School of Pharmacy

Faculty of Medicine & Health Sciences

Evaluation of drug combinations to sensitize ovarian cancer cells to chemotherapy

Mohammed Najim Abed Abo Donia

Ph.D.

March 2018

Keele University

Abstract

Ovarian cancer is a complex disease characterized by low incidence, accounting for about 4% of cancer diagnoses in women, but with rapid progression and higher mortality rate than any other gynecological malignancy. Approximately 70 % of the cases are diagnosed late in the course of the disease due to unawareness of subtle symptoms and failure of screening methods for detecting the disease at early-stage. Although most patients respond well to standard treatment, which involves surgery followed by platinum/taxane combination therapy, relapse seems unavoidable and the majority of patients presented with chemoresistant disease, which is considered as the main obstacle to successful treatment. Reduced susceptibility to apoptosis is one of the major causes for the development of resistance to chemotherapy, one cause of it is overexpression of pro-survival members of Bcl-2 family. Advances in understanding of the molecular mechanisms of the involvement of pro-survival proteins in the emergence of resistance to chemotherapy has made them attractive targets for the development of new therapies to treat ovarian cancer. BH3 mimetics are agents that antagonize the apoptosis inhibitors of Bcl-2 family, and have been shown to potentiate the activity of carboplatin against a panel of ovarian cancer cell lines. However, in clinical trials, agents that antagonize Bcl-X_L were associated with life-threatening thrombocytopenia. To avoid this venetoclax was developed to selectively inhibit Bcl-2 and avoid inhibition of Bcl-X_L. Unfortunately, Bcl-X_L appears to be more frequently deregulated than Bcl-2 in ovarian cancer. In this study, the ability of venetoclax, and the Bcl-X_L selective compound WEHI-539, to potentiate the activity of carboplatin were assessed in ovarian cancer cell lines.

In addition to the genes encoding the Bcl-2 family of proteins, a number of other genes have shown to be overexpressed in drug resistant ovarian cancer. These are potential targets for the development of new therapeutic agents to overcome drug resistance. Work from our group

previously reported an RNAi screen to assess whether knockdown of some of these genes increases the sensitivity to carboplatin or paclitaxel. In the present study, some of these hits were validated and branched-chain amino acid dehydrogenase kinase emerged as a potential new target for developing chemosensitizing agents.

Contents

Figures

Tables

Abbreviations

ABC	ATP-Binding cassette
ATP	Adenosine triphosphate
Bak	BCL2 antagonist killer 1
Bax	BCL2 associated X protein
BCAA	Branched chain amino acid
BCKDH	Branched-chain alpha-keto acid dehydrogenase complex
BCKDK	Branched-chain alpha-keto acid dehydrogenase kinase
BCAT	Branched-chain amino transferase
BCKA	Branched-chain keto acid
Bcl-2	B-cell lymphoma protein 2
Bcl-X_L	BCL2 related protein, long isoform
Bid	BH3 interacting domain death agonist
Bim	BCL2 interacting protein BIM
Caspase	Cysteinyl aspartic acid-protease
CI	Combination Index
CT	Cycle threshold
CMVA	(S)-2-Chloro-4-methylvaleric acid
DCBC	3,6-Dichloro-1-Benzothiophene-2-Carboxylic acid
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HOE	Human ovarian epithelial
IC_{50}	Half maximal inhibitory concentration

IGF	Insulin-like growth factor
KIC	Keto- isocaproate
LC3	Microtubule-associated protein light chain 3
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MOMP	Mitochondrial outer membrane permeabilization
Mcl-1	Induced myeloid leukemia cell differentiation protein
mTOR	Mammalian target of rapamycin
NF-$_K$B	Nuclear transcription factor-$_K$B
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
OSE	Ovarian surface epithelium
PARP	Poly (ADP) ribose polymerase
PBS	Phosphate buffered saline
PET	Positron emission tamography
PI3K	Phosphatidylinositol 3-kinase
PP2cm	Protein phosphatase 2Cm
qPCR	Quantitative polymerase chain reaction
RIPA	Radio-immunoprecipitation assay
RNAi	RNA interference
SDS	Sodium dodecyl sulphate
siRNA	Small interfering ribonucleic acid
TSGs	Tumour suppressor genes
TNFα	Tumour necrosis factor alpha
XIAP	X chromosome-linked inhibitor of apoptosis

Publications

1. **Abed, M. N.**, Abdullah, M. I. and Richardson, A. (2016) 'Antagonism of Bcl-X_L is necessary for synergy between carboplatin and BH3 mimetics in ovarian cancer cells', Journal of Ovarian Research, 9(1), p. 25.

2. **Abed, M. N.** and Richardson, A. (2016) 'Bcl-X_L antagonism is essential for sensitisation of ovarian cancer cell lines to carboplatin', European Journal of Cancer Supplements, 61(1), p. s189.

3. **Abed, M. N.** and Richardson, A. (2016) 'Inhibition of BCKDK increases the sensitivity of ovarian cancer cells to paclitaxel', European Journal of Cancer Supplements, 68(1), p. s20.

4. Abdullah, M. I., **Abed, M. N.** and Richardson, A. (2017) 'Inhibition of the mevalonate pathway augments the activity of pitavastatin against ovarian cancer cells', Scientific Reports. Springer US, 7(1), p. 8090.

5. Uche, F. I., **Abed, M. N.**, Abdullah, M. I., Drijfhout, F. P., McCullagh, J., Claridge, T. W. D., Richardson, A. and Li, Wu.-W. (2017) 'Isochondodendrine and 2′-norcocsuline: additional alkaloids from Triclisia subcordata induce cytotoxicity and apoptosis in ovarian cancer cell lines', RSC Adv. Royal Society of Chemistry, 7(70), pp. 44154–44161.

6. Abdullah, M. I., **Abed, M. N.** and Richardson A. (2017). 'The interplay between TP53 and mevalonate pathway in ovarian cancer', Annals of Oncology, 28 (suppl-5), 22p.

7. Uche, F. I., **Abed, M. N.**, Abdullah, M. I., Drijfhout, F. P., McCullagh, J., Claridge, T. W. D., Richardson, A. and Li, Wu.-W. (2017) 'Isolation, identification and anti-cancer activity of minor alkaloids from Triclisia subcordata Oliv', Biochemical Pharmacology, 139 (1), pp112

8. **Abed, M. N.**, Ibrahim, S.L., Abdullah, M. I. and Richardson, A. (2017). 'Targeting the branched-chain alpha-keto acid dehydrogenase kinase augments the sensitivity of ovarian and breast cancer cells to paclitaxel'. Submitted.

9. Al-Shakarchi W., Alsuraifi A., **Abed M. N.**, Abdullah M. I., Richardson A, Curtis A .D. M., Hoskins C. (2018). 'Combined effect of anticancer agents and cytochrome C decorated hybrid nanoparticles for liver cancer therapy'. Submitted.

Acknowledgments

I would like to express my sincere gratitude to my supervisor Dr. Alan Richardson for his continuous support of my Ph.D. study; you have been a tremendous mentor for me. I appreciate your patience, motivation, and immense knowledge and would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your precious advices and guidance helped me in all the time of research and writing of this thesis. I would also like to thank my thesis advisor Prof. Dr. Nicholas Forsyth, the director of ISTM, School of Medicine, Keele University, for his continuous support throughout my study. A very special gratitude goes out to my sponsors at MOHESR for providing the funding for the work, my study would not have been possible without their support. Words cannot express how grateful I am to Prof. Dr. Trevor Greenhough for his endless support in providing the offer of Ph.D and making my life easier during the whole period of study. I would also like to thank Dr. Emad Hani, Dr. Maher Ibrahim, Dr. Loay Ahmed and Dr. Mohanad Alfahad, for supporting me to strive towards my study. Many thanks to Chris Hargreaves and Kiren at the student services centre, the door to their offices was always open whenever I had a question or needed help. My sincere appreciation to my group mates, Marwan Ibrahim, Natalie, Mohammed MJ, Suad and Emily for helping me every time I needed. I am also grateful to the members of the Guy Hilton Research Centre for providing a best research area. My thanks extend to Prof. Dr. Ying Yang, who was always keen on our safety in the laboratory and to Katy Cressy and John Misra, the lab technicians, for supporting me in overcoming numerous technical obstacles. I must express my very profound gratitude to my colleagues, Hamza Abu Owida, Rashid Siddique, Lanxin, Zaid Mowafaq, Yanny, Sandhya, Michelle Chen, David Carrasco, Pan, Guo, Kaarjel, Sudeshna, Hati and Mathew, I spent the best moments in my life with you and I am lucky to have great friends like you. Most of all, I would like to express my very profound gratitude to my beloved wife Israa, your patience, sense of humour, wisdom

and quiet willingness provided me with unfailing support and encouragement throughout the years of my study. My children, Rzan and Ibrahim, I love you so much and thank you for being patient and agreeing to sacrifice your holidays when I was busy at work. A special thanks to my brothers Nashwan, Marwan and Omar, my sisters Liqaa and Shaimaa, my mother-in-law and everyone wished good luck for me, for all of your prayers which sustained me and supported me spiritually thus far. I dedicate this thesis to my parents, who would have been so proud to see me achieving this degree, I know million tears and words would not bring you back but your teachings and advices will always keep me strong and motivated.

Mohammed, 2018

Chapter 1

Introduction

1.1 Overview of the female reproductive system and ovaries

The ovaries are part of the female reproductive system which includes the Fallopian tubes, uterus, vagina, vulva, breasts and the mammary glands (figure 1.1). These organs are variously responsible for the formation and transportation of gametes (ova) as well as the production of sex hormones (estrogen and progesterone). The ovaries are a pair of small oval shaped glands located on the both sides of the pelvic cavity lateral to the upper part of the uterus and supported by the mesovarium and are attached to the uterus by ovarian ligament. Suspensory ligaments extend from the mesovarium, and contain the ovarian lymph and blood vessels. The main types of cells that form the ovaries are the ovarian surface epithelium, the stromal cells, and the germ cells (oocytes). The ovarian surface epithelium is composed of a single cell layer of cuboidal-to-flat cells. During ovulation, the ovaries release an ovum, which is then travel to the Fallopian tube, where it may be fertilized before further moving into the uterus.

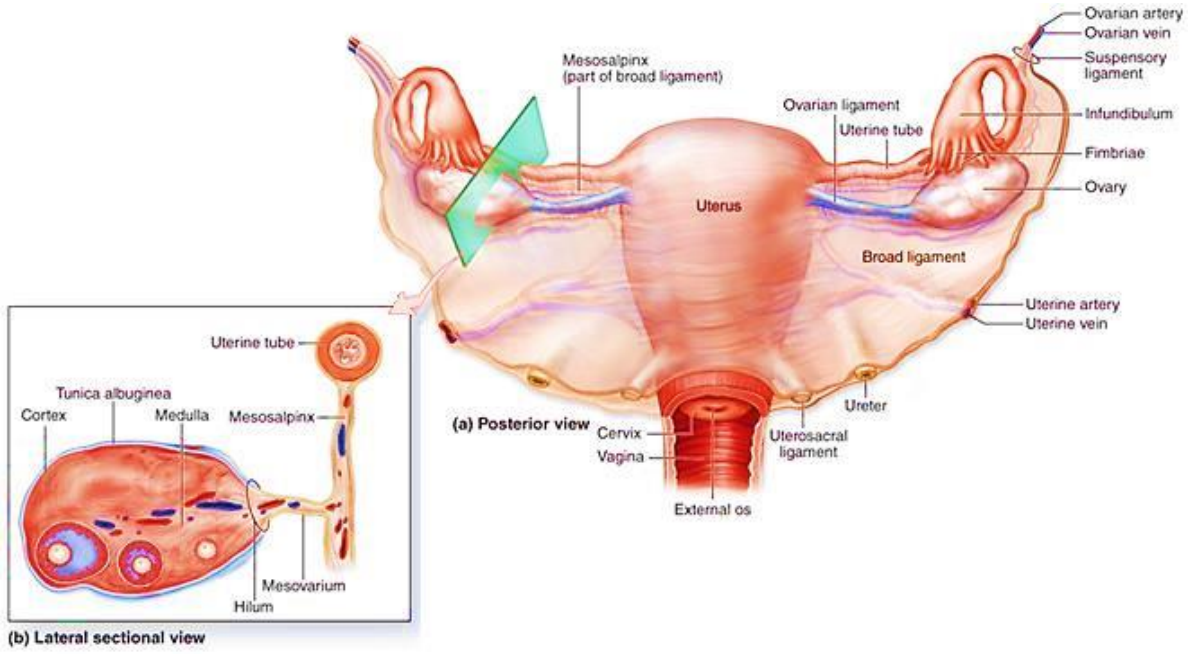


Figure 1.1: The female reproductive system and the ovary

From. http://humananatomylibrary.com/ovary-female-reproductive-system/

1.2 Introduction to Ovarian cancer

Ovarian cancer (figure 1.2) comprises a growth of the cancerous cells arising from tissues in and nearby the ovary. The disease results in high mortality rates because most of patients are diagnosed at an advanced stage (where the disease has invaded other parts of the body), and therefore, treatment for this pathology is very difficult, prolonged, complex and multimodal (1).

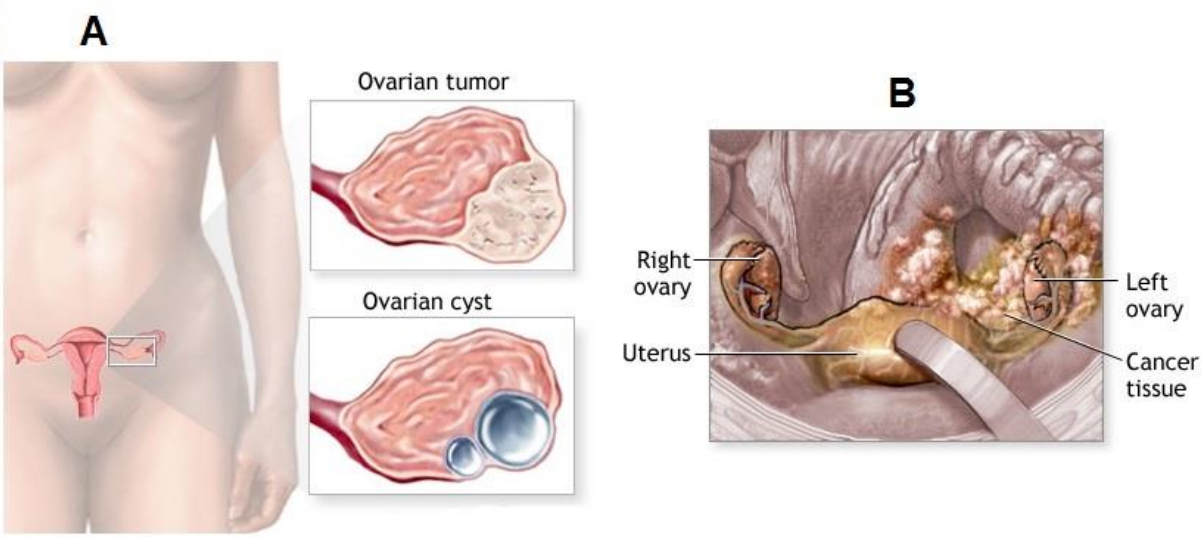


Figure 1.2: Ovarian Cancer

A. A mass in the ovary that is less than 2 cm is a follicle cyst that will resolve spontaneously. However, if the growth is persistent and doesn't disappear during the course of a repetitive menstrual cycles, then surgery is needed to remove it. B. A typical ovarian cancer, which is very dangerous due to the location of ovaries that are in close proximity to various organs and therefore, it carries high risk of metastasis. From http://keckmedicine.adam.com.

When compared to other cancers such as prostate or breast cancer, the survival rate of patients with ovarian cancer is disappointingly low. One of the main causes of poor survival rate is the development of drug resistance following repeated rounds of anti-cancer medications. Accordingly, the disease places a real and substantial load on patients and their families (2). To improve survival, the disease should be diagnosed at an early stage, so increasing awareness of disease symptoms among health professionals, women and patients is crucial.

Improved treatment strategies and using targeted therapy and novel drug combinations that reduce drug resistance also have the potential to improve survival (3).

1.3 Epidemiology and incidence

Ovarian cancer accounts for 4% of all new cases of cancer, and is considered to be the 5th most common form of cancer among women, with about 7,000 new cases across the United Kingdom and more than 240,000 cases each year worldwide. The incidence rate has progressively risen over the past 20 years, especially in those aged 65 and above (4–6). Ovarian cancer is regarded as the second most common malignancy of the female genitalia following uterine corpus cancer, and unfortunately, it is the most deadly and results in higher mortalities than any other gynaecological form of cancer. More than two-thirds of ovarian cancer patients are diagnosed with stage III or IV and consequently the disease kills annually more than 152,000 patient worldwide with more than 4400 patients in the UK (7–9).

The incidence of ovarian cancer usually increases with age (figure 1.3), and the incidence rates of the disease are highest in countries from north America as well as Europe; rates are about 10-folds lower in most parts of Africa and Eastern Asia. Additionally, within each country, the incidence rates may differ among ethnic groups (10,11).

The 5-year survival rate for patients diagnosed with advanced ovarian cancer is very low, around 45% and it is less than 30% in patients with late stages of the disease. This variation in survival is probably linked to various factors, including the time of diagnosis and the effectiveness of treatment (12).

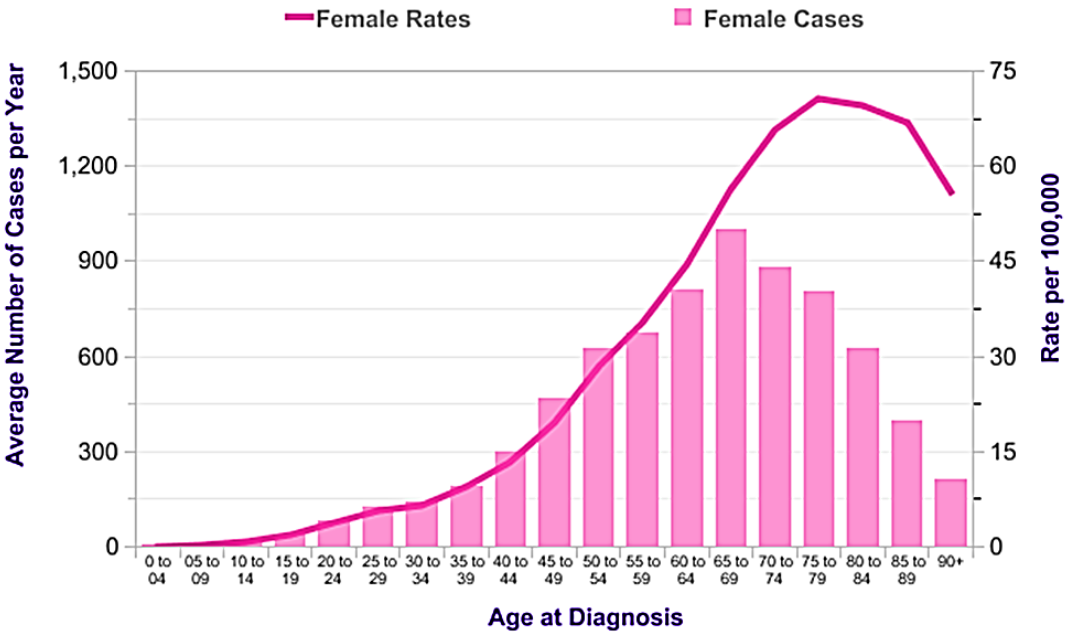


Figure 1.3: Average number of new cases each year and age-specific incidence rates of ovarian cancer per 100,000 population, females, UK. 2012-2014.

From (Cancer Research UK, 2014).

The incidence of ovarian cancer markedly increases after the age of 40 and patients in this age group account for over 90% of ovarian cancer cases, of these more than half are over 63 years. The incidence keeps rising with age to reach its peak in those aged 80-84 years. Incidence is high in the age group of 50-70 year, with 75% of cases diagnosed in women aged more than 55 years (13,14). Additionally, the 5-year survival rates of the disease varies according to the age of patient, with higher survival rates in young age group – there is an 84% survival rate in those less than 39 years old compared to less than 14% survival rate for patients over 85 years (15).

1.4 Aetiology of ovarian cancer

Ovarian cancer is a heterogeneous disease. It can be classified into two broad types comprising the low-grade tumours that develop through a gradual process of mutation, grow more slowly and which are less responsive to anti-cancer therapy. This type of ovarian cancer shares molecular features with low malignant potential neoplasms. On the other hand, high-grade carcinomas show higher genetic instability, rapid metastasis, sensitivity to chemotherapy and absence of distinct detectable precursor lesions (16). Accordingly, the heterogeneity among ovarian cancer classes is evident in differences in risk factors, somatic and germline mutations, genetic expression and responsiveness to chemotherapy (17).

Numerous factors are associated with the initiation and subsequent progression of ovarian cancer, such as family history, age, overweight, lack of use of a contraceptive pill, not having children or not breastfeeding, and early menarche or menopause started later than average. Taking fertility drugs and hormone replacement therapy can also increase the risk of developing ovarian cancer (18–20).

The risk of developing ovarian cancer is increased in women who have a close relative diagnosed with this disease when compared with those with no family history of the disease (21). Even though only about 10% of women with a family history of ovarian cancer develop the disease, it has been found that the presence of inherited mutations in the breast and ovarian cancer susceptibility genes (BRCA1 and BRCA2) can increase the risk of ovarian cancer. The risk of the disease is approximately 40% by 70 years of age in case of mutations in BRCA1, while risk is only 10% in BRCA2 mutation carriers (22).

A specific inherited mutation in one allele of the human breast cancer tumour suppressor genes, BRCA1 or BRCA2, accounts for about 15% of ovarian cancer cases. BRCA1 mutations are usually associated with female breast, fallopian tube, ovarian and peritoneal cancer. In addition, carriers of BRCA2 mutation are at increased risk of a variety of cancers at other sites of the body (23). Moreover, females with a family history of breast or ovarian cancer are at a two-fold higher risk of developing ovarian cancer than those without this history (24). Mutations in the hereditary nonpolyposis colorectal gene constitutes another inherited genetic susceptibility to develop ovarian cancer (figure 1.4) (25).

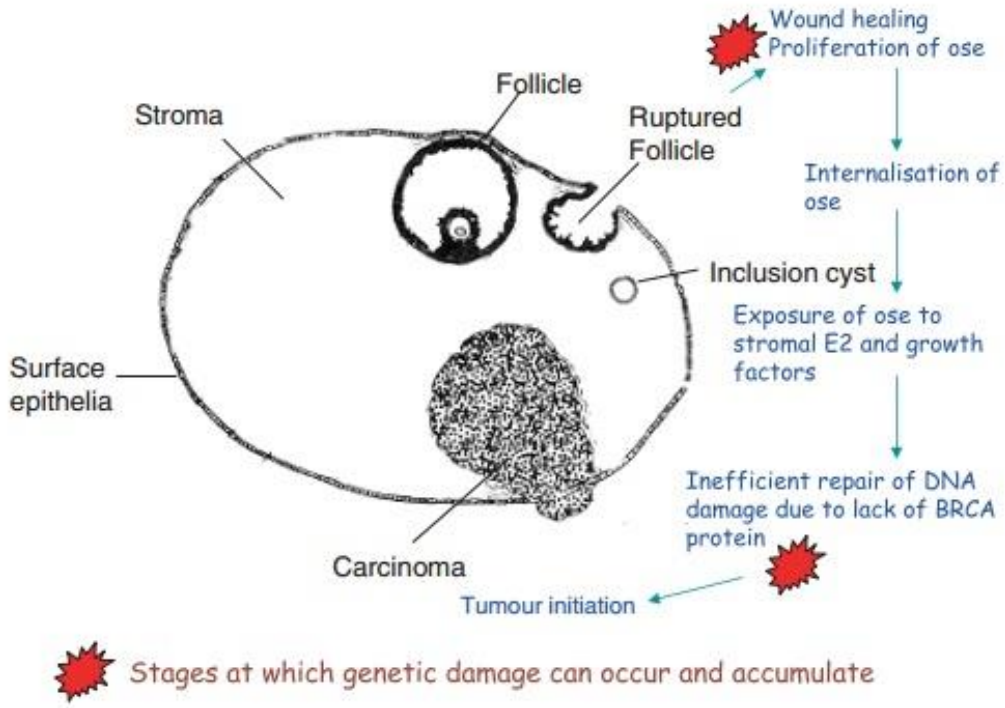


Figure 1.4: Development of ovarian cancer in persons with BRCA mutation

The OSE forms a single layer of cells that are adjacent to the peritoneal mesothelium. The process of ovulation causes OSE proliferation and agitation of wound healing to repair the ruptured membrane during the release of the follicle. With repeated ovulation and repair inclusion cysts can be formed. Such inclusion cysts are a rich environment for the development of ovarian cancer, because of their exposure to the ovarian stroma. Those with a mutation in BRCA1 or BRCA2 genes are less able to repair such DNA damage inflicted on the cells and so less able to cope with the accumulation of mutations during estrogen exposure and proliferation, leading to the initiation of epithelial ovarian tumours (23).

1.4.1 Theories of ovarian cancer aetiology

The clear relationship between patient's ovulations history and the risk of developing ovarian cancer has prompted several theories to explain the causes of ovarian cancer.

1.4.1.1 Hypothesis 1: The incessant ovulation hypothesis

This first hypothesis for explaining the aetiology of ovarian cancer was proposed by Fathalla in 1971. It speculates that repetitive injury of the ovarian surface epithelium (OSE) during ovulation followed by proliferation of cells results in successive accumulation of DNA mutations and so results in ovarian cancer. Depending on this hypothesis, serial rounds of programmed cell death and repair of OSE at the site of ovulation trigger genetic instability, which makes this cell layer more prone to develop into neoplasms (26,27). Murdoch and colleagues provided evidence that supported this hypothesis where the cells of the OSE within the formative site of ovulation undergo DNA oxidative damage, p53 expression and apoptosis. A genetically-altered progenitor cell, with damaged DNA, that is not committed to death, could give rise to a transformed phenotype that is hence propagated upon the healing of ovulatory wound, Therefore, with each ovulation cycle, there is increased likelihood of generating genetically modified and carcinogenic cells in the OSE and this raises the risk of ovarian tumour formation (Murdoch *et al.*, 2001).

1.4.1.2 Hypothesis 2: The pituitary gonadotropin theory

The gonadotropins, FSH and LH, are essential hormones for regulation of ovarian cells function, and a potential role in the pathogenesis of ovarian carcinoma has been suggested. Ovarian cancer has been reported to express specific gonadotropin receptors. The role of endocrine factors in controlling the normal growth of ovaries may also provide suitable conditions for malignant transformation. Furthermore, the presence of high levels of FSH and LH in the fluid of ovarian cancer suggests a role for these hormones in the transformation and progression of ovarian cancers (29,30). Gonadotropins cause activation of mitogenic

pathways, including the extracellular signal-regulated kinase (Erk) pathway, and stimulate proliferation and invasion of the ovarian cancer cell (31). During ovulation, gonadotropins activate proteolytic enzymes and cytokines to generate an inflammatory-like process, which causes rupturing of the OSE layer for the release of the ovum. After rupturing of the follicle and the release of the ovum at the OSE, the estrogen-producing follicle is converted to a progesterone-producing corpus luteum, which then provides feedback inhibition that reduces gonadotropin levels (32).

During menopause, there is entire exhaustion of the germ cells in the ovary accompanied by loss of the follicular structure that surrounds the germ cells, and this cause an absence of corpus luteal feedback, leading to high serum gonadotropin and proinflammatory cytokines levels. Therefore, high levels of gonadotropin in postmenopausal women might encourage an environment of inflammation that cannot contribute to ovulation, but might lead to a high risk of developing ovarian cancer, by causing morphological changes in the OSE, and support transformation of genetically compromised cells into ovarian cancer (32).

Failure of apoptosis in the cells of the granulosa and theca following the ovulatory period may promote carcinogenesis because the ability of these cells to produce steroid hormones is retained (33). Epidemiologic evidence supports this hypothesis and has shown that the risk of developing ovarian cancer is reduced in women with multiple pregnancies, breast feeding, and those that use oral contraceptives, which has been interpreted as being due to the suppression of pituitary gonadotropin secretion in these situations (31).

1.4.1.3 Hypothesis 3: The androgen/progesterone theory

The hormonal stimulation theory, also known as the androgen/progesterone theory, proposes that androgens (which are elevated after menopause) may support the generation of ovarian cancer. In contrast to androgens, progestins protect against the development of the disease.

Progesterone contraceptives can reduce the risk of ovarian cancer by reduction of ovarian testosterone levels (34,35). Additionally, the proliferation of cells in ovarian surface epithelium may be driven by androgen receptors. It is supposed that the follicles produce androgens and create a rich environment of androgen around the epithelial cells. Known hyperandrogenic conditions like acne, hirsutism, and polycystic ovarian syndrome, have been shown to be associated with increased risk of developing ovarian neoplasms (36).

1.4.1.4 Hypothesis 4: The inflammation theory

As a cause for the development of ovarian cancer, the inflammatory theory suggests that inflammation may work in conjugation with steroid hormones and ovulation, to increase the possibility of developing ovarian epithelium carcinoma (37). According to this theory, repair of damaged DNA is impaired, particularly in those with defective function of BRCA1 and BRCA2, resulting in high risk of carcinogenesis. In addition, stimulation of inflammation as a result of endometriosis, talc or asbestos exposure, or as a result of ovulation itself, may work to support the development of ovarian cancer (38). Inhibition of inflammation caused a reduction in the incidence of cancer in a study that used anti-inflammatory drugs, where it has reported a statistically significant inverse association between anti-inflammatory drugs use and cancer risk (39).

Many mechanisms have been anticipated to explain the beneficial effects of anti-inflammatory drugs in the reduction of the risk of this disease, including induction of apoptosis, inhibition of prostaglandin synthesis mediated by cyclo-oxygenase, or improvement of cellular immune response (39).

The biomarkers of inflammation can be used as a means to monitor the progression of ovarian cancer. Such biomarkers can also help to prevent and treat cancer by developing a new anti-

inflammatory drugs that can also be used as adjuvant to radiotherapy and chemotherapy, which by themselves activate NF-kB and mediate resistance (39).

1.4.1.5 Hypothesis 5: The tubal origin hypothesis

This is the most recent hypothesis and suggests that the vast majority ovarian cancers originate in the fallopian tube, in both high-risk women and in the general population, and from where there is no barrier to peritoneal spread (40). Most of the ovarian cancers detected in their early stages in women carrying BRCA mutations arise in the fallopian tube (41). Pre-invasive changes in the fimbriated end of the fallopian tube are usually associated with early HGSOC (42). Additionally, disabling of PTEN, a key negative regulator of the PI3K pathway, and Dicer, an essential gene for microRNA synthesis, with Amhr2-Cre in mice resulted in subsequent spread of fallopian tube cancers to the ovary and then aggressively metastasize to the abdominal cavity, leading to ascites and death (43). Research is in progress to explore the molecular and genetic basis critical to the development of ovarian cancer that originats from fallopian tube (44).

1.5 Types of ovarian cancer

Ovarian cancer can present in different forms that can be categorized by the type of cells and tissue from which they originated. Understanding ovarian cancer type, its stage and grade has a great importance in the diagnosis and treatment options of the disease. Age as well can affect the susceptibility to different kinds of tumours (45). Generally, the ovaries have three types of cells that are able to develop into different types of cancer (figure 1.5): the ovarian surface epithelium cells (which is composed of one layer of cells and often times is described as modified peritoneal mesothelium) undergo a cancerous conversion to be the origin of epithelial ovarian cancer, the germ cells, which give rise to germ cell tumours, and the stromal

cells, which grow to form ovarian sex cord-stromal tumours. In addition, as discussed above, ovarian cancer may originate from epithelial cells in the fallopian tube.

Most of ovarian malignancies originate from epithelial cells and so account for about 90% of all ovarian cancers and approximately 3% of all cancer types in women (45,46).

Germ cell tumours develop in the reproductive cells of the ovary. This type of cancer is very rare, it comprises around 2-3 % of all ovarian neoplasms and is characterized by rapid growth. This kind of tumour usually starts in girls, adolescents, and women of reproductive age. Indeed, tumours of ovarian germ cells account for the vast majority of ovarian cancers in adolescent women and should always be taken into account in any young woman presented with solid mass in the ovaries (47,48).

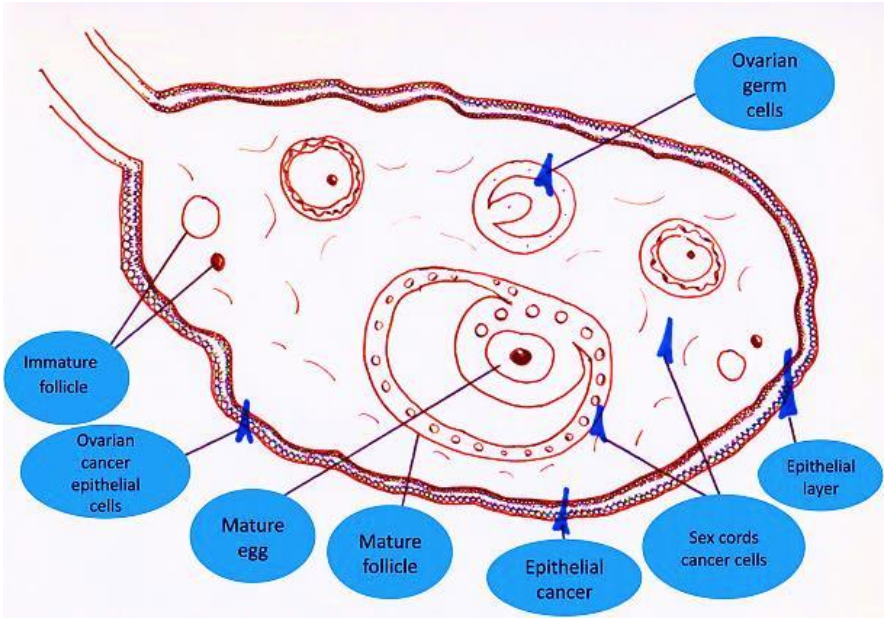


Figure 1.5: Types of ovarian cancer.

Types of ovaries cancer according to the origin of cells. Different types of cells in the ovaries may develop into different types of cancer cells. The abnormal presence of BRCA1 is associated in women with more probability of a predisposition to ovarian cancer that arises from these cells. From (49).

Ovarian sex cord-stromal tumours are also a very uncommon kind of tumour that constitute about 1-8 % of all primary ovarian cancers. Usually, it develops in adolescents and young adults. Clinically, these are slow-growing heterogeneous tumours that include several pathologic types which originate in the structural tissue that holds the ovaries together and produces the female ovarian hormones. The involvement of cells of this tumour type in the production of steroid hormones (e.g. estrogens, androgens and corticoids) leads to the association of sex cord-stromal tumours with many hormone-mediated syndromes and consequently a wide range of clinical symptoms varying from hyperestrogenic manifestations to hyperandrogenic virilising states (50–52).

Ovarian epithelial neoplasms are sub-classified into ovarian serous carcinoma (30–70%), ovarian endometrial carcinoma (10–20%), ovarian mucinous carcinoma (5–20%) and clear cell carcinoma (3–10%) (53) (figure 1.6), in addition to undifferentiated (1%) (54), and transitional cell (Brenner) carcinoma (2%) (55).

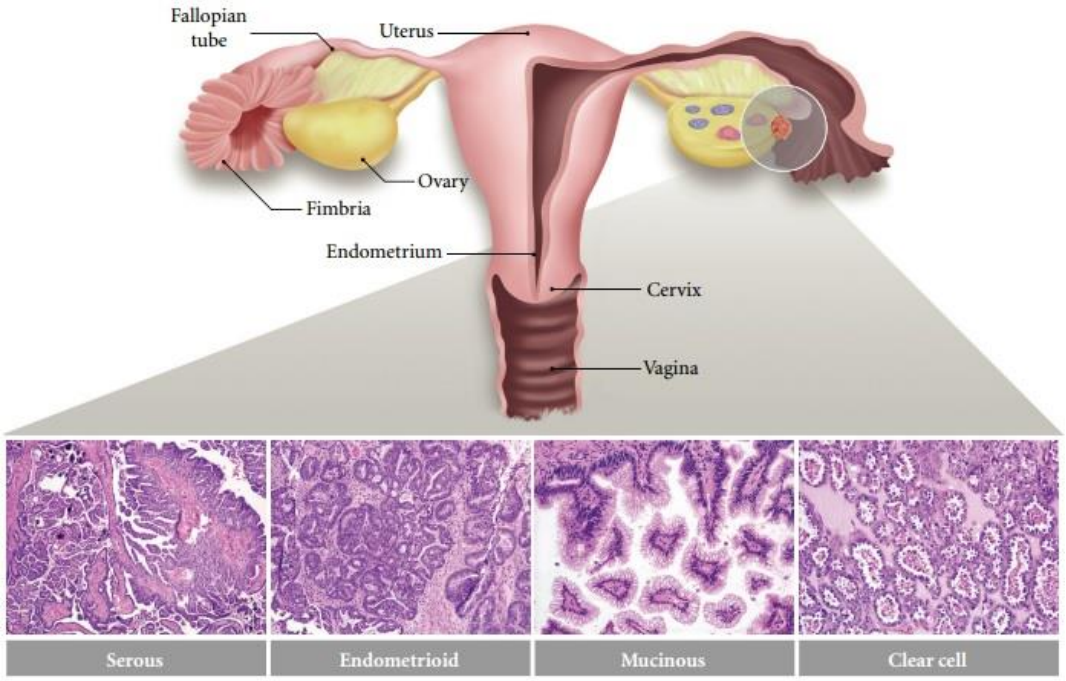


Figure 1.6: The four major types of epithelial ovarian cancer

Epithelial ovarian cancer includes: Serous, endometrioid, mucinous and clear cell carcinomas. The photos represent tumour sections which are stained with hematoxylin and eosin. From (56).

As the most common histotype of epithelial ovarian cancer, the high-grade serous ovarian carcinoma (HGSOC) is also considered as an aggressive form of this disease (57). Generally, almost all cases of HGSOC have TP53 mutations, which potentially occur in an early stage disease. In addition, about 10-15 % of HGSOCs have BRCA mutation (58).

The vast majority of HGSOCs are likely to originate from fallopian tube epithelia, especially the dysplastic lesions in the distal fallopian tube. Therefore, what has been often regarded "ovarian" cancer may in fact be tubal in origin (59). Recently, salpingectomy alone has been proposed as a prophylactic option to avoid the future development of ovarian cancer (60).

In addition to these, primary peritoneal cancer arises in the tissue that lines the pelvic and abdominal cavities and is characterized by its ability to spread widely inside the peritoneal cavity and in most cases it involves the omentum. This type of cancer is very uncommon,

however, it shares various histological, pathological and clinical features with epithelial ovarian cancer, so it is often described as a type of ovarian cancer and it is often treated in the same way (61).

1.6 Clinical Symptoms

Patients with ovarian cancer are often diagnosed late in the course of the disease because the symptoms are subtle and women frequently remain unaware of the disease until it reaches an advanced stage; therefore, the disease continues to be known as a "silent killer" (62). However, some non-specific and somewhat vague symptoms (table 1.1) may be present such as persistent pain in the pelvis or abdomen, increased abdominal size with persistent bloating, feeling full quickly after eating and increased urinary output. These symptoms are often misdiagnosed as irritable bowel disease or changes that are associated normally with past pregnancies, aging and menopause (63,64). However, in the United Kingdom, recent guidance has urged clinicians to consider ovarian cancer in patients with these symptoms, especially if they are of recent onset and are persistent (65).

