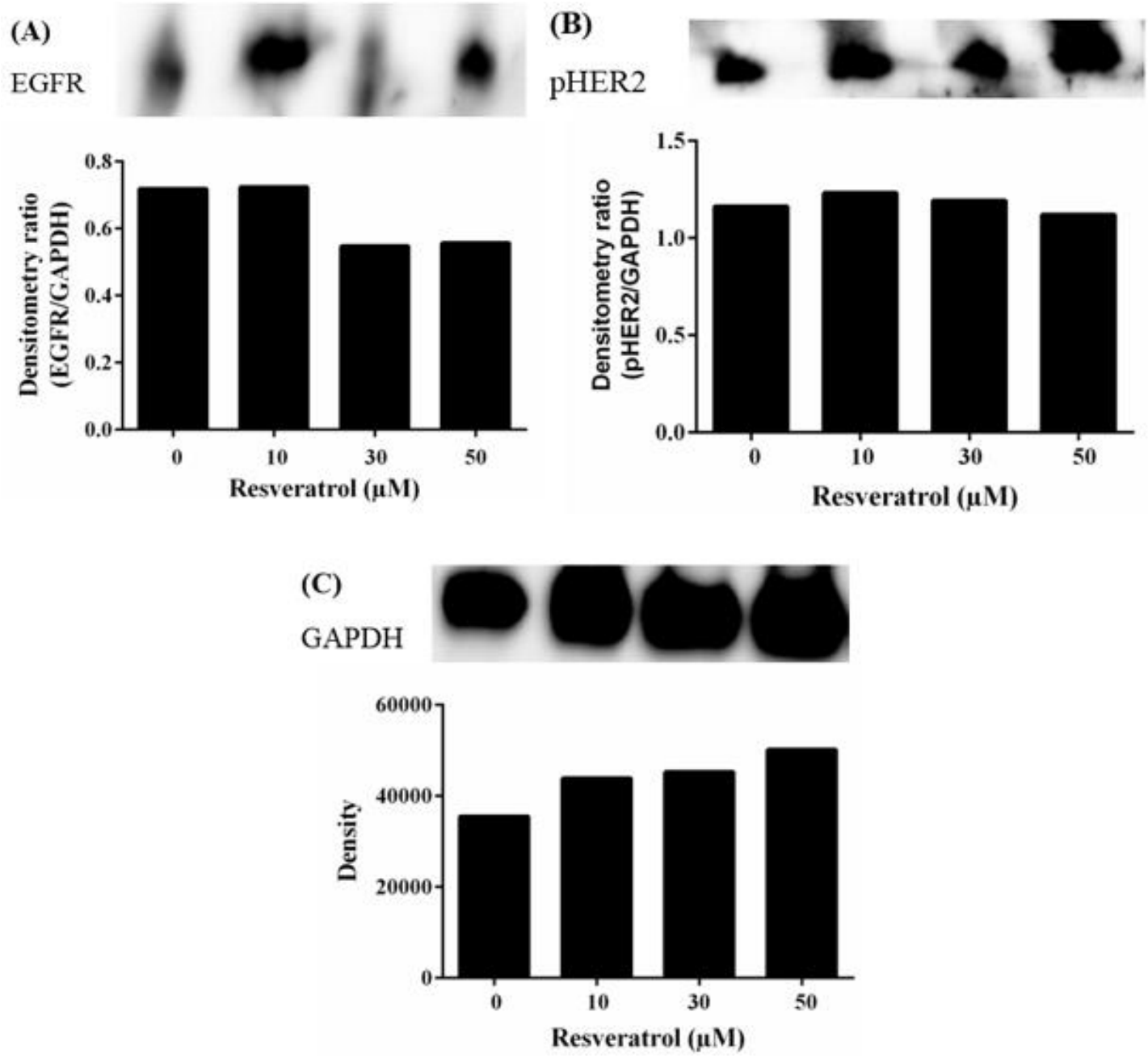


Figure 5.4. Western blot for protein expression of (A) EGFR; (B) pHER2 and (C) GAPDH in OVCAR-5 spheroids after resveratrol treatments at 0µM, 10µM, 30µM and 50µM. 10µg of protein sample was loaded for each sample and compared to a reference protein, glycolytic enzyme GAPDH.