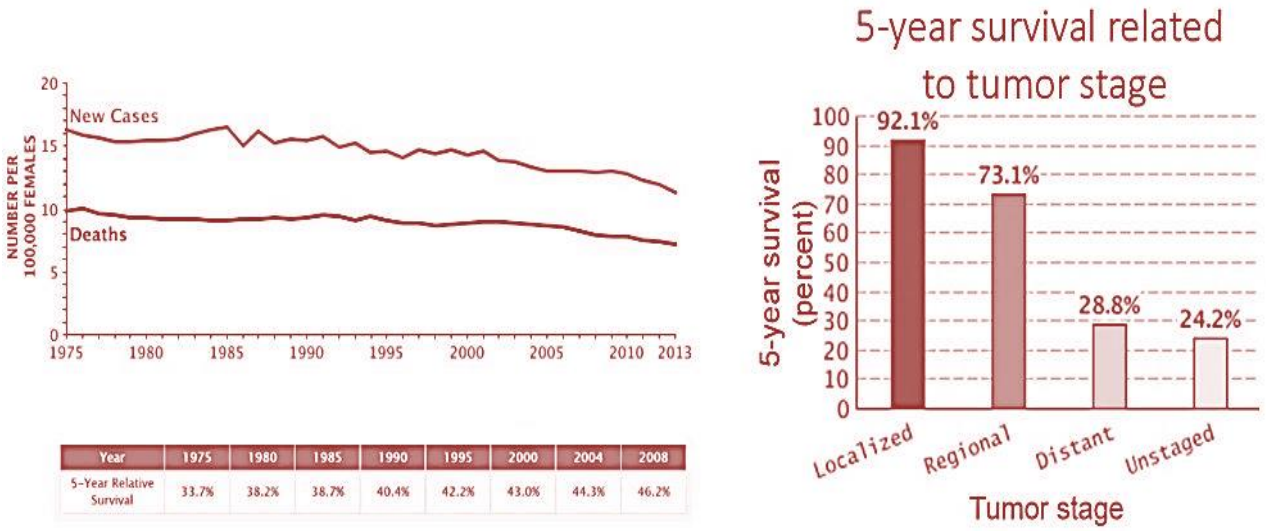
Table 1.1: Main symptoms of ovarian cancer

Symptoms	Frequency	Positive predictive value
Abdominal pain	53%	0.3%
Fatigue	39%	Unknown
Abdominal distension	36%	2.5%
Diarrhea	27%	Unknown
Bloating	17%	0.3
Pelvic pain	16%	Unknown
Increased urinary frequency	14%	0.2
Abnormal vaginal bleeding	13%	0.5
Weight loss	10%	Unknown

Positive predictive values for most symptoms are low because the incidence of ovarian cancer is relatively low (65).

1.7 Staging, screening and diagnosis

The vast majority of patients with ovarian cancer are diagnosed at a progressive stage where they may develop extra-ovarian disease. While over 90% patients diagnosed with stage I ovarian cancer survive for 5 years or more, only about 24-28 % of patients with progressive stages (stages III and IV) survive 5 years after diagnosis (Figure 1.7), so improving early diagnosis gives a great opportunity for improvements in ovarian cancer survival (66).



Year	1975	1980	1985	1990	1995	2000	2004	2008
5-Year Relative Survival	33.7%	38.2%	38.7%	40.4%	42.2%	43.0%	44.3%	46.2%

Figure 1.7: Percent of cases and 5-years relative survival by stage at diagnosis. From (67)**.**

1.7.1 Staging of ovarian cancer

Two diagnostic challenges arise when an ovarian mass is detected. The first one is the detection of ovarian malignancy and the second is an assessment of tumour spread, so accurate diagnosis is significant to avoid unnecessary surgery as most of ovarian masses are non-cancerous cysts. Furthermore, the decision of which type of surgery to be done (laparoscopy or laparotomy) is influenced by ovarian mass information. Therefore, The International Federation of Gynaecology and Obstetrics (FIGO) issued a recommendation for staging of ovarian cancer in accordance with laparotomy (table 1.2 (68,69)). Early identification of ovarian cancer symptoms can improve prognosis and in this regard it has been reported that symptoms of ovarian cancer have diagnostic and predictive importance. In addition, patient age and family history might help with estimation of the malignant nature and risk of ovarian cancer (70).

Table 1.2: FIGO staging and prognosis of ovarian cancer

Staging	Characteristics
I	Growth limited to the ovaries.
Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumour on the external surface; capsule intact.
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumour on the external surfaces; capsules intact.
Ic	Tumour either stage Ia or Ib, but with tumour on the surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
II	Growth involving one or both ovaries with pelvic extension.
IIa	Extension and/or metastases to the uterus and/or tubes.
IIb	Extension to other pelvic tissues.
IIc	Tumour either stage IIa or IIb, but with tumour on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
III	Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive regional lymph nodes. Superficial liver metastases equals stage III. The tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum.
IIIa	Tumour grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery.
IIIb	Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative.
IIIc	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive regional lymph nodes.
IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to stage IV. Parenchymal liver metastasis equals stage IV.

1.7.2 Methods of ovarian cancer diagnosis

Adnexal mass palpitation during examination of the patient's pelvis is routinely used to initiate a diagnostic assessment of ovarian cancer (68). An ultrasound examination is commonly used as a diagnostic technique to assess the disease, to aid in planning surgery and it can be helpful in the differentiation between a borderline ovarian tumour and ovarian cancer. It is rapid non-invasive tool for evaluation of multiple parameters of vascular supply and tumour structure (71).

In patients with advanced disease, cancer antigen 125 (CA125) level in serum is elevated by 80% or more, however, this biomarker has a controversial role in diagnosis because it is inadequate for the detection of early-stage disease. Additionally, CA125 is not specific for ovarian cancer and it can be elevated in conditions such as pelvic inflammatory disease, endometriosis in addition to other malignancies such as pancreatic and endometrial carcinoma. Consequently, in many medical disorders, false-positive results have been noted (72). Other studies proposed that the sensitivity and specificity of CA 125 in the diagnosis of ovarian cancer are poor, because CA125 is only raised by approximately 50% in stage I of the disease and in 75-90% in patients with advanced ovarian cancer (73).

Various models have been predicted to improve the precision of ovarian cancer diagnosis and to clarify whether ovarian disease is benign or malignant. In this regard, the risk of malignancy index (RMI) has been developed which is an index including evidence of serum CA 125 concentration, menopausal status of the patient and ultrasonography findings; it has been concluded that the RMI was the best available tool to test triage patients with potential ovarian tumours for referral to tertiary oncology units (73).

A new diagnostic tool for ovarian cancer is positron emission tomography/computed tomography (PET/CT). This is superior to, and more accurate than, Doppler ultrasound examination of the pelvis and abdomino-pelvic CT scan or pelvic MRI for diagnosis and

differentiation between different cases of benign and marginal ovarian malignancies. In addition, stage IV disease and co-existing neoplasms can be diagnosed by PET/CT and used to inform disease management and prognosis (74).

Another new diagnostic technique that has recently emerged is the exosomes that are actively released by tumours into the peripheral circulation. In general, exosomes are a stable source of miRNA. miRNA expression is often deregulated in cancer cells and can be detected by distinct exosomal miRNA profiles obtained from the bodily fluids of patients, suggesting that exosome-microRNA profiling could be used as substitute diagnostic markers for biopsy profiling (75,76).

1.8 Ovarian cancer therapy

The standard treatment of ovarian cancer involves histopathological diagnosis and staging, followed by cytoreduction (surgical resection) of the tumours and then repeated cycles of IV anti-cancer therapy with carboplatin/paclitaxel. Approximately, 70% of patients respond to chemotherapy. The main aim of therapy here is to prolong progression free survival and overall survival (77). Patients with chemosensitive disease may receive repeated rounds of therapy. Once the disease is resistant to further forms of therapy, the goal shifts to palliative care with an aim to improve the quality of patient's life by minimization of the symptoms of ovarian cancer (78,79).

1.8.1 Surgery

In spite of significant efforts in developing new strategies for chemotherapy and biologic treatment of ovarian cancer, surgery remains the cornerstone in the treatment of this disease. It aims to obtain a pathological diagnosis, precisely determine the stage of disease and to achieve cancer cytoreduction (80). Debulking or cytoreduction surgery is followed by washing of the peritoneal cavity with normal saline to remove as many cancer cells as

possible. Generally, patients have bilateral oophorectomy (removal of both ovaries) and/or a hysterectomy (removal of the uterus) (81). Surgery alone may be sufficient only in patients diagnosed with early disease, but in late stage disease, debulking surgery is generally followed by chemotherapy.

1.8.2 Chemotherapy

The current gold standard for chemotherapy of ovarian carcinoma is a combination of carboplatin with paclitaxel. Studies have revealed that this combination increases survival rates with median progression free survival of about 17-30 months and median overall survival of 36-65 months (82). However, in early stage patients, the adverse outcomes of chemotherapy drastically outweigh beneficial effects and use of conventional chemotherapy is not advised in patients with stage I disease (83). In patients with more advanced disease, the initial response is often followed by relapse. Patients who relapse within 6 months of initial treatment are considered to have primary chemoresistance (84). Patients relapsing after 6 months are more likely to respond to repeated chemotherapy, but ultimately most tumours become chemoresistant and at this point widely effective therapy is lacking. The development of drug resistance leads to a poor overall survival rate for ovarian cancer patients (84). Thus, chemosensitization strategies and or alternative therapeutics are required to overcome the mortality caused by ovarian tumours (85).

1.8.2.1 Platinum chemotherapy

The platinum-based drugs cisplatin, carboplatin, and the new members (oxaliplatin, satraplatin and picoplatin) are frequently used for the treatment of lung, ovarian, colorectal, breast, testicular head, neck, and bladder cancers. In 1978, cisplatin received approval for use in the treatment of both testicular and ovarian cancer. The second generation platinum drug carboplatin was approved in 1989 for use in ovarian cancer treatment. Carboplatin is a more

stable alternative to cisplatin, with similar activity in some types of cancer and less toxicity. Adverse effects of platinum-based agents include bone marrow suppression, anaemia, neutropenia, decrease immunity to infections, allergic reactions, gastrointestinal disorders, neurotoxicity and nephrotoxicity (86,87). It considered as a part of the first line therapy for ovarian cancer, especially when combined with a taxane. In 2002, a third-generation platinum-based drug, oxaliplatin, was approved by FDA for use in colorectal cancer (88,89).

Platinum-based antineoplastic agents induce covalent cross-linking of nitrogen nucleophiles in DNA nucleotides and result in DNA damage, primarily at N-7 nitrogen atoms of adenine and guanine residues, generating monoadducts and DNA interstrand or intrastrand crosslinks. The major DNA adduct formed by carboplatin results from the linkage of two adjacent guanine bases by platinum to form an intrastrand crosslinks. In contrast, cisplatin mostly causes interstrand crosslinks. This prevents strand separation, inhibiting gene transcription, DNA repair and/or DNA synthesis in cancer cells. Carboplatin causes cell cycle arrest in the S phase and induces apoptosis if the DNA damage is not properly repaired. The ability to form intrastrand crosslinks correlates with the cytotoxic effect of the particular drug (90–92).

Although the initial response to platinum compounds is high in ovarian cancer, most of patients relapse with chemoresistance (93). Different mechanisms of resistance to platinum-based chemotherapy have been illustrated. These mechanisms can be summerized in two major classes: those that prevent cell death occurring following platinum-DNA adduct formation and those that limit the cytotoxic platinum-DNA adducts formation. A better understanding of the molecular mechanisms of platinum resistance may lead to new anticancer strategies that could sensitize ovarian tumours to platinum-based chemotherapy (94,95).

1.8.2.2 Taxanes

Taxanes are important family of drugs that were introduced into the oncology therapy field in the early 1990s. They are prescribed for the treatment of several types of cancers (96). The commonly available taxanes, paclitaxel (Taxol®) and docetaxel (Taxotere®), have become widely accepted as very active chemotherapeutic agents with established beneficial effect in the treatment of ovarian, breast, head, neck, bladder, lung and oesophagus carcinomas (97–99). Taxanes produce their cytotoxic effect through inhibition of microtubule function. Microtubules play a key role in cell division and are considered as essential components of the cytoskeleton. They comprise α and β-tubulin subunits that undergo longitudinal assembly into filaments. They can undergo cycles of polymerization and depolymerisation. Taxanes bind to β-tubulin, stabilize them by preventing depolymerisation. This inhibits the normal formation of mitotic spindles, leading to chronic activation of the spindle-assembly checkpoint, mitotic arrest and ultimately cell death,. Thus, taxanes are considered as mitotic poisons (100–102). β-tubulin is the target of this class of compounds so, it has been considered as a biomarker for taxane activity and it has been reported that β-tubulin III overexpression is associated with resistance to taxanes (103).

Taxanes also decrease tumour angiogenesis and cell migration, while stimulating the immune system against cancers. This latter effect is mediated by increasing the levels of tumour necrosis factor alpha (TNF-α) in macrophages. TNF-α is an integral membrane cytokine (mTNF-α) usually formed in activated macrophages and monocytes, then released in a soluble form (sTNF-α) by the action of the metalloproteinase ADAM-17. Release of sTNF-α from cells can then cause either cell death or a cell-survival response, depending on the receptor to which it binds (104,105). The antiangiogenic activity of taxanes at low doses was also reported in some *in vivo* models of neovascularization, where administration of low doses of

paclitaxel to the chick embryo chorioallantoic membrane resulted in significant antiangiogenic activity (106).

Recently, it has been reported that taxanes can induce a dose-dependent production of soluble tumour necrosis factor alpha (sTNF-α) in cancer cells at clinically relevant concentrations, and this can contribute to their cytotoxic effect. Deregulation in the cytotoxicity pathway of TNF or induction of TNF-dependent NF-kappa B survival genes may, on the other hand, contribute to taxane resistance in cancer cells (105).

Like many other chemotherapeutic agents, therapy with taxanes is accompanied by adverse effects such as nausea, vomiting, diarrhoea, bradycardia, hypotension and bone marrow suppression. Peripheral neuropathy, myelo-suppression, arthralgia and myalgia are usually observed in patients receiving taxanes and where this toxicity accumulates throughout the course of treatment, it can be a dose-limiting. Adverse effects of taxanes may be increased by disorders that affect drug metabolism and excretion, such as hepatic or renal failure, by increasing serum levels of drugs. Additionally, drugs that affect the metabolism and excretion of taxanes can increase their toxicity if they are used simultaneously with such drugs (107–109). Patients quality of life may be adversely affected by taxane-induced cutaneous toxicity and skin reactions that includes nail pigmentation, erythema and desquamation, involving primarily the hands (110).

1.8.3 Chemotherapy resistance

Although there have been extensive advances in current chemotherapeutic strategies, clinical drug resistance remains a major obstacle to successful cancer treatment and is still a limiting factor in patient survival, a problem that is particularly obvious in the treatment of ovarian cancer (111,112). Resistance to chemotherapy can be classified as either intrinsic or acquired. Intrinsic resistance reflects the presence of cancer cells that are drug-resistant prior to chemotherapy treatment and following treatment these cells become the dominant population

(113). In contrast, acquired chemoresistance reflects the development of chemoresistant cells after chemotherapy treatment.

1.8.3.1 Mechanisms of resistance to chemotherapeutic agents

Resistance to chemotherapy prevents effective therapy and can ultimately result in patient death; therefore, it is important to understand mechanisms of drug resistance (114). Drug resistance is now recognised as being multifactorial including both genetic and epigenetic alterations in the cancer cells (figure 1.8) (115). These include decreased uptake of a drug into cells and/or increased its efflux and this is regarded as one of the major causes of resistance in many kinds of tumours (116). Additional mechanisms include increased repair of damaged DNA, loss of p53 function and failure to undergo apoptosis. Furthermore, because of heterogeneity of cancer cells in a tumour, more than one mechanism of resistance to chemotherapy may be present in a particular tumour (117–119).

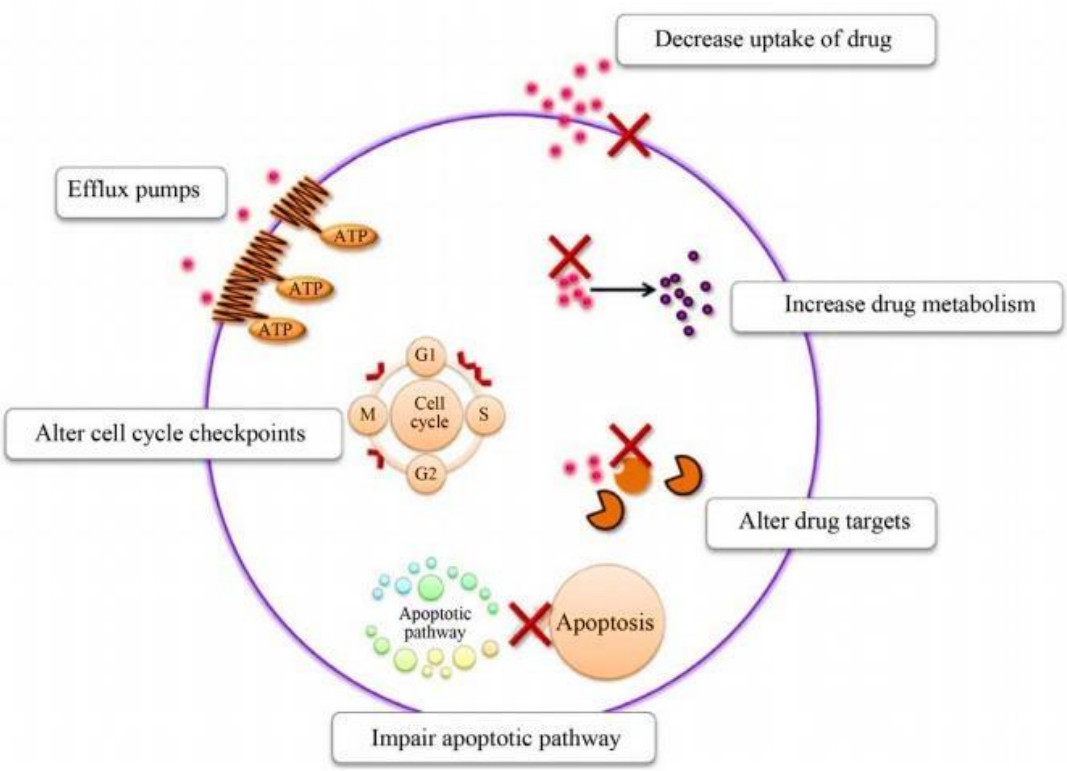


Figure 1.8: Cellular mechanisms of resistance to chemotherapy

Cells can develop resistance to chemotherapy by different pathways like augmentation of the activity of efflux pumps (such as ATP-dependent transporters) to reduced drug influx and this results in removal of the drug, triggering of the de-toxifying proteins (such as. cytochrome P-450), repairing their damaged DNA, disrupting and evading apoptotic signalling pathways, in addition to alteration of the cell cycle checkpoints (120).

The ATP-binding cassette (ABC) family constitute a major mechanism of multidrug resistance (MDR). ABC family proteins encode enzymes which function as drug transporters, actively pumping drug out of tumour cells (121). The transmembrane drug efflux transporter P-glycoprotein "PgP", encoded by the *MDR1* gene, is the most studied and well-characterized ABC transporter, which reduces the exposure of the cancer cells to chemotherapeutic drugs (122). P-glycoprotein efflux pump has a wide spectrum of substrates, allowing resistance to different types of drugs, such as anthracyclines, vinca alkaloids, podophyllotoxins, and taxanes, thus restricting their effective use (123).

Multiple changes in the DNA methylation can be associated with the development of drug resistance. In recent years, it has become evident that cancer cells can gain resistance to chemotherapy when abnormal DNA methylation of CpG islands and associated epigenetic silencing occurs (124).

Translational studies involving the prospective collection of tumour samples from the same patient before treatment and on clinical relapse are likely to be integral to identifying clinically relevant mechanisms of resistance. Circulating blood has been shown to contain free tumour DNA and tumour cells in most cancer patients. Detection of such DNA has the potential to identify specific genetic and epigenetic changes which highlight as yet unknown drug resistance mechanisms (125,126). Aberrant expression of drug resistance-related genes is considered as an important contributor to the development of drug resistance. Among all these genes, tumour suppressor genes (TSGs) are some of the key players (127). Tumour suppressor genes are wild-type alleles of genes that provide essential regulatory roles in different cellular activities, including cell cycle checkpoint responses, cell proliferation, differentiation, migration, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signalling, and tumour angiogenesis, where loss of TSGs function contributes to cancer development (128–130). It has been demonstrated that TSGs contribute to chemoresistance in many types of solid cancers. For instance, TSGs including p53, Fhit, E1A, IL-24, BiKDD and Fus1 are associated with chemoresistance in ovarian, pancreatic and lung cancer (127).

Genes that are associated with epithelial to mesenchymal transition (EMT) have also been shown to be responsible for drug resistance in patients with ovarian cancer (131,132). The EMT is an important differentiation process during embryogenesis involving effects on extracellular matrix components regulation, loss of intracellular cohesion, augmentation of cellular migration, in addition to resistance to apoptosis. During cancer progression, tumour

cells appear to undergo a form of EMT which supports tumour cell invasion and migration, as well as to drug resistance (133).

1.8.3.2. Effect of BRCA1/2 mutations and upregulation of 53BP1 on resistance to carboplatin and other chemotherapeutics

Since the mechanisms of cellular DNA repair play an essential role in determining the sensitivity of cells to DNA damaging agents, it is rational to speculate whether a DNA repair has a contribution in the emergence of resistance to DNA damaging anti-cancer agents (134). Cells are equipped with a wide range of DNA repair pathways to deal with any damage that affects the DNA structure caused by exogenous agents or normal metabolic processes. Genetic instability is promoted by loss of DNA repair integrity, which can result in carcinogenesis (135).

An additional outcome of defective DNA repair is a hypersensitivity of cells to DNA damaging substances, that has been exploited in the extensive use of DNA damaging chemotherapy. DNA damaging drugs are potent in patients with known cellular defects in DNA repair mechanisms. A commonly studied group of such are those with mutations in the tumor suppressor genes, BRCA1 and BRCA2 (136–138).

BRCA1 and BRCA2 are essential proteins for the first step of DNA double-strand break (DSB) /repair by homologous recombination (HR) (139). BRCA1 directly work by binding to the phosphorylated CtIP protein and localizes CtIP to DSBs, which then accelerates resection of DSB ends to generate 3' overhangs of single-stranded DNA (ssDNA). Subsequently, the ssDNA is coated by replication protein A, which is then displaced by the action of recombinase protein RAD51 (140). BRCA2 plays a critical role in this step, it directly binds to RAD51 and facilitates loading of RAD51 on ssDNA. RAD51 makes a nucleoprotein filament on ssDNA and induces the search for a homologous sequence and stimulates strand

invasion, that is accompanied by production and resolution of intermediate recombination structures (141,142). In addition to binding with BRCA2, BRCA1 forms a protein complex with the partner and localizer of BRCA2 (PALB2). Furthermore, BRCA1 has also been shown to play a vital role in checkpoint function, chromatin remodeling, transcriptional regulation and ubiquitination. Loss of the function of BRCA1/2 promotes chromosomal instability, which can result in tumorigenesis (143,144).

Notably, BRCA1/2 deficiency can result in cellular sensitivity to interstrand DNA crosslinking agents such as platinum based compounds in addition to PARP inhibitors (145). Interstrand crosslinks are primarily recognized during S phase of the cell cycle as they are essential for approaching replication forks to stall, even though the repair of interstrand crosslink also occurs in G1 phase (146). Repair of the interstrand crosslink is a complex process that occurs after several steps, starting with one strand endonucleolytic excision with subsequent translesion synthesis through the interstrand crosslink followed by removal by excision. The resulting DSB should be resected to produce ssDNAs followed by repair by HR. Since HR is malfunctioning in BRCA1/2-deficient cells, it renders these cells hypersensitive to interstrand crosslinking substances (147,148).

Platinum-based compounds (cisplatin and carboplatin) are effectively used for ovarian carcinomas, where patients with BRCA1 and BRCA2 mutations show longer recurrence-free intervals than patients with sporadic ovarian tumours, especially when treated with platinum compounds (149). However, recurrence of the disease does occur in the vast majority of individuals with BRCA1/2-associated ovarian tumour and the recurrent cancer ultimately acquire resistance to cisplatin and carboplatin. Several mechanisms have been suggested to result in carboplatin resistance including changes in its transport by altering the expression of copper transporters and augmented glutathione expression (150).

Cells lacking BRCA1 and BRCA2 are highly responsive to PARP inhibitors, where several clinical trials of using PARP inhibitors for ovarian cancer patients with BRCA1/2 mutations are ongoing (151,152). PARP-1 attaches ADP-ribose to itself and other molecules to accelerate repair of single strand breaks (SSBs). PARP inhibition leads to accumulation of SSBs, which then can be transformed into DSBs in S phase. HR plays an important role in the repair of DSBs during S phase. Thus, PARP inhibition in HR-deficient cells, like BRCA1/2-deficient cells, leads to cell death (153,154). However, not all of the cancers with BRCA1 and BRCA2 mutations are sensitive to the PARP inhibitor, and even for women who respond to the therapy, the duration of response is limited (155). The mechanism of clinical resistance to PARP inhibitors is not clear, however in ovarian carcinomas with mutated BRCA1 and BRCA2 that are treated with a PARP inhibitor, there is a link between the drug benefit rate and platinum-free interval, proposing that there may be a common pathway of resistance to PARP inhibitors and platinum based compounds (156).

Additionally, the therapeutic response of cancers with BRCA1/2 deficiency to anti-cancer agents may be influenced by other genetic or epigenetic modifications (157). It is possible that mutations which suppress other genes in BRCA1 and BRCA2-deficient cells can reverse sensitivity of tumour to PARP inhibitors and carboplatin, without restoring BRCA1 and BRCA2 themselves (158). It has been reported that the loss of the DNA-damage response protein 53P1 in mice with BRCA1-null or BRCA1 exon11 deletion mutation has resulted in rescuing the embryonic mortality of BRCA1-deficient mice (159). Furthermore, BRCA1/53BP1-deficient cells showed increased growth and proliferation, HR efficiency and chromosomal stability relative to BRCA1-deficient cells (160).

Essentially, 53BP1 deficiency in cells with BRCA1 mutation also restored resistance to PARP inhibitor and platinum compounds (161). Some HER2-non-overexpressing (triple negative), estrogen receptor-negative, progesterone receptor-negative, BRCA1 and BRCA2-associated

breast tumours have reduced 53BP1 expression. This suggests that suppression of BRCA1 deficiency by the loss of 53BP1 can probably modify the clinical progression of BRCA-associated neoplasms (161,162).

The most profound clinical implication of BRCA1/2 and 53BP1 is that secondary BRCA1 and BRCA2 mutations may predict whether a cancer patient will respond to platinum based compounds (163). This may also be applicable for patients treated with PARP inhibitors, although there is an additional complication of P-glycoprotein pumps upregulation, which contributes to resistance to this class of drugs (164). Moreover, restoration of DNA repair plays an important role in acquiring resistance to DNA damaging agents (165). Therefore, drug-resistant tumours with secondary BRCA1 and BRCA2 mutations may be re-sensitized to those drugs if HR can be inhibited by another agent, such as proteasome inhibitors, HSP90 inhibitors and CDK inhibitors that have been shown to inhibit RAD51 foci formation. These agents may provide a therapeutic option for the treatment of platinum/PARP inhibitor-resistant cancers in combination with PARP inhibitors and platinum-based compounds (166–168).

1.8.3.3 Strategies for the treatment of drug resistant disease

1.8.3.3.1 Pharmacokinetic approaches

Pharmacokinetic approaches depend on the hypothesis that resistance to chemotherapy occurs as a result of inadequate exposure of cancer cells to a therapeutic drug; therefore, increasing drug exposure may improve the patient outcome by preventing the emergence of chemoresistant cells (169). High local doses of anti-cancer agents can be achieved by direct intraperitoneal administration of drugs to overcome the problem of low tumour-cell drug exposure and to increase local drug concentration (170,171). Several randomized phase II and III clinical trials have shown that intraperitoneal administration of anti-cancer drugs is more

effective than standard intravenous chemotherapy in the treatment of advanced ovarian cancer, but this is accompanied by increased toxicity (172–174). A major problem with the acceptance and usage of the intraperitoneal route of administration is the support required to manage toxicities and complications related to therapy (175,176).

To achieve increased cancer-cell-specific drug delivery, one solution is to use liposomes to deliver drugs because they show increased permeability in cancer vasculature relative to normal tissue (177). Accordingly, encapsulation of drugs in these substances can be used as an alternative way of improving efficiency, non-immunogenicity, biocompatibility and solubility of chemotherapeutic agents to deliver them effectively to the site of tumour, such as the use of lipid-, polymer- or poly(L)glutamic-acid conjugated paclitaxel (178,179).

1.8.3.3.2 Combination therapy

The combination of multiple chemotherapeutic agents constitutes a leading strategy for targeting drug-resistant cancer. In most types of cancer, using a single-drug may lead to the activation of alternative molecular pathways in tumour cells which overcome the effect of the drug. Since the evolution of cancer chemotherapy, the combination of anti-cancer agents has been shown to improve clinical outcome, induce synergistic effect and delay the onset of chemoresistance. Therefore, drug combinations have become the standard first-line therapy of many types of cancers (180,181). For example, the combination of carboplatin and paclitaxel is largely accepted as the gold standard regimen for the treatment of ovarian cancer. (182,183).

The current identification of driver oncogenic pathways has led to the development of a large number of targeted agents that are directed to oncogenic drivers to which cancers are potentially addicted. This has improved the survival of cancer patients for various types of tumour treated with the matched targeted agents. (184,185). There are currently multiple

clinical trials for combination of molecular substances that target the main signalling pathways, comprising the ErbB family, PI3K-AKT, mTOR kinase, IGF, HGF–c-MET, ALK, Wnt, FGFR and MAPK pathways for the treatment of various cancers (186).

Although therapeutic regimens which combine carboplatin with other cytotoxic drugs are the current standard of care for patients with advanced ovarian cancer, the cumulative toxicities of these combinations can present barriers for their long-term use (187). There are three main limitations for using carboplatin based combinations, including allergy to carboplatin, ototoxicity and nephrotoxicity (188). Additionally, knowledge of the status of BRCA1 and BRCA2 should be considered as a part of the standard of care for patients diagnosed with ovarian carcinoma (189). Interestingly, there is a large body of evidence suggesting benefits of targeting pathways that are involved in maintaining DNA integrity, including BRCA1/2 signaling. Harboring a germline mutations in BRCA1 and BRCA2 is can predict sensitivity to carboplatin. Futhermore, depending on the concept of synthetic lethality, sensitivity to carboplatin can be enhanced by the simultaneous promotion of DSBs and hindrance of DSB repair by inhibition of the expression of PARP protein (190). Inspite of these strategies intratumour genetic heterogeneity can limit sensitivity of tumours to anti-cancer agents and can be considered as the main contributor to therapeutic failure. Also it is becoming increasingly known that individual cancers may achieve chemoresistance via multiple routes simultaneously (polyclonal resistance) (191). Ttumour heterogeneity can be observed at several different levels, and may be caused by a number of different factors. Intracellular heterogeneity is the heterogeneity that generates first at the cellular level, however continual selective outgrowth of any given cell clone give rise to variable degrees of clonal heterogeneity (192). Subclones may grow and develop in a sequential linear fashion, or may keep diverging, following branched evolutionary paths (193).

Tumours diversity is evident at the genetic, epigenetic, proteomic and transcriptomic levels. Genomic instability has an effect on DNA sequence, chromosome number and structure with some modes of instability compromising genome integrity at different levels simultaneously (194). Genomic instability, a common feature of the vast majority of solid tumours including ovarian cancer, is responsible for high proportion of intracellular genetic heterogeneity and has been related with both poor prognosis in cancer patients in addition to chemoresistance (195). Epigenetic, proteomic and transcriptomic heterogeneity may arise because of underlying genotypic variation, however it can also reflect stochastic variation between cells, cell cycle stage or hierarchical organisation of cells according to the theory of cancer stem cells. Moreover, diverse phenotypes can arise from extrinsic factors which include hypoxia, pH and paracrine signalling interactions with cancer and stromal cells (196–198).

The degree to which metastatic tumors further develop by continual accumulation of additional mutations and copy number alterations is still unclear and has yet to be taken into account extensively using next-generation sequencing of HGSOC in order to further predict successful use of combination therapy (199).

1.8.3.3.3 Sequential therapy

Sequential therapy is designed to minimize resistance to chemotherapy. One drug is used initially and then followed by a second drug. This is designed to eliminate cells that may have become resistant to the first therapy. Possible advantages are that the full dose of the most potent drug can be administered at first, and then differential sensitivities of cancer cells to certain agents can be exploited in subsequent rounds of therapy. Such strategies have shown evidence of successful reduction of chemoresistance and provided better tolerability and minimal risk of negative interactions between drugs (200,201).

1.8.3.3.4 Chemotherapy dosing schedule

The abolition of treatment-free intervals by administration of continuous doses of anti-cancer agents has been shown to result in better efficacy and overcoming of resistance (202). This has led to the development of ”dose-dense chemotherapy schedules” and improved overall and disease-free survival. Administration of dose-dense chemotherapy has been facilitated by the introduction of granulocyte colony-stimulating factor, which allowed condensation of the chemotherapy courses without producing unwanted toxicity (203).

1.8.3.3.5 Improvement of targeted epigenetic therapies

The discovery of how cancer cells alter the epigenetic machinery to their own advantage has broadened the understanding of how tumours develop resistance to the existing agents. This has led to the idea of using “epidrugs” to re-sensitize resistant tumours to chemotherapy. (204,205). Modest clinical results have been obtained following the use of epigenetic inhibitors (including DNA methylation and histone modifications) in hematological cancer (205,206). Second-generation epigenetic inhibitors (such as azanucleoside) have intrinsically wider selectivity for their targets (207). Solid malignancies have more genetic and epigenetic complexity than hematological cancers, however the epigenome and transcriptome biomarkers have been studied for many of these tumour types, and the aberrant expression profile of these tumours may be modified by means of epidrugs together in combination with conventional and innovative chemotherapeutic agents (208).

Background information on apoptotic pathways are explained in chapters 3 and 5.

Chapter 2

Aims

2.1 Aims

Chemoresistance is one of the major obstacles in the way of effective ovarian cancer treatment. The research presented here seeks to improve the efficacy of chemotherapy by increasing the sensitivity of ovarian cancer cells to chemotherapies by two different strategies.

1. Previous work in our group had shown that the "BH3 mimetics" ABT-737 and navitoclax, drugs which stimulate the intrinsic apoptosis pathway. These drugs inhibit the Bcl-2 family of proteins including Bcl-2 and Bcl-X_L and can sensitize ovarian cancer cell to carboplatin. Venetoclax is a selective BH3 mimetic agent that was developed to inhibit the apoptosis regulators Bcl-2 and which avoids inhibiting Bcl-X_L which is associated with thrombocytopenia. Venetoclax, but not navitoclax, has received approval for the treatment of leukemia. This raised the question whether venetoclax would also be effective in ovarian cancer. To test this hypothesis, venetoclax was compared with WEHI-539, a new selective Bcl-X_L inhibitor to see which agent would most likely be useful as a chemosensitizer in ovarian cancer.

2. Previously, our group has conducted an siRNA screen to identify genes which contribute to drug resistance with the goal of using these as targets to develop novel chemosensitizers. Several hits from this screen increased the sensitivity of ovarian cancer cells to carboplatin and/or paclitaxel. Although one of the hits, Autotaxin, has already been pursued by our group, several of the hits still required validation. Consequently, these hits were validated by more detailed siRNA studies (chapter 5) and BCKDK, one of the validated hits explored in more detail (chapter 6).

Chapter 3

Materials and methods

3.1 Ovarian Cancer Cell Lines

Several ovarian cancer cell lines, in addition to normal human epithelial cells and gene-edited cells were used in this research (table 3.1).

Table 3.1: Description of cell lines

Cell line	Description
FUOV-1	This cell line was obtained from the tumour tissue of a woman with high grade serous ovarian cancer after hysterectomy[1] (209).
OVCAR-8	This cell line was obtained from a high grade ovarian serous adenocarcinoma from carcinoma of the ovaries that was resistant to carboplatin[1] (209).
OVCAR-4	This cell line was established from the ascites of a woman with high grade ovarian serous adenocarcinoma that was resistant to cisplatin[1] (210).
Igrov-1	This cell line was derived from an ovarian carcinoma of a woman that was sensitive to chemotherapy treatment[1] (211).
OVCAR-3	This cell line was developed from the malignant ascites of a 60 years female with progressive ovarian adenocarcinoma following treatment with cisplatin, cyclophosphamide and adriamycin[1] (212).
Ovsaho	This cell line was derived from high grade ovarian serous papillary adenocarcinoma[1] (213).
COV318	This cell line was established from a peritoneal ascites of woman with ovarian epithelial-serous carcinoma[1] (214).
COV362	This cell line was established from a pleural effusion of woman with epithelial-endometroid carcinoma [1] (214).
HOE	Human Ovarian Epithelial cell line (HOE) was derived from normal human ovary cells and it was and immortalised using SV40 large T antigen[2].
HAP-1	Haploid cell line derived from the KBM-7 cells[3].
BDK	Gene edited haploid cell to lack BCKDK[3].

[1]Supplied from the American Tissue Culture Collection (ATCC). [2]Supplied from Applied Biological Materials (ABM) Inc. [3]Supplied from ThermoFisher

3.2 Cell growth mediums and conditions

OVCAR-3, OVCAR-4, OVCAR-8, Ovsaho and HOE cell lines were grown in Roswell Park Memorial Institute medium (RPMI 1640; Lonza), while COV318, COV362 and Igrov-1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Lonza). FUOV-1 cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; Lonza). HAP-1 and BDK cells were grown in Iscove's Modified Dulbecco's Medium (IMDM; Lonza). Growth media were supplemented with 10% fetal bovine serum (FBS; Lonza), 50 µg/ml (50 IU/ml) penicillin/streptomycin (Lonza) and 2 mM L-Glutamine (Lonza). The growth medium of Ovcar-3 cell line was further supplemented with 0.01 mg/ml insulin (Lonza) and 0.11 g/L sodium pyruvate (Lonza). Each cell line was incubated in its specific culture medium at 37 ºC in 5% CO2 until the cells became confluent.

3.3 Seeding and harvesting of cells

When the cells were more than 80% confluent, the growth medium was removed and then cells were rinsed with 1 ml of phosphate buffered saline (PBS; Lonza). To detach the cells 1 ml 0.01% trypsin (Lonza) in PBS was added and the cells incubated at 37 ºC with gentle agitation until the cells detached, and 1 ml growth medium containing FBS was added to neutralize the trypsin. The cells were collected by centrifugation (150 g, 3 minutes) at room temperature. The supernatant was aspirated and the cell pellets were re-suspended in 5 ml of growth medium, then transferred into T25 or T75 flask containing suitable volume of culture medium and allowed to grow again for routine passage. For cell proliferation and other assays, the cells were counted using a haemocytometer to determine the number of cells required for each experiment and then cells were transferred into culture plates as described for each experimental procedure.

3.4 Cryopreservation of adherent cell lines

Before cryopreservation, the cells were checked for bacterial, fungal or any other contamination and also to ensure the cells were grown in logarithmic phase. The cells to be frozen were of low passage number and they were collected by trypsinization as described above. The cells were collected by centrifugation (150 g, 3 minutes, 4 °C). The supernatant was aspirated and the pellets re-suspended in growth medium containing 8% dimethyl sulfoxide (DMSO, Sigma-Aldrich) and 10% FBS, then 0.2 ml was transferred into cryovials and frozen slowly overnight before being transferred to liquid nitrogen.

3.5 Thawing cells

The cryovials were removed from the liquid nitrogen and rapidly thawed at 37 °C and then its content was added gently into 10 ml pre-warmed growth medium and cells collected by centrifugation (150 g, 3 minutes). The supernatant was removed and the pellets were re-suspended in a culture medium and transferred into T25 flask and kept in incubator for 24 hours. Next day, the growth medium was replaced and the cells were allowed to grow to be used for ordinary sub-culture or experimentation.

3.6 Chemical agents

Dimethylsulfoxide was used to dissolve the drugs (except PBS for carboplatin) to the following final stock concentrations: 20 mM for each of ABT-737 (Abbot Laboratories), venetoclax (Abbot Laboratories), WEHI-539 (Medchem Express), 3,6-Dichloro-1-Benzothiophene-2-Carboxylic acid (DCBC; Sigma-Aldrich) and (S)-2-Chloro-4-methylvaleric acid (CMVA; Tokyo Chemical Industry “TCI”); 13.5 mM for carboplatin (SigmaAldrich) and 5 mM for paclitaxel (Sigma-Aldrich). Cell culture grade branched chain

amino acids (leucine, isoleucine and valine; Sigma-Aldrich) were dissolved in growth medium at 10 mM. siRNAs (appendix 2) were prepared by dissolving each individual siRNA in siRNA buffer (Dharmacon) at a concentration of 20 µM.

3.7 Spheroid (3 Dimensional) culture

The Spheroid culture was performed using GravityTRAP™ (Tissue Re-aggregation and Assay Plate) Ultra-Low Attachment (ULA) plate (InSphero), figure 3.1.

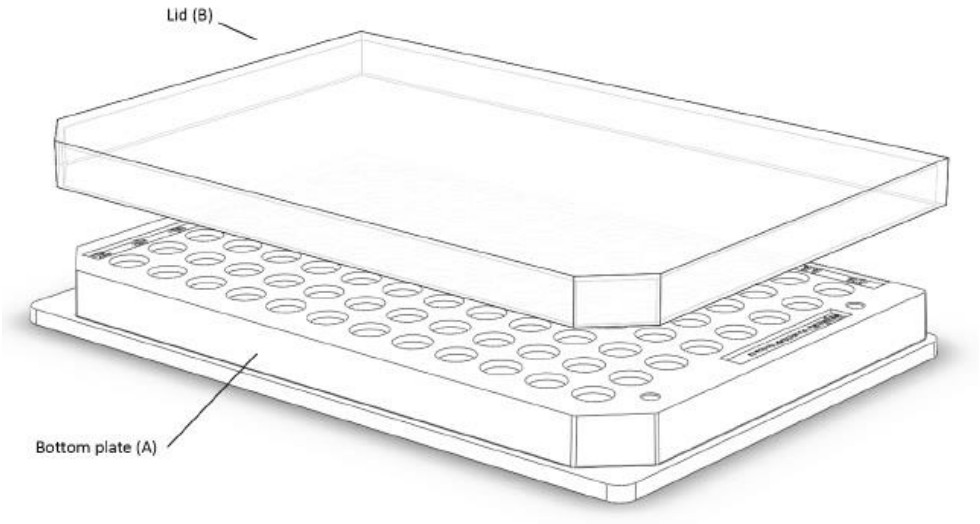


Figure 3.1: Components of the GravityTRAP™ ULA Plate

Before seeding of cells, the well of the plate were pre-wet with 40 µl of culture medium to prevent any air bubbles forming. 70 µl cell suspension, containing an optimized number of cells (2500 cell/well for COV318, 1000 cell/well for FUOV-1 and Ovsaho and 250 cell/well for OVCAR-4, HAP-1 and BDK cells), was seeded to the bottom of the well (figure 3.2), and

then the whole plate was centrifuged (1000 rpm, 2 minutes) to encourage aggregation of cells and spheroid formation. The plate was incubated at 37 °C with a routine daily check to observe spheroid maturation.

Three dimensional aggregates were typically observed within 2-5 days of seeding based on the culture conditions and the type of cells and used for experiments as described below.

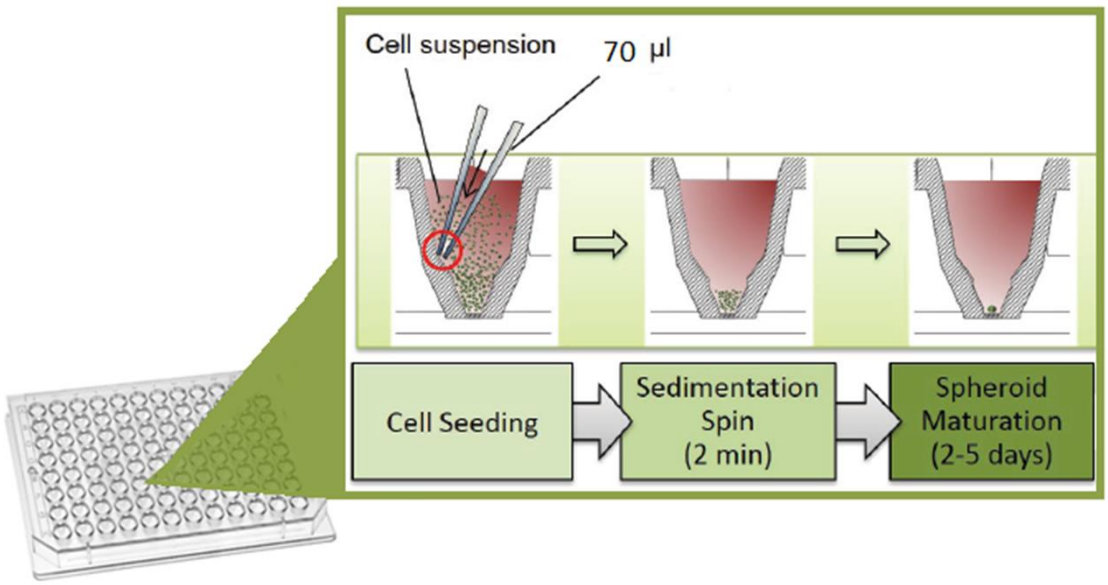


Figure 3.2: Seeding of cells

3.7.1 The CellTiter-Glo cell viability assay

The CellTiter-Glo luminescent was used to determine the cell viability in three dimensional culture and cytotoxicity assays (Promega). This method depends on the measurement of the ATP in cells as an indicator of the number of metabolically active cells. The CellTiter-Glo reagent was prepared according to manufacturer's instructions. The plates containing the cells were allowed to equilibrate to room temperature. For spheroid cultures, the spheroids with the growth medium were transferred to opaque-walled 96 well plate and equal volume of CellTiter-Glo reagent was added to each well and mixed gently for 2 minutes to allow full penetration of the reagent into the three dimensional structure to induce cell lysis. The plate was incubated for 10 minutes at room temperature to stabilize the luminescent signal, before measurement using a BioTek Synergy 2 multi-mode microplate reader.

3.7.2 Statistical analysis of 3D culture

The Z-factor or Z-prime value (commonly written as Z') was used to assess the suitability of 3D cultures for testing drugs (215). The mean signal (μ) and standard deviations (σ) of both the samples (s) and negative controls (n) were used to determin Z.

$$\text{Z-factor} = 1 - \frac{3(\sigma_s + \sigma_n)}{3(\mu_s - \mu_n)}$$

Z-factor approach 1 is an ideal value, while a value between 0.5 and 1.0 is an excellent assay, between 0 and 0.5 is marginal. A Z-factor values less than 0 is unlikely to produce reliable results.

3.8 Cell proliferation assay

Cells were collected by trypsinization as described above (section 3.3) and suspended in culture medium (62,500 cell/ml, except for OVCAR-8, HAP-1 and BDK cell lines which were prepared at 25,000 cell/ml). 80 µl of the suspension was seeded per each well of 96 well plate (to obtain 5000 cells/well for all cell lines except OVCAR-8, HAP-1 and BDK cells, which were seeded at 2000 cell/well) and incubated at 37 ºC in 5% CO_2 for 24 hours. The next day, cells were treated with drugs by adding 20 µl of the drug being tested (typically 18 different concentrations were used), prepared in growth medium at five times the desired final concentration. After 72 hours incubation, the growth medium was removed and the cells were fixed using 100 µl of cold trichloroacetic acid 10% (TCA) and incubated for 30 minutes on ice. Subsequently, the plates were washed with water, then dried and stained with 100 µl of 0.4% sulforhodamine B (SRB; Sigma-Aldrich) in 1% acetic acid (Sigma-Aldrich) for 30 minutes at room temperature. After incubation, the SRB was tipped off and the plates washed with 100 µl of 1% acetic acid and allowed to air dry. Then the dye was solubilized by adding 100 µl of 10 mM/pH10 Tris (Sigma-Aldrich). The optical density was measured at 570 nm by using a BioTek Synergy 2 multi-mode microplate reader.

3.8.1 Statistical Analysis and determination of IC_{50} Value

The data from cell growth assays was analyzed by using Graphpad Prism software. Non-linear regression was used to fit a four parameters Hill equation to determine the IC_{50}. The mean and standard deviation of IC_{50} were calculated from at least 3 experimental replicates.

3.8.2 Drug combination studies

Drug combination studies were performed using different ovarian cancer cell lines by exposing them to different concentrations of traditional anti-cancer drugs (either carboplatin or paclitaxel) combined with the drugs under evaluation. Drug combinations with carboplatin and either ABT-737, venetoclax or WEHI-539 were performed by treating the cells simultaneously with a range of concentrations of carboplatin combined with ABT-737, venetoclax or WEHI-539 at a 5% ratio of their IC_{50} values previously determined from single agent activity studies. The combination studies of paclitaxel with DCBC or CMVA were performed by treating the cells at the same time with a range of concentrations of paclitaxel combined with DCBC or CMVA used at either 30 or 100 µM. In each experiment, the viability of cells was determined 72 hours after treatment with the given drugs, by fixing the cells in 10% TCA, then using the SRB assay described previously.

3.8.2.1 Evaluate of the drug combinations efficacy

In order to evaluate the effectiveness of the drug combinations, a combination index (CI) was calculated using Chou and Talalay equation which provides a quantitative assessment of the synergistic, additive or antagonistic effects between two drugs (216,217). Synergy is indicated when the CI value between two drugs is less than one (CI<1). However, CI values equals to or more than 1, indicates additive and antagonistic effect respectively (table 3.2).

The CI value for two drugs is defined as

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad \text{..... (1)}$$

This equation is valid when the two drugs have similar mechanism of action or mutually exclusive effect. $(Dx)_1$ is the concentration of the first drug as a single agent that inhibits the

cell growth by x%. $(Dx)_2$ is the concentration of the second drug as a single agent that inhibits the growth by x%. $(D)_1$ and $(D)_2$ represent the concentration of the first and the second drugs in the combination which inhibit the growth of cells by x%. The values of $(Dx)_1$ and $(Dx)_2$ were calculated from the nonlinear regression results by using Graphpad Prism.

When the combined drugs have a dissimilar mode of action or reciprocally non-exclusive mechanism of action, the equation may be modified to:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1\,(D)_2}{(D_x)_1(D_x)_2}$$

Table 3.2: Symbols and description of synergism, additive or antagonism in drug combination studies determined by the combination index method.

(218)

Range of Combination Index	Description	Graded Symbols
<0.1	Very strong synergism	+++++
0.1–0.3	Strong synergism	++++
0.3–0.7	Synergism	+++
0.7–0.85	Moderate synergism	++
0.85–0.90	Slight synergism	+
0.90–1.10	Nearly additive	±
1.10–1.20	Slight antagonism	−
1.20–1.45	Moderate antagonism	− −
1.45–3.3	Antagonism	− − −
3.3–10	Strong antagonism	− − − −
>10	Very strong antagonism	− − − − −

3.9 Caspase 3/7 activity assay

Caspase-Glo Assay (Promega; Madison, USA) was used to quantify apoptosis by measurement of the executor caspases, 3 and 7 activities (figure 3.3). The required number of cells (62,500 cell/ml, except for OVCAR-8, HAP-1 and BDK cell lines which were seeded at 25,000 cell/ml) was seeded in 96 well plate and incubated overnight at 37 ºC. The next day, cells were exposed to carboplatin alone (final concentration 2 x IC_{50} measured in cell growth assays) and in combination with ABT-737, venetoclax and WEHI-539, and then incubated at 37 ºC for 48 hours. For experiments with BCKDK inhibitors, cells were exposed to 12 nM paclitaxel alone and in combination with 100 µM final concentrations of DCBC or CMVA and incubated for 48 hours.

To measure caspase 3/7 activity assay in siRNA experiments, the cells were transfected with 100 nM of individual siRNAs (including non-targeting siRNA, which was served as a control as described in section 3.14) and incubated for 24 hours. Then, the growth medium was removed and replaced with 80 µl of fresh growth medium and the cells were treated with 12 nM paclitaxel.

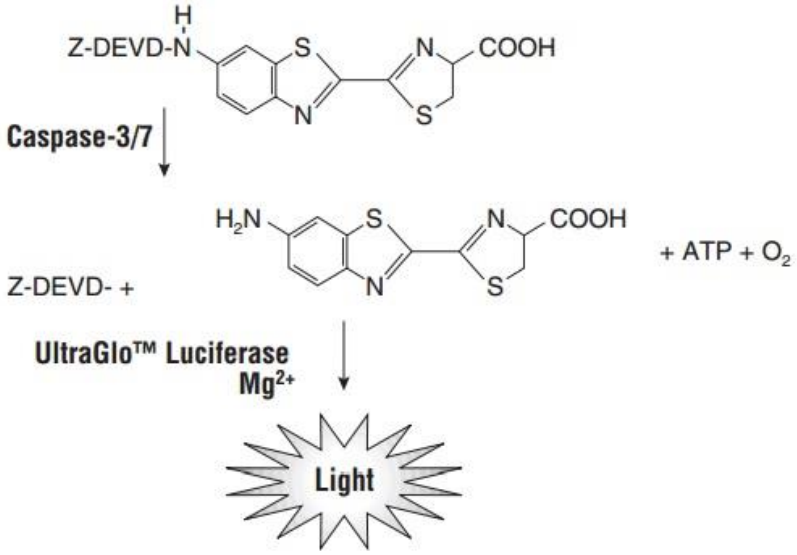


Figure 3.3: Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence

Caspase 3/7 reagent was prepared according to the manufacturer's instructions and then kept at -80 ºC. To measure the activity of caspase 3/7, 25 µl of Caspase-Glo 3/7 reagent was added directly to the cells in each well. The plate was protected from direct light by covering with foil, the contents gently mixed and incubated on a rocker for 30 minutes at room temperature. Subsequently, 100 µl from each well was transferred to opaque-walled 96 well plate to and shielded from light prevent any loss of the luminescence signal. The luminescence was measured using a BioTek Synergy 2 multi-mode microplate reader. The compounds being tested may significantly inhibit cell growth resulting in significant variation in cell number between each condition after treatment;to control for any difference in cell number, parallel plates were also prepared and subjected to the same treatment and relative cell number estimated by staining with SRB and used normalized to the caspase 3/7 signal.

3.10 Trypan blue assay

Trypan blue is a diazo dye that can be used to measure drug induced cell death due to its ability to differentiate between viable and dead cells. (219). 100,000 cell/well were seeded in 12 well plate and incubated overnight. The next day, drugs were added. For experiments using BH3 mimetics, 1, 3 and 0.3 µm of ABT-737, venetoclax and WEHI-539 respectively, were added to the cells alone and in combination with 66 µm carboplatin. A duplicate of each plate were prepared; one was incubated for 24 hours and the other for 48 hours. After incubation, the supernatants were collected into 15 ml tubes, and adherent cells were detached by trypsin and mixed with the corresponding supernatant, then centrifuged (150 g, 3 minutes), the supernatant was removed and the collected cells were gently re-suspended in 500 µl growth medium. Each sample was mixed with an equal volume of trypan blue (SigmaAldrich, 0.4% in PBS) and the number of live (exclude stain) and dead (blue stained) cells were counted on a haemocytometer. The percentage of viability was calculated according to the following equation.

Calculated % viability = 100 x number of dead cells/(number of dead cells + number of live cells)

3.11 Measurement of annexin V/PI labelling

This assay was performed to detect viable, apoptotic and dead cells. The mechanism by which this assay detects the viable or dead cells is shown in figure 3.4

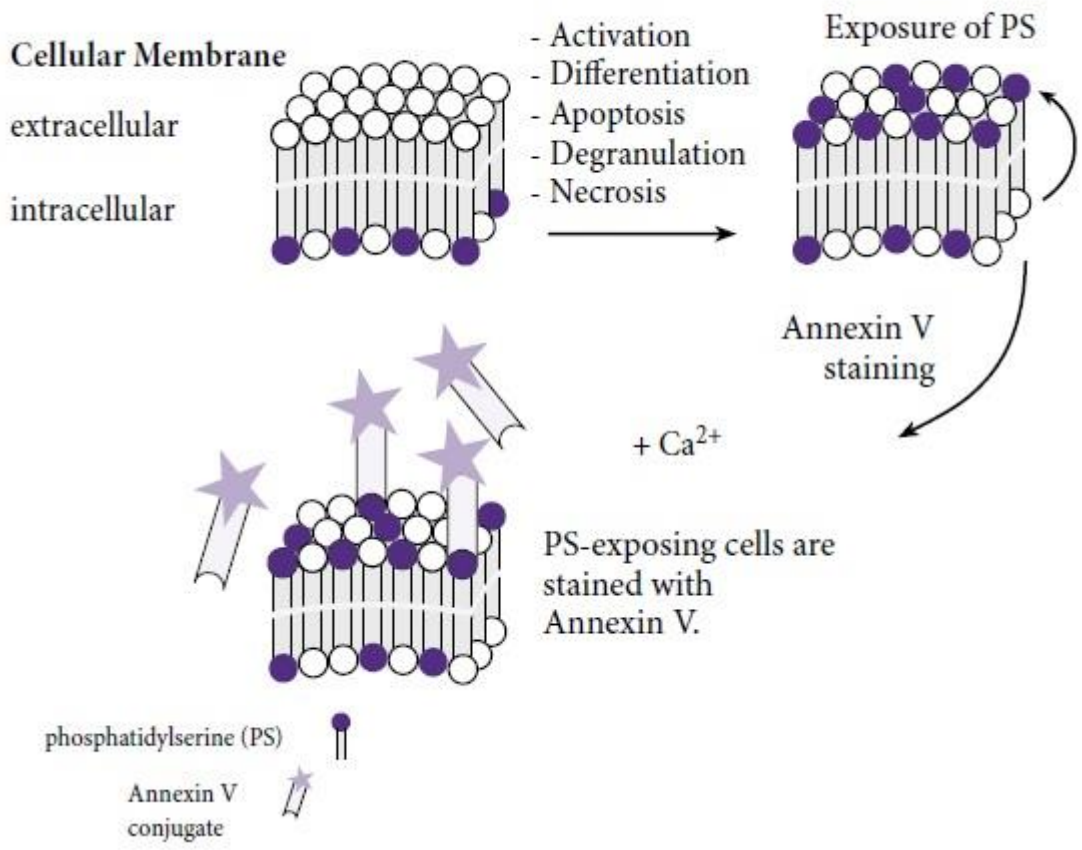


Figure 3.4: Annexin V staining mechanism

Cells were plated and treated in the same way as in the trypan blue assay. After incubation, cells were washed with 1 ml of 1 × annexin V binding buffer (Miltenyi Biotec), and collected by centrifugation (300 g, 10 minutes). The supernatant was aspirated completely and the cells were re-suspend in 100 µl of 1 × annexin V binding buffer and 10 µL of annexin V

fluorochrome (Miltenyi Biotec) was added and mixed gently and incubated in the dark for 15 minutes at room temperature. After incubation the cells were washed with 1 ml of 1 × annexin V binding buffer and collected by centrifugation (300 g, 10 minutes). The supernatant was removed and the cells re-suspended in 500 µl of 1 × annexin V binding buffer. For detection of dead cells 1 µg/ml of PI solution was added immediately prior to analysis by flow cytometry.

3.12 Bliss independence criterion

The Bliss independence criterion was used to determine the expected effect of drug combinations in caspase 3/7, trypan blue and annexin V/PI assays. This was used in place of the combination index in studies where the full dose response relationship curves were not determined, but this method still allows quantification of synergy between two drugs. The criterion allows calculation of the expected effect of a drug combination, if the two drugs combine additively. This value may be compared to the observed effect of a drug combination to evaluate synergy or antagonism between the drugs (220).

The Bliss independence criterion for two toxic substances is expressed by the following equation:

$$\mathbf{E_{(x,y)} = E_{(x)} + E_{(y)} - E\,(x)\ x\ E_{(y)}}$$

Where $E_{(x,y)}$ is the expected effect of the combination and $E_{(x)}$ and $E_{(y)}$ are the effect of individual drugs. Paired t-tests were used to compare the expected effect calculated by the Bliss independence criterion and the experimentally observed effect of the drug combination. The level of significance was at $P \leq 0.05$.

3.13 Western blot assay

Western blotting was used for detection of different intracellular proteins from normal human epithelial cell line and ovarian cancer cells. Cells were seeded in 12 wells plate and treated with drugs as described for the trypan blue and annexin V assays or left untreated to measure the basal expression of some proteins. Following incubation of cells, the growth media was collected, the adherent cells were detached by trypsinizatoin, mixed with corresponding supernatant and centrifuged (250 g, 3 minutes, 4ºC), then the growth medium was removed and the cell was washed gently with chilled PBS, centrifuged, aspirated and the obtained pellets were kept frozen at -80ºC. To lyse the cell pellets, RIPA (Radio-Immunoprecipitation Assay) buffer was used comprising 20 mM Hepes (CalbioChem), 150 mM sodium chloride (Sigma-Aldrich), 2 mM EDTA (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 1% NP40 (Sigma-Aldrich) and freshly supplemented with 18µl of 20mM leupeptin (Sigma-Aldrich), 15 µl of 10 mM pepstatin (Sigma-Aldrich) and 20 µl of 150 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich). Phosphatase inhibitors comprising 0.8 M sodium fluoride and 0.5 M sodium pyrophosphate were added to the mixture as required when protein phosphorylation was measured. 100 µl of RIPA buffer was added to each sample, mixed and centrifuged (14000 RPM, 10 minutes, 4 ºC). The supernatant was collected and frozen at -80 ºC.

5- 15 µg protein samples (measured using the BCA assay, described below) was mixed with 5 µl of SDS-Nupage sample buffer (Invitrogen) supplemented with 5% β-mercaptoethanol incubated at 80 ºC for 15 minutes. The samples were loaded into 4-20% Tris-Glycine (Nusep) polyacrylamide gradient gel using running buffer (100 mM Tris, 100 mM hepes and 1% sodium dodecyl sulphate) and separated at 70 V for 1.5 hours. A prestained protein ladder (PageRuler Plus; Thermo Scientific) was loaded on the first wall of each gel for estimating the size of each detected protein.

The separated proteins were then electro-transferred onto poly(vinylidene difluoride) (PVDF, Amersham Hybond P 0.45 µm polyvinylidene difluoride; GE Healthcare Life Sciences) membrane using transfer buffer solution (200 mM glycine, 25 mM Tris (SigmaAldrich), 0.075% SDS and 10% methanol (Sigma-Aldrich)) at 30 V for 1.5 hours.

Each PVDF membrane was blocked by incubating it in 5% blocking milk solution in Tris Buffered Saline (50 mM Tris hydrochloride (pH 7.4), 150 mM NaCl, 0.1% Tween-20) for 1 hour at room temperature with gentle rocking. The membrane was incubated overnight at 4°C with the primary antibody (appendix 1) with gentle rocking. The next day, the membrane washed three times with TBST, then incubated, with gentle rocking, for 1 hour at room temperature in a solution containing 1:1000 HRP-conjugated anti-rabbit or anti-mouse antibodies, then washed three times in TBST. Immuno-reactive bands in the membrane were detected in FluorChem M Imager by using UptiLight HRP chemiluminescent substrate (Uptima). AlphaView SA software (Protein Simple) was used to quantify the obtained bands by measurement the overall number of pixel grey levels in each band area and then normalize it to the loading control.

3.13.1 Protein assay

Protein concentration was estimated with a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Bovine serum albumin (Sigma-Aldrich) was used as standard and prepared at eight different concentrations from 0.1 and 2 mg/ml. The BCA reagent was prepared by mixing BCA solution with 4% copper (II) sulphate pentahydrate solution (Sigma-Aldrich) in a ratio of 50:1. 100 µL of the prepared reagent was added to 10 µL of the protein lysates and BSA standards and incubated at 37ºC for 30 minutes. The optical density was measured at 570nm by using a BioTek Synergy 2 multi-mode microplate reader. A calibration curve for the protein standard was obtained from the absorbance values of the BSA standard and then analyzed by linear regression.

3.14 Small interfering RNA transfections

Suspensions of OVCAR-4 or COV362 cells in antibiotic-free growth medium were seeded in 96 well plates (5000 cell per well) and incubated overnight at 37 ºC in 5% CO_2. The next day, 1% Dharmafect-1 was prepared in a serum-free modified eagle's minimum essential media (Opti-MEM, Invitrogen) and incubated for 5 minutes at room temperature before mixing with an equal volume of siRNA prepared in serum free Opti-MEM and incubated for 20 minutes at room temperature. The growth medium of the previously seeded cells was replaced with 80 µl of fresh and pre-warmed antibiotic-free medium and 20 µl of the siRNA/DharmaFECT 1 mixture was added wells in the 96 well plate. A non-targeting siRNA (NT-1siRNA), which is designed not to target known genes, was included as control in all transfections experiments. After the addition of transfection mixture, the cells were incubated overnight, the growth medium was replaced and the cells were either exposed to a range of concentrations of different drugs or left untreated. Cell number was assessed by staining with SRB, or capase-3/7 activity was measured as described above. Alternatively proteins or mRNA were measured by western blotting or qPCR.

3.15 Extraction of RNA from cells

RNA was isolated from cells using RNeasy Plus Mini Kit (QIAGEN). Cells were harvested from 96 well plate either as a lysate or cell pellet and mixed with 350 µl Buffer RLT Plus, vortexed for 30 seconds and centrifuged for 3 minutes at high speed. The supernatant was carefully removed by pipetting and transferred to a gDNA Eliminator spin column, centrifuged (10,000 RPM, 30 seconds) and 350 µl 70% ethanol was added to the flow-through. The samples were mixed, transferred to an RNeasy spin column, then centrifuged (10,000 RPM, 15 seconds) followed by discarding the flow-through and addition of 700 µl of RW1 buffer to the RNeasy Mini spin column, centrifuged (10,000 RPM, 15 seconds) and the

flow-through was discarded. Then, 500 µl RPE buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 RPM. The flow-through was removed and another 500 µl RPE buffer was added to the RNeasy spin column and centrifuged for 2 minutes at 10,000 RPM. The RNeasy spin column was centrifuged again at high speed for 1 min to dry the matrix, then the RNeasy spin column was placed in a new 1.5 ml collection tube and 30 µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 10,000 RPM to elute the RNA. The samples were checked for the presence of RNA by using NanDrop reader and kept at -80 ºC.

3.16 Preparation of complementary DNA (cDNA) from RNA

To prepare cDNA, 1.5 µl of oligo-dT (0.5 µg/ µl) was added to 12 µl of each RNA sample and heated for 5 minutes at 65 ºC to denature RNA secondary structure, then chilled for 2 minutes and spun for 10 seconds at 4 ºC. For each sample, 6.5 µl of a mixture containing 4 µl reverse transcriptase buffer, 1 µl of 10 mm dNTP (mixture of dATP, dCTP, dGTP and dTTP), 1 µl nuclease free water and 0.5 µl reverse transcriptase was added, mixed well and centrifuged for 10 seconds at 4 ºC and then heated for 30 minutes at 50 ºC, then for 5 minutes at 85 ºC. cDNA was quantified by reading the ssDNA using a NanoDrop reader and the samples were stored at -80 ºC.

3.17 Quantitative polymerase chain reaction (qPCR)

qPCR was performed to measure the level of gene expression. To duplicate wells, 5 µl of each cDNA template or nuclease-free water as a control was added with 1.25 µl of 1 µM forward and reverse primers (appendix 3) and with 6.25 µl of 1X absolute qPCR SYBR Green ROX Mix (Thermo Scientific) and nuclease-free water were added to each corresponding well of

the tubes that contain cDNA. The reaction mixtures in the 8-tube strips were gently mixed and centrifuged to ensure collection of the samples at the bottom of the tubes and get rid of any air bubbles. Amplification was performed using the following profile: enzyme activation cycle (1 cycle, 15 minutes, 95 ºC), in addition to 40 cycles of denaturation (15 second, 95 ºC), annealing (15 second, 50-60 ºC) and extension (15 second, 72 ºC). At the end of the reaction, the dissociation curve was assessed to confirm that only one amplicon was detected. Results were analyzed according to the comparative cycle threshold (CT) method (ΔΔCT Method). The amount of targeted DNA was normalized to an endogenous reference gene and relative to a calibrator: $2^{-\Delta\Delta C}{}_{T}$

3.18 Optimization of transfection conditions

Transfection conditions were optimized using several different cell lines. COV318, COV362 and FUOV-1 are seeded at 3000, 4500 or 6000 cell per wells in 10% FCS growth medium containing no antibiotics and incubated overnight at 37 ºC in 5% CO_2 before transfection. The next day, transfection reagent was prepared by mixing different ratios of Lipofectamine2000 in antibiotic-free Opti-MEM with DsRed plasmid encoding red fluorescent protein to optimize the best ratio of transfection. Conditions tested included different DNA concentrations ranged from 0.1 to 0.3 µg per well and 1:1-3:1 for Liopfectamine2000:DNA ratio. Lipofectamine2000 combined with the diluted DNA, and incubated at room temperature for 30 minutes, then 50 µl of the DNA:Lipofectamine mixture was added to each well, then the plate was shaken gently and kept in an incubator for 6 hours. After 6 hours, the growth medium was aspirated and changed with 100 µl of growth medium containing no antibiotics and incubated for 48 hours. Transfection was assessed by fluorescent microscopy.

Chapter 4

Antagonism of Bcl-X_L is necessary for synergy between carboplatin and BH3 mimetics in ovarian cancer cells

4.1 Introduction

Despite many endogenous and pharmacological cues to die, cancer cells have developed several mechanisms to block the mitochondrial pathway of apoptosis. Importantly, such alterations in the apoptosis pathway are considered as a major contributor to tumourigenesis (221,222). In the context of cancer therapy, suppression of apoptosis can lead to resistance to anti-cancer agents. Consequently, promoting apoptosis provides a potential avenue to treat drug-resistant disease (223–225).

Apoptosis was first described in 1972, when it was appeared to be implicated in physiological turnover of cells during development and aging, where it was shown to be responsible for pivotal cells elimination during normal embryogenesis (226). Since then apoptosis had been established as a distinct and important form of programmed cell death, which comprises the directed removal of cells. It is characterized by a sequence of changes, including cell shrinkage, nuclear condensation and fragmentation, chromatin aggregation, endonucleolytic DNA degradation, membrane blebbing and disruption of the of organelle structures, which lead to the formation of apoptotic bodies that are then engulfed by neighbouring cells or phagocytes (figure 4.1) (227,228). Apoptosis can also protect tissues in case of immune reactions or when cells are subjected to damage by disease or harmful agents, like irradiation or anti-cancer drugs that leads to DNA damage in some cells. This can result in apoptosis through both *p53*-dependent and independent pathways (229,230). The Bcl-2 family of proteins are critical regulators of the intrinsic apoptosis pathway that eventually affect the integrity of the mitochondrial outer membrane (231).

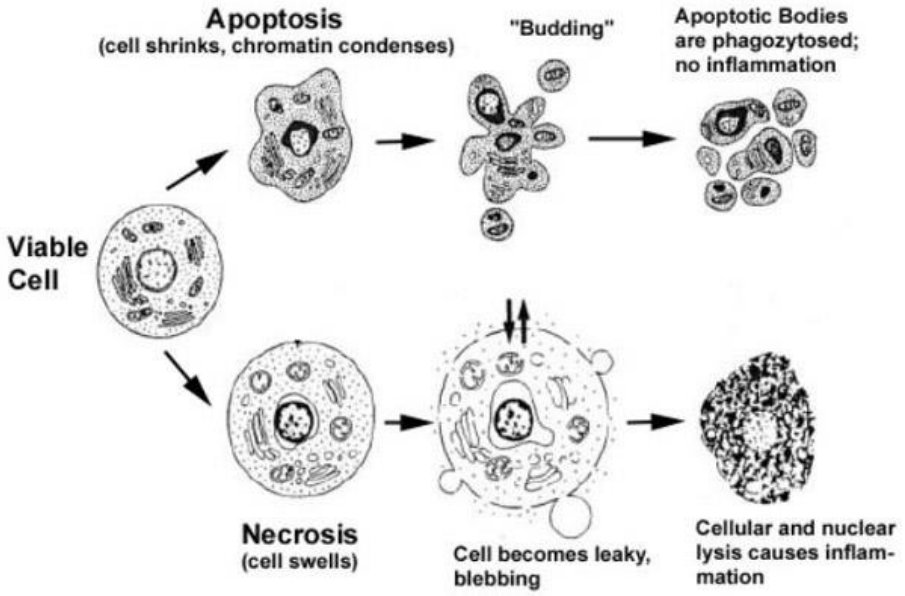


Figure 4.1: Morphological changes during apoptosis compared to necrosis

Necrosis is characterized by cellular swelling, with cellular and nuclear lysis, which eventually proceed to inflammation, while apoptosis is marked by blebbing of the cell membrane with disruption of the structures of organelle, which eventually leads to the formation of apoptotic bodies that are engulfed by the process of phagocytosis, so it does not cause inflammation (232).

4.2 Pathways of apoptosis

Apoptosis can be activated by two main mechanisms, the “intrinsic” pathway which depends on changes in mitochondrial permeability and the “extrinsic” pathway that is mediated by “death receptors” on the cell surface. These pathways trigger a series of enzymes that belong to cysteine aspartyl-specific proteases (caspases), and result in cleavage of the vital proteins and dismantle the cell (figure 4.2) (233,234).

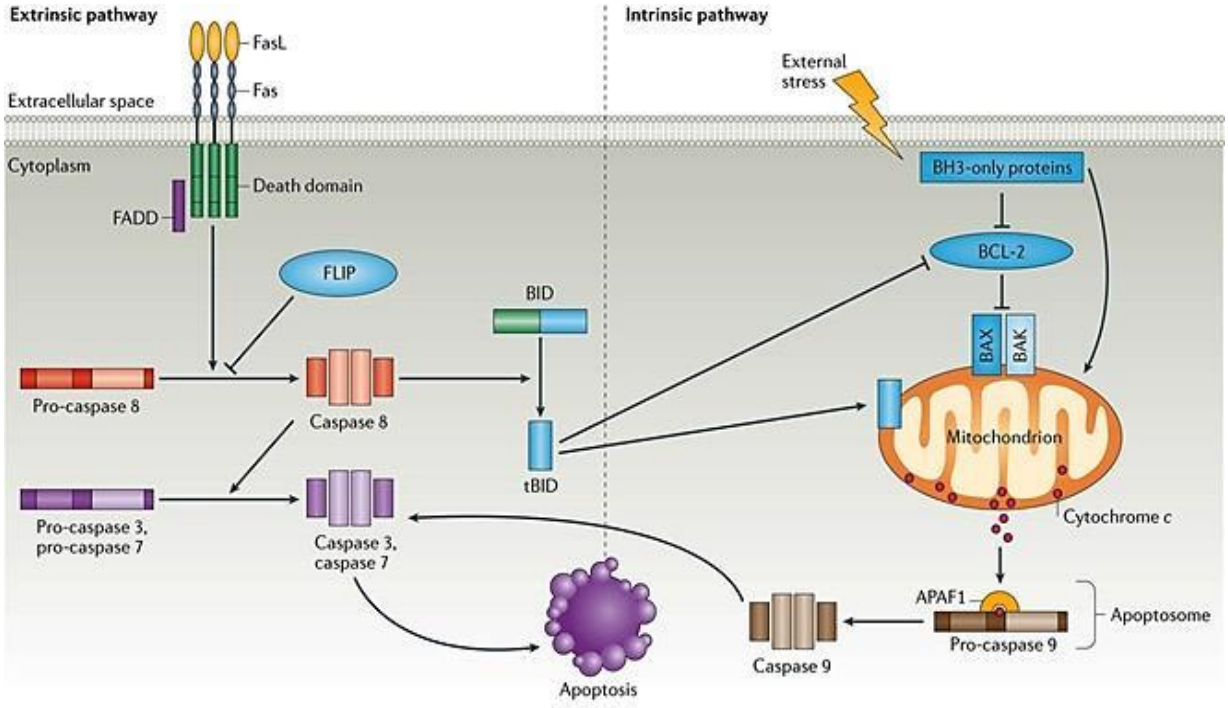


Figure 4.2: The extrinsic and intrinsic pathways of apoptosis From (235)

4.2.1 Extrinsic apoptosis pathway

The typical examples of death receptors that activates the extrinsic pathway are Fas (CD95) or TNF-α receptor 1. Interaction of Fas with its ligand (FasL) or an agonistic antibody generates a death-inducing signalling complex (DISC) that includes the critical adaptor molecule FADD. DISC activates the initiator protease caspase-8 that is activated by autoproteolytic cleavage which in turn can trigger the activation of downstream effector caspases like caspase-3 and caspase-7 that drives apoptosis (236,237).

4.2.2 Intrinsic apoptotic pathway

The intrinsic pathway of apoptosis, which is frequently deregulated in cancer, is engaged by several intracellular stimuli including DNA damage, hypoxia, oxidative stress and the endoplasmic reticulum stress. Such apoptotic stress conditions converge to stimulate

mitochondrial outer membrane permeabilization (MOMP). The pathway depends on the activation of B cell lymphoma-2 (Bcl-2) family proteins, Bax and Bak, which form a pore in the mitochondrial outer membrane (238,239). Increasing MOMP allows the release of soluble proteins (e.g., cytochrome c) from the intermembrane space (IMS) of the mitochondrion into the cytosol, where it binds to apoptotic protease activating factor-1 (APAF-1) to form the apoptosome. The apoptosome attaches and enhances the activation of initiator caspase-9, which then activates caspases-3 and 7. After MOMP and caspase activation, apoptosis occurs often within minutes (240). Additionally, second mitochondria-derived activator of caspase (SMAC) is also released from the mitochondrion in response to cellular stress, which then interferes with the ability of X chromosome-linked inhibitor of apoptosis protein (XIAP) and other inhibitors of apoptosis (IAPs) which, otherwise, block the activation of caspase-3, 7 and 9. Thus, in general, XIAP inhibition has been thought to facilitate apoptosis (241).

4.3 Apoptosis regulation by Bcl-2 protein family

The Bcl-2 family of proteins constitute the key regulators of apoptosis which have shown to be over-expressed in many human malignancies and considered amongst the most important groups of programmed cell death regulators in cancer (242). The complex interactions between pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins controls the commitment to cell death, where they undergo either activation or inhibition depending on MOMP (243–246).

4.3.1 Members of the Bcl-2 family

Three functionally different groups comprise the Bcl-2 family of apoptosis regulators. These are: anti-apoptotic proteins (such as Bcl-2, Bcl-X_L, Mcl-1, Bcl-W and Bcl-2A1), that block the mitochondrial outer membrane permeability and inhibit the intrinsic apoptosis pathway by sequestering the pro-apoptotic proteins (247,248); the pro-apoptotic effector proteins (Bax,

Bak and Bok), which directly increase the permeability of the mitochondrial outer membrane (249,250); the pro-apoptotic "BH3-only" regulator proteins (Bim, Bid, Puma, Bad, Hrk, Noxa and Bmf), which promote activation of the pore-forming members (251). Bcl-2 family members have up to four conserved Bcl-2 homology (BH) domains described as BH1, BH2, BH3, and BH4, which correspond to a-helical segments (figure 4.3) (252–254).