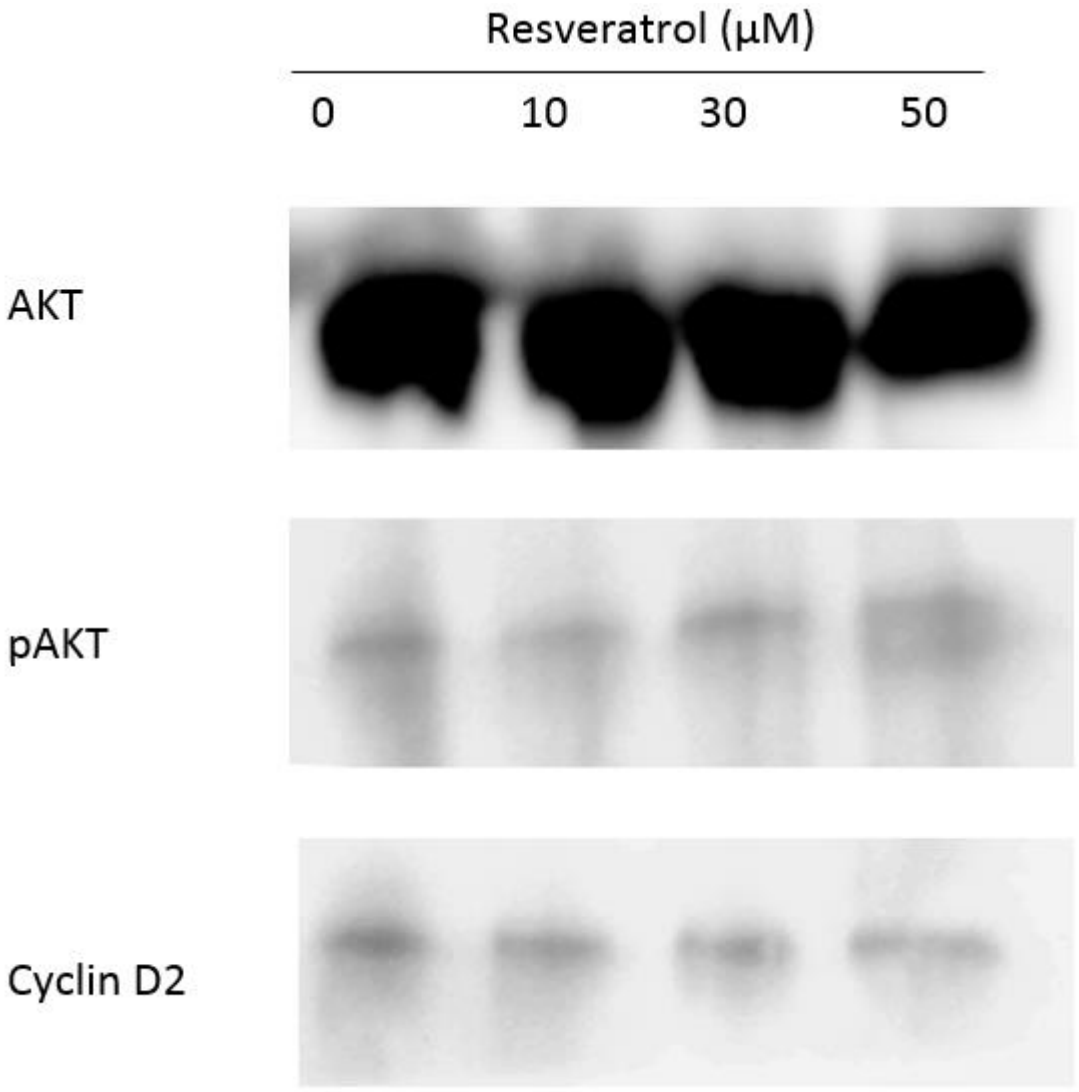


Figure 5.5. Western blot of protein expression of AKT, pAKT and cyclin D2 in SKOV-3 spheroids after resveratrol treatments (0μM, 10μM, 30μM and 50μM).

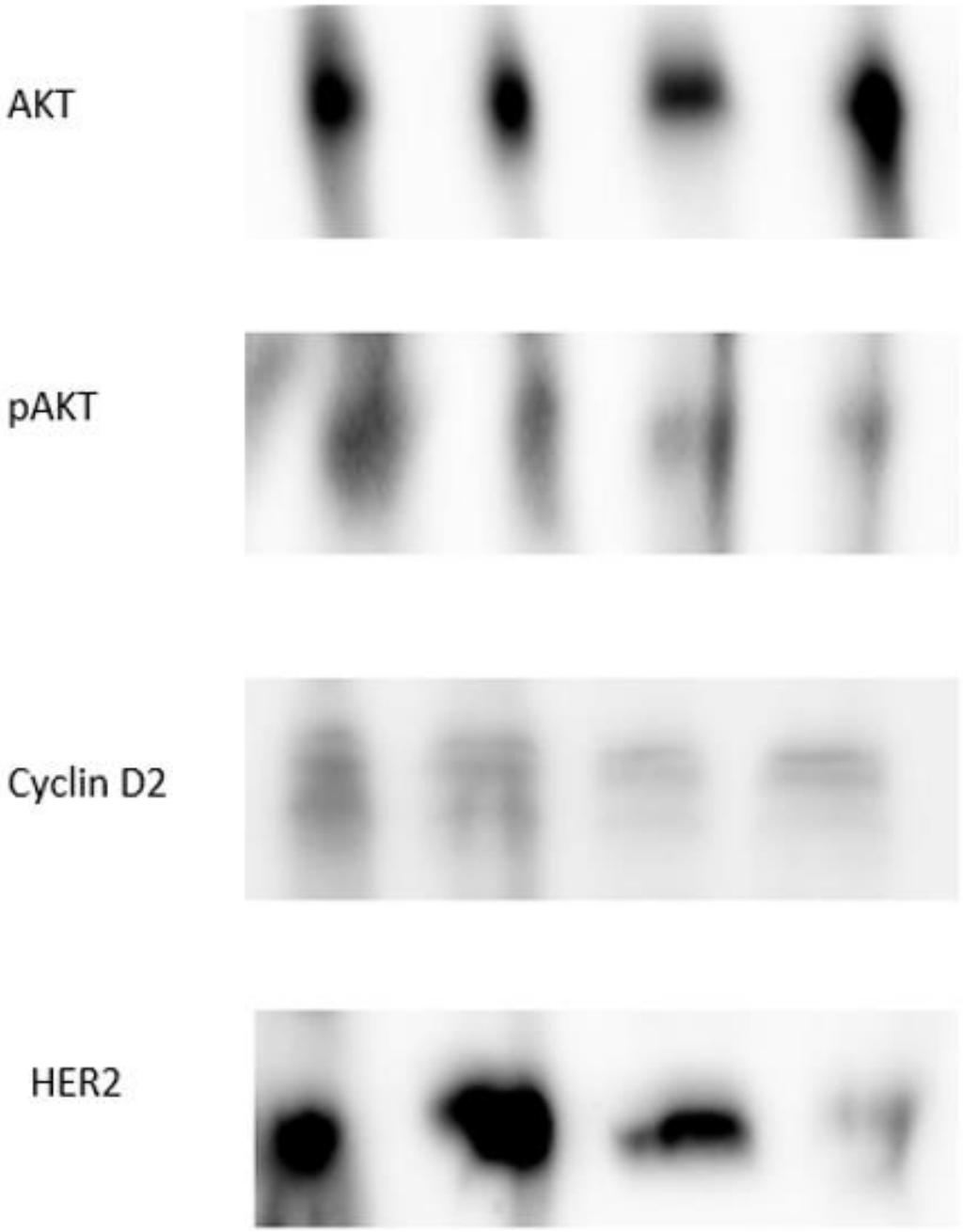


Figure 5.6. Western blot of protein expression of AKT, pAKT, cyclin D2 and HER2 in OVCAR-5 spheroids after pterostilbene treatments (0μM, 10μM, 30μM and 50μM).