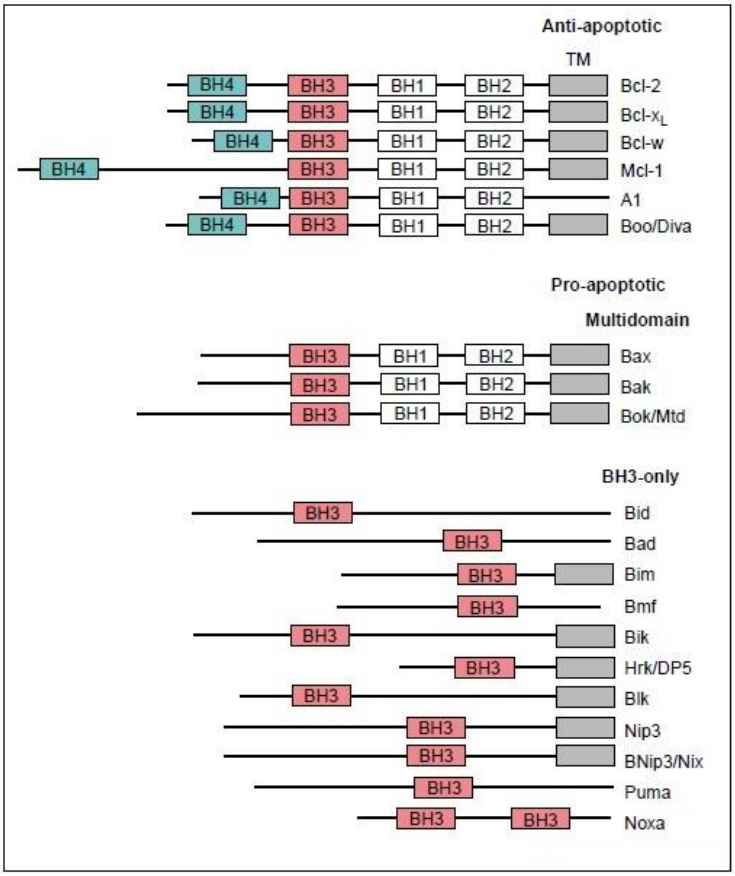


Figure 4.3: The members of Bcl-2 family of proteins

'TM' refers to a hydrophobic region in the carboxyl terminus of several of these proteins that were originally assumed to be a transmembrane domain. From (255).

The anti-apoptotic members of Bcl-2 family contain all of four BH domains subtypes (BH 1-4). They mostly localize at the mitochondrial outer membrane because it possesses a hydrophobic carboxyl terminal end, although they can be found in other sub-cellular

membranes or present in the cytosol (256). Anti-apoptotic Bcl-2 proteins have been shown to have an important cell survival protection role because they can inhibit the response of cells to different apoptotic stimuli (257–259).

The pro-apoptotic members of Bcl-2 family or the "multidomain" proteins (Bax and Bak) contain three BH regions (BH 1-3) and lack the fourth one. They are synthesized as inactive proteins that can be present either at the mitochondria (for Bak) or in the cytosol (for Bax). Following activation, they oligomerize in the mitochondrial membrane and stimulate MOMP without the requirement for any other Bcl-2 proteins member. The essential role of Bax and Bak in MOMP makes their presence crucial for apoptosis and their combined absence generates resistance to MOMP and this results in more cell survival (260). Bcl-2-related ovarian killer (Bok), is similar to the multi-BH domain pro-apoptotic proteins with less widespread expression and has been proposed to function like Bax/Bak (261,262).

The pro-apoptotic BH3-only proteins, contain only the third BH domain and are known as BH3-only proteins. BH3-only proteins bind to multidomain Bcl-2 family members and/or Bax or Bak (222,263). They are considered to promote apoptosis by either directly stimulating Bax or Bak or by preventing Bcl-2 apoptosis inhibitors from sequestering and inhibiting Bax or Bak. Bcl-2 family members also have a role in non-apoptotic processes, such as mitochondrial function, mitochondrial fission and fusion and autophagy (264,265). The properties of the most well-studied members of Bcl-2 family are further summarized in table 4.1 (241).

Two contrasting models have been proposed to explain the mechanism by which Bax and Bak are activated by BH3-only proteins. Both models have in common the concept that the anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-X_L can bind to Bax and Bak, preventing their activation. The direct activation model proposes that BH3-only proteins can be considered as activators or sensitizes. Activators, Bim and the cleaved form of Bid (tBid),

trigger the activation of Bax and Bak by directly binding to Bak or Bax. BH3-only proteins that work as sensitizers (such as Bad) bind to the pro-survival Bcl-2 proteins, antagonising their pro-survival activity (266). The indirect activation model proposes that Bak and Bax can intrinscially activate unless they are continually inhibited by the Bcl-2 family apoptosis inhibitors. In this model, BH3-only proteins trigger the activation of Bax and Bak in an indirect manner by engagement with multiple pro-survival Bcl-2 proteins and prevent them from binding and inhibiting Bax and Bak (267).

Table 4.1: Properties of the Bcl-2 family members

Members	Action	Mechanism of action	Subcellular localization
Bcl-2	Anti-apoptotic	Inhibits apoptosis by preservation of mitochondrial membrane integrity	(i) Outer mitochondrial membrane (ii) Nuclear envelope (iii)Membrane of the endoplastic reticulum(ER)
Bcl-X_L	Anti-apoptotic	Inhibits cytochrome c release, that inhibits activation of the cytoplasmic caspase cascade by cytochrome c	Transmembrane molecule in the mitochondria
Bcl-W	Anti-apoptotic	Reduced cell apoptosis under cytotoxic conditions	Exclusively on the mitochondrion
Mcl-1	Anti-apoptotic	Interaction with Bak1, Noxa, Bcl-2L11, Bcl-2-associated death promoter	Mitochondria, nucleus
Bax	Pro-apoptotic	Release of apoptogenic factors like cytochrome c, activation of caspases	Cytosol
Bak	Pro-apoptotic	Undergoes conformational changes during apoptosis	Integral mitochondrial membrane protein
Bid	Pro-apoptotic	Direct activator of Bax	Cytosol and membrane
Bim	Pro-apoptotic	Free Bim binds to Bcl-2 or Bcl-X_L and inactivates their functions	Free BIM in mitochondria
Bad	Pro-apoptotic	Dephosphorylated Bad forms a heterodimer with Bcl-2 and Bcl-X_L, inactivating them and thus allowing Bax/Bak-triggered apoptosis	Free BAD in mitochondria

A modulator of apoptosis-1 (MAP-1) is another BH3 domain-only pro-apoptotic protein. It interacts with Bcl-2, Bax and Bcl-X_L and itself to form dimers in mammalian cells. MAP-1 is linked with Bax through its BH3 domain, and this association assumed to be responsible for its caspase-dependent pro-apoptotic function, which is obvious upon overexpression (268).

4.4 Evasion from apoptosis

Escaping from apoptosis constitutes one of the major hallmarks of cancer, where it can be a part of cellular responses to stress in order to ensure the survival of cells upon experiencing a stressful stimuli. Such resistance to death may contribute to uncontrolled cell proliferations, tumour progression, carcinogenesis and resistance to anti-cancer agents (269,270).

There are several routes by which cancer cells can suppress apoptosis and these include the imbalance between pro-apoptotic and anti-apoptotic proteins (e.g. over-expression of anti-apoptotic proteins like Bcl-2 or downregulation of pro-apoptotic proteins such as Bak and Bax) or a reduction of caspase function and impaired signalling of death receptor (271). Accordingly, the ratio of pro-apoptotic and pro-survival proteins in each cell plays a crucial role in the determination of death or survival. (272). In particular, increased expression of anti-apoptotic proteins may sequester the BH3 proteins to prevent Bax/Bak activation or they may directly inhibit Bax and Bak (267). Figure 4.4 illustrates mechanisms by which cancer cells can suppress apoptosis (224). Each defect in the pathways of apoptosis can commit pre-neoplastic and neoplastic cells to proliferate, survive in addition to enhancement of the tumour pathogenesis through the activation of proto-oncogenes (273,274).

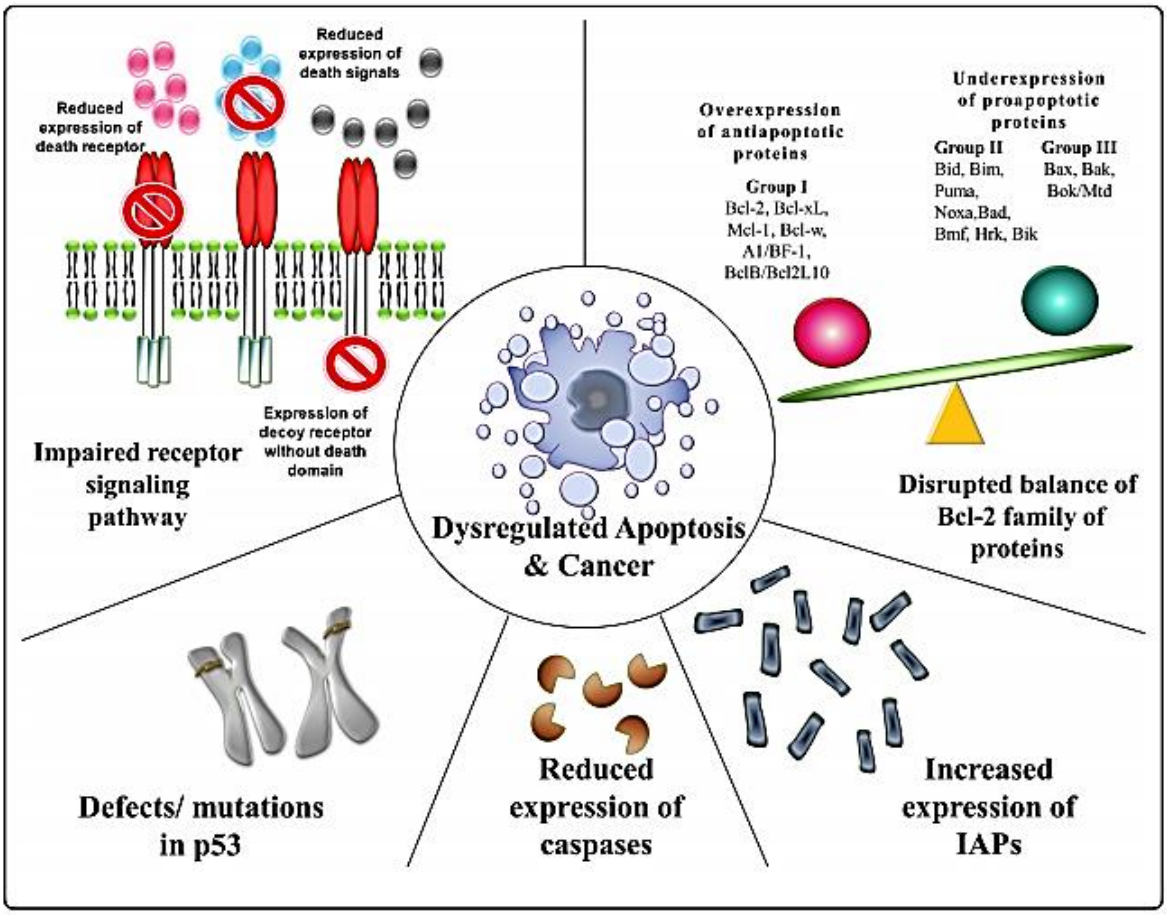


Figure 4.4: Mechanisms contributing to evasion of apoptosis and carcinogenesis

From (224).

4.5 Therapeutic implications of Bcl-2 family proteins

A dampened response to apoptotic stimuli in cancer cells is associated with resistance to chemotherapy (275). The balance between the pro- and anti-apoptotic Bcl-2 family proteins regulate cell survival. For example, augmentation in the expression of pro-survival proteins of Bcl-2 family, Bcl-X_L and Mcl-1 has been observed in different forms of haematopoietic tumours and in certain solid malignancies (268,276). Generally, high expression of the apoptosis inhibitor Bcl-X_L has been linked in many cases to drug resistance (277). High

expression of Bcl-X_L in ovarian cancers is accompanied with shorter disease free intervals after sequential rounds of chemotherapy (278). Additionally, some studies have revealed that overexpression of Bcl-X_L confers resistance to apoptosis stimulated by a different chemotherapeutic agents, including paclitaxel and cisplatin (278,279).

4.5.1 The prospects for therapeutic use of BH3 mimetics

In an effort to develop drugs that can inhibit the pro-survival Bcl-2 family proteins, structural analysis revealed a hydrophobic groove on their surface. This groove serves as a binding site for the protected BH3 domain of BH3-only proteins and plays a crucial role in the ability of pro-survival proteins to bind to and inhibit the pro-apoptotic counterparts. This suggested an approach to develop antagonists of Bcl-2 survival protein by synthesizing compounds that occupy the hydrophobic groove (280). Bcl-2 homology domain 3 mimetics (BH3 mimetics) are the result of extensive work in this field and represent a new class of anti-cancer drugs that mimics the function of BH3-only proteins, by binding to Bcl-2 family proteins. These drugs prevent the anti-apoptotic Bcl-2 family members from binding the BH3 domains of pro-apoptotic proteins and consequentially sensitize cells to apoptosis (281). The introduction of BH3 mimetics in the treatment of cancer is regarded as a (partial) fulfilment of the dream of many oncologists in the development of drugs that are capable of direct promoting apoptosis in cancer cells (282) (figure 4.5).

The best studied BH3 mimetics are ABT-737 (figure 4.6 A) and its orally bioavailable analogue ABT-263 (navitoclax; figure 4.6 B). Both drugs inhibit Bcl-2, Bcl-X_L and Bcl-w but not Mcl-1 or Bcl-2A1. These drugs prevent sequestration of BH3 domain-containing proteins by anti-apoptotic Bcl-2 proteins, and in this manner initiate MOMP and unleash the proteolytic cascade that leads to apoptosis (283,284).

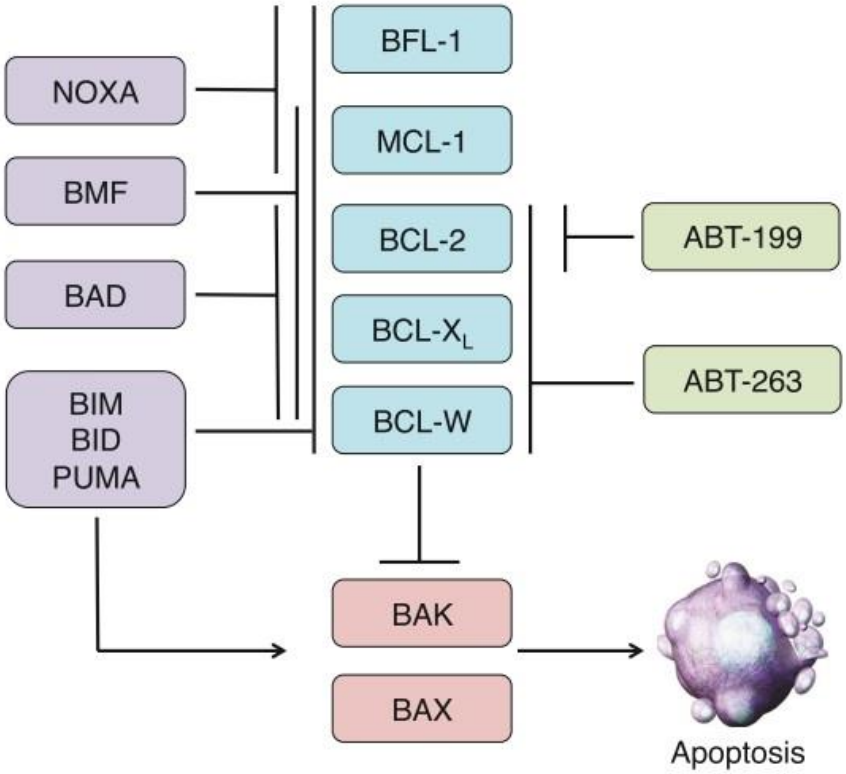


Figure 4.5: The effect of BH3 mimetic drugs on pro-survival and pro-apoptotic proteins

When there is cellular stress, BH3-only proteins either bind to the pro-survival proteins and unleash the pro-apoptotic proteins from sequestration or directly activate Bak and Bax. Once activated, Bak and Bax oligomerize and stimulate MOMP, causing release of other pro-apoptotic substances and eventual apoptosis. BH3 mimetics imitate the actions of the BH3-only proteins (285).The diagram illustrates the selectivity of various BH3 only proteins and BH3 mimetics for different Bcl-2 family apoptosis inhibitors.

Preclinical studies of both ABT-737 and navitoclax are consistent with a BH3-mimetic mechanism of killing (286,287). ABT-737 was effectively used as a single agent in xenograft studies in immunodeficient mice, and the *in vivo* sensitivity to ABT-737 significantly correlated with high expression of the pro-apoptotic protein Bim, and to the extent of its association with Bcl-2 (288). Navitoclax has been shown to induce cell death in lymphoma and leukaemia cell lines, in addition to tumour regression in xenograft studies of multiple myeloma and B-cell lymphoma (289,290). In the majority of these cancers, cells depend on

anti-apoptotic Bcl-2 family proteins for survival and the BH3-only proteins cannot induce apoptosis because they are sequestered by anti-apoptotic proteins. However, these agents displayed potent single agent activity because they unleashed the pro-apoptotic proteins and hence induced cell death. If BH3-only proteins are not highly expressed, these agents display weak single agent activity due to low dependence of the cells on Bcl-2 family apoptosis inhibitors for continual survival (291,292).

Using *in vitro* and xenograft models of ovarian cancer, our group has previously shown that ABT-737 and navitoclax can potentiate cytotoxicity induced by carboplatin. In addition, our research group demonstrated a successful *in vitro* combination of navitoclax with carboplatin and paclitaxel (293,294). These latter drugs are first line chemotherapy for ovarian cancer. This is of great importance in ovarian cancer because the emergence of resistance to chemotherapy prevents successful treatment. This notion suggests BH3-mimetics may be used to treat patients with drug resistant ovarian cancer, especially when Bcl-2 family of apoptosis inhibitors are over-expressed in a drug-resistant form of this disease. However, some studies have shown that cancer cells with elevated levels of Mcl-1, an apoptosis inhibitor that does not bind these drugs with high affinity, exhibited decreased sensitivity to ABT-737 and navitoclax. Likewise, the cells that are initially sensitive to ABT-737 and navitoclax can become resistant following over-expression of Mcl-1 (295–297). This suggests that ABT-737 and navitoclax may be more effective in cancers in which Bcl-2 and/or Bcl-X_L are over-expressed, but not for those that show high expression of Mcl-1. Alternatively they could be used in combination with other anti-cancer drugs that inhibit Mcl-1 function (282). ABT-737 or navitoclax were shown to increase the anti-cancer potency of carboplatin, paclitaxel or PARP inhibitors when used in combination, and this suggests their use in combination with other anti-cancer drugs for the treatment of different kinds of tumour including ovarian cancer (294,298–300).

Navitoclax has entered to clinical trials in human hematologic and solid malignancies (301,302). However, dose-limiting thrombocytopenia was observed in patients. This was likely to be due to the antagonism of the pro-survival function of Bcl-X_L in platelets, resulting in Bak/Bax-dependent platelet apoptosis. Considering that many cancer patients presented with pre-existing thrombocytopenia, particularly in the case of blood malignancies, this created a significant clinical obstacle to further development of navitoclax and quenched the enthusiasm for Bcl-2 inhibitors that bound to several Bcl-2 family members (303,304).

In order to solve this problem, re-engineering of navitoclax to develop a compound that selectively inhibits Bcl-2 protein but not Bcl-X_L led to the synthesis of ABT-199 (venetoclax, figure 4.6 C), which is a highly potent, orally bioavailable drug (305). The anti-cancer activity of venetoclax has been reported in different types of hematologic malignancies, including multiple myeloma, acute myeloid leukaemia, and chronic lymphocytic leukaemia (306–308) and as anticipated, this drug had minimal effect on circulating platelets (309). In addition, further clinical studies in patients with refractory chronic lymphocytic leukaemia have shown that venetoclax does not result in significant thrombocytopenia in cancer patients (310–313), where a single dose of venetoclax resulted in immediate antileukemic activity within one day, which allowed its successful development for the treatment of chronic lymphocytic leukemia. This support the hypothesis that "selective inhibition of Bcl-2 has the potential for the treatment of Bcl-2 dependent hematological tumours without triggering thrombocytopenia" (310). Additionally, venetoclax suppressed the growth of cancer in several preclinical xenograft models of human blood malignancies and showed additive efficacy when used in combination with standard chemotherapy (310). *In vitro* cell culture models showed that venetoclax used the mitochondrial pathway of apoptosis to elicit its anti-cancer effect in hematopoietic cells with high Bcl-2-expression, but it was not effective in Bcl-X_L dependent cancer cells. Also, the *in vivo* potency of venetoclax for solid tumours with high Bcl-X_L

expression is still unknown (310). Furthermore, this drug has not been used for the treatment of ovarian cancer. Initial studies in our laboratory suggested relatively low expression of Bcl-2 protein in ovarian cancer cell lines compared to other members of anti-apoptotic proteins, particularly Bcl-X_L and Mcl-1, which are not potently inhibited by venetoclax. This expression pattern has also been reported in clinical samples of ovarian cancer (279) and in the cancer genome atlas which shows amplified Bcl-2 or upregulated mRNA in less than 3 % of all cases in comparison to Bcl-X_L (14%), Mcl-1 (14%) and Bcl-W (12%) (314). This qustioned whether venetoclax would be useful in the treatment of ovarian cancer. Instead, we postulated that a selective Bcl-X_L inhibitor would be preferable, although strategies to overcome the expected thrombocytopenia are likely to be required for clinical use.

WEHI-539 (figure 4.6 D), a selective Bcl-X_L inhibitor (315), was therefore considered for evaluation in ovarian cancer. WEHI-539 is a new BH3 mimetic that has 400-fold greater affinity for Bcl-X_L than other members of anti-apoptotic Bcl-2 family. It provides the first highly selective Bcl-X_L antagonist that does not potently inhibit Bcl-2 and Bcl-W. This distinguishes it from other inhibitors such as navitoclax and ABT-737, which inhibit both Bcl-2 and Bcl-X_L. WEHI-539 also can evoke Bax/Bak-dependent apoptotic responses (316). It was hypothesized that WEHI-539 would potentiate the activity of carboplatin in ovarian cancer cells, but venetoclax would not. ABT-737, which is less selective Bcl-2 family antagonist (Bcl-2, Bcl-X_L and Bcl-w inhibitor) was also included in this study as a comparator because we have already demonstrated its synergy with carboplatin.

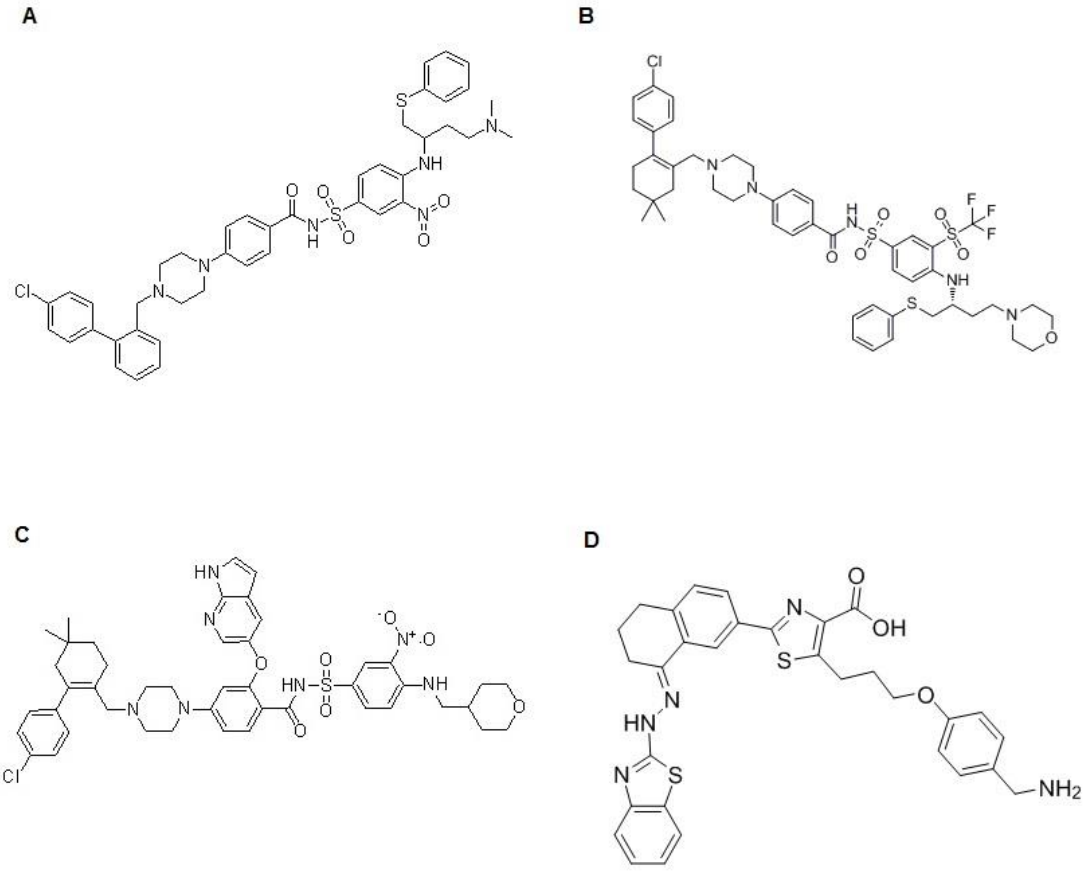


Figure 4.6: Chemical structures of some BH3 mimetics

Chemical structures of some BH3 mimetics including A. ABT-737, B. navitoclax, C. venetoclax and D. WEHI-539 (317–320).

4.6 Aim of the study

The study in this chapter was to evaluate different BH3 mimetics, which differ in their Bcl-2 family target's selectivity. In particular, the goal was to confirm the hypothesis that WEHI-539, but not the platelet sparing BH3 mimetic venetoclax, would synergize with carboplatin.

4.7 Results

4.7.1 Expression of BCL-2 family proteins and single agent activity

Before exploring the efficacy of drug combinations of carboplatin and BH3 mimetics, the expression of Bcl-2 family proteins (Bcl-2, Bcl-X_L, Mcl-1 and Bcl-w) and the activity of single agent of the three BH3 mimetics (ABT-737, venetoclax and WEHI-539) in cell growth assays were evaluated. The expressions of Bcl-2, Bcl-X_L, Mcl-1 and Bcl-w were measured in 8 ovarian cancer cell lines including OVCAR-4, OVCAR-8, OVSAHO, Igrov-1, OVCAR-3, FUOV-1, COV362 and COV318 (figure 4.7). In three of these cell lines (OVCAR-8, OVCAR-3 and Igrov-1) synergy between carboplatin and ABT-737 has previously been observed (294). These cells were supplemented with five other cell lines, which have recently been reported to show close similarity to the high grade serous ovarian carcinoma profile (321). OVSAHO, COV362, OVCAR-4, COV318 and FUOV-1 cells are likely HGSOC cells, whereas Igrov-1 cells are hypermutated and definitely do not represent HGSOC cells. Full description of each cell line is illustrated in materials and methods, table 3.1.

Bcl-2 expression was highly variable; although it was detectable in five cell lines it was only prominent in three cell lines, OVCAR-4, FUOV-1 and COV362. Bcl-X_L was readily detected in all cell lines. Mcl-1 showed notable expression in five cell lines to a varying degree, and Bcl-w was mostly expressed significantly in Igrov-1 cell line (figure 4.7).

In cell growth assays, ABT-737, venetoclax and WEHI-539 inhibited the growth of cultured cells of each cell line with microMolar potencies (table 4.2). There was not a substantial variation in potency of these drugs between the different cell lines, with the most prominent difference being a six-fold difference in IC_{50} in COV318 and COV362 cells.

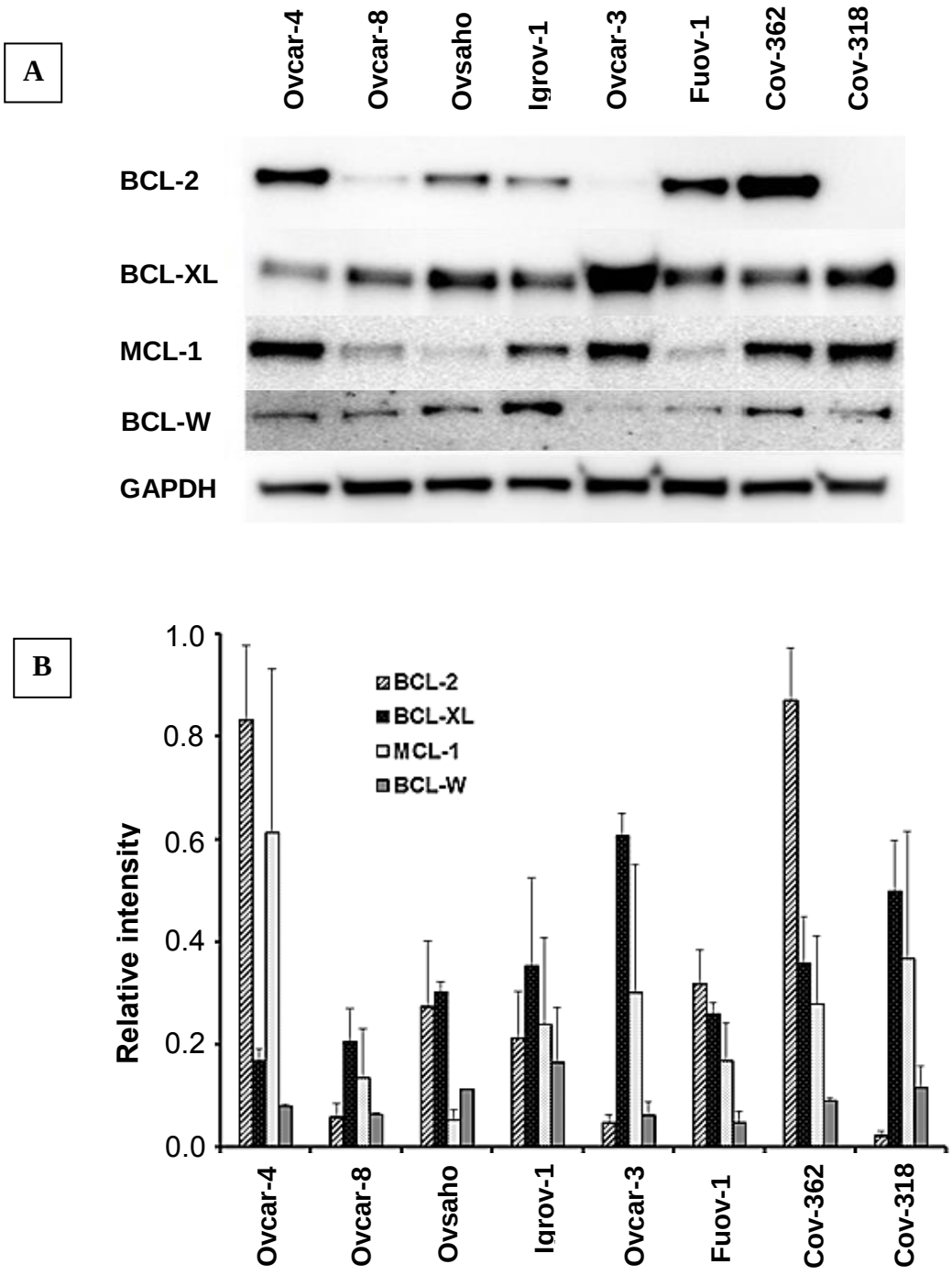


Figure 4.7: Expression of Bcl-2 family members

A. Lysates were obtained from the eight indicated ovarian cancer cell lines and the expressions of Bcl-2, Bcl-X_L, Mcl-1 and Bcl-w were determined by immunoblotting. The experiment shown in the figure is representative of three different experiments. B. The proteins in western blot experiments were quantified (mean ± S.D., n = 3) by normalization of the intensity of the signal for each band to the corresponding signal intensity of GAPDH in the cell line and in the same blot.

Table 4.2: Activity of BH3 mimetics in ovarian cancer cell lines cell growth assays

	IC_{50} (μM)		
Cell line	**ABT-737**	**venetoclax**	**WEHI-539**
OVCAR-8	11 ± 5 (12)	18 ± 6 (13)	9 ± 5 (11)
OVCAR-3	10 ± 4 (11)	15 ± 5 (12)	9 ± 4 (14)
IGROV-1	11 ± 1 (12)	14 ± 8 (14)	16 ± 12 (12)
COV-362	17 ± 3 (10)	4 ± 2 (9)	12 ± 2 (9)
OVCAR-4	14 ± 5 (8)	15 ± 2 (7)	26 ± 9 (7)
OVSAHO	7 ± 1 (10)	17 ± 9 (6)	14 ± 1 (6)
COV-318	20 ± 3 (5)	24 ± 2 (3)	23 ± 5 (3)
FUOV-1	13 ± 1 (4)	14 ± 6 (3)	27 ± 4 (3)

The activity of ABT-737, venetoclax and WEHI-539 in ovarian cancer cell lines cell growth assays. Cells were exposed to ABT-737, venetoclax or WEHI-539 for 72 hours and the surviving cells were estimated by staining with SRB stain. The results are expressed as mean ± S.D. for the indicated number of experiments shown in parentheses.

4.7.2 Drug combination studies

To explore whether ABT-737, venetoclax and WEHI-539 were synergistic when used in combination with carboplatin in cell growth assays, drug combination studies were performed in six ovarian cancer cell lines: OVCAR-8, OVCAR-3, Igrov-1, COV362, OVCAR-4 and OVSAHO. In these studies, the BH3 mimetic was used at a concentration equal to 5% of IC_{50} measured previously and combined simultaneously with a range of carboplatin concentrations. The BH3 mimetics were used at a fixed concentration because at high concentrations these agents are cytotoxic through a mechanism which does not depend on inhibition of Bcl-2 family members (294). Additionally, ABT-737 is insoluble at high concentrations (>80 μM).

ABT-737 (which inhibits Bcl-2 and Bcl-X_L proteins) was synergistic when used with carboplatin in three cell lines (figure 4.8) including, as observed previously, in OVCAR-8 and Igrov-1, in addition to COV362. Significant synergy has also previously been reported in OVCAR-3 cells (294), but in this study this did not reach statistical significance. WEHI-539, the Bcl-X_L selective agent, also resulted in significant synergy when combined with carboplatin in the same cell lines in addition to OVCAR-3. In OVCAR-4 and OVSAHO cells, synergy between BH3 mimetics and carboplatin was not observed. On the other hand, the Bcl-2 selective agent, venetoclax, did not show synergistic effect when used with carboplatin in any of the studied cell lines and was even antagonistic with carboplatin in three cell lines (OVCAR-8, Igrov-1 and OVSAHO).

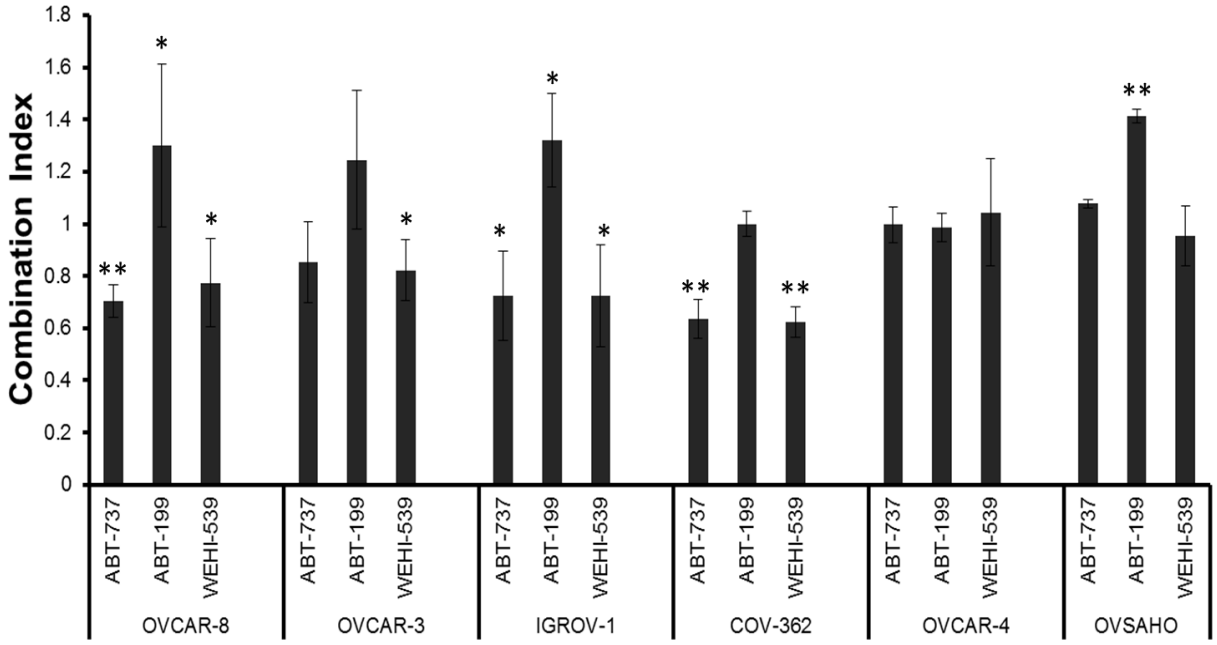
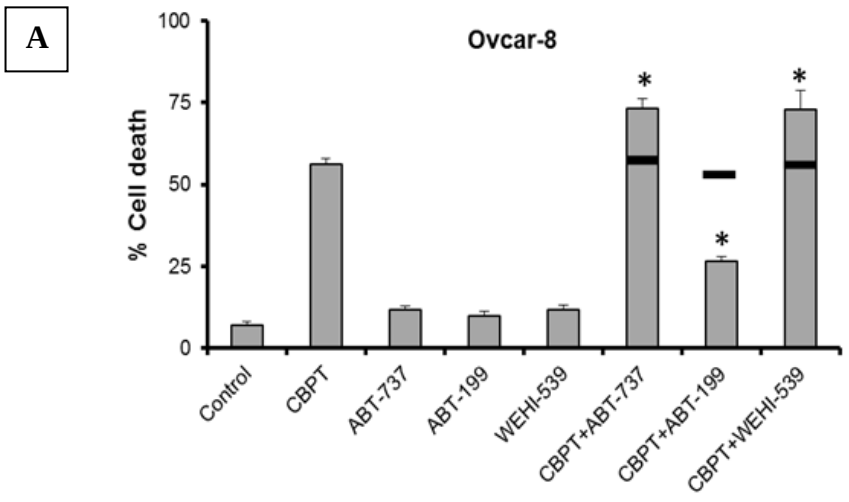


Figure 4.8: Evaluation of drug combinations in cell growth assays

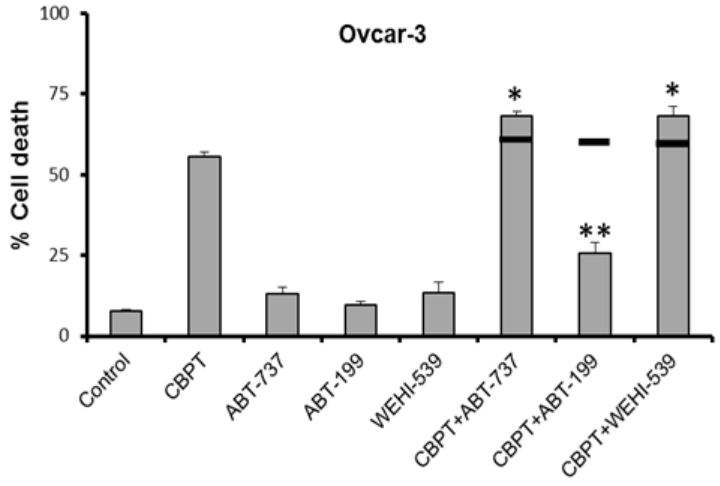
The indicated cell lines were exposed to a combination of carboplatin and either ATP-737, ATP-199 or WEHI-539 for 72 hours. The surviving number of cells was determined by staining with SRB stain. Combination indices (mean ± S.D., no. of experiments=3) at fraction affected = 0.5 were calculated using Chou and Talalay equation and were shown to be significantly different from additivity (CI = 1.0) where indicated (*P ≤ 0.05 and **P ≤ 0.01; paired t-test).