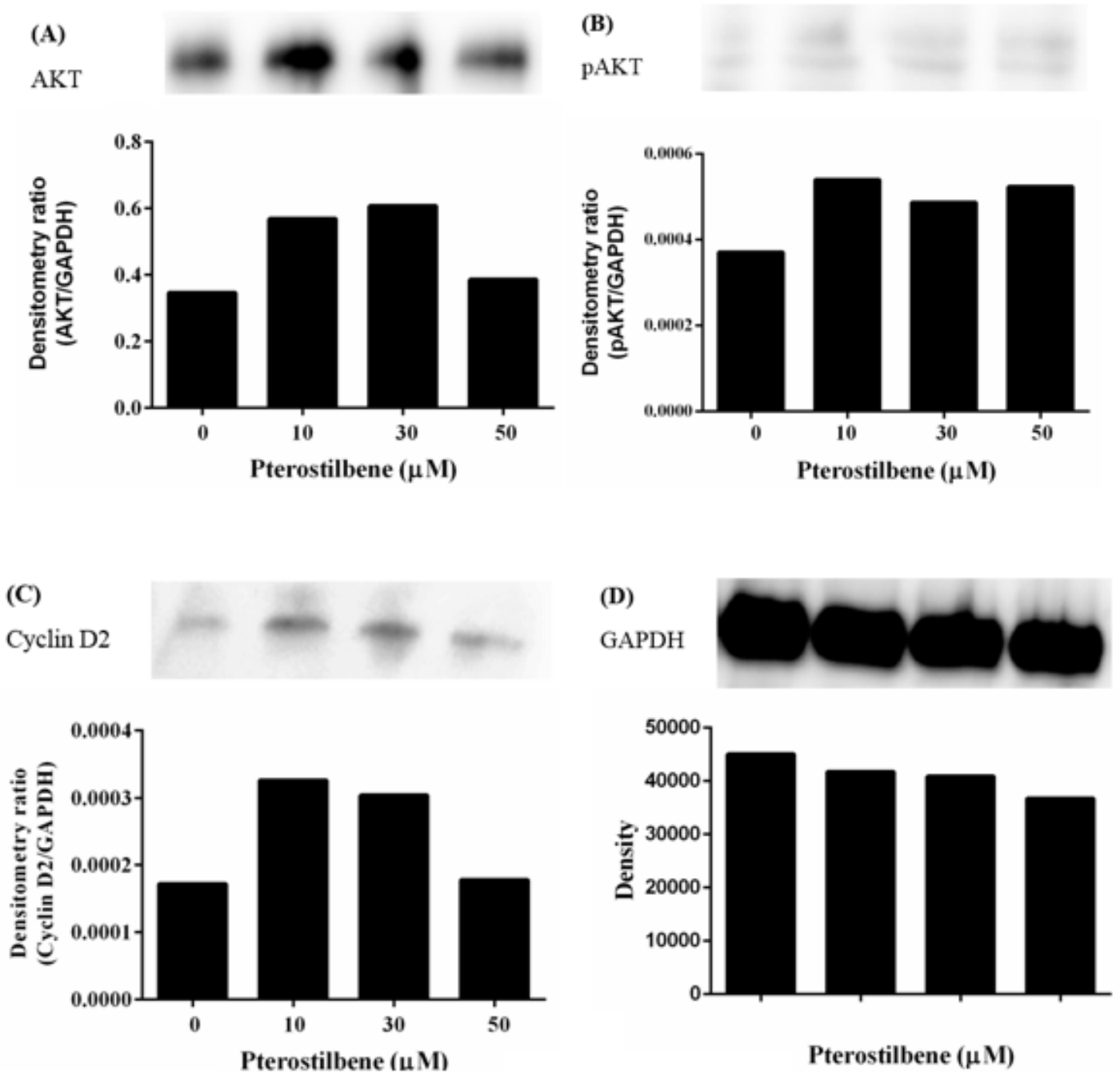


Figure 5.7. Western blot of protein expression of (A) AKT; (B) pAKT; (C) cyclin D2 and (D) GAPDH in SKOV-3 spheroids after pterostilbene treatments at concentrations 0μM, 10μM, 30μM and 50μM). 10μg of protein sample was loaded for each sample and compared to a reference protein, glycolytic enzyme GAPDH.

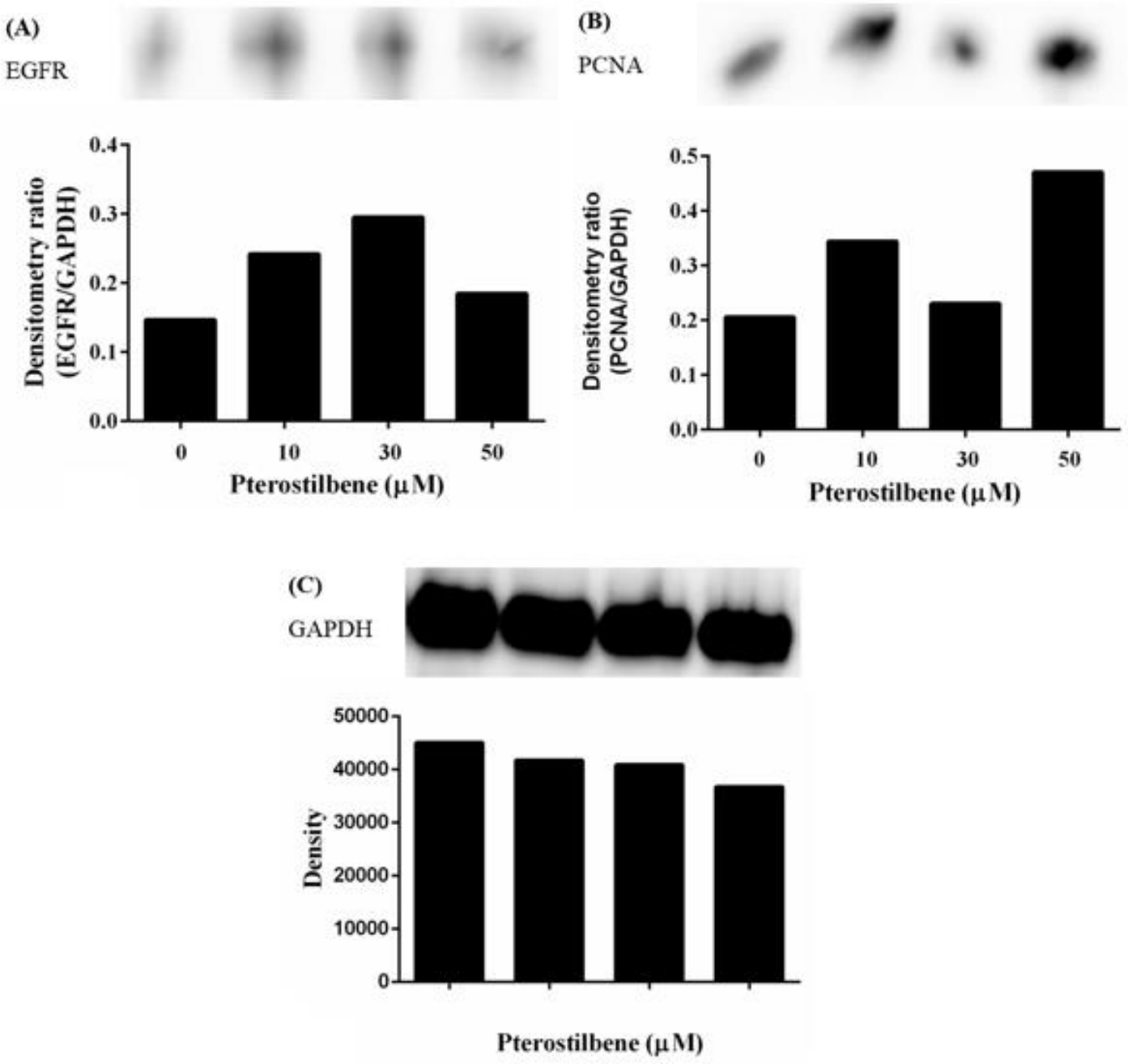


Figure 5.8. Western blot of protein expression of (A) EGFR; (B) PCNA and (C) GAPDH in SKOV-3 spheroids after pterostilbene treatments (0μM, 10μM, 30μM and 50μM). 10μg of protein sample was loaded for each sample and compared to a reference protein, glycolytic enzyme GAPDH.

5.4. Discussion

The PI3K/AKT signalling pathway in cancer is important for angiogenesis, tumourigenesis and the inhibition of apoptosis. Therefore this is a desirable pathway to target for cancer treatment. Using western blot analysis, antibodies specific to AKT and pAKT-serine 473 were used to determine expression levels of these proteins. The changes in the expression of AKT and increased activation of pAKT after 10 and 50µM in OVCAR-5 spheroids after resveratrol treatment seems unusual as no dose dependant pattern has emerged. Due to time and resource constraints, the western blots were repeated once (n=1) for each protein in each cell line. Therefore it is difficult to conclude anything with certainty with respect to the results of pAKT in the OVCAR-5 spheroids after resveratrol, and an error in the western blot process could have resulted in the weaker band for the control. The same applies for the increase in pAKT after 50µM resveratrol to SKOV-3 spheroids. Further replicates would be needed to determine this. Following 50µM resveratrol treatment in SKOV-3 spheroids a decrease in AKT protein expression occurred. However, an increase was observed in the phosphorylated form, suggesting that after resveratrol treatment, the AKT protein became more active, and thus resveratrol may cause activation of AKT in the SKOV-3 cell line. This activation seems an unusual result and to the best of my knowledge this has not been observed in any prior study looking at the effects of resveratrol on AKT/pAKT in cancer. Vergara, Simeone et al. (2012) demonstrates AKT expression in response to resveratrol as observed in previous studies. With the OVCAR-3 cell line, no change was observed in AKT expression, however a decrease in the activation from pAKT occurred with concentrations as low as 25µM.

Pterostilbene treatment in SKOV-3 does not have a strong impact on the expression of AKT, however it may prevent further activation and expression of pAKT, suggesting any anti-cancerous effect in this cell line may be due to the ability to shut down the function of the AKT protein. In contrast, pAKT expression in OVCAR-5 spheroids was decreased, suggesting pterostilbene may be capable of exhibiting anti-cancerous traits in this cell line. To the best of my knowledge, many studies have focussed on resveratrol and derivatives on AKT/pAKT, however none of which has used pterostilbene. Overall, this suggests that the response of AKT/pAKT to resveratrol and pterostilbene may be cell line specific. The widening of this study to other cell lines would be of interest to investigate this point further.

The role of AKT in angiogenesis is significant as it is a downstream binding partner of VEGFR2. Following activation of this receptor, PI3K can bind, activating the subsequent AKT pathway, eventually resulting in the production of the VEGF protein due to the transcriptional activation in this pathway. Therefore, there is a correlation between the level of VEGF and AKT expression (Trinh, Tjalma et al. 2009, Tsutsui, Matsuyama et al. 2010). In the current study, resveratrol affected activation of AKT, where increases in protein expression of pAKT were observed. This could be a factor in the VEGF secretion results discussed in chapter 4, where no significant decreases in VEGF occurred following resveratrol treatment. The resveratrol treatments in both the OVCAR-5 and SKOV-3 spheroids both resulted in an increase in the pAKT expression, suggesting the AKT/pAKT is still able to function to activate the VEGF. Although non-significant, there was a decreasing trend in the VEGF secretion. It is possible this may be caused by resveratrol causing alterations in the Ras pathway which has previously been shown to have a role in VEGF production and angiogenesis. OVCAR-5 has a mutated *Kras* gene so further research into this pathway's response to resveratrol in VEGF signalling may give further insight (Karar and Maity 2011). Following pterostilbene treatment, pAKT expression was decreased for OVCAR-5 and had no change for SKOV-3 spheroids. This does not correlate with the decrease in VEGF secretion observed in chapter 4 for SKOV-3 after pterostilbene treatment of 50µM, and therefore suggests that pterostilbene may affect the VEGF signalling via a different pathway. The results observed in this study contrast with a previous study where resveratrol caused a dose dependant decrease in activated AKT following treatment with 25µM, 50µM and 100µM resveratrol, which correlated to the decrease in VEGF (Cao, Fang et al. 2004). It is difficult to know what has caused this difference without further research and more replicates.

There are many proteins involved in the regulation of the cell cycle and the resulting proliferation. Of particular interest in this study was cyclin D2. Following treatment with resveratrol in the OVCAR-5 spheroids, expression of cyclin D2 displayed a similar trend to the pAKT. This was to be expected due to the PI(3)K/AKT signalling pathway being responsible for its upregulation of cyclin D2 (Muise-Helmericks, Grimes et al. 1998). In contrast, resveratrol in SKOV-3 spheroids resulted in a slight decrease in the expression of cyclin D2, suggesting the response to resveratrol may vary between cell lines. It has been previously shown that a decrease in cyclin D2 can occur in the presence of active AKT, through the overexpression of

the FOXO Forkhead transcription factors. The study showed that these transcription factors reduce cyclin D2 expression and subsequently the phosphorylation of the retinoblastoma protein, resulting in the E2F not being released, regardless of the activation level of $p27^{kip1}$ (Schmidt, de Mattos et al. 2002). It is possible that this is occurring following exposure to resveratrol in the SKOV-3 spheroids, however further replicates would be required to back up this conclusion. Further research could examine the effects of FOXO transcription factors that are relevant to cyclin D2. These changes to cyclin D2 expression do not mimic the pattern observed in the proliferation experiments discussed in chapter 3, where significant decreases were observed. For this reason, more cell cycle proteins should be examined in their response to resveratrol to determine which proteins would make better targets to shut down the cell cycle, especially cyclin D1, due to the high percentage (14-59%) of ovarian cancers which have an increased expression of the cyclin D1 mRNA (Bali, O'Brien et al. 2004).