4.7.3 Cell viability assay and morphology of cells after treatment

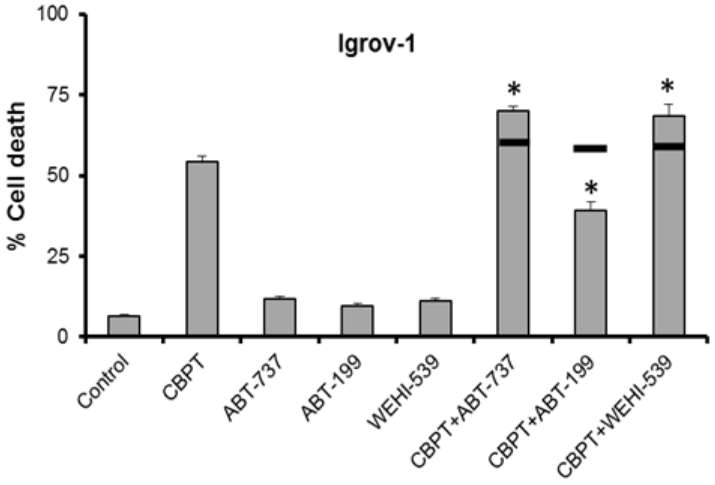
To confirm the previous results, the influence of the drug combinations on the viability of cells was assessed by trypan blue staining (figure 4.9). The results were broadly consistent with those obtained in cell growth assays. In OVCAR-8, COV362, Igrov-1 and Ovcar-3 cells, using carboplatin with ABT-737 or WEHI-539 resulted in significantly more cell death than that expected effect if the drugs acted additively and calculated by the Bliss independence criterion. Although synergy was not observed in OVSAHO cells in cell growth assays, supra-additive effects of carboplatin and ABT-737 or WEHI-539 were observed in cell viability assays with this cell line. Notably, OVSAHO cells express a reasonably high level of Bcl-X_L protein in comparison to Mcl-1 (figure 4.7). In contrast, the combination of venetoclax and carboplatin did not result in supra-additive effects in any of the studied cell lines and in OVCAR-8, Igrov-1 and OVSAHO the carboplatin and venetoclax drug combinations resulted in an antagonistic effect. Morphological evaluation of the cells by microscopy after 48 hours of treatment with drugs (figures 4.10 A, B, C, D, E and F) was consistent with the results obtained by trypan blue staining.



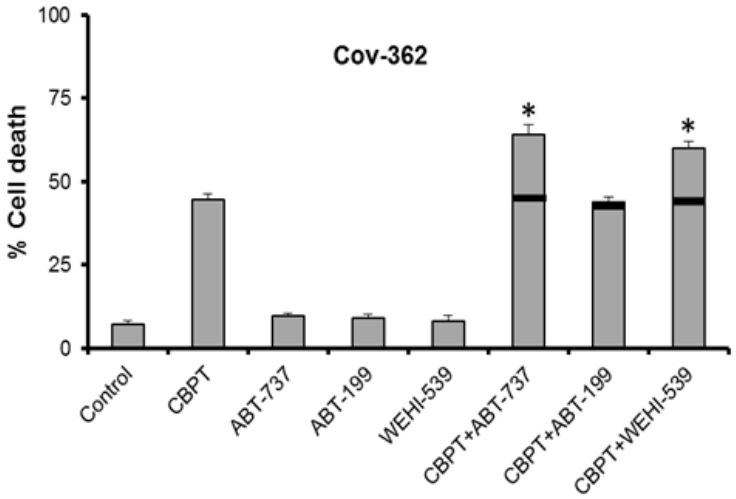
B


Ovcar-3
% Cell death
100
75
50
25
0
*
**
*
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539

C


Igrov-1
% Cell death
100
75
50
25
0
*
*
*
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539

D


Cov-362
% Cell death
100
75
50
25
0
*
*
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539

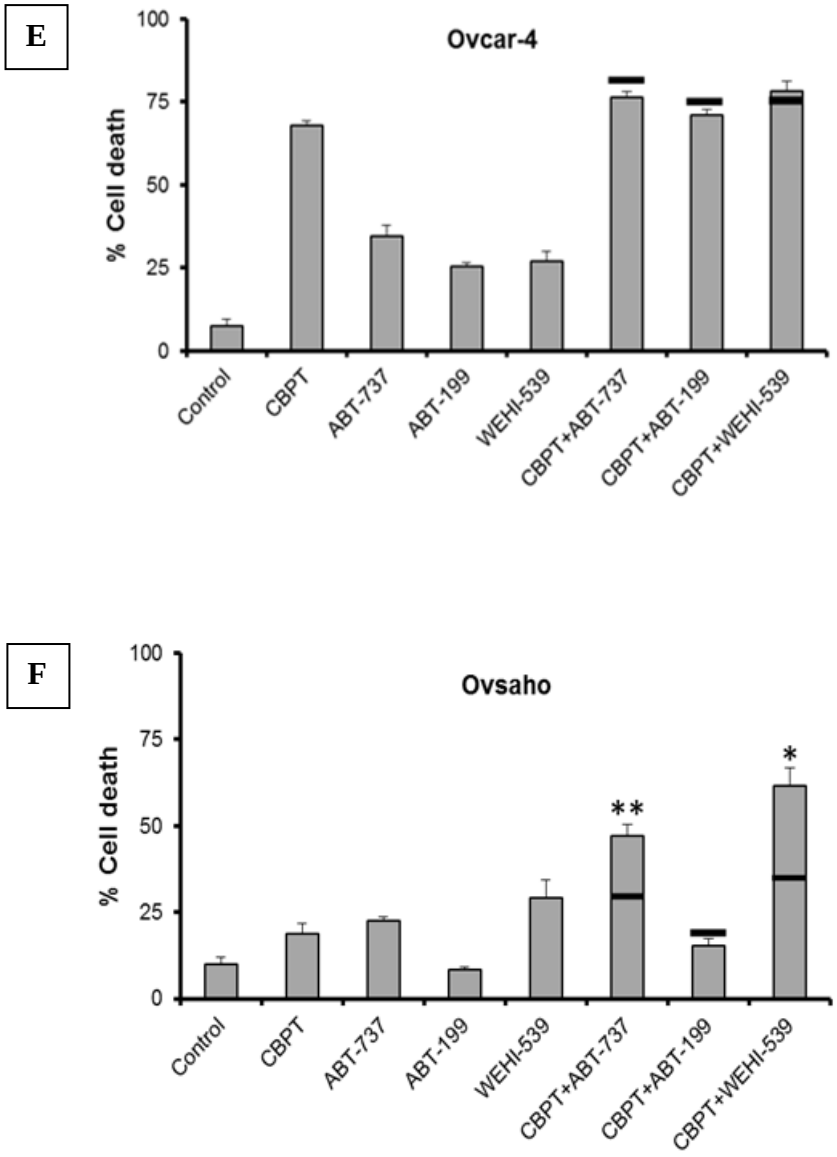
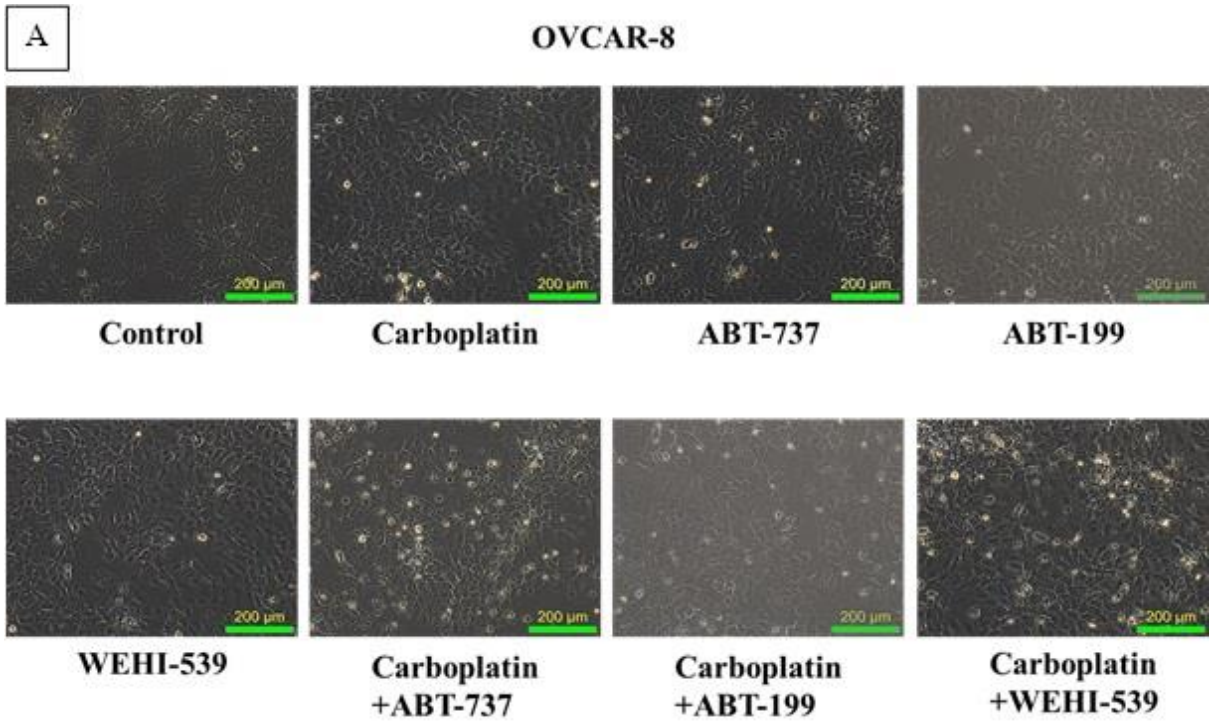
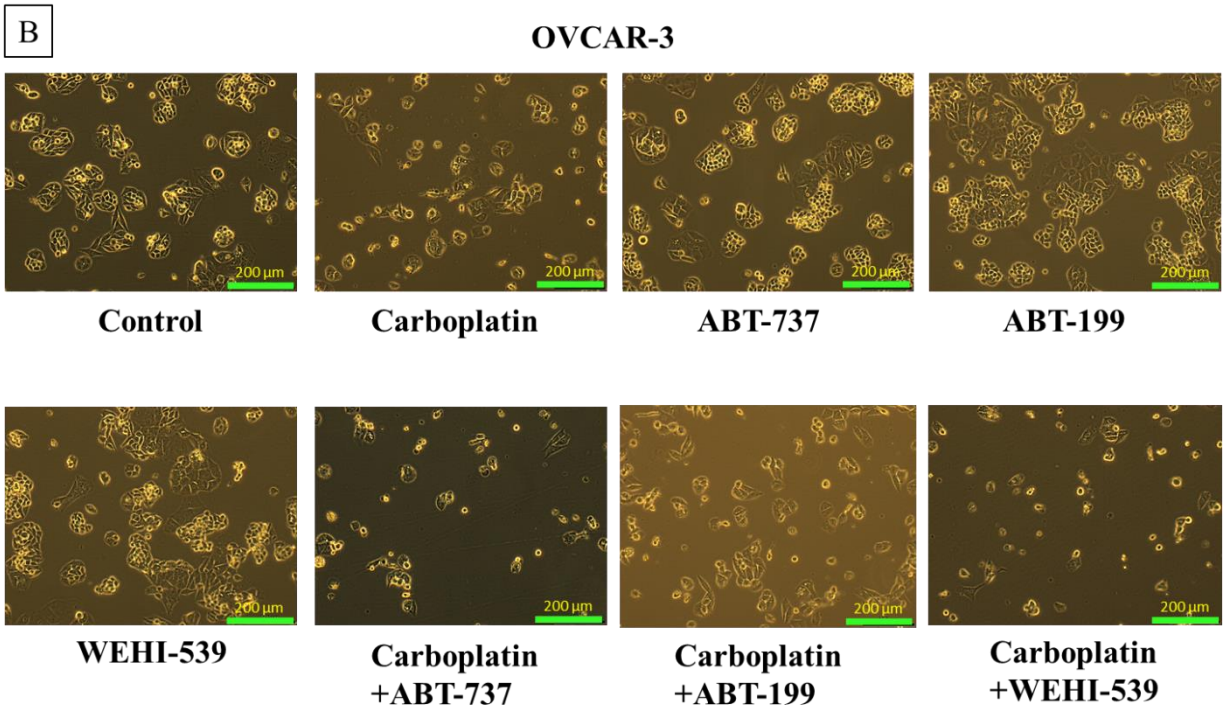


Figure 4.9: The effect of BH3 mimetic combinations on cell viability

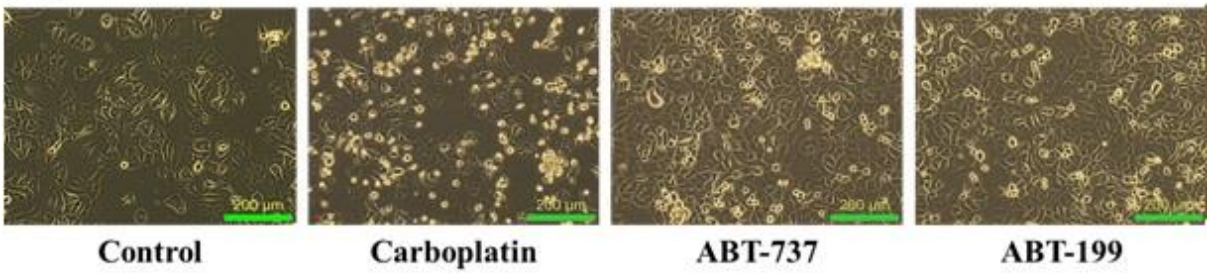
The indicated cells (A. OVCAR-8, B. OVCAR-3, C. Igrov-1, D. COV362, E. OVCAR-4 and F. OVSAHO) were treated with 66 µM carboplatin, 1 µM ABT-737, 3 µM venetoclax or 0.3 µM WEHI-539 or a combination of carboplatin and the indicated BH3 mimetics as shown, for 48 hours (24 hours for OVCAR-4). Cell death was determined by microscopy after staining of the cells with trypan blue. The data (mean ± SD, n = 3) are expressed as a fraction of the number of live cells that were measured after exposure to with drug vehicle. The expected effect of the combination assuming the drugs interacted additively is indicated as a solid horizontal line on the bar chart and was calculated using the Bliss independence criterion. There is a significant difference between the expected additive effect and the experimental results where indicated (*$P \leq 0.05$ and **$P \leq 0.01$, paired t-test).


A
OVCAR-8
200 µm
Control
Carboplatin
ABT-737
ABT-199
WEHI-539
Carboplatin
+ABT-737
Carboplatin
+ABT-199
Carboplatin
+WEHI-539

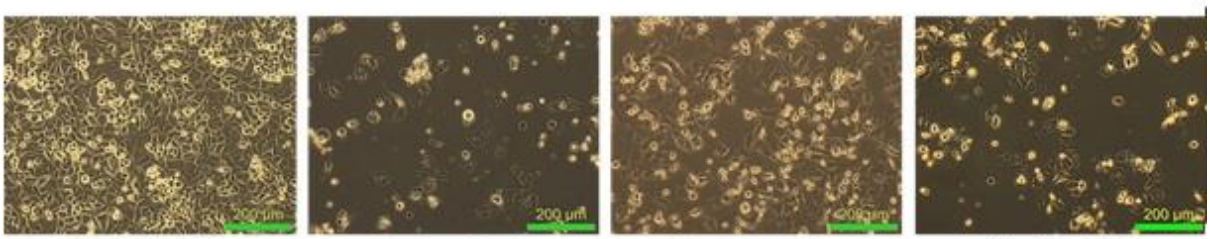

B
OVCAR-3
200 µm
Control
Carboplatin
ABT-737
ABT-199
WEHI-539
Carboplatin
+ABT-737
Carboplatin
+ABT-199
Carboplatin
+WEHI-539

C

Igrov-1



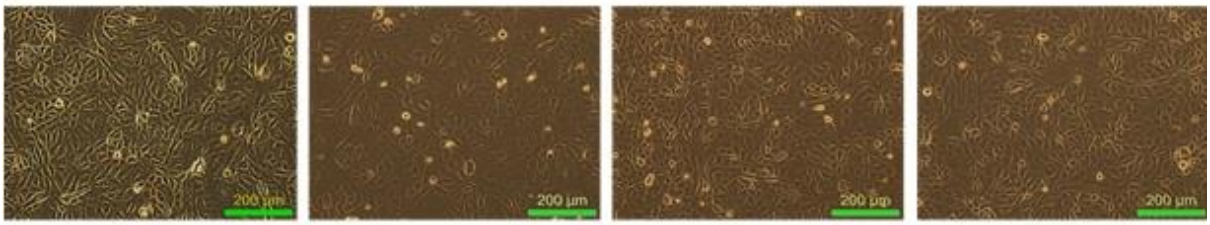
Control | Carboplatin | ABT-737 | ABT-199



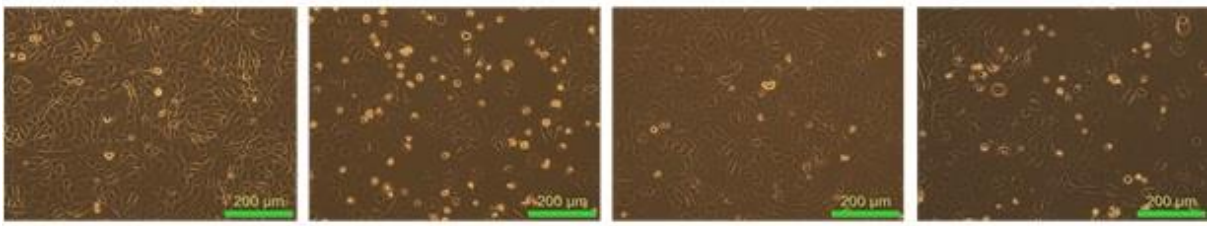
WEHI-539 | Carboplatin +ABT-737 | Carboplatin +ABT-199 | Carboplatin +WEHI-539

D

COV362



Control | Carboplatin | ABT-737 | ABT-199



WEHI-539 | Carboplatin +ABT-737 | Carboplatin +ABT-199 | Carboplatin +WEHI-539

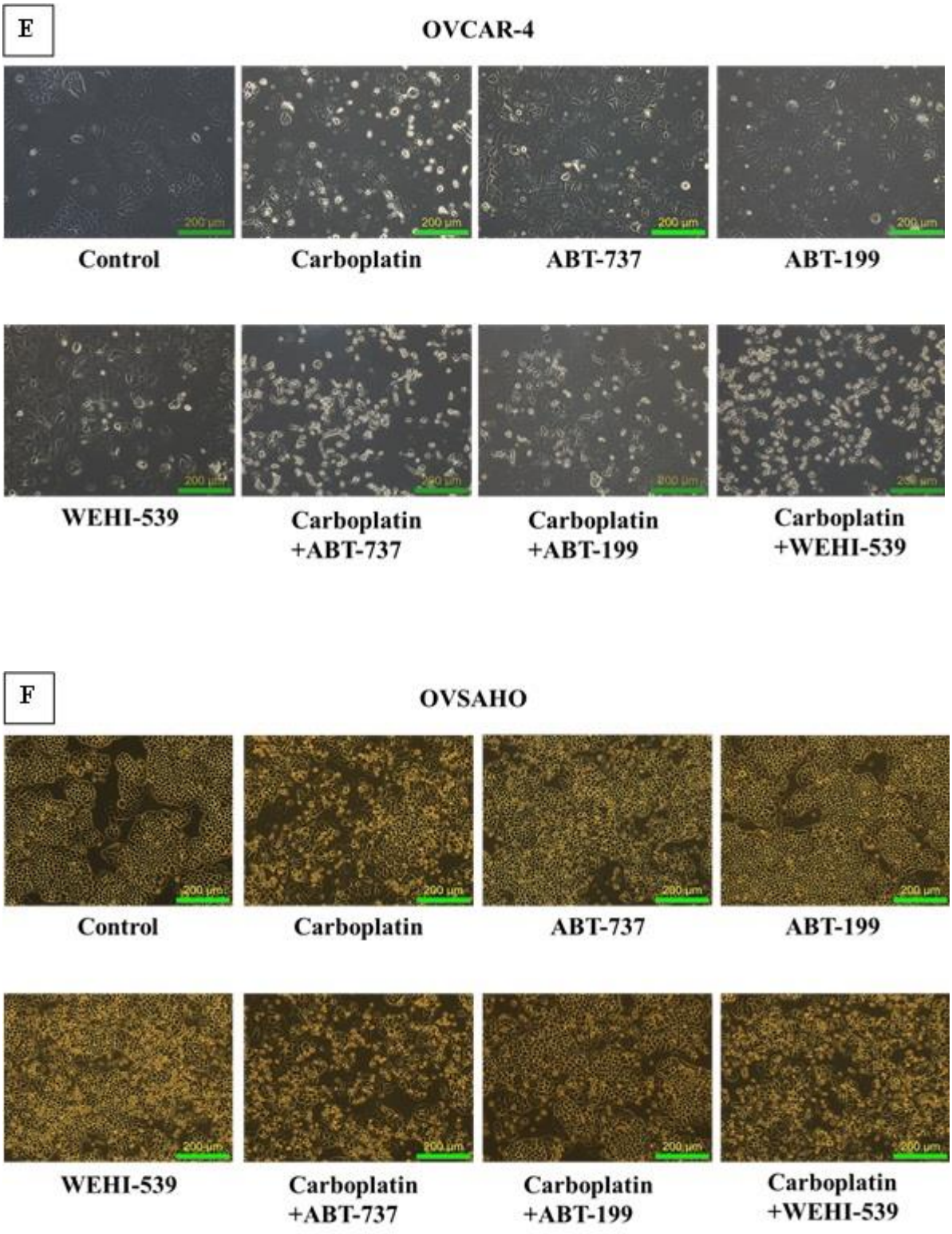
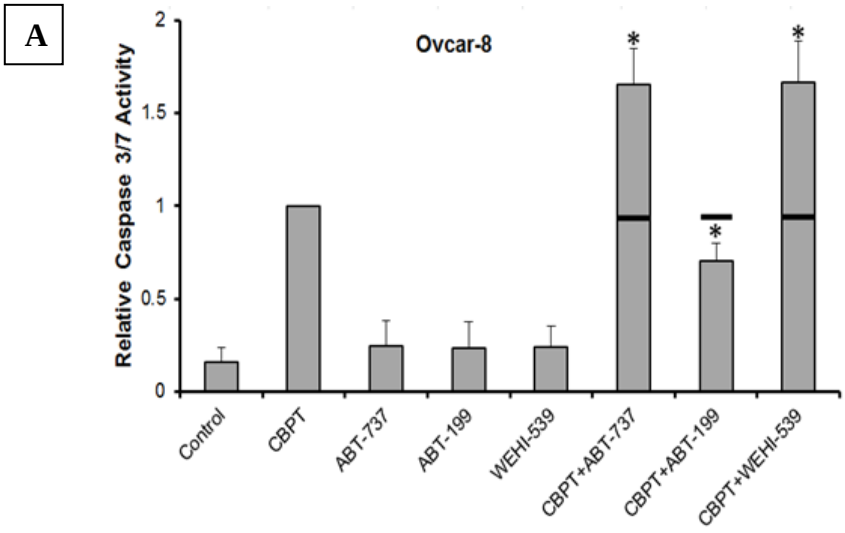


Figure 4.10: Morphological appearance of cells under light microscope

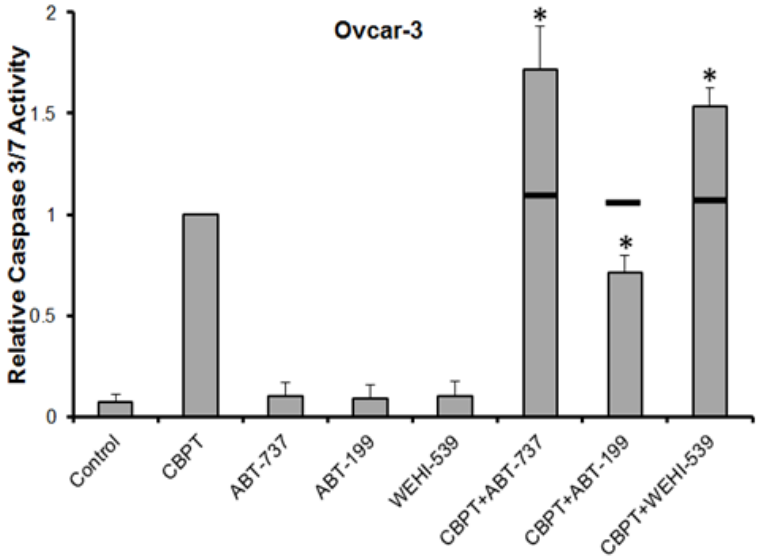
The indicated cells were treated with drugs as described in figure 4.9 Photographs were taken after 48 hours of drug exposure.

4.7.4 Caspase 3/7 activity assay

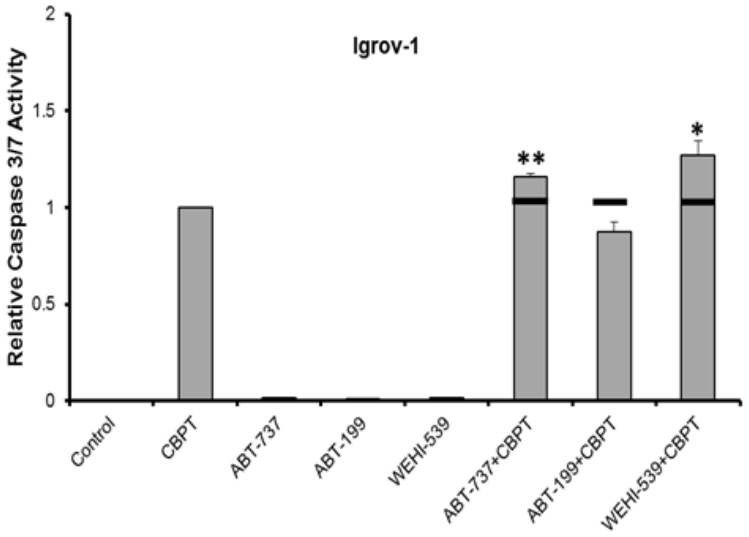
In addition to the synergistic effect between carboplatin and the less selective Bcl-2 family inhibitors in cell growth assays, previous work in our lab has shown that navitoclax and ABT-737 potentiate apoptosis induced by carboplatin. To explore the effect of the selective BH3 mimetics in combination with carboplatin, caspase 3/7 activity, Annexin V/Propidium iodide staining and PARP cleavage were assessed. Caspase 3/7 activation by the combination of drugs was reminiscent of the activity of drugs on cells. In five out of six cell lines, the combinations of either ABT-737 or WEHI-539 with carboplatin significantly augmented caspase-3/7 activity more than the expected additive effect of combination calculated using the Bliss independence criterion. In the OVCAR-4 cell line, which has relatively low expression of Bcl-X_L protein (figure 4.7) and in which in cell growth assays synergy was not observed (figure 4.8), the effect of combinations of carboplatin with ABT-737 or WEHI-539 on caspase-3/7 activity did not result in significant differences from additivity. In contrast to the synergy observed between ABT-737 and WEHI-539 (which both inhibit Bcl-X_L), synergistic activation of caspase 3/7 was not observed with combinations of carboplatin and the Bcl-2 selective inhibitor venetoclax in any of the studied cell lines (figure 4.11). Indeed, antagonism was observed in OVCAR-8 and OVCAR-3 cells.



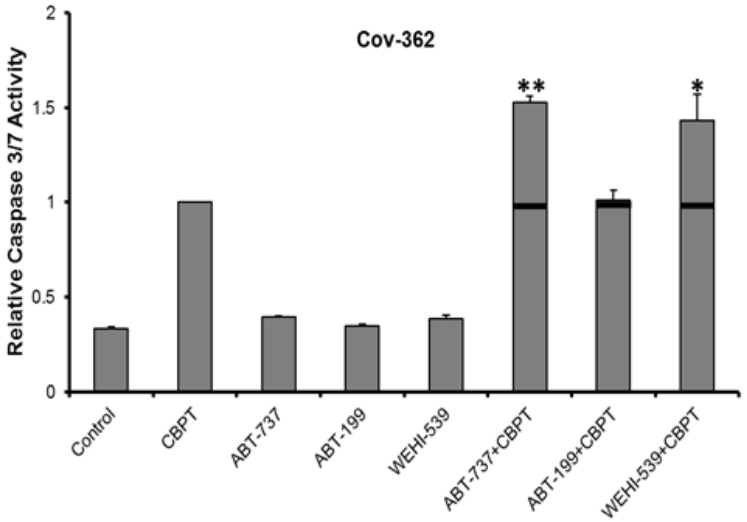
B


Ovcar-3
Relative Caspase 3/7 Activity
2
1.5
1
0.5
0
*
*
*
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539

C


Igrov-1
Relative Caspase 3/7 Activity
2
1.5
1
0.5
0
**
*
Control
CBPT
ABT-737
ABT-199
WEHI-539
ABT-737+CBPT
ABT-199+CBPT
WEHI-539+CBPT

D


Cov-362
Relative Caspase 3/7 Activity
2
1.5
1
0.5
0
**
*
Control
CBPT
ABT-737
ABT-199
WEHI-539
ABT-737+CBPT
ABT-199+CBPT
WEHI-539+CBPT

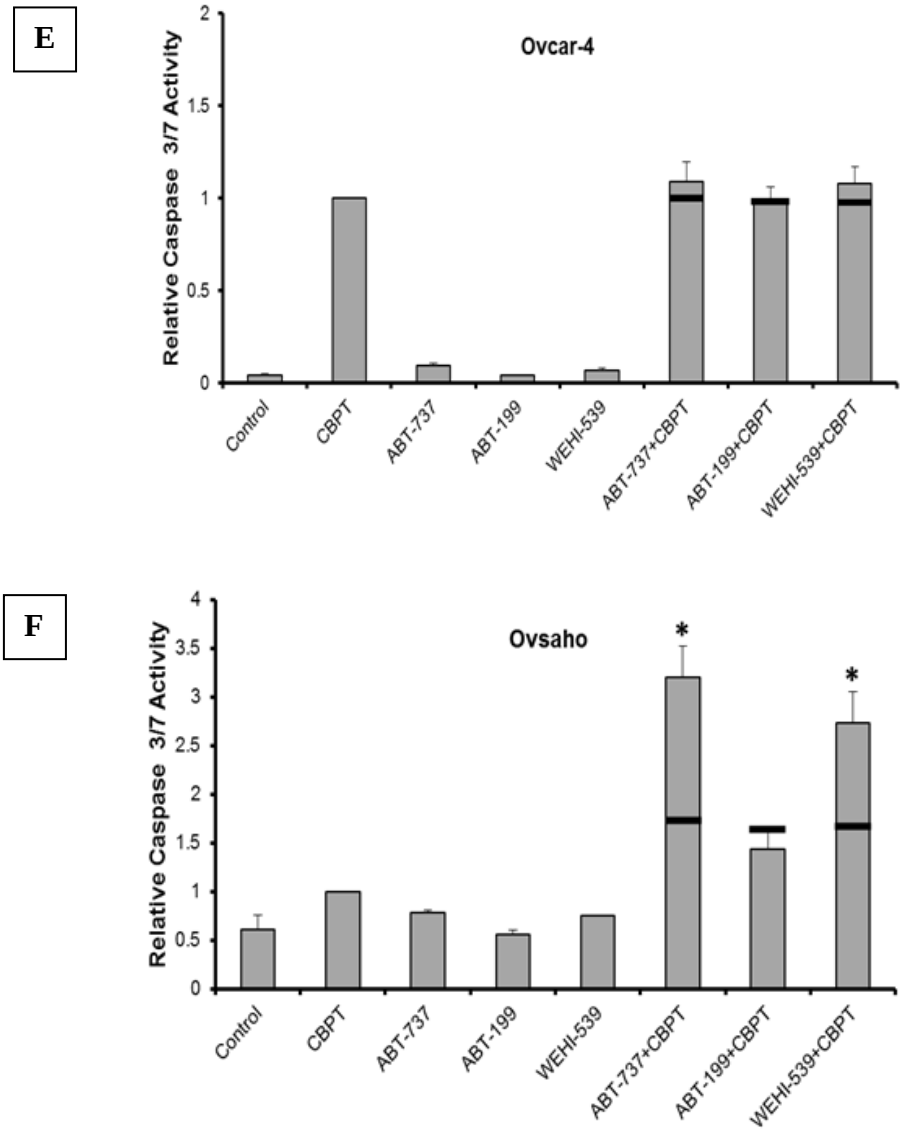


Figure 4.11: The effect of drug combinations on caspase 3/7 activity

The indicated cell lines (A. OVCAR-8, B. OVCAR-3, C. Igrov-1, D. COV362, E. OVCAR-4 and F. OVSAHO) were treated with 66 µM carboplatin, 1 µM ABT-737, 3 µM venetoclax or 0.3 µM WEHI-539 or a combination of carboplatin and the indicated BH3 mimetics as shown, for 48 hours, then the activity of caspase 3/7 was measured. The results in the figures are expressed as a fraction of the activity of caspase 3/7 measured in cells treated with carboplatin only and were normalized to the viable cell number determined by a cell growth assay by staining a parallel sample of cells with SRB stain. The experimental results (mean ± S.D., n = 3) were significantly different from the expected additive effect of the drug combination (indicated with a horizontal solid line on the bar chart) and calculated from the Bliss independence criterion and the effect of the single agents where indicated (*$P \leq 0.05$ and **$P \leq 0.01$, paired t-test).

4.7.5 PARP cleavage assay

As single agents, ABT-737, venetoclax or WEHI-539 did not induce noticeable PARP cleavage in OVCAR-8, Igrov-1, OVCAR-3, and COV362 cells, (figure 4.12). However, venetoclax (Bcl-2 selective) induced cleavage of PARP in only OVCAR-4 cell line, which express the least amount of Bcl-X_L of the cell lines tested (figure 4.7). ABT-737 and WEHI-539 (which both inhibit Bcl-X_L) as single agents resulted in obvious PARP cleavage in OVSAHO and OVCAR-4 and cells (figure 4.12). Notably, in OVSAHO cells, in comparison to other cell lines, the ratio of Bcl-X_L expression in comparison to Mcl-1 was relatively high (figure 4.7).

When used in combination with carboplatin, ABT-737 and WEHI-539 increased the cleavage of PARP cleavage induced by carboplatin, especially in the cells (e.g. OVCAR-8, Igrov-1, COV-362) where the synergistic effect with carboplatin was prominent in the cell growth assay, trypan blue or caspase 3/7 activation assays. The combination of ABT-737 and carboplatin also induced very significant PARP cleavage in OVCAR-4 cells; however, the single agents have also resulted in significant PARP cleavage in these cells (figure 4.12). In contrast to ABT-737 and WEHI, venetoclax (Bcl-2 selective) was unsuccessful in augmenting the cleavage of PARP induced by carboplatin.