The reduction in cyclin D2 expression in OVCAR-5 after pterostilbene treatment at concentrations of 30µM and 50µM is similar to the expression observed for pAKT. This suggests that pterostilbene may be affecting cyclin D2 through interruption of the PI(3)K/AKT signalling pathway. In addition, the level of expression also correlates with the changes to proliferation discussed in chapter 3, where a significant decrease occurred after treatment with 30µM and 50µM pterostilbene in OVCAR-5 spheroids. This suggests that pterostilbene may be more potent in affecting the cell cycle via cyclin D2 in OVCAR-5 spheroids. In contrast, there was limited change in the expression of cyclin D2 after pterostilbene treatment in SKOV–3 spheroids. This suggests the effect of pterostilbene on cyclin D2 may be cell line specific. Similarly to OVCAR-5, proliferation was significantly reduced despite the relatively constant levels of cyclin D2, suggesting the importance of other cell cycle regulator proteins and pathways. The effect of pterostilbene on cyclin in previous studies appear to be limited to the cyclin D1 rather than D2.

Resveratrol treatment in OVCAR-5 spheroids led to a decrease in EGFR protein expression after the 30µM and 50µM treatments. Using antibodies specific for pEGFR and performing a western blot, the resulting images appeared to have worked due to the presence of the ladder, however there was little to no bands present for either cell line. This could be due to no pEGFR expression, or possibly a faulty western blot, as there were no repeats (n=1). Therefore this interpretation must be taken with caution and further replicates required to

determine this. Similarly, a decrease in pEGFR and limited change in EGFR expression has been observed in a previous study, using SKOV-3 and PA-1 ovarian carcinomas after 25µM resveratrol (Jeong, Cho et al. 2013). This demonstrated the anti-proliferative effect of resveratrol, a factor that may be responsible for the decreased proliferation observed in chapter 3 of the current study. A second recent study which used similar techniques as the present study determined the effect of resveratrol on the SKOV-3 spheroids expression of EGFR/pEGFR. In contrast the study by Jeong, Cho et al. (2013) a decrease in pEGFR only occurred following 100µM resveratrol, and a non-significant decrease occurred in the EGFR after resveratrol treatment (Hogg, Chitcholtan et al. 2015). Due to this studies observation that pEGFR is downregulated after non-physiological levels of resveratrol and at concentrations not tested in the present study, creates doubt that it was bands for pEGFR appearing in the western blot image. Further replicates are required. Similarly to OVCAR-5, pEGFR was not observed in the SKOV-3 spheroids after pterostilbene treatment, regardless of the ladder appearing in the image. The increase in EGFR however could occur due to the downregulation of pEGFR, however the lack of replicates and no pEGFR bands in the image makes this conclusion difficult to make, and again should be treated with caution.

This study observed an increase in PCNA expression, where 50µM pterostilbene treatment in SKOV-3 spheroids resulted in the largest increase observed. This suggests that the role in DNA replication, epigenetic maintenance and DNA repair can take place, to assist in the proliferation of the tumour cells. This is not represented by the actual proliferative effect of pterostilbene, as discussed in chapter 3, where a significant decrease in cell number was observed after 30µM and 50µM pterostilbene. In contrast to the current study, Paul, DeCastro et al. (2010) observed a significant decrease in PCNA expression in human colon carcinoma HT-29 cells following both western blot and PCNA staining. This difference could be due to different cancer types, or the result in this study may not be accurately reflecting the level of PCNA in the SKOV-3 spheroids due to only having one repeat (n=1).

The use of monoclonal antibodies for use in the western blot is advantageous as it only binds to one epitope on an antigen, creating high specificity resulting in less background staining. However, in this research polyclonal antibodies, where antibodies an bind multiple epitopes on the antigen, were used for the detection of pHER2, PCNA, and GAPDH. Results produced may be more accurate if a monoclonal antibody was used in future, with the exception of

GAPDH. Housekeeping proteins typically result in high protein levels so it is unlikely the polyclonal antibody had a limiting effect here.

Although this study demonstrated decreases in cell activity, due to the bioavailability limitation *in vivo*, future studies would need to assess how resveratrol and pterostilbene affect spheroid growth in the body, as the results obtained in the current study may not be replicated.

Chapter 6: Conclusions

As hypothesised, both resveratrol and pterostilbene exhibited anti-cancerous properties on cell metabolism and cell number, however there was some cell line dependency observed. In contrast to what was expected, pterostilbene did not always show the most potent effect, specifically on the metabolic activity and cell number of OVCAR-5. Although anti-cancerous properties were exhibited, it is difficult to conclude what the signalling pathways were that were directly responsible for these changes. VEGF was not affected by resveratrol in either cell line and was affected only at the highest concentration of pterostilbene for the SKOV-3 cell line. This effect may be linked with the AKT pathway after resveratrol treatment as neither VEGF nor pAKT had significantly decreased expression. Pterostilbene decreased the expression of cyclin D2 in both cell lines, however this is not enough to cause the observed decreases in proliferation. It was hypothesised that both resveratrol and pterostilbene would cause potent anti-cancerous effects on EGFR, HER2 and PCNA expression, however this was no observed in this study.

Chapter 7: Limitations and Future Directions

Using a Crystal Violet assay for cellular quantification requires many washing steps which results in the loss of cells with each wash. Although this research obtained results demonstrating a decreasing trend following the drug treatments when using this assay, a potentially unequal number cells may be lost from the samples which could affect the results. If this experiment were to be carried out again, using a different cell quantification method may provide more representative results. One such method could be by the use of Annexin V, a phospholipid binding protein capable of binding to phosphatidylserine. Although phosphatidylserine in normal cells is present in the cytoplasmic side of the cell membrane, during cell death via apoptosis it becomes exposed due to structural changes of the membrane where it can then bind to Annexin V and fluorescently tested to determine the number of dead cells (Van Engeland, Nieland et al. 1998). A second option could be to use propidium iodide in a fluorescence assay which focuses on the proliferation in different cell lines and eliminates the assumption that the cancer cells die via apoptosis alone rather than autophagocytosis as has been observed previously in cancer cells (Opipari, Tan et al. 2004). Both methods are advantageous because they do not require any washing steps where sample cells could get lost and neither require a lot of cells to obtain results (Dengler, Schulte et al. 1995).

Due to the cell line specificity in the VEGF response to resveratrol by ovarian carcinomas observed in the current research and prior studies, it is important that further research focusses on a wider range of ovarian cancer cell lines to determine how they respond. From there, future research could focus on determining why the cell lines react differently to the different concentrations of resveratrol.

Due to the importance of the cell cycle in cellular proliferation, further study deciphering the effect on resveratrol and pterostilbene on a wider range of cell cycle proteins is important, specifically, the cyclin D proteins. Further research on cyclin D2 could focus on the FOXO transcription factors with resveratrol and pterostilbene to see how these may be affecting cyclin D2 expression. Although cyclin D1 and D2 are often expressed in ovarian cancer, there is more research literature examining cyclin D1. This suggests this protein may have a more

important role than D2 in ovarian cancer and therefore future research should consider testing the effect of resveratrol and pterostilbene on cyclin D1.