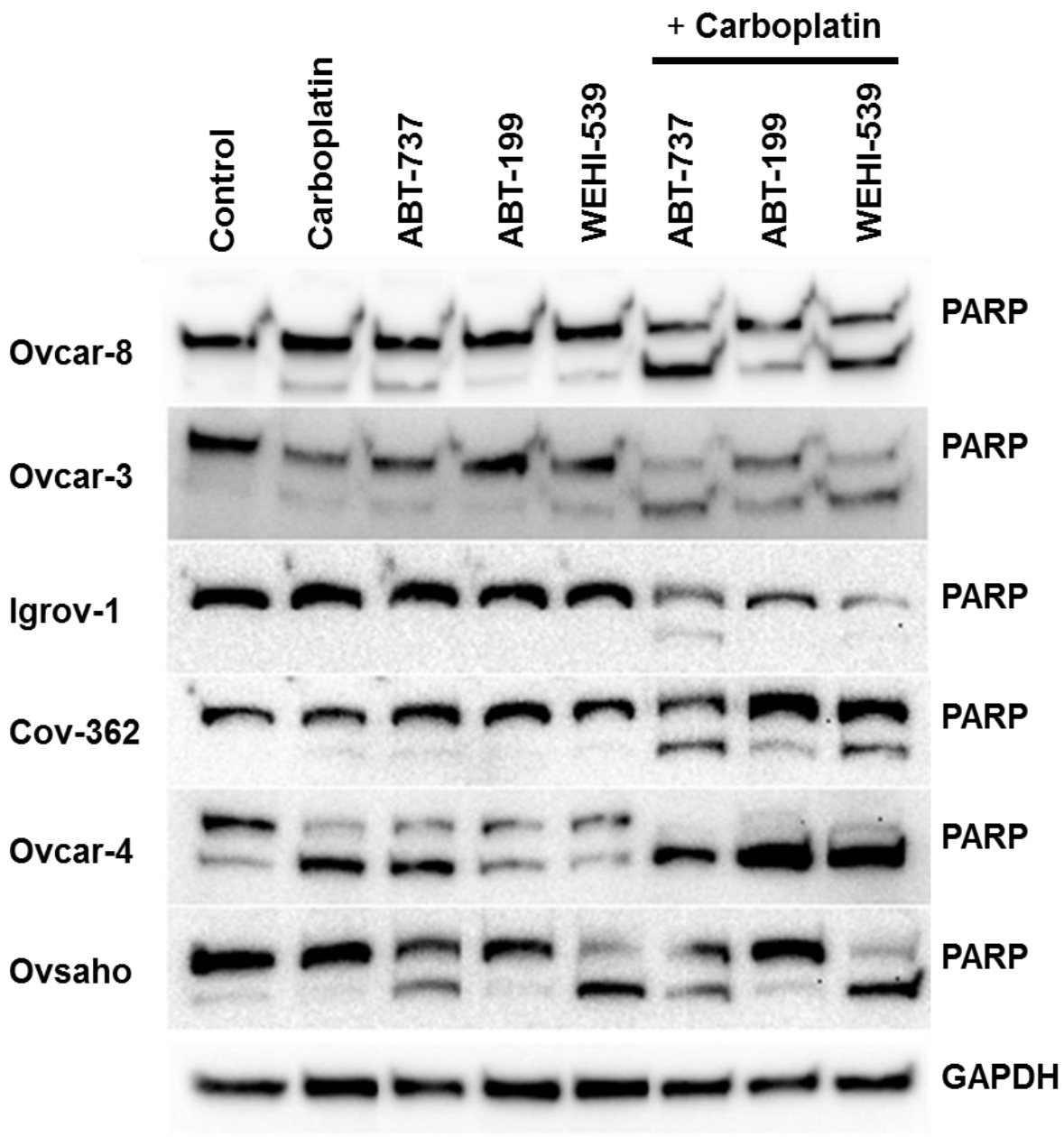
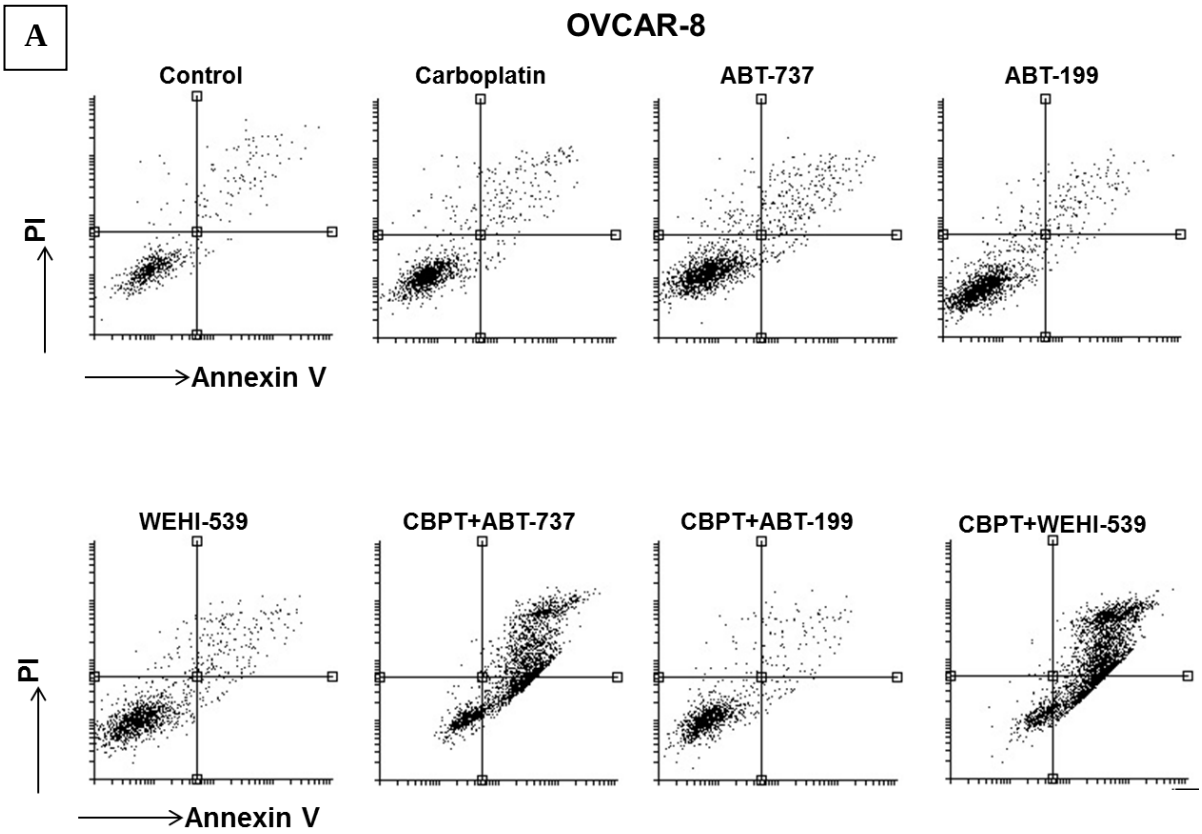


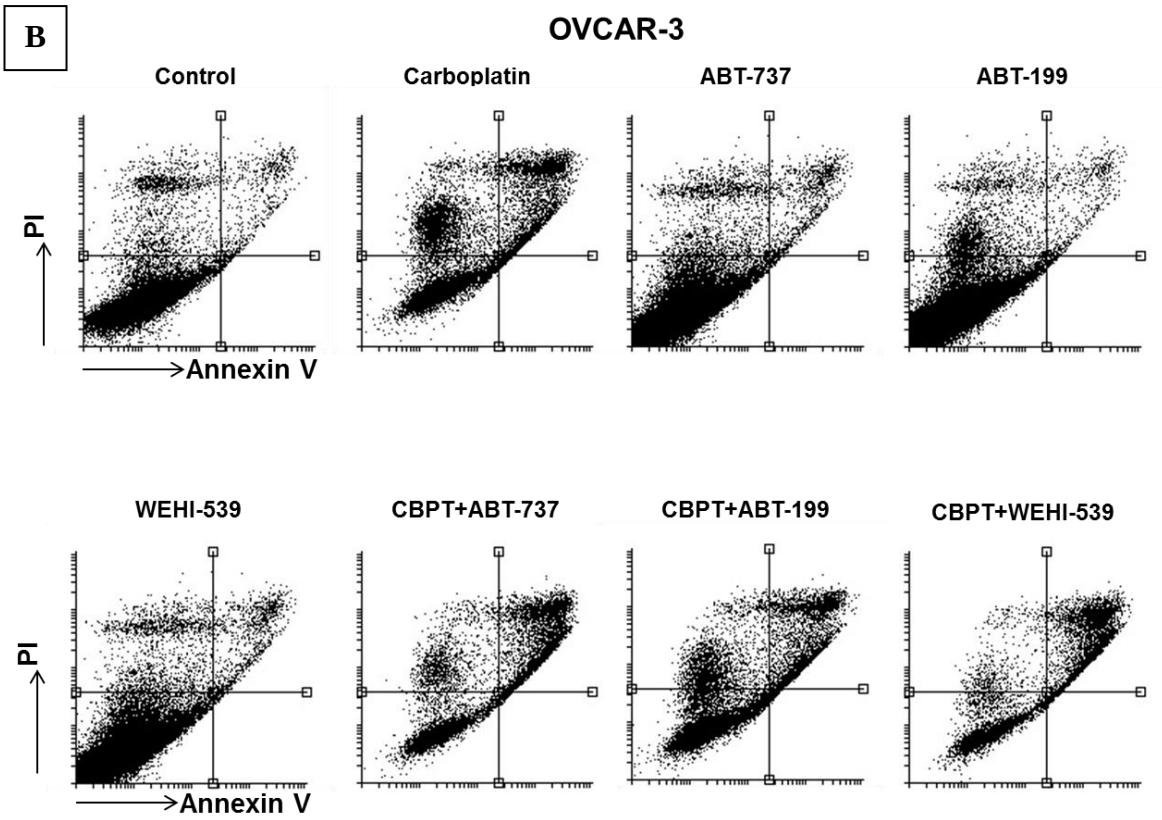
Figure 4.12: The effect of BH3 mimetic and carboplatin combinations on PARP cleavage

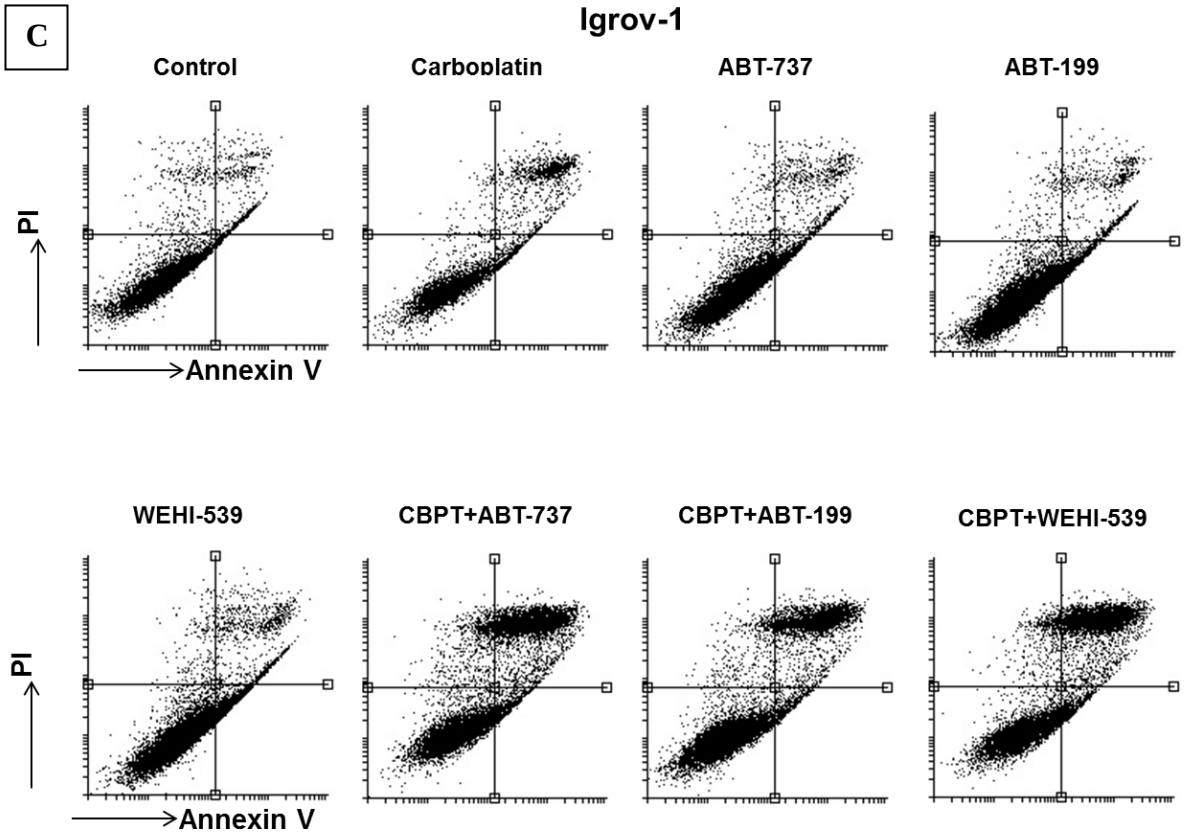
The indicated cell lines were treated with 66 μM carboplatin or the indicated BH3 mimetic alone and in combination for 48 hours (concentration as indicated in figure 4.9) and the cleavage of PARP was assessed by immunoblotting. The results are representative of 3 independent experiments.

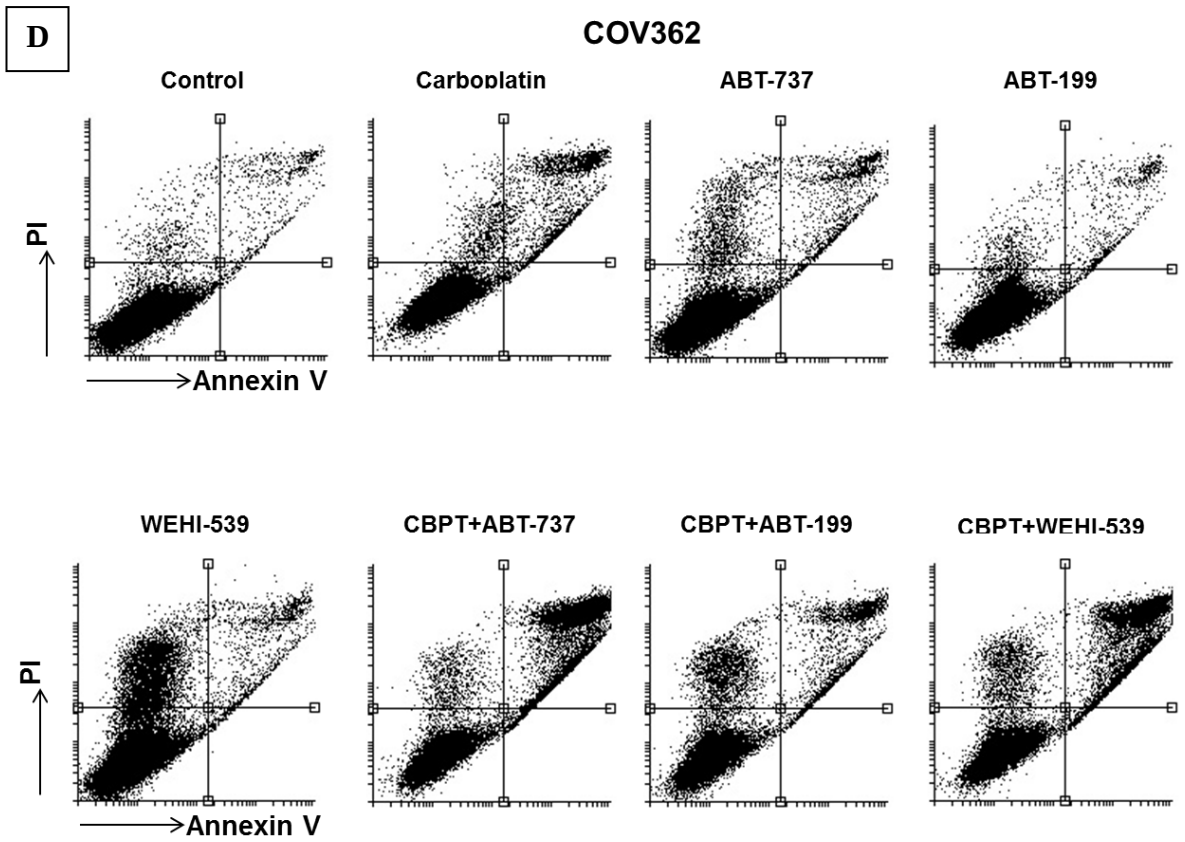
4.7.6 Annexin V/PI

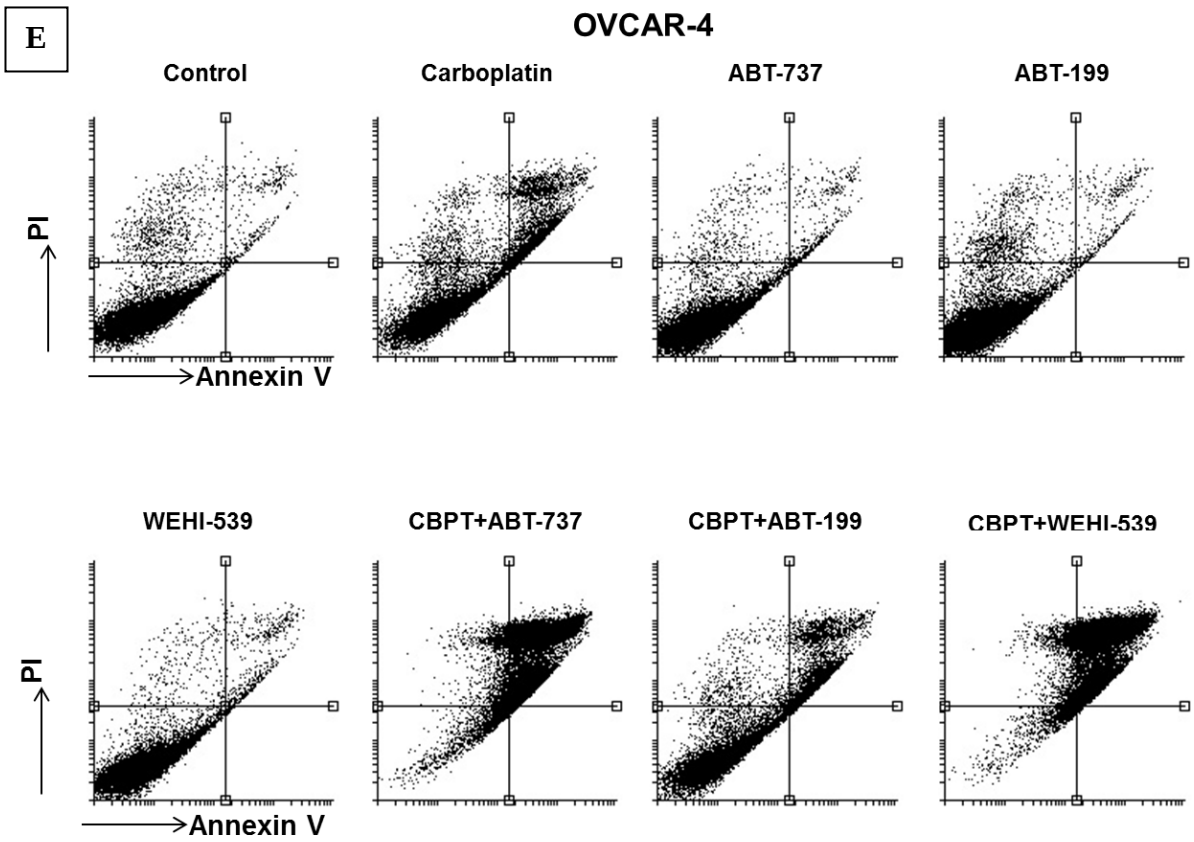
Experiments assessing annexin V/propidium iodide staining, assessed by flow cytometry (figures 4.13 A, B, C, D, E and F), confirmed the induction of apoptosis by the BH3 mimetic drug combinations in a manner consistent with the previous experiments. Carboplatin in combination with ABT-737 and WeHi-537, which both inhibit Bcl-X_L, resulted in significantly more apoptosis than the expected additive effect of the drugs in OVCAR-8, Igrov-1, OVCAR-3 and COV-362 cells, but not in OVCAR-4 cells. However, venetoclax (Bcl-2 selective) once again failed to augment carboplatin activity in all of the studied cell lines as shown in figures 4.14 A, B, C, D, E and F.




B
OVCAR-3
Control
Carboplatin
ABT-737
ABT-199
PI
Annexin V
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539
PI
Annexin V


C
Igrov-1
Control
Carboplatin
ABT-737
ABT-199
PI
Annexin V
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539
PI
Annexin V


D
COV362
Control
Carboplatin
ABT-737
ABT-199
PI
Annexin V
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539
PI
Annexin V


E
OVCAR-4
Control
Carboplatin
ABT-737
ABT-199
PI
Annexin V
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539
PI
Annexin V

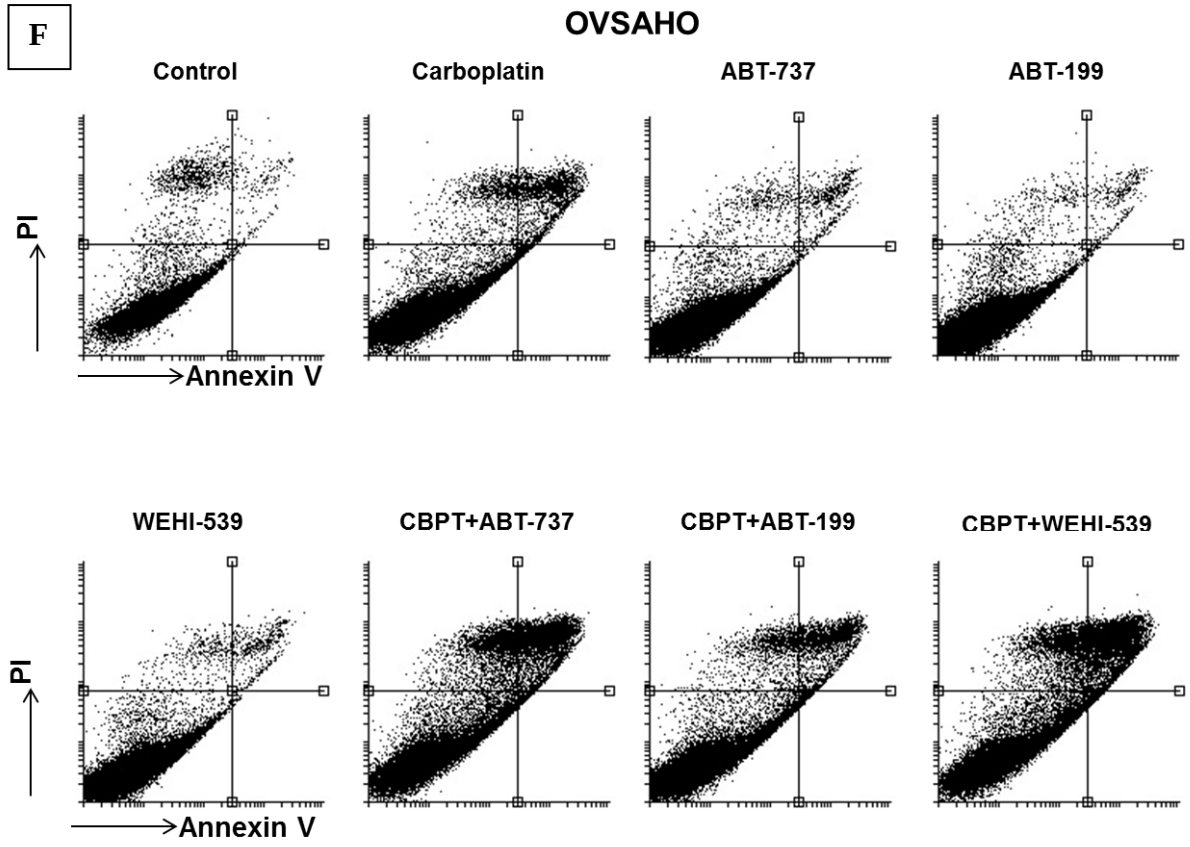
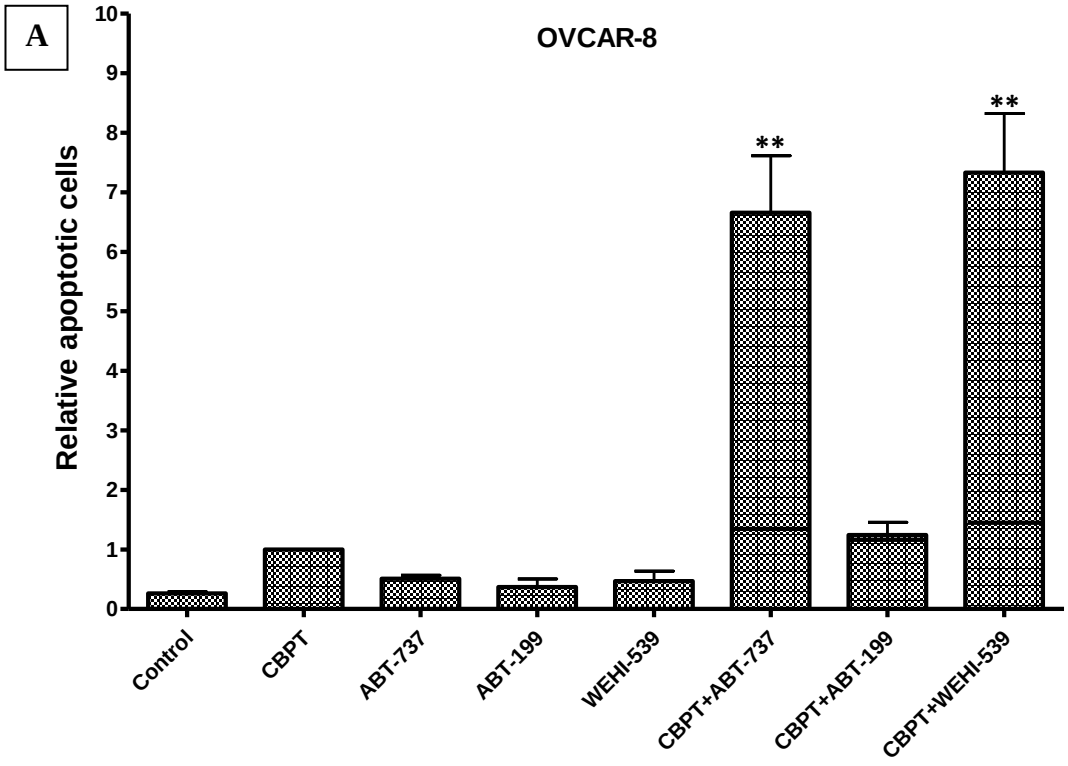
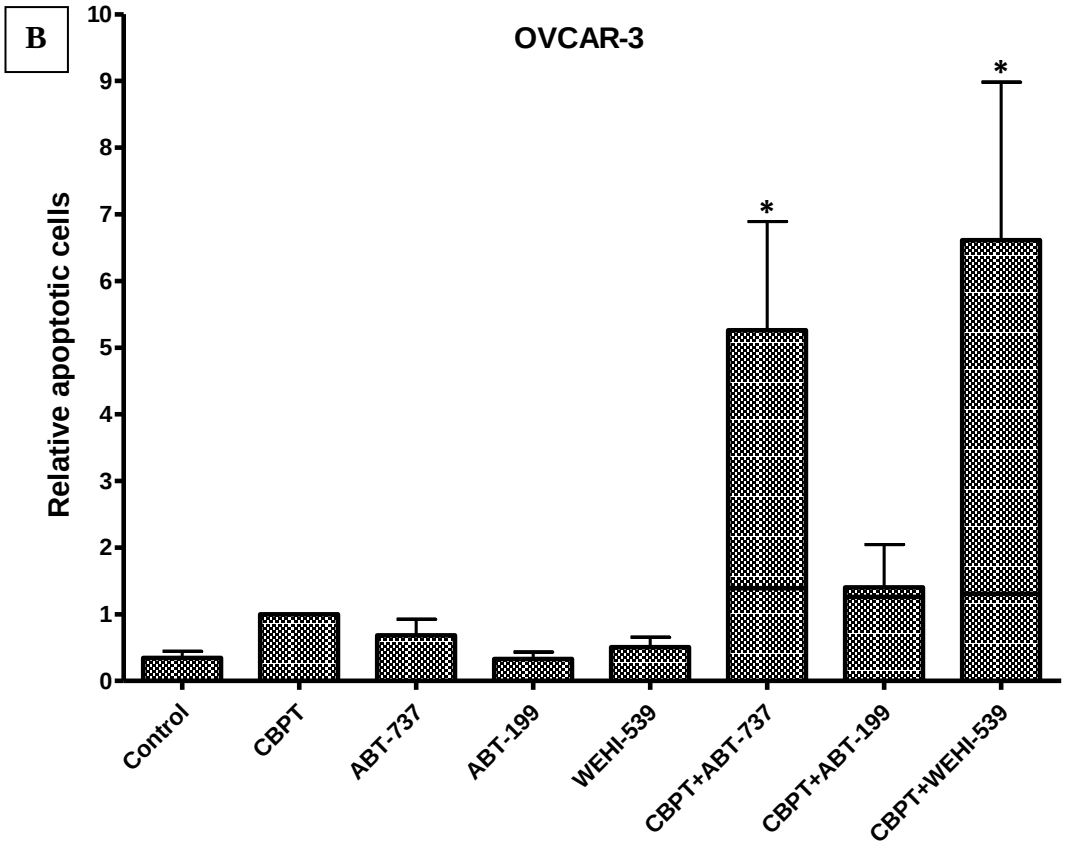
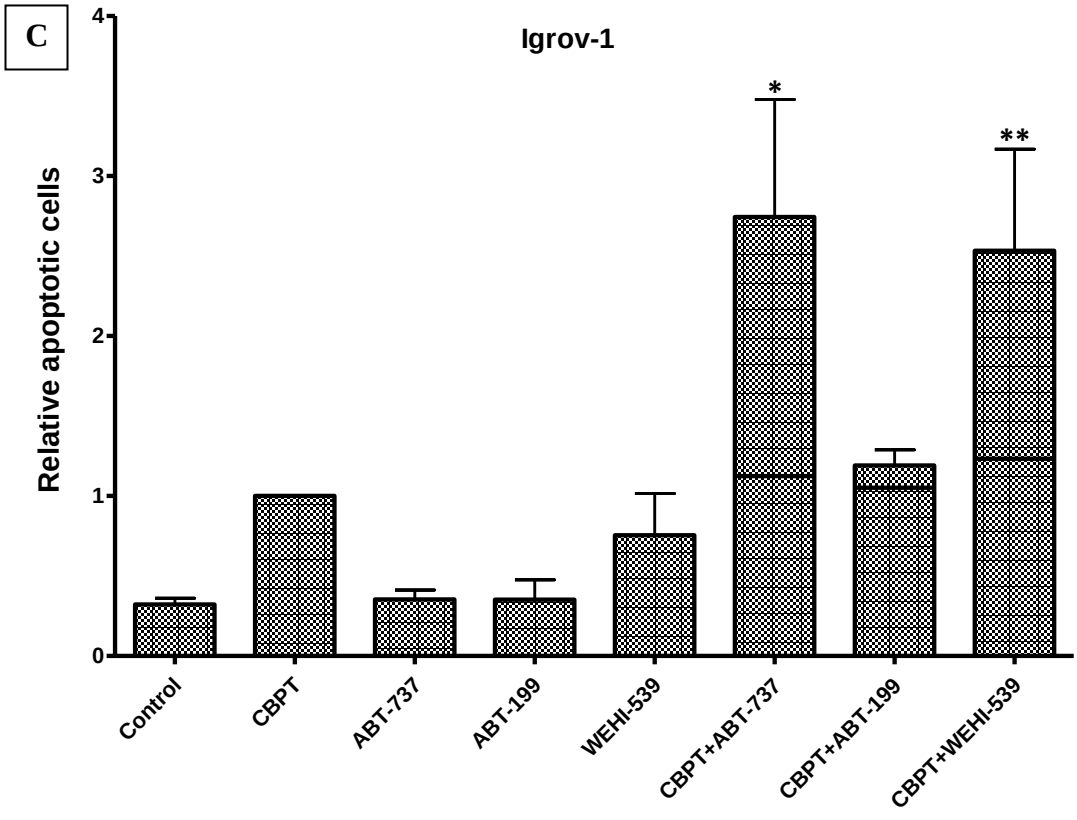


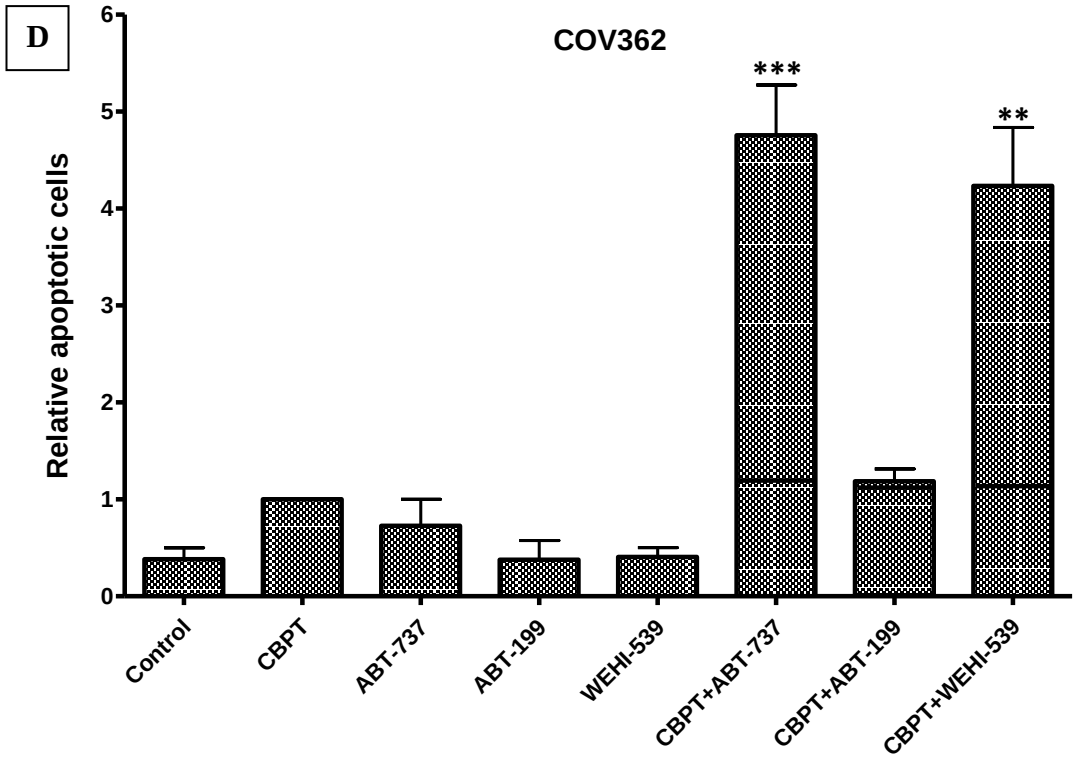
Figure 4.13: The effect of BH3 mimetic combinations on annexin V/propidium iodide staining

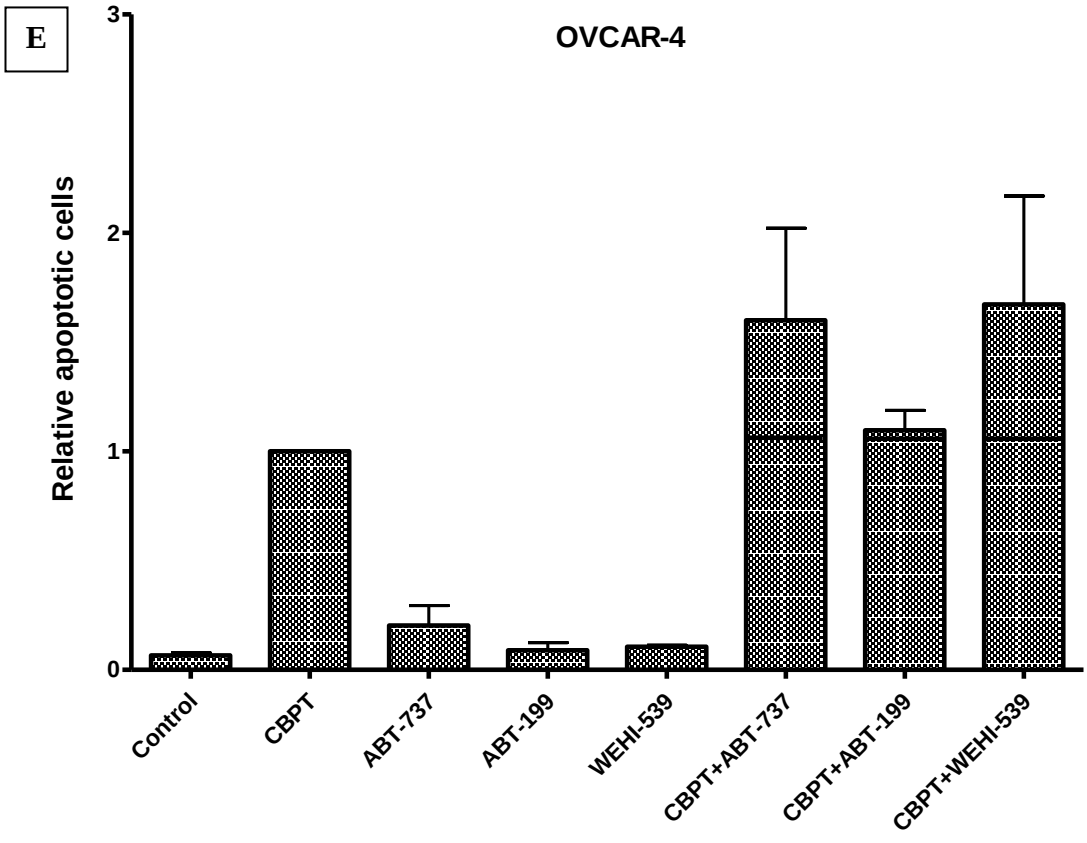
OVCAR-8 (A), OVCAR-3 (B), Igrov-1 (C), COV362 (D), OVCAR-4 (E) and OVSAHO (F) cells were treated with the indicated BH3 mimetic (1 µM ABT-737, 3 µM venetoclax or 0.3 µM WEHI-539), carboplatin (66 µM) or a combination of carboplatin and BH3 mimetic for 48 hours, the cells were labelled with annexin V and propidium iodide and analyzed by flow cytometer. The results shown in the figures are representative of 3 experiments. The results are quantified in figure 4.14.


A
OVCAR-8
Relative apoptotic cells
10
9
8
7
6
5
4
3
2
1
0
**
**
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539


B
OVCAR-3
Relative apoptotic cells
10
9
8
7
6
5
4
3
2
1
0
*
*
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539


C
Igrov-1
Relative apoptotic cells
4
3
2
1
0
*
**
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539


D
COV362
Relative apoptotic cells
0
1
2
3
4
5
6
***
**
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539


E
OVCAR-4
Relative apoptotic cells
0
1
2
3
Control
CBPT
ABT-737
ABT-199
WEHI-539
CBPT+ABT-737
CBPT+ABT-199
CBPT+WEHI-539

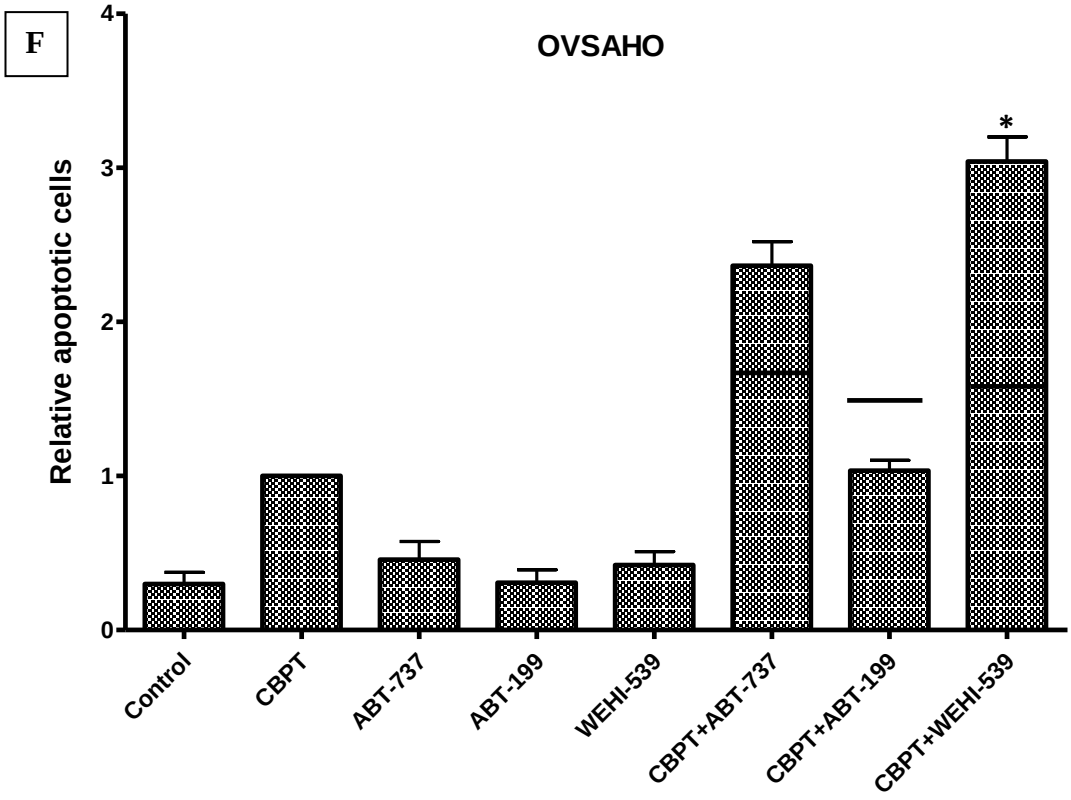


Figure 4.14: The effect of BH3 mimetic combinations on annexin V/propidium iodide staining

The indicated cells were exposed to 66 μM carboplatin or the indicated BH3 (1 μM ABT-737, 3 μM venetoclax or 0.3 μM WEHI-539) mimetic for 48h, the cells were labelled with annexin V and propidium iodide and assessed by flow cytometery. The percentage of cells that stained positive with both annexin V and propidium iodide is shown (mean ± S.D., n = 3). This was significantly different where shown (paired t-test, *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$;) from the percentage of annexin V/propidium iodide positive cells expected from the activity of the single agents and calculated from the Bliss independence criterion.

4.8 Discussion

The efficacy of many anti-cancer agents, such as taxol and platinum-based compounds, principally depends on activation of apoptosis (322). Notably, cancer cells often modify and suppress their apoptotic mechanisms to survive and dysregulation of apoptosis is considered as one of the main contributors to failure of chemotherapeutic agents. This may be caused by overexpression of the pro-survival Bcl-2 proteins (323,324). Therefore, one strategy to overcome resistance to chemotherapy is pharmacological inhibition of the overexpressed Bcl-2 family proteins (325,326).

Previous work in our lab has shown that navitoclax and ABT-737, (Bcl-2, Bcl-w and Bcl-X_L inhibitors), can potentiate the cytotoxicity of carboplatin in several ovarian cancer cell lines (293,294). The increased sensitivity to carboplatin observed in these studies after combination with either ABT-737 or navitoclax was complemented by a reduction in the time at which apoptosis was detected by measurement of the number of viable cells, nucleosome formation and PARP cleavage. In addition to this *in vitro* synergy, navitoclax augmented the inhibitory effect of carboplatin on tumour xenograft growth in mice beyond that observed by using carboplatin alone.

In the present study, analysis revealed that the three drugs, when tested as single agents, produced cytotoxicity in all of the investigated ovarian cancer cell lines, but only at relatively high conentrations. These results are consistent with most studies that evaluated BH3 mimetics in solid tumours (303,327). However, these results are different from those that showed massive cell death after using BH3 mimetics in hematological malignancies (307,308). A plausible explanation for this could be the "priming of cells for death" by the expression of BH3 only proteins and the consequent high dependency of malignant blood cells on Bcl-2 (oncogene addiction) for survival (328). Cancer cells often are forced to survive in an environment that induces stress. This can cause the cells to constitutively express BH3-

only proteins. This would induce apoptosis, but cells which also express high levels of anti-apoptotic Bcl-2 family proteins to sequester the BH3-only proteins, have a survival advantage. However, the liberation of BH3-only proteins from Bcl-2 apoptosis inhibitors would induce cell death and so the cells are considered to be "primed for death" (329). This can be considered a form of oncogene addiction and correlates with sensitivity to BH3 molecules that bind the anti-apoptotic Bcl-2 protein. Thus, addiction of the cells to Bcl-2 family protein will result in sensitivity to a BH3 mimetic that can bind to it. (330) The BH3 mimetics release the pro-apoptotic BH3 only proteins and consequently are effective as single agents. "Priming" has consequently been regarded as the best explanation for responsiveness of cancer cells to single agent BH3 mimetic therapy (331). The evasion of apoptosis due to priming can be identified by evaluating the sensitivity of mitochondria to the members of apoptosis by an approach called "BH3 profiling". The ability of peptides corresponding to the BH3 domain of BH3 mimetics to induce apoptosis is diagnostic of priming. By considering the selectivity of the peptides for different Bcl-2 family members, the identity of the Bcl-2 protein that is suppressing apoptosis can be deduced (332,333). This can potentially be used as a clinical test to identify tumours that are addicted to a particular oncogene and so this test may be useful for patient selection.