References

Adams, M., et al. (1989). "A comparison of the toxicity and efficacy of cisplatin and carboplatin in advanced ovarian cancer." Acta Oncologica **28**(1): 57-60.

Agarwal, R. and S. B. Kaye (2003). "Ovarian cancer: strategies for overcoming resistance to chemotherapy." Nature Reviews Cancer **3**(7): 502-516.

Aggarwal, B. B., et al. (2009). "Models for prevention and treatment of cancer: problems vs promises." Biochemical pharmacology **78**(9): 1083-1094.

Ahmed, N., et al. (2005). "Role of integrin receptors for fibronectin, collagen and laminin in the regulation of ovarian carcinoma functions in response to a matrix microenvironment." Clinical & experimental metastasis **22**(5): 391-402.

Aletti, G. D., et al. (2006). "Aggressive surgical effort and improved survival in advanced-stage ovarian cancer." Obstetrics & Gynecology **107**(1): 77-85.

Alosi, J. A., et al. (2010). "Pterostilbene inhibits breast cancer in vitro through mitochondrial depolarization and induction of caspase-dependent apoptosis." Journal of Surgical Research **161**(2): 195-201.

Altomare, D. A. and J. R. Testa (2005). "Perturbations of the AKT signaling pathway in human cancer." Oncogene **24**(50): 7455-7464.

Anand, P., et al. (2008). "Cancer is a preventable disease that requires major lifestyle changes." Pharmaceutical research **25**(9): 2097-2116.

Ang, C., et al. (2011). "Ultra-radical (extensive) surgery versus standard surgery for the primary cytoreduction of advanced epithelial ovarian cancer." The Cochrane Library.

Arboleda, M. J., et al. (2003). "Overexpression of AKT2/protein kinase Bβ leads to up-regulation of β1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells." Cancer research **63**(1): 196-206.

Aziz, M. H., et al. (2003). "Cancer chemoprevention by resveratrol: In vitroand in vivo studies and the underlying mechanisms (Review)." International journal of oncology **23**: 17-28.

Bader, A. G., et al. (2005). "Oncogenic PI3K deregulates transcription and translation." Nature Reviews Cancer **5**(12): 921-929.

Bali, A., et al. (2004). "Cyclin D1, p53, and p21Waf1/Cip1 expression is predictive of poor clinical outcome in serous epithelial ovarian cancer." Clinical cancer research **10**(15): 5168-5177.

Banerjee, S. and S. B. Kaye (2013). "New strategies in the treatment of ovarian cancer: current clinical perspectives and future potential." Clinical cancer research **19**(5): 961-968.

Barnard, R. J. (2004). "Prevention of cancer through lifestyle changes." Evidence-based Complementary and Alternative Medicine **1**(3): 233-239.

Baselga, J. (2002). "Why the epidermal growth factor receptor? The rationale for cancer therapy." The oncologist **7**(Supplement 4): 2-8.

Berman, M. L. (2003). "Future directions in the surgical management of ovarian cancer." Gynecologic oncology **90**(2): S33-S39.

Bookman, M. A., et al. (2003). "Evaluation of monoclonal humanized anti-HER2 antibody, trastuzumab, in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2: a phase II trial of the Gynecologic Oncology Group." Journal of Clinical Oncology **21**(2): 283-290.

Brabletz, T. (2012). "EMT and MET in metastasis: where are the cancer stem cells?" Cancer cell **22**(6): 699-701.

Breslin, S. and L. O'Driscoll (2013). "Three-dimensional cell culture: the missing link in drug discovery." Drug discovery today **18**(5): 240-249.

Cai, Y., et al. (2004). "Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer." Life sciences **74**(17): 2157-2184.

Cao, Z., et al. (2004). "trans-3, 4, 5'-Trihydroxystibene inhibits hypoxia-inducible factor 1α and vascular endothelial growth factor expression in human ovarian cancer cells." Clinical cancer research **10**(15): 5253-5263.

Cardenes, H. and M. E. Randall (2000). Integrating radiation therapy in the curative management of ovarian cancer: current issues and future directions. Seminars in radiation oncology, Elsevier.

Carmeliet, P. (2005). "VEGF as a key mediator of angiogenesis in cancer." Oncology **69**(Suppl. 3): 4-10.

Carmeliet, P. and R. K. Jain (2000). "Angiogenesis in cancer and other diseases." Nature **407**(6801): 249-257.

Chang, F., et al. (2003). "Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy." Leukemia **17**(3): 590-603.

Chen, J., et al. (1998). "Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells." Molecular cell **2**(3): 317-328.

Cho, K. R. and I.-M. Shih (2009). "Ovarian cancer." Annual review of pathology **4**: 287.

Choi, Y. J., et al. (2012). "The requirement for cyclin D function in tumor maintenance." Cancer cell **22**(4): 438-451.

Christian, J. and H. Thomas (2001). "Ovarian cancer chemotherapy." Cancer treatment reviews **27**(2): 99-109.

Ciardiello, F. and G. Tortora (2003). "Epidermal growth factor receptor (EGFR) as a target in cancer therapy: understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs." European journal of cancer **39**(10): 1348-1354.

Collins, I. and M. D. Garrett (2005). "Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors." Current opinion in pharmacology **5**(4): 366-373.

D'Andrilli, G., et al. (2004). "Cell cycle genes in ovarian cancer steps toward earlier diagnosis and novel therapies." Clinical cancer research **10**(24): 8132-8141.

DeBerardinis, R. J., et al. (2008). "Brick by brick: metabolism and tumor cell growth." Current opinion in genetics & development **18**(1): 54-61.

Dengler, W. A., et al. (1995). "Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays." Anti-cancer drugs **6**(4): 522-532.

Dhar, K., et al. (1999). "Expression and subcellular localization of cyclin D1 protein in epithelial ovarian tumour cells." British journal of cancer **81**(7): 1174.

Duthie, G. G., et al. (2000). "Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants." Nutrition Research Reviews **13**(01): 79-106.

Einhorn, N., et al. (2003). "A systematic overview of radiation therapy effects in ovarian cancer." Acta Oncologica **42**(5-6): 562-566.

English, D. P., et al. (2013). "HER2 expression beyond breast cancer: therapeutic implications for gynecologic malignancies." Molecular diagnosis & therapy **17**(2): 85-99.

Fang, X., et al. (1998). "Expression of p16 induces transcriptional downregulation of the RB gene." Oncogene **16**(1): 1-8.

Ferrara, N. (2004). "Vascular endothelial growth factor as a target for anticancer therapy." The oncologist **9**(Supplement 1): 2-10.

Ferrara, N. (2005). "VEGF as a therapeutic target in cancer." Oncology **69**(Suppl. 3): 11-16.

Ferrara, N., et al. (2003). "The biology of VEGF and its receptors." Nature medicine **9**(6): 669-676.

Giacinti, C. and A. Giordano (2006). "RB and cell cycle progression." Oncogene **25**(38): 5220-5227.

Godwin, A. K., et al. (1992). "Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer." Journal of the National Cancer Institute **84**(8): 592-601.

Greenblatt, M., et al. (1994). "Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis." Cancer research **54**(18): 4855-4878.

Griffiths, C. T. (1975). "Surgical resection of tumor bulk in the primary treatment of ovarian carcinoma." National Cancer Institute Monograph **42**: 101-104.

Grun, B., et al. (2009). "Three-dimensional in vitro cell biology models of ovarian and endometrial cancer." Cell proliferation **42**(2): 219-228.

Han, X., et al. (2007). "Dietary polyphenols and their biological significance." International journal of molecular sciences **8**(9): 950-988.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." cell **100**(1): 57-70.

Herbst, R. S. (2004). "Review of epidermal growth factor receptor biology." International Journal of Radiation Oncology* Biology* Physics **59**(2): S21-S26.

Hilton, J. L., et al. (2002). "Inactivation of BRCA1 and BRCA2 in ovarian cancer." Journal of the National Cancer Institute **94**(18): 1396-1406.

Hogg, S. J., et al. (2015). "Resveratrol, Acetyl-Resveratrol, and Polydatin Exhibit Antigrowth Activity against 3D Cell Aggregates of the SKOV-3 and OVCAR-8 Ovarian Cancer Cell Lines." Obstetrics and gynecology international **2015**.

Holmes, D. and I. Zachary (2005). "The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease." Genome Biol **6**(2): 209.

Huang, W.-Y., et al. (2009). "Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention." Nutrition and cancer **62**(1): 1-20.

Israels, E. and L. Israels (2000). "The cell cycle." The oncologist **5**(6): 510-513.

Ivascu, A. and M. Kubbies (2006). "Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis." Journal of biomolecular screening **11**(8): 922-932.

Jeong, K. J., et al. (2013). "EGFR mediates LPA-induced proteolytic enzyme expression and ovarian cancer invasion: Inhibition by resveratrol." Molecular oncology **7**(1): 121-129.

Joe, A. K., et al. (2002). "Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines." Clinical cancer research **8**(3): 893-903.

Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." The Journal of clinical investigation **119**(6): 1420-1428.

Karar, J. and A. Maity (2011). "PI3K/AKT/mTOR pathway in angiogenesis." Frontiers in molecular neuroscience **4**: 51.

Kelland, L. (2007). "The resurgence of platinum-based cancer chemotherapy." Nature Reviews Cancer **7**(8): 573-584.

Kieran, M. W., et al. (2012). "The VEGF pathway in cancer and disease: responses, resistance, and the path forward." Cold Spring Harbor perspectives in medicine **2**(12): a006593.

Kim, K. K., et al. (2003). "Overexpression of p21, cyclin E and decreased expression of p27 in DMBA (7, 12-dimethylbenzanthracene)-induced rat ovarian carcinogenesis." Pathology international **53**(5): 291-296.

Kroemer, G. and J. Pouyssegur (2008). "Tumor cell metabolism: cancer's Achilles' heel." Cancer cell **13**(6): 472-482.

Kueck, A., et al. (2007). "Resveratrol inhibits glucose metabolism in human ovarian cancer cells." Gynecologic oncology **107**(3): 450-457.

Kurman, R. J., et al. (2008). "Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume of disease based on a new model of carcinogenesis." American journal of obstetrics and gynecology **198**(4): 351-356.

Lebwohl, D. and R. Canetta (1998). "Clinical development of platinum complexes in cancer therapy: an historical perspective and an update." European journal of cancer **34**(10): 1522-1534.

Lee, M.-H., et al. (2009). "Resveratrol suppresses growth of human ovarian cancer cells in culture and in a murine xenograft model: eukaryotic elongation factor 1A2 as a potential target." Cancer research **69**(18): 7449-7458.

Lengyel, E. (2010). "Ovarian cancer development and metastasis." The American journal of pathology **177**(3): 1053-1064.

Li, L., et al. (2004). "Correlation of serum VEGF levels with clinical stage, therapy efficacy, tumor metastasis and patient survival in ovarian cancer." Anticancer research **24**(3B): 1973-1979.

Ludwig, T., et al. (1997). "Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos." Genes & development **11**(10): 1226-1241.

Luo, J., et al. (2003). "Targeting the PI3K-Akt pathway in human cancer: rationale and promise." Cancer cell **4**(4): 257-262.

Machida, S., et al. (2014). "Radiation therapy for chemotherapy-resistant recurrent epithelial ovarian cancer." Oncology **86**(4): 232-238.

Maga, G. and U. Hübscher (2003). "Proliferating cell nuclear antigen (PCNA): a dancer with many partners." Journal of cell science **116**(15): 3051-3060.

Manach, C., et al. (2004). "Polyphenols: food sources and bioavailability." The American journal of clinical nutrition **79**(5): 727-747.

Marques, F. Z., et al. (2009). "Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits." The international journal of biochemistry & cell biology **41**(11): 2125-2128.

McCormack, D. and D. McFadden (2012). "Pterostilbene and cancer: current review." Journal of Surgical Research **173**(2): e53-e61.

McGuire, W. r. and M. Markman (2003). "Primary ovarian cancer chemotherapy: current standards of care." British journal of cancer **89**: S3-S8.

McMahon, G. (2000). "VEGF receptor signaling in tumor angiogenesis." The oncologist **5**(Supplement 1): 3-10.

Muise-Helmericks, R. C., et al. (1998). "Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway." Journal of Biological Chemistry **273**(45): 29864-29872.

Munoz-Pinedo, C., et al. (2012). "Cancer metabolism: current perspectives and future directions." Cell death & disease **3**(1): e248.

Nakayama, K., et al. (2012). "Mechanisms of ovarian cancer metastasis: biochemical pathways." International journal of molecular sciences **13**(9): 11705-11717.

Naryzhny, S. (2008). "Proliferating cell nuclear antigen: a proteomics view." Cellular and molecular life sciences **65**(23): 3789-3808.

Neufeld, G., et al. (1999). "Vascular endothelial growth factor (VEGF) and its receptors." The FASEB journal **13**(1): 9-22.

Nguyen, H. N., et al. (1993). "National survey of ovarian carcinoma part V. The impact of physician's specialty on patients' survival." Cancer **72**(12): 3663-3670.