In blood malignancies, the overexpressed Bcl-2 is strongly primed with pro-apoptotic Bim protein, conferring sensitivity to drugs that provoke its release to initiate apoptosis (figure 4.15) (330,334). Conversely, in solid tumours, including ovarian cancer, although there is a relatively high expression of Bcl-X_L the lack of sensitivity to BH3 mimetics as single agents suggests that these cells are ''not primed'' for death. Rather, the potentiation of apoptosis by Bcl-X_L is likely to require an additional pro-apoptotic stimulus, such as a chemotherapeutic agent that induces the expression of a BH3-only protein.

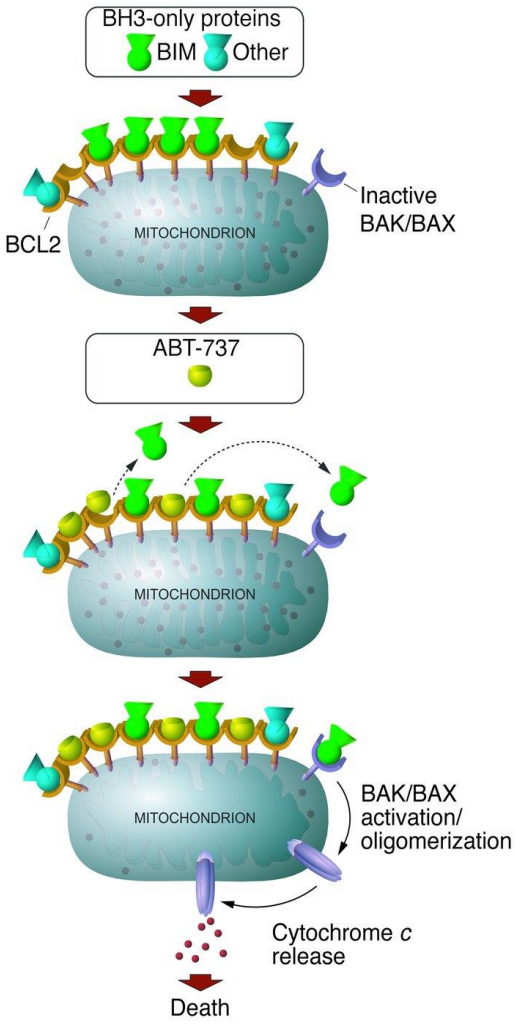


Figure 4.15: Model of BH-3 mimetics induced intrinsic apoptosis

Bcl-2 sequesters Bim in the mitochondria of cancer cells. Upon exposure to ABT-737, Bim displaces and ABT-737 occupies Bcl-2. Free Bim then interacts with Bak or Bax, inducing oligomerization leading to the release of cyto c and irreversible commitment to apoptosis. Bcl-2 primed with activator BH3-only proteins renders the tumour cells sensitive to BH3-mimetics and possibly other chemotherapeutics (334).

The ability of selective inhibitors of Bcl-2 and Bcl-X_L, venetoclax and WEHI-539 to potentiate the activity of carboplatin in ovarian cancer cells has been compared in this study. Both ABT-737 and WEHI-539 inhibit Bcl-X_L, resulted in potentiation of carboplatin activity in several ovarian cancer cell lines using different assays, including cell growth inhibition, cell death, activation of caspase 3/7 activity, PARP cleavage and Annexin V/propidium iodide staining. ABT-737 and WEHI-539 clearly increased the cell death induced by carboplatin and

conferred synergy in several cell lines, whereas when carboplatin was combined with venetoclax an additive or even antagonistic interaction was obtained.

A plausible reason for the synergy may again involve priming of cells to death. Probably, the over-expressed Bcl-X_L caused sequestration of the apoptosis activators, whose expression was induced by the carboplatin, until the apoptotic signal overwhelmed the apoptosis inhibitors. In this scenario, synergy is observed because the BH3 mimetic prevents the sequestration of the BH3 only protein that is induced by chemotherapy and which would otherwise be sequestered by the Bcl-2 family members present. ABT-737 and WEHI-539 are likely to have prevented Bcl-X_L from sequestering the activators and as a result primed cancer cells closer to the threshold for cell death. An alternative possible cause for synergy between carboplatin and ABT-737 or WEHI-539, is because Bcl-X_L inhibition with these agents was accompanied by inhibition of the pro-survival protein Mcl-1 by carboplatin inducing expression of pro-apoptotic BH3 only proteins. In this scenario, BH3 mimetics which do not bind to Mcl-1, may synergize with carboplatin even in cells which express Mcl-1. However, this may not be possible in cells that express very high levels of Mcl-1. Previous studies have linked the resistance to ABT-737 in several cancer cell lines to high Mcl-1 level (335,336). In this study, Mcl-1 was expressed in almost all cell lines with the lowest level in OVSAHO cells and higher levels in OVCAR-4 (the cell line that showed the least response to the combination of carboplatin with ABT-737 or WEHI-539). OVCAR-4 cells are consequently less likely to be dependent on Bcl-X_L for survival and may depend on Mcl-1 instead.

The pre-eminent exception to the synergy observed between carboplatin and BH3 mimetics in this study was OVCAR-4 cell line, where the combination of these agents was additive in all assays. This could be explained by the relatively low level of Bcl-X_L in OVCAR-4 cells. These cells are likely to be less dependent on Bcl-X_L, suggesting that synergy may not have been observed with carboplatin and drugs inhibiting BclX_L because these cells are dependent

on other anti-apoptotic members of the Bcl-2 family. However, an alternative explanation is suggested by the observation that ABT-737 and WEHI-539, on their own, in OVCAR-4 cells induced apoptosis, although with modest potency. Thus, the lack of a synergistic effect between carboplatin and BH3 mimetic in OVCAR-4 cell line may reflect the low level of Bcl-X_L, which allowed these compounds to induce apoptosis when they were used as single agents, so synergy has not readily been observed.

The lack of synergy between venetoclax and carboplatin, and the limited expression of Bcl-2 in ovarian cancer cells, strongly supports the claim that inhibition of Bcl-X_L is crucial if BH3 mimetics are to be effectively used in the management of ovarian cancer. Such a notion has fortified by the more common deregulation of Bcl-X_L than Bcl-2 in ovarian tumours reported in the cancer genome atlas (314). It has also been reported that Bcl-X_L overexpression in ovarian cancer cells could be responsible for the development of chemoresistance in ovarian cancer cells rather than Bcl-2 (278). Also, when Bcl-X_L level was evaluated in ovarian tumour tissue from a group of patients, tumours with high level of Bcl-X_L were less sensitive to taxane treatment in comparison to patients with low Bcl-X_L level (318).

In this research, venetoclax failed to augment the cell death induced by carboplatin in some assays and was even slightly antagonistic in others. The cause of antagonism between venetoclax and carboplatin, which has been noted in this study using several ovarian cancer cell lines is uncertain. Venetoclax may inhibit a target other than a Bcl-2 family protein. Alternatively, Bcl-2 family proteins have been implicated in cell cycle regulation, and this may antagonise the cell cycle dependant effect of carboplatin (337). Whatever the cause of antagonism, any proposal for a clinical trial for the evaluation of carboplatin and venetoclax combination of ovarian cancer should be viewed with considerable caution. OVCAR-8, OVCAR-3 and Igrov-1 cell lines, which show relatively low amounts of Bcl-2, showed the most evident antagonism, whereas additivity between venetoclax and carboplatin was

observed in COV-362 and OVCAR-4 cells which expressed higher levels of Bcl-2 (figure 4.7).

Consequently, inhibition of Bcl-X_L is more likely to be effective than inhibition of Bcl-2 for the chemosensitization of ovarian cancer cells. Unfortunately navitoclax, has been closely linked to dose dependent thrombocytopenia, and this is also anticipated to occur with WEHI-539 because both compounds inhibit Bcl-X_L. It is possible that a small therapeutic window for these compounds may be identified by careful dose escalation studies. Alternatively, novel strategies may be developed to minimize thrombocytopenia, such as encapsulation of WEHI-539 or other Bcl-X_L inhibitors in nanoparticles, which has previously shown to be effective in enhancement of ABT-737 anti-tumour effects while minimizing thrombocytopenia and other systemic side effects (338). As an alternative, it is possible to exploit the "platelet sparing" properties of paclitaxel to use it in combination with Bcl-X_L inhibitors in ovarian cancer especially after the supra-additive effect that has been observed in our lab when paclitaxel was combined with navitoclax and carboplatin (293). This may create an adequate medical window to make the use of navitoclax possible in patients with ovarian carcinoma.

In summary, although Bcl-2 has shown to be a vital target in hematological malignancies, which depend on Bcl-2 for their survival, the lack of Bcl-2 expression in ovarian cancer and lack of synergy of carboplatin with venetoclax raise concerns over whether BH3 mimetics will be of use in the treatment of ovarian cancer unless suitable platelet-sparing strategies are developed. However, by using a personalized therapeutic approach, it is possible that a limited number of patients whose tumour overexpress Bcl-2, may benefit from drugs such as venetoclax. This will require measurement of Bcl-2 level in the tumour and this is particularly desirable according to the findings of this research to avoid treatment failure.

Chapter 5

Validating potential drug-resistance genes

5.1 Introduction

One of the major causes of the ineffectiveness of chemotherapeutic agents in patients with ovarian cancer is the development of drug resistance that can be acquired during treatment course or be inherent (exist prior to treatment) (339). Both types can be caused by multiple different mechanisms including modifications to the drug targets, alterations in drug metabolism, cellular uptake or efflux, altered sensitivity to apoptosis and altered DNA repair (340–342). Resistance to chemotherapy in patients has the features of multidrug resistance, representing reduced sensitivity of tumour cells not only to previously utilized anti-cancer agents but also to various other agents that have different structure and/or mechanisms of action (343,344). Moreover, because cancer is a heterogeneous disease, more than one mechanism of chemoresistance may be present simultaneously in any particular case (345). Multidrug resistance could occur as a result of the involvement of multiple proteins or genes via multistep processes, or multifactorial cross-reactivity. Diverse regulatory factors are associated with the development of drug resistance, including both genetic and epigenetic alteration (346).

The effective treatment of chemoresistant disease is likely to benefit from consideration of the resistance mechanisms involved and this requires that the resistance mechanisms are identified (347,348). It has been reported that gene expression profiles can be identified and correlated with states of chemoresistance (349). A wide variety of genes and epigenetic modifications are linked to drug resistance in ovarian cancer, affecting tumour suppressor genes modifications and oncogenes. The resistance may be caused by activation of oncogenes or downregulation of tumour suppressor genes (350). Increasingly, it is being recognized that therapeutic targeting of only one gene or protein that the cells are dependent on can lead to ineffective treatment, because another parallel pathway may become active to support tumour growth and survival. In such case, simultaneous targeting of two pathways or

using drug combinations can result in synergistic lethal relationship (351). Accordingly, verifying the causative pathways of platinum and taxane resistance, especially the genes that could be responsible for it, may offer a novel strategy for the development of targeted therapies to re-sensitize cancer cells to chemotherapy. The combination of a drug which inhibits the mechanism of drug resistance with carboplatin or paclitaxel has great therapeutic potential to treat ovarian cancer patients (95,352).

Alterations in the p53 pathway within cancer cells provide an example of genetic modifications that may lead to chemresistance (353). The primary role of p53 protein is to maintain genomic integrity by regulating cell cycle progression and mediating the responses of cells to DNA damage by initiating DNA repair, or activating apoptosis. These functions make it an important tumour suppressor protein (354). It has been reported that genetic mutations in the *p53* are the main causes for unresponsiveness to high-dose of cisplatin in patients with ovarian cancer (355). In contrast to multidrug resistance, it has been shown that some cancer cells that have acquired taxane or platinum resistance, can exhibit an inverse chemoresistance relationship, developing collateral chemosensitivity to other drugs. This has been confirmed by preclinical studies which have revealed marked response to taxane therapy in ovarian carcinoma cells that have *p53* mutations and are resistant to cisplatin (356). Subsequent studies have shown the likely genetic roles *BRCA1* in this relationship. Tumours with *BRCA1* mutations exhibit reduced efficiency in repairing DNA adducts but increased sensitivity to platinum-based compounds. Conversely, the response to taxanes in these cells is reduced while the opposite is true for cancers with functional BRCA1 (357).

The past decade has seen novel drug targets being identified by utilizing high-throughput screens, functional genomic studies and new sequencing technologies (358–360). Furthermore, several microarray studies have previously identified numerous genes with altered expression in chemoresistant cancer and which deserve investigation to determine

their contribution to drug resistance. Many groups have used RNA interference (RNAi) technology to identify genes affecting cell growth and survival, and similarly functional genomic screens to identify genes regulating drug resistance are also feasible. This has been facilitated by the availability of exogenous short interfering RNA (siRNA) to downregulate the expression of targeted gene temporarily to provide an effective technique for performing large-scale screens (361,362).

Previous work in our laboratory has focused on identifying potential determinants of carboplatin or paclitaxel sensitivity in order to develop novel drug targets that could be used to potentiate the activity of carboplatin or paclitaxel in ovarian cancer cells. This was achieved by making use of a report which identified genes whose expression was increased in either primary chemoresistant or acquired chemoresistant tumours, compared to chemonaive tumours (363). In 2005, our group designed a screen using RNAi technology to knock down the expression of some genes and measure the impact on chemosensitivity. Several goals were taken in consideration: use a limited number of drug concentrations (two) to attain a reasonable throughput; obtain an estimate of the change in sensitivity to rank the hits from the screen; use several ovarian cancer cell lines because different cells may acquire resistance via different pathways; and detect modest (2-fold) changes in drug sensitivity. Ninety genes were selected for knock down in a panel of 6 ovarian cancer cell lines and the change in sensitivity to carboplatin or paclitaxel was evaluated in cell growth assays. To validate the methods used in the screen, it was shown that inhibition of FLIP expression increased the sensitivity of ovarian cancer cells to paclitaxel and the inhibition of Bcl-X_L expression increased the sensitivity of cells to carboplatin (364).

One of the hits that was identified by the screen was *ENPP2*. *ENPP2* encodes autotaxin, which is an enzyme that functions as a lysophospholipase D to catalyse the production of the survival factors, sphingosine 1- phosphate and lysophosphatidic acid. It has been reported that

ectopic expression of autotaxin is responsible for induction of mammary tumourigenesis, invasion, and metastases (365). Likewise, it has been shown that autotaxin overexpression can protect melanoma and breast cancer cells against paclitaxel-induced cell death (366). Additionally, a combination of autotaxin and lysophosphatidic acid-receptor antagonist was able to inhibit tumour growth and angiogenesis in non-small cell lung cancer xenografts. In our laboratory, we have observed that inhibition of autotaxin by means of siRNA or a drug inhibitor enhances cell death induced by carboplatin, whereas overexpression of autotaxin delays it (364).

Another important hit that was identified by the screen was *CYR61,* This was significant because it was subsequently reported to be involved in chemoresistance. CYR61 is a secreted matricellular protein located on cell surface and in the extracellular matrix, it binds to integrins and regulates numerous cellular activities including cell differentiation, proliferation, migration and apoptosis (367). In ovarian cancer, *CYR61*mRNA is overexpressed in about 60% of patients (from 2 to 10 fold more than normal ovarian tissues expression). Its upregulation is associated with advanced disease stages, where it found to be responsible for induction of cell growth and proliferation, as well as resistance to carboplatin (368).

These results gave confidence that the screen had identified valid regulators of chemosensitivity. However, the screen only involved two technical replicates at each drug concentration and only one experimental replicate in each of the cell lines. Thus the hits required confirmation in additional experiments, and several hits appeared worthy of investigation. Thus, the screen contained a potential list of unexplored drug-resistance genes that required confirmation before further validation of their role in chemoresistance.

5.2 Aims

The goal of study in this chapter was to confirm that a selection of the hits from the screen were valid and then identify which genes to pursue in greater detail. The hits from the screen selected for investigation were: *CYR61*, *FABP4*, *FBLN5*, *FOXC1*, *TAGLN*, *MAPK*, *FBLN1*, *FOXO1A*, *LAMA2*, *EFEMP1*, *CFHL*, *PLA2R1* and *ENTPD*.

5.3 Validation of hits previously obtained from a screen to identify genes contributing to ovarian cancer drug resistance

5.3.1. Candidate carboplatin-resistance genes

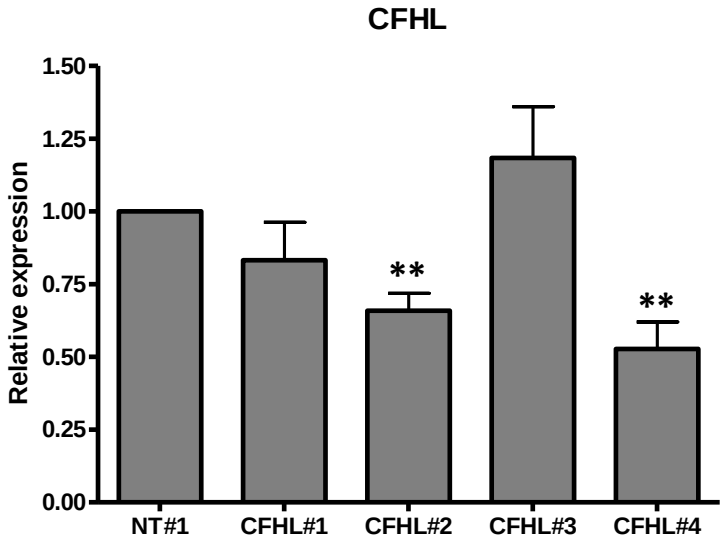
5.3.1.1 Knock-downs of the candidate carboplatin-resistance genes

The contribution of candidate genes to drug resistance was assessed in OVCAR-4 cells and in some experiments COV-362 cells. These cells have been shown to be a good models of ovarian cancer (321) and were derived from patients resistant to chemotherapy. OVCAR-4 but not COV-362 cells were used in the original screen.

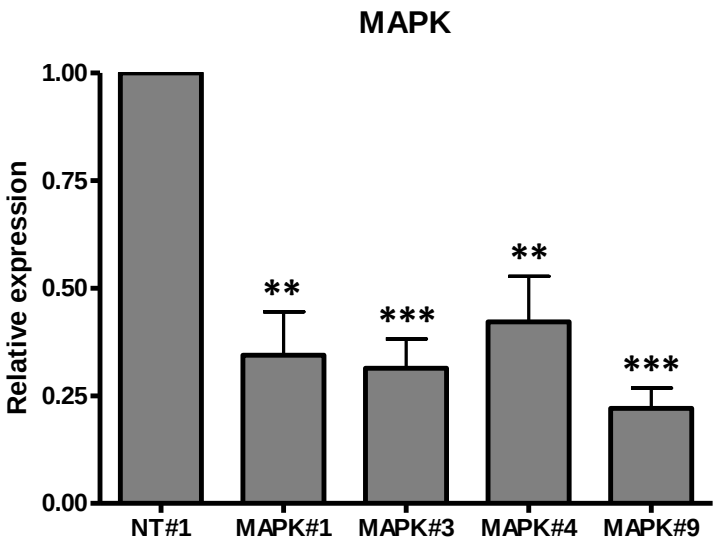
Prior to evaluating the effect of knock-down, the expression of candidate drug resistance genes and the transfection conditions for each siRNA were optimized to ensure efficient transfection without notable toxicity. To ensure that knock-down of each of the genes was not cytotoxic, OVCAR-4 cells were transfected with siRNA for each gene (mRNA) and after 72 hours, the relative number of cells present estimated by staining with SRB and compared to cells transfected with non-targeting siRNA.

The efficiency of gene knock-down was analyzed for 4 separate siRNAs for each of the candidate genes. The results revealed variable extent of knock-down of the different targeted genes, but at least 2 siRNAs were identified in almost all the genes which caused significant knock-down. Although more than 50% knock-down was achieved with most of the genes, only 40% knock-down was achieved with *CFHL* and *ENTPD* (figure 5.1). In the case of *PLA2R1*, no siRNAs were identified which caused knock-down for this gene.

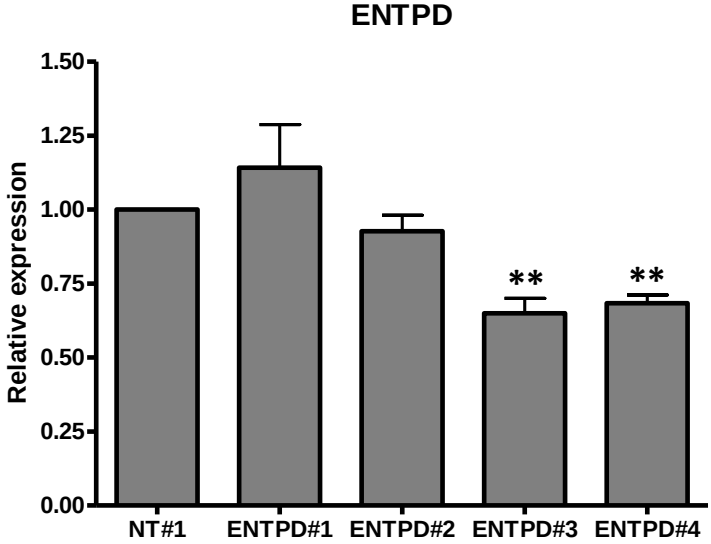
A


CFHL
Relative expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
**
NT#1
CFHL#1
CFHL#2
CFHL#3
CFHL#4

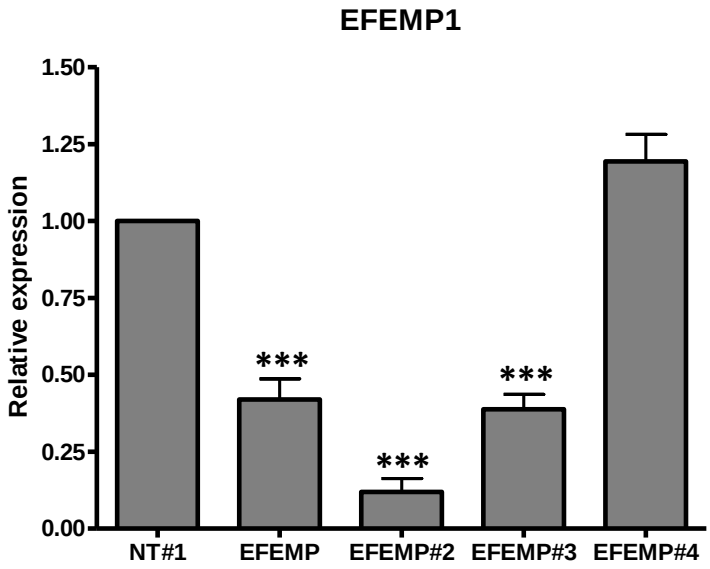
B


MAPK
Relative expression
1.00
0.75
0.50
0.25
0.00
**
***
**
***
NT#1
MAPK#1
MAPK#3
MAPK#4
MAPK#9

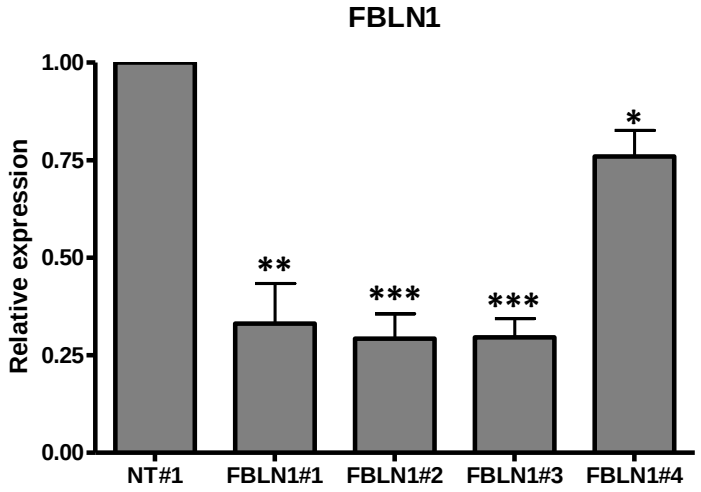
C


ENTPD
Relative expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
**
NT#1
ENTPD#1
ENTPD#2
ENTPD#3
ENTPD#4

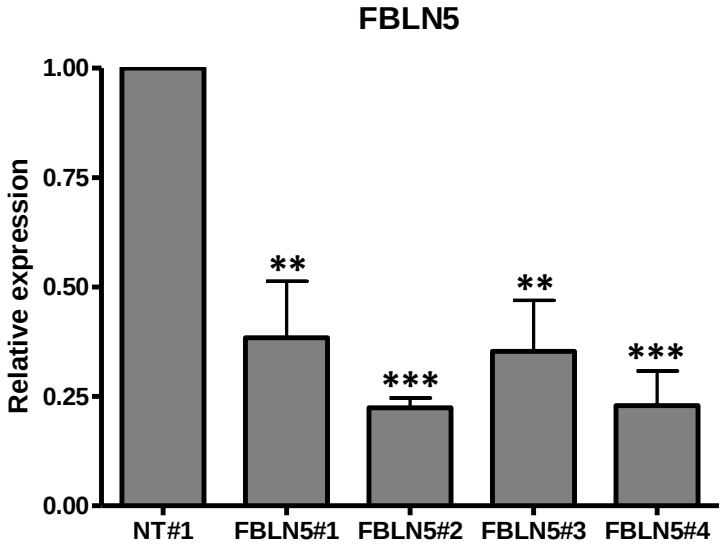
D


EFEMP1
Relative expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
***
***
***
NT#1
EFEMP
EFEMP#2
EFEMP#3
EFEMP#4

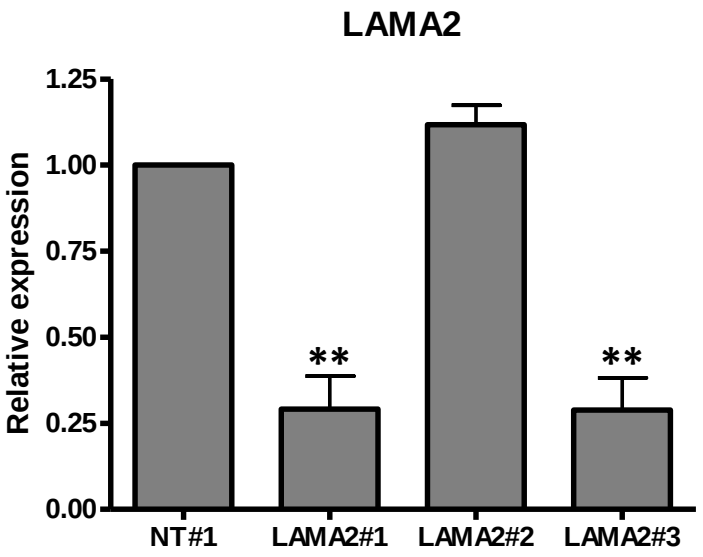
E


FBLN1
Relative expression
1.00
0.75
0.50
0.25
0.00
**
***
***
*
NT#1
FBLN1#1
FBLN1#2
FBLN1#3
FBLN1#4

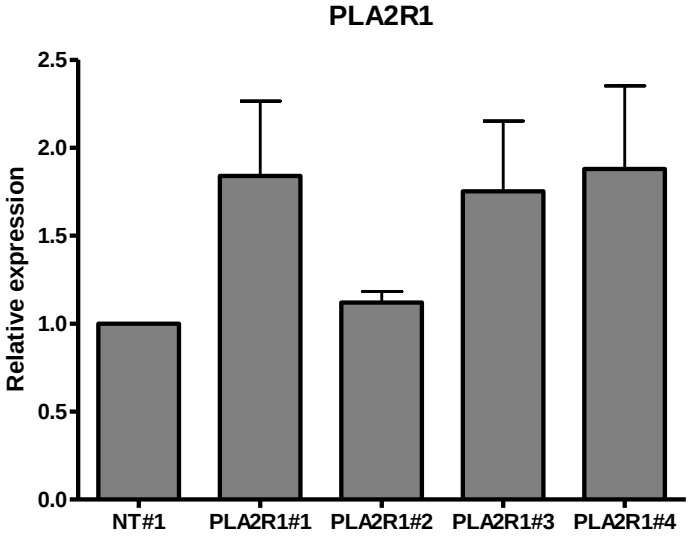
F


FBLN5
Relative expression
1.00
0.75
0.50
0.25
0.00
**
***
**
***
NT#1
FBLN5#1
FBLN5#2
FBLN5#3
FBLN5#4

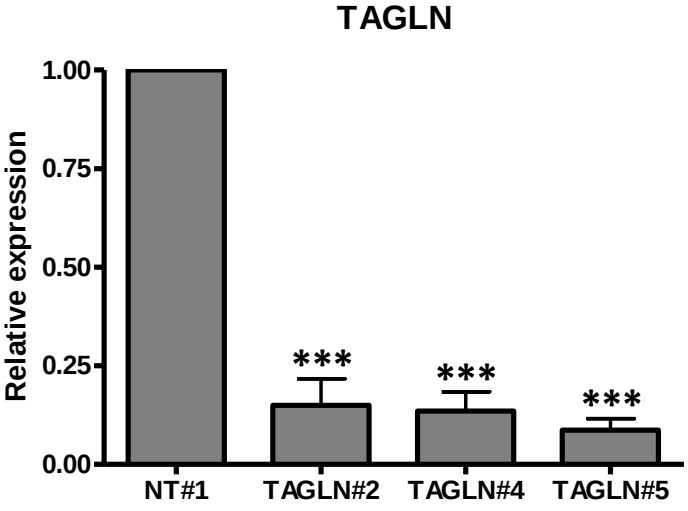
G


LAMA2
Relative expression
1.25
1.00
0.75
0.50
0.25
0.00
**
**
NT#1
LAMA2#1
LAMA2#2
LAMA2#3

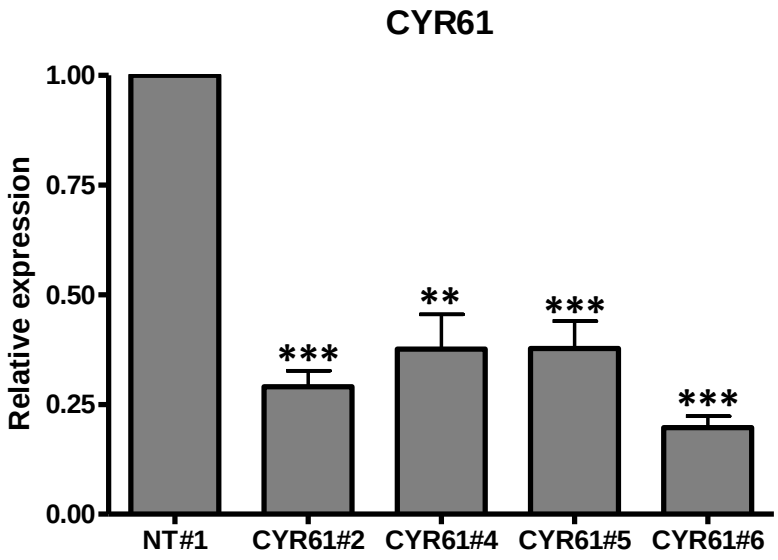
H


PLA2R1
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
NT#1
PLA2R1#1
PLA2R1#2
PLA2R1#3
PLA2R1#4

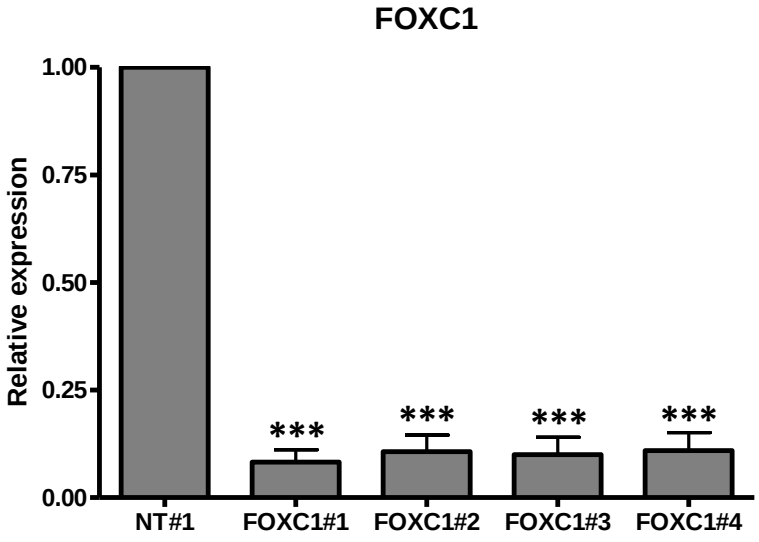
I


TAGLN
Relative expression
1.00
0.75
0.50
0.25
0.00
***
***
***
NT#1
TAGLN#2
TAGLN#4
TAGLN#5

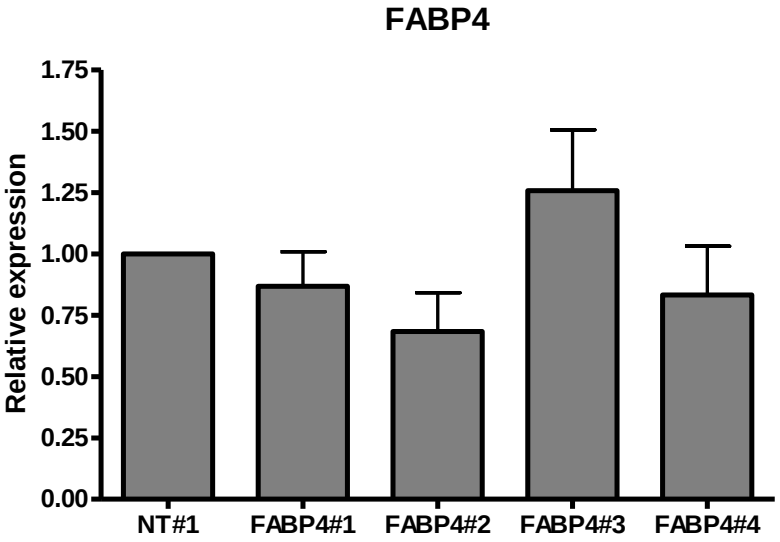
J


CYR61
Relative expression
1.00
0.75
0.50
0.25
0.00
***
**
***
***
NT#1
CYR61#2
CYR61#4
CYR61#5
CYR61#6

K


FOXC1
Relative expression
1.00
0.75
0.50
0.25
0.00
***
***
***
***
NT#1
FOXC1#1
FOXC1#2
FOXC1#3
FOXC1#4

L


FABP4
Relative expression
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
NT#1
FABP4#1
FABP4#2
FABP4#3
FABP4#4

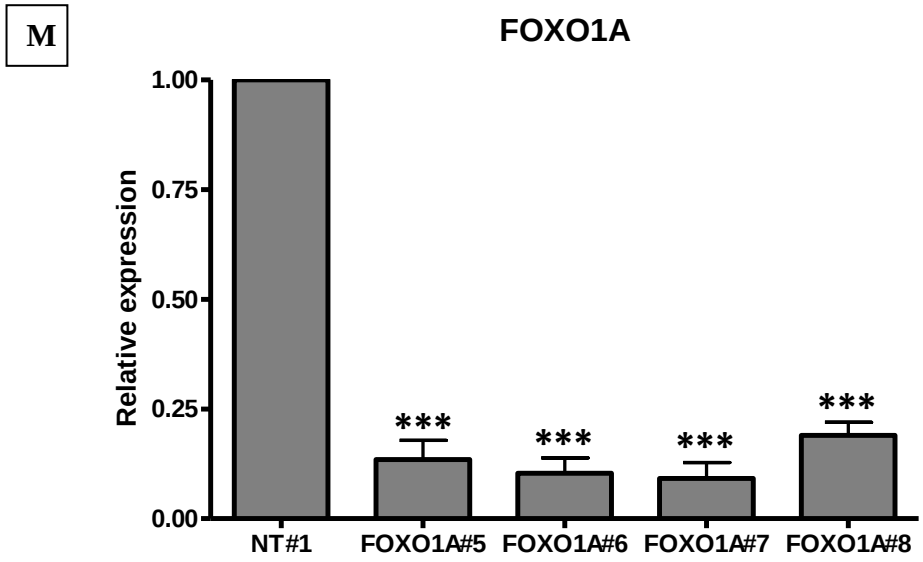


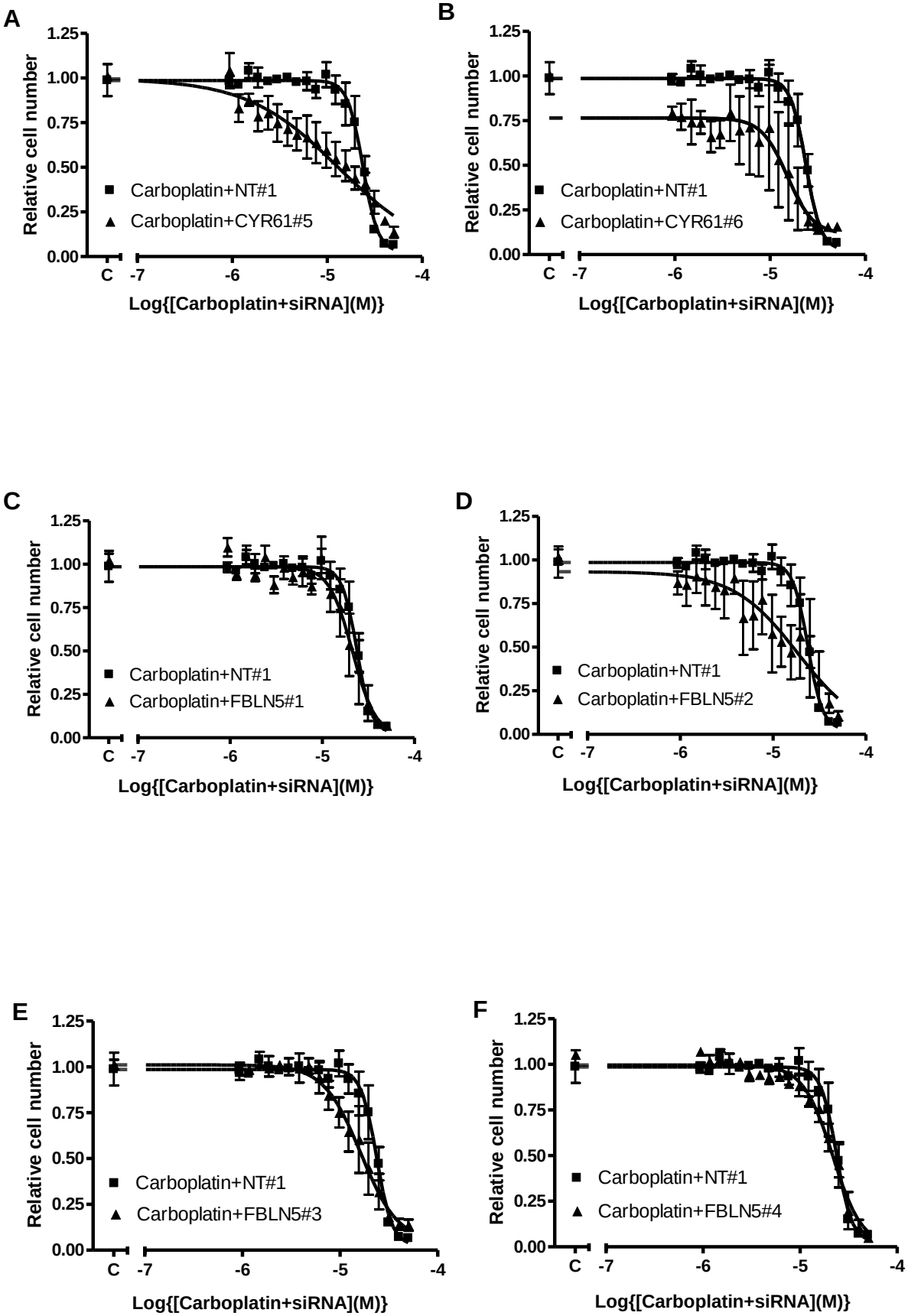
Figure 5.1: Expression of each gene in OVCAR-4 cell line after knock down with different individual siRNAs