Normanno, N., et al. (2006). "Epidermal growth factor receptor (EGFR) signaling in cancer." Gene **366**(1): 2-16.

Nutakul, W., et al. (2011). "Inhibitory effects of resveratrol and pterostilbene on human colon cancer cells: a side-by-side comparison." Journal of agricultural and food chemistry **59**(20): 10964-10970.

Nyati, M. K., et al. (2006). "Integration of EGFR inhibitors with radiochemotherapy." Nature Reviews Cancer **6**(11): 876-885.

Opipari, A. W., et al. (2004). "Resveratrol-induced autophagocytosis in ovarian cancer cells." Cancer research **64**(2): 696-703.

Osaki, M., et al. (2004). "PI3K-Akt pathway: its functions and alterations in human cancer." Apoptosis **9**(6): 667-676.

Pan, M.-H., et al. (2007). "Pterostilbene induces apoptosis and cell cycle arrest in human gastric carcinoma cells." J. of Agricultural and Food Chemistry **55**(19): 7777-7785.

Pan, M.-H., et al. (2009). "Pterostilbene inhibited tumor invasion via suppressing multiple signal transduction pathways in human hepatocellular carcinoma cells." Carcinogenesis **30**(7): 1234-1242.

Paul, S., et al. (2010). "Dietary intake of pterostilbene, a constituent of blueberries, inhibits the β-catenin/p65 downstream signaling pathway and colon carcinogenesis in rats." Carcinogenesis **31**(7): 1272-1278.

Pines, J. (1995). Cyclins, CDKs and cancer. Seminars in cancer biology, Elsevier.

Prat, J. (2014). "Staging classification for cancer of the ovary, fallopian tube, and peritoneum." International Journal of Gynecology & Obstetrics **124**(1): 1-5.

Reedijk, J. (2003). "New clues for platinum antitumor chemistry: kinetically controlled metal binding to DNA." Proceedings of the National Academy of Sciences **100**(7): 3611-3616.

Reichert, J. M. and E. Dhimolea (2012). "The future of antibodies as cancer drugs." Drug discovery today **17**(17): 954-963.

Ren, Y., et al. (2015). "Radical surgery versus standard surgery for primary cytoreduction of bulky stage IIIC and IV ovarian cancer: an observational study." BMC cancer **15**(1): 583.

Richards, J. S. and S. A. Pangas (2010). "The ovary: basic biology and clinical implications." The Journal of clinical investigation **120**(4): 963.

Rochet, N., et al. (2010). "Intensity-modulated whole abdominal radiotherapy after surgery and carboplatin/taxane chemotherapy for advanced ovarian cancer: phase I study." International Journal of Radiation Oncology* Biology* Physics **76**(5): 1382-1389.

Roncoroni, L., et al. (2008). "Resveratrol inhibits cell growth in a human cholangiocarcinoma cell line." Liver International **28**(10): 1426-1436.

Rosenberg, B., et al. (1965). "Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode." Nature **205**(4972): 698-699.

Rowinsky, M., Eric K (1997). "The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents." Annual review of medicine **48**(1): 353-374.

Samuels, Y. and K. Ericson (2006). "Oncogenic PI3K and its role in cancer." Current opinion in oncology **18**(1): 77-82.

Schmidt, M., et al. (2002). "Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D." Molecular and cellular biology **22**(22): 7842-7852.

Serrano-Olvera, A., et al. (2006). "Prognostic, predictive and therapeutic implications of HER2 in invasive epithelial ovarian cancer." Cancer treatment reviews **32**(3): 180-190.

Seshacharyulu, P., et al. (2012). "Targeting the EGFR signaling pathway in cancer therapy." Expert opinion on therapeutic targets **16**(1): 15-31.

Shield, K., et al. (2009). "Multicellular spheroids in ovarian cancer metastases: Biology and pathology." Gynecologic oncology **113**(1): 143-148.

Singer, G., et al. (2003). "Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma." Journal of the National Cancer Institute **95**(6): 484-486.

Stakleff, K. S., et al. (2012). "Resveratrol exerts differential effects in vitro and in vivo against ovarian cancer cells." Asian Pacific Journal of Cancer Prevention **13**(4): 1333-1340.

Strzalka, W. and A. Ziemienowicz (2011). "Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation." Annals of botany **107**(7): 1127-1140.

Swain, S. M., et al. (2015). "Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer." New England Journal of Medicine **372**(8): 724-734.

Trapp, V., et al. (2010). "Anti-angiogenic effects of resveratrol mediated by decreased VEGF and increased TSP1 expression in melanoma-endothelial cell co-culture." Angiogenesis **13**(4): 305-315.

Trinh, X. B., et al. (2009). "The VEGF pathway and the AKT/mTOR/p70S6K1 signalling pathway in human epithelial ovarian cancer." British journal of cancer **100**(6): 971-978.

Tsutsui, S., et al. (2010). "The Akt expression correlates with the VEGF-A and-C expression as well as the microvessel and lymphatic vessel density in breast cancer." Oncology reports **23**(3): 621-630.

van der Burg, M. E., et al. (1995). "The effect of debulking surgery after induction chemotherapy on the prognosis in advanced epithelial ovarian cancer." New England Journal of Medicine **332**(10): 629-634.

Van Engeland, M., et al. (1998). "Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure." Cytometry **31**(1): 1-9.

Vega-Avila, E. and M. K. Pugsley (2011). An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proc West Pharmacol Soc.

Vergara, D., et al. (2012). "Resveratrol downregulates Akt/GSK and ERK signalling pathways in OVCAR-3 ovarian cancer cells." Molecular BioSystems **8**(4): 1078-1087.

Verri, E., et al. (2005). "HER2/neu oncoprotein overexpression in epithelial ovarian cancer: evaluation of its prevalence and prognostic significance." Oncology **68**(2-3): 154-161.

Walle, T., et al. (2004). "High absorption but very low bioavailability of oral resveratrol in humans." Drug metabolism and disposition **32**(12): 1377-1382.

Wang, Y., et al. (2012). "Pterostilbene simultaneously induces apoptosis, cell cycle arrest and cyto-protective autophagy in breast cancer cells." American journal of translational research **4**(1): 44.

Weiderpass, E. and F. Labrèche (2012). "Malignant tumors of the female reproductive system." Safety and health at work **3**(3): 166-180.

Yamada, K. M. and E. Cukierman (2007). "Modeling tissue morphogenesis and cancer in 3D." cell **130**(4): 601-610.

Yamamoto, S., et al. (1997). "Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels." British journal of cancer **76**(9): 1221.

Yarden, Y. (2001). "The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities." European journal of cancer **37**: 3-8.

Yuan, Z. Q., et al. (2000). "Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer." Oncogene **19**(19): 2324-2330.