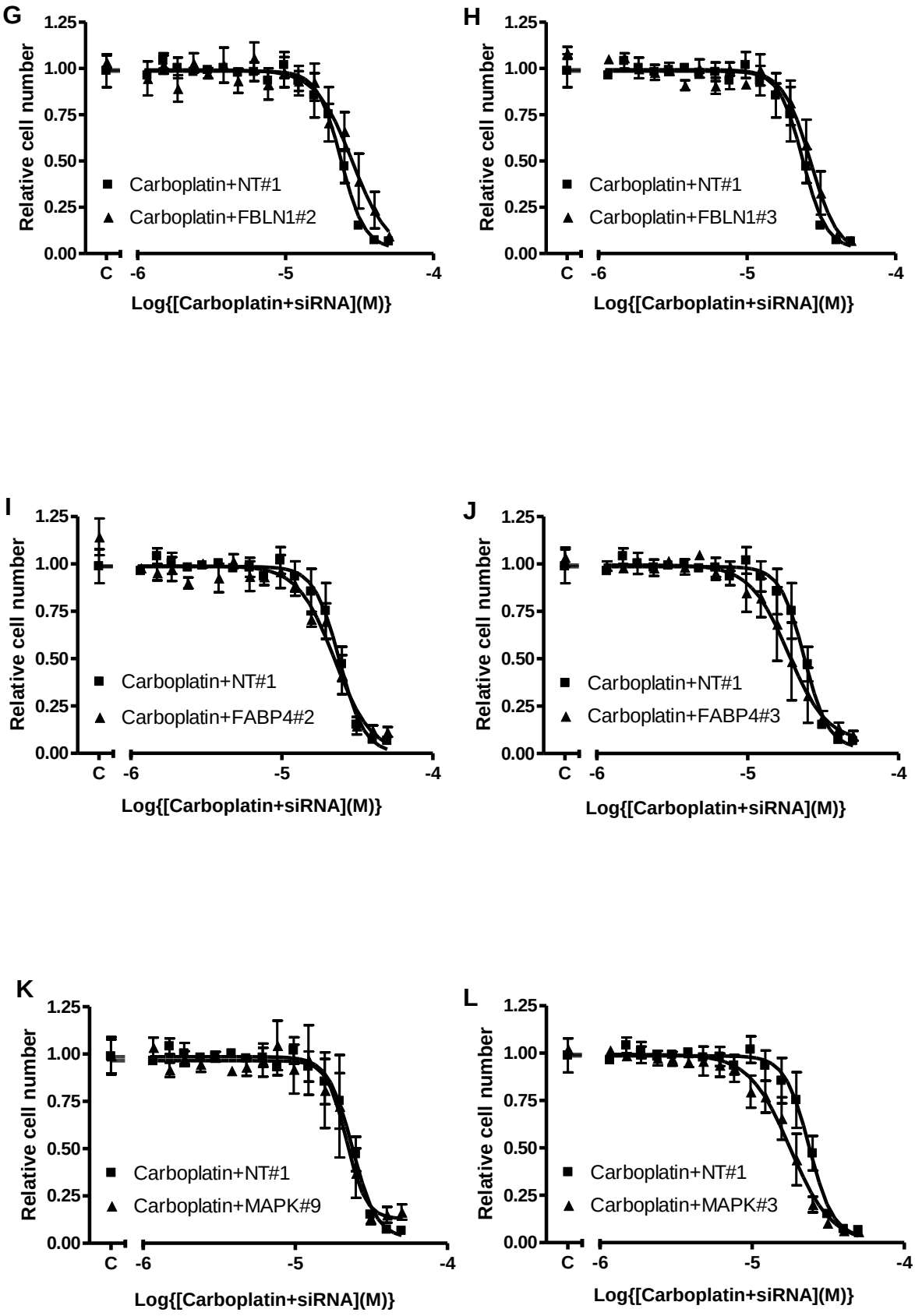
OVCAR-4 cells were transfected with each of the indicated siRNA (100nM) and after 72 hours mRNA isolated from the cells. Knock-down of gene expression was measured by Q-PCR. The results (n=3, mean±S.D.) are expressed as a fraction of that measured in cells transfected with a non-targeting siRNA (NT-1) and normalized to actin. The results were significantly different from the expression measured in cells transfected with a NT-1 where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

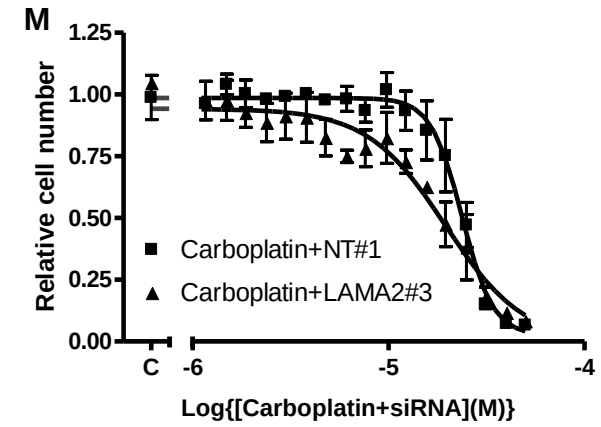
5.3.1.2 Effect of genes knock-down on the sensitivity to carboplatin in cell growth assays

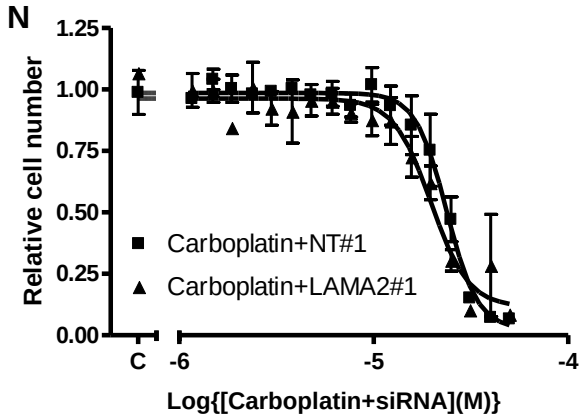
To confirm that the hits from the screen, the hits regulating sensitivity to carboplatin, the expression of the selected genes were knocked-down in OVCAR-4 cells using the siRNA identified in the previous experiment that caused more than 50% knock-down. The impact on sensitivity to carboplatin measured in cell growth assays. Complete concentration response curves were obtained and the potency of carboplatin (IC_{50}) compared in cells transfected with siRNA targeting the hits or a non-targeting siRNA (figure 5.2 and table 5.1).

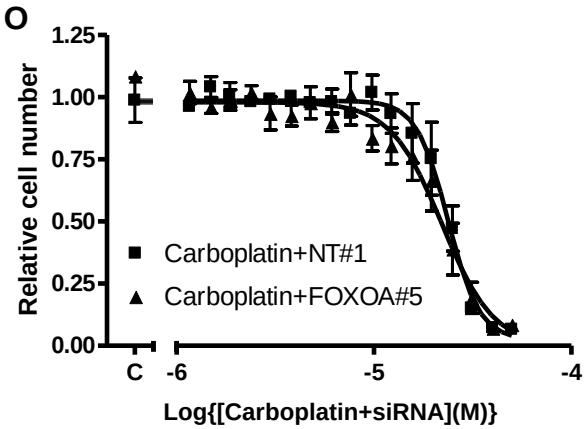
In the case of *CYR61* and *TAGLN,* all the siRNAs tested increased the sensitivity of the cells to carboplatin significantly, giving confidence that these were valid hits. For most of the remaining genes, a more mixed picture was obtained with some siRNA apparently increased sensitivity to carboplatin significantly and some failing to do so, despite all of these siRNA giving robust knock-down in the previous experiments. In the case of *FOXC1, FOXO1A, FBLN5* and *EFEMP1,* at least two siRNA increased sensitivity to carboplatin, suggesting that these also might be valid hits. For *MAPK* and *LAMA2*, one siRNA augmented the potency of carboplatin. However, for these genes only two siRNAs were used and further work with additional siRNA is desirable to make a final decision about the validity of these hits. In the case of *FABP4* and *FBLN1,* none of the siRNA increased sensitivity to carboplatin, suggesting these are unlikely to be valid hits. In summary, of ten genes tested, two genes were identified where all the tested siRNA increased sensitivity to carboplatin and an additional three genes were identified where multiple siRNA increased sensitivity.

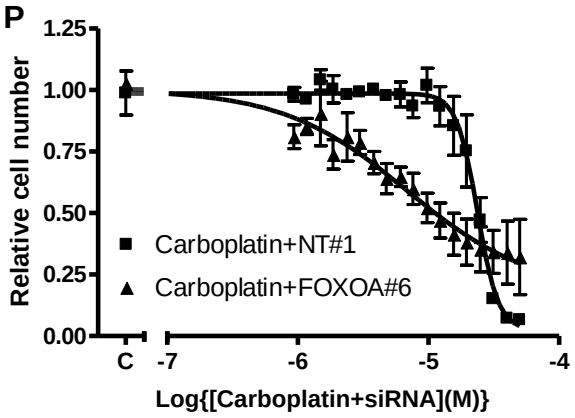

A
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+CYR61#5
C
-7
-6
-5
-4
Log{[Carboplatin+siRNA](M)}
B
Relative cell number
Carboplatin+NT#1
Carboplatin+CYR61#6
Log{[Carboplatin+siRNA](M)}
C
Relative cell number
Carboplatin+NT#1
Carboplatin+FBLN5#1
Log{[Carboplatin+siRNA](M)}
D
Relative cell number
Carboplatin+NT#1
Carboplatin+FBLN5#2
Log{[Carboplatin+siRNA](M)}
E
Relative cell number
Carboplatin+NT#1
Carboplatin+FBLN5#3
Log{[Carboplatin+siRNA](M)}
F
Relative cell number
Carboplatin+NT#1
Carboplatin+FBLN5#4
Log{[Carboplatin+siRNA](M)}

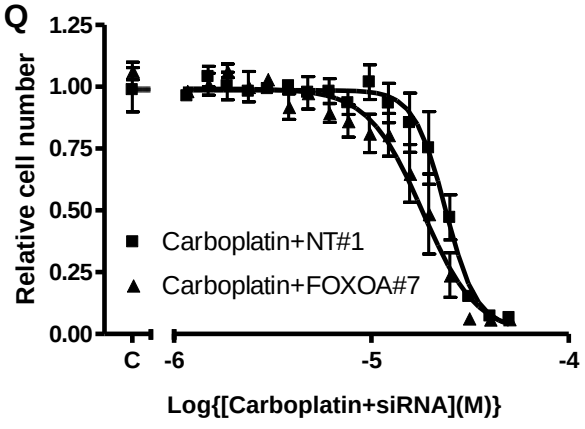

G
H
I
J
K
L
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}
Carboplatin+NT#1
Carboplatin+FBLN1#2
Carboplatin+FBLN1#3
Carboplatin+FABP4#2
Carboplatin+FABP4#3
Carboplatin+MAPK#9
Carboplatin+MAPK#3

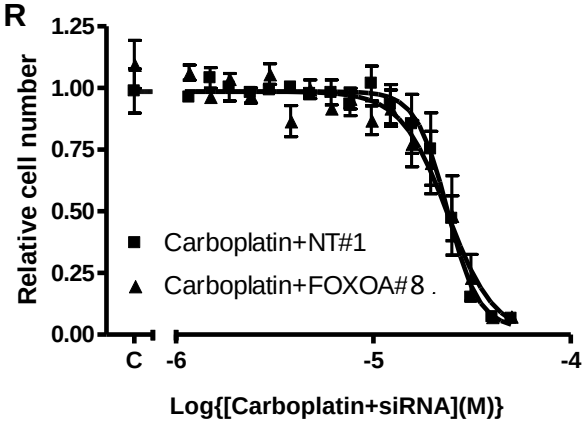

M
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+LAMA2#3
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}

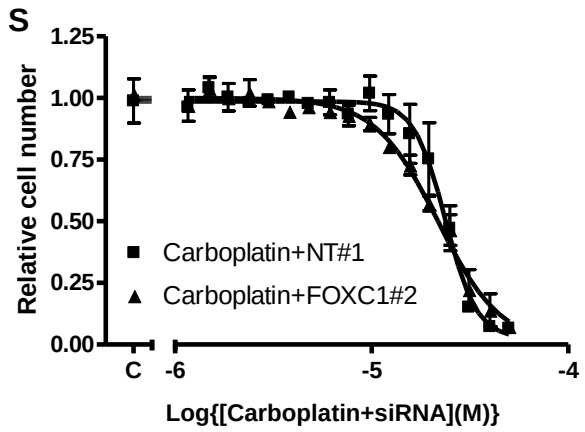

N
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+LAMA2#1
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}

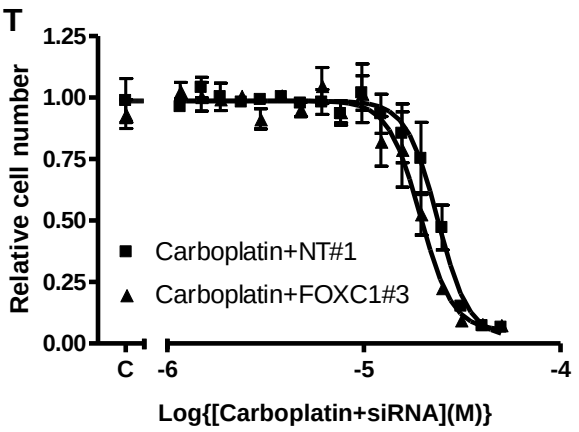

O
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+FOXOA#5
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}

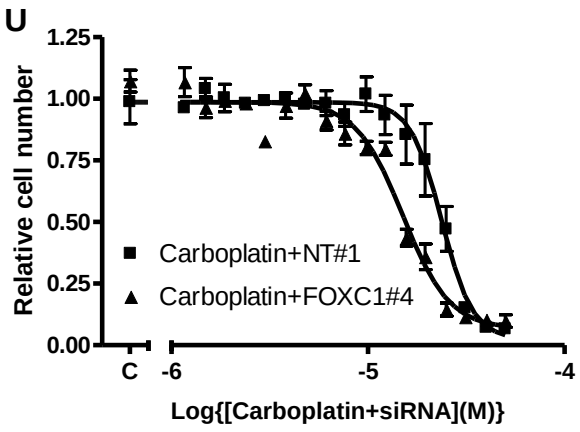

P
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+FOXOA#6
C
-7
-6
-5
-4
Log{[Carboplatin+siRNA](M)}

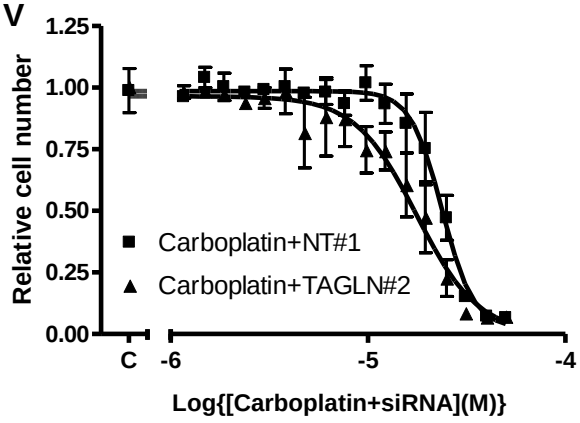

Q
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+FOXOA#7
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}

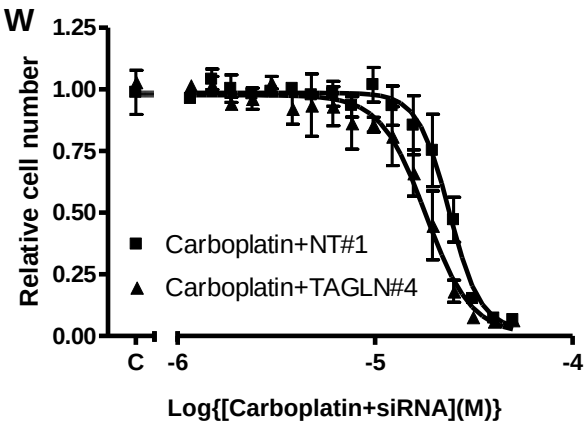

R
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+FOXOA#8
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}

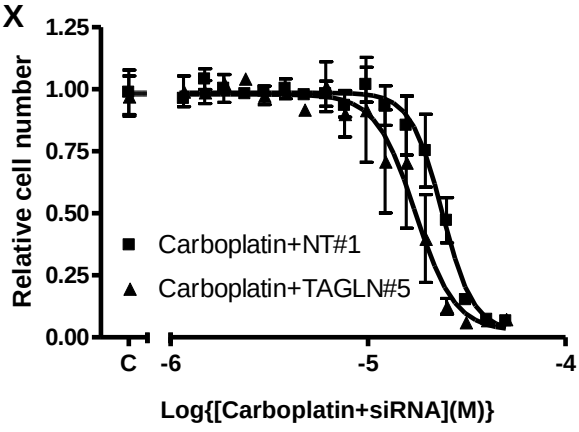

S
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+FOXC1#2
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}


T
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+FOXC1#3
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}


U
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+FOXC1#4
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}


V
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+TAGLN#2
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}


W
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+TAGLN#4
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}


X
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
Carboplatin+NT#1
Carboplatin+TAGLN#5
C
-6
-5
-4
Log{[Carboplatin+siRNA](M)}

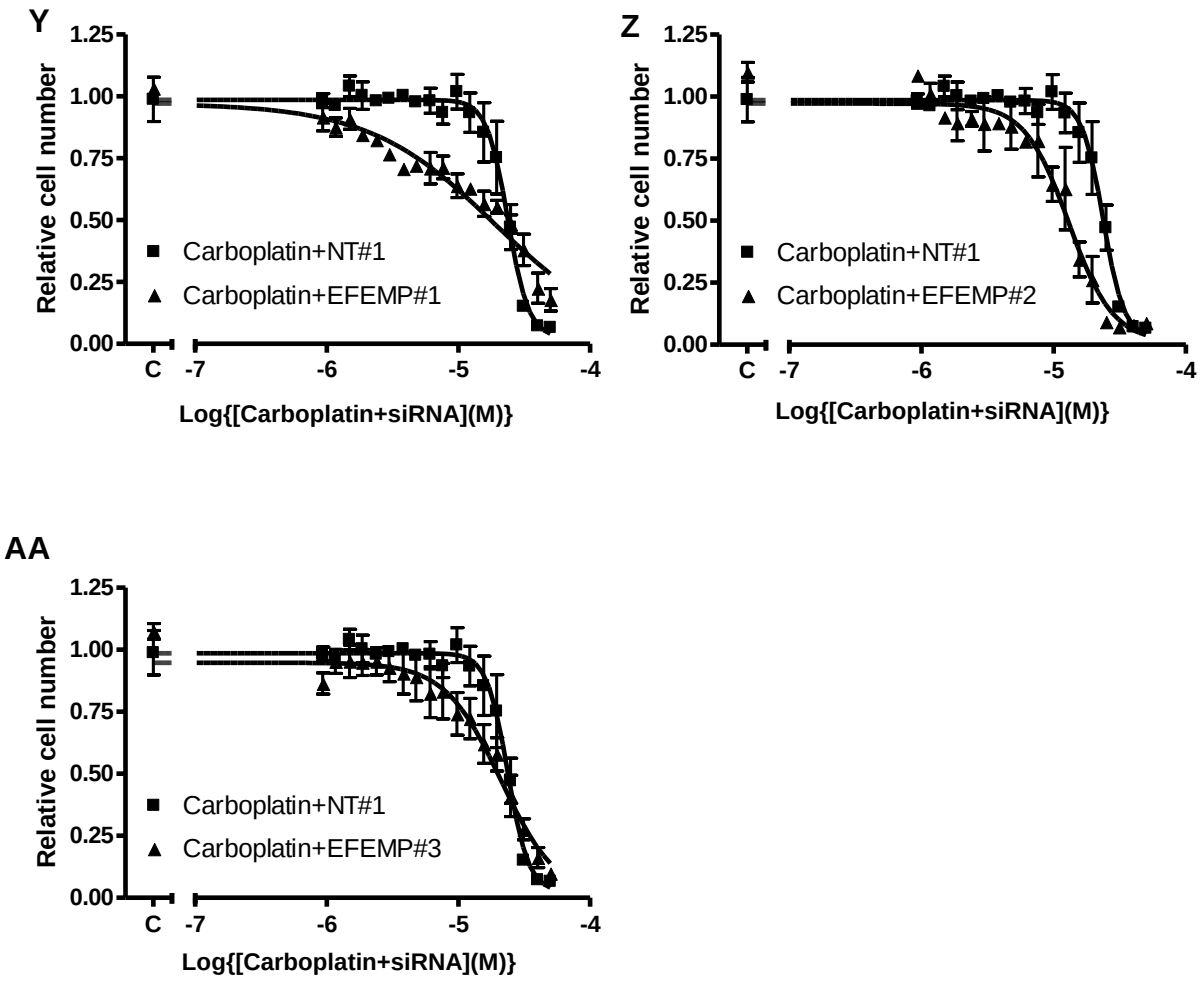


Figure 5.2: Effect of genes knock-down on the sensitivity of OVCAR-4 cell line to carboplatin in cell growth assays

OVCAR-4 cells were transfected with 100 nM NT-1 or 100 nM of different individual siRNAs as indicated. Cell sensitivity to carboplatin was then evaluated in cell growth assays by staining with SRB. The results (n=3, mean± S.D.) are expressed as a fraction of the relative number of cells measured in the absence of drug (labelled "C").

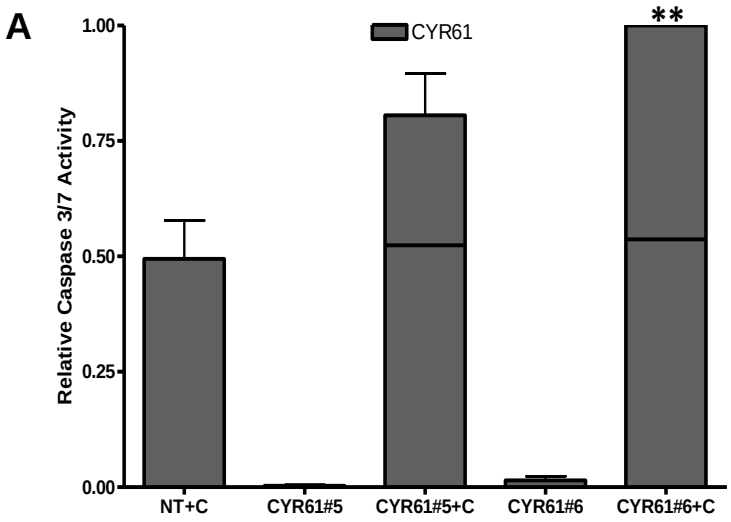
Table 5.1: IC_{50} values of carboplatin following knockdown of selected candidate drug resistance genes

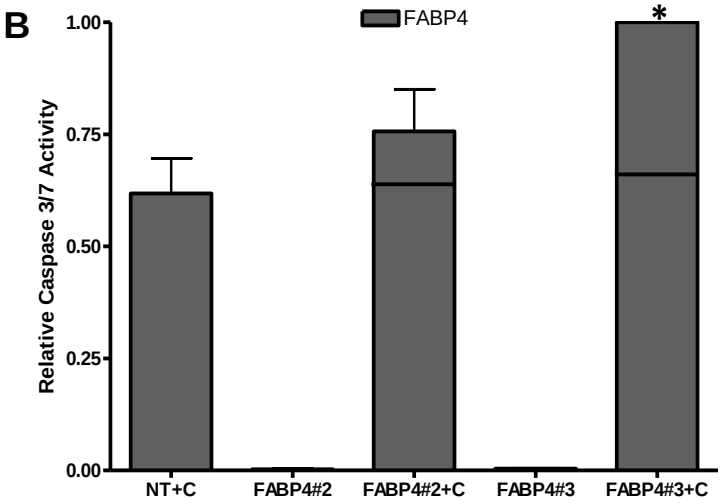
siRNA	Carboplatin IC_{50} (μM)
NT#1	23.7±3.3
CYR61#5	13.1±7.1***
CYR61#6	9.6±8.3***
FABP4#2	21.8±2.8
FABP4#3	19.0±5.7
FBLN5#1	20.8±6.9
FBLN5#2	17.9±1.2*
FBLN5#3	15.8±6.2**
FBLN5#4	22.0±4.6
FOXC1#2	22.1±2.7
FOXC1#3	18.6±1.4*
FOXC1#4	15.1±0.6***
TAGLN#2	16.8±5.4**
TAGLN#4	17.4±3.9**
TAGLN#5	16.3±5.1**
MAPK#3	17.3±3.5**
MAPK#9	20.3±6.3
FBLN1#2	28.2±5.8
FBLN1#3	26.5±4.5
FOXO1A#5	21.6±4.3
FOXO1A#6	6.9±1.8***
FOXO1A#7	18.2±4.3*
FOXO1A#8	23.4±5.1
LAMA2#1	20.7±6.2
LAMA2#3	16.9±2.6**
EFEMP#1	18.0±4.5*
EFEMP#2	12.6±3.2***
EFEMP#3	19.0±5.6

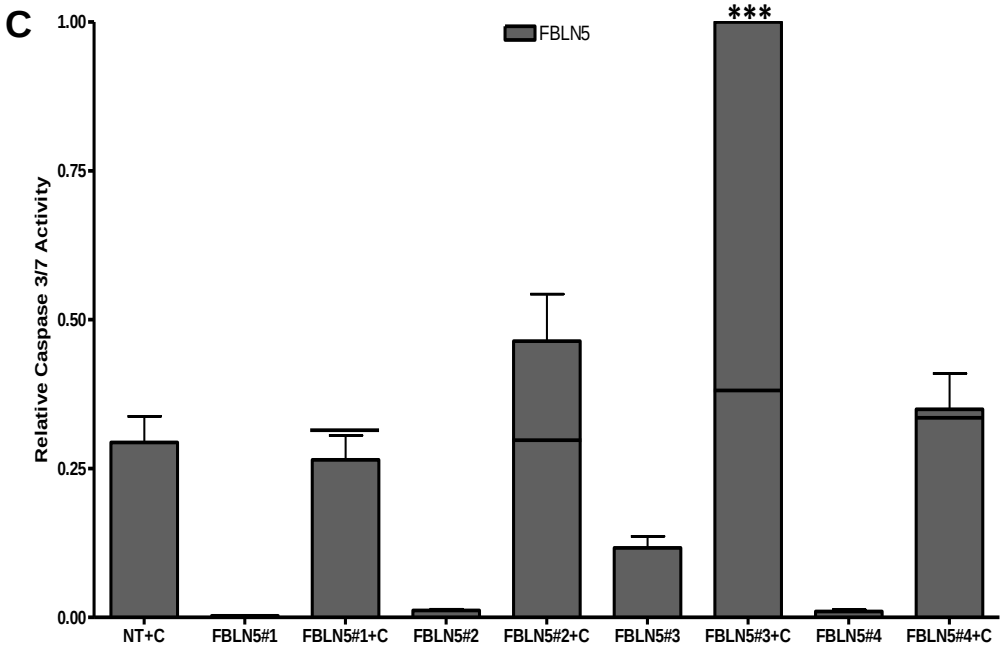
The activity of siRNAs in combination with carboplatin was determined in OVCAR-4 cells using the SRB cell growth assay and are significantly different from that measured in the same cell line treated with carboplatin after transfection with NT-1 where indicated by using paired *t*-test (*, $P<0.05$; **, $P< 0.01$; *** $P< 0.001$). Results are expressed as mean±S.D.; n=3.

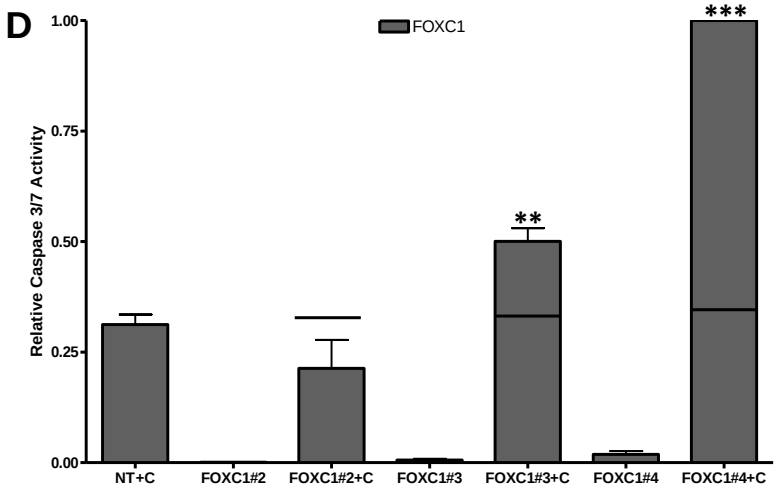
5.3.1.3 Caspase 3/7 assay

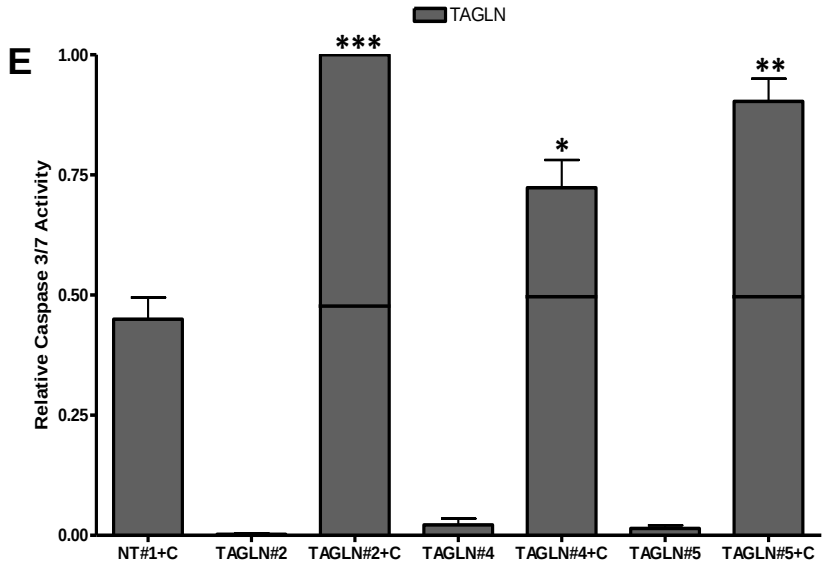
To confirm the results obtained using cell growth assays, the effect of knocking-down the expression of genes on the activation of caspase 3/7 by carboplatin was assessed. The siRNA on their own did not induce significant activation of caspase 3/7, whereas both carboplatin on its own and combinations of siRNA and carboplatin caused robust activation of caspase 3/7. To calculate the expected additive effect of the drug and the siRNA, the Bliss independence criterion was used. This requires calculation of the effect of each agent expressed as a fraction of the maximum caspase activity. Caspase activity is transient in nature and may vary according to the potency of the drug being used. This complicates analysis so the maximum effect was estimated to be the maximum caspase activity measured in each experiment and the fractional effect of each agent calculated from this. In general the caspase 3/7 activity observed in cells transfected with siRNA that increased carboplatin potency in the cell growth assays significantly exceeded that observed in cells transfected with NT-1 and treated with carboplatin. (figure 5.3). Exceptions to this included experiments with siRNAs EFEMP#1, LAMA2#3, CYR61#5 and MAPK1#3 where the siRNA previously were found to increase sensitivity to carboplatin but did not significantly augment caspase 3/7 activity. In the case of FABP4#3, the siRNA augmented the caspase activity although it has previously failed to increase sensitivity to carboplatin in cell growth assays, Importantly, however, all three TAGLN siRNA augmented activation of caspase 3/7 and all three siRNA augmented caspase 3/7 activity.

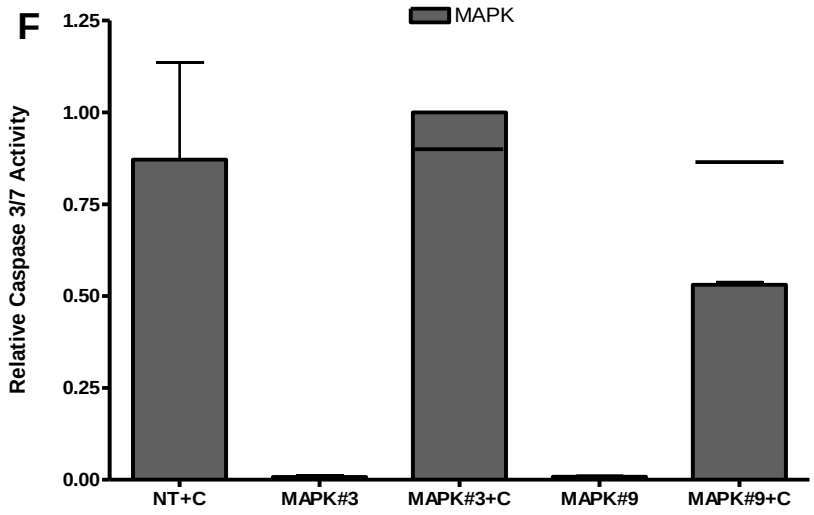

A
CYR61
**
Relative Caspase 3/7 Activity
1.00
0.75
0.50
0.25
0.00
NT+C
CYR61#5
CYR61#5+C
CYR61#6
CYR61#6+C

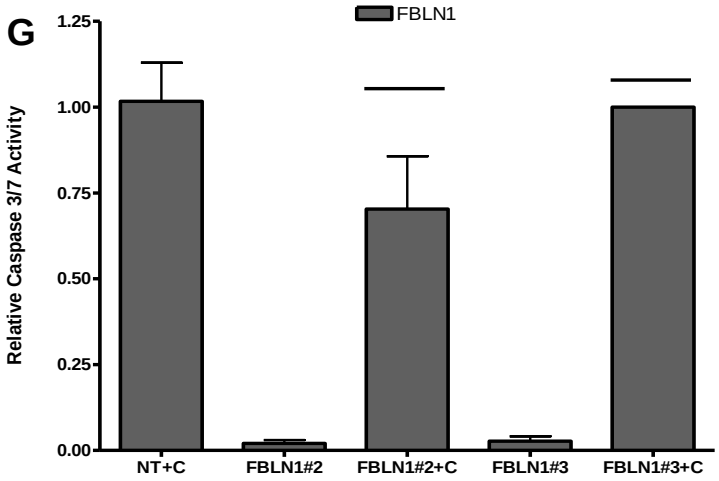

B
FABP4
*
Relative Caspase 3/7 Activity
1.00
0.75
0.50
0.25
0.00
NT+C
FABP4#2
FABP4#2+C
FABP4#3
FABP4#3+C

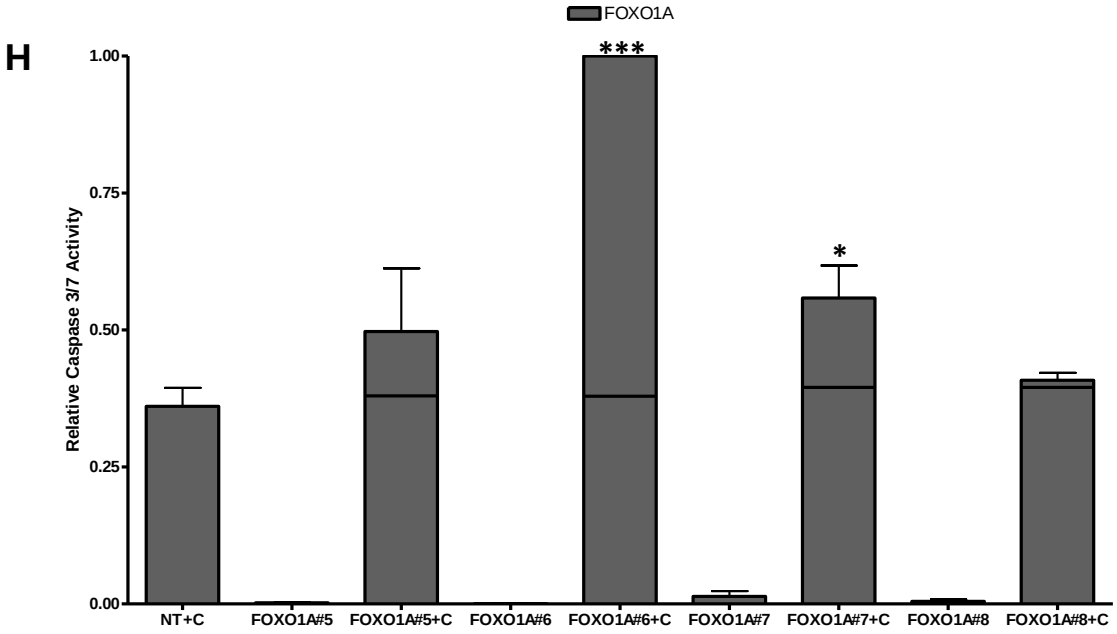

C
FBLN5
***
Relative Caspase 3/7 Activity
1.00
0.75
0.50
0.25
0.00
NT+C
FBLN5#1
FBLN5#1+C
FBLN5#2
FBLN5#2+C
FBLN5#3
FBLN5#3+C
FBLN5#4
FBLN5#4+C

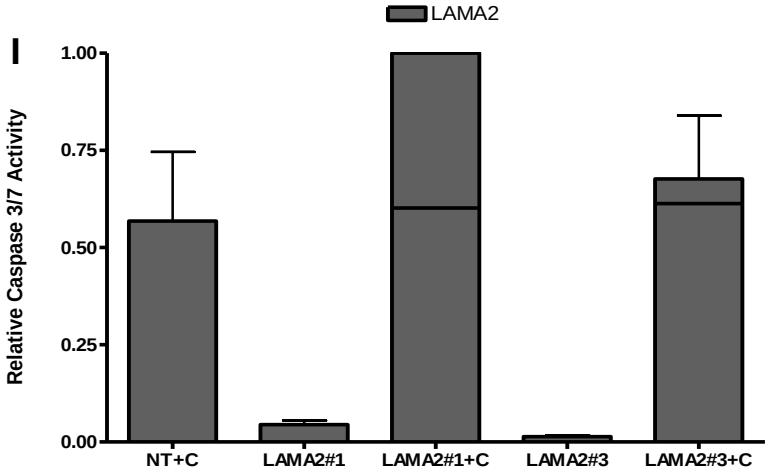

D
FOXC1
Relative Caspase 3/7 Activity
1.00
0.75
0.50
0.25
0.00
***
**
NT+C
FOXC1#2
FOXC1#2+C
FOXC1#3
FOXC1#3+C
FOXC1#4
FOXC1#4+C


E
TAGLN
Relative Caspase 3/7 Activity
1.00
0.75
0.50
0.25
0.00
***
*
**
NT#1+C
TAGLN#2
TAGLN#2+C
TAGLN#4
TAGLN#4+C
TAGLN#5
TAGLN#5+C


F
MAPK
Relative Caspase 3/7 Activity
1.25
1.00
0.75
0.50
0.25
0.00
NT+C
MAPK#3
MAPK#3+C
MAPK#9
MAPK#9+C


G
FBLN1
Relative Caspase 3/7 Activity
1.25
1.00
0.75
0.50
0.25
0.00
NT+C
FBLN1#2
FBLN1#2+C
FBLN1#3
FBLN1#3+C


H
FOXO1A
Relative Caspase 3/7 Activity
1.00
0.75
0.50
0.25
0.00
***
*
NT+C
FOXO1A#5
FOXO1A#5+C
FOXO1A#6
FOXO1A#6+C
FOXO1A#7
FOXO1A#7+C
FOXO1A#8
FOXO1A#8+C


I
LAMA2
Relative Caspase 3/7 Activity
1.00
0.75
0.50
0.25
0.00
NT+C
LAMA2#1
LAMA2#1+C
LAMA2#3
LAMA2#3+C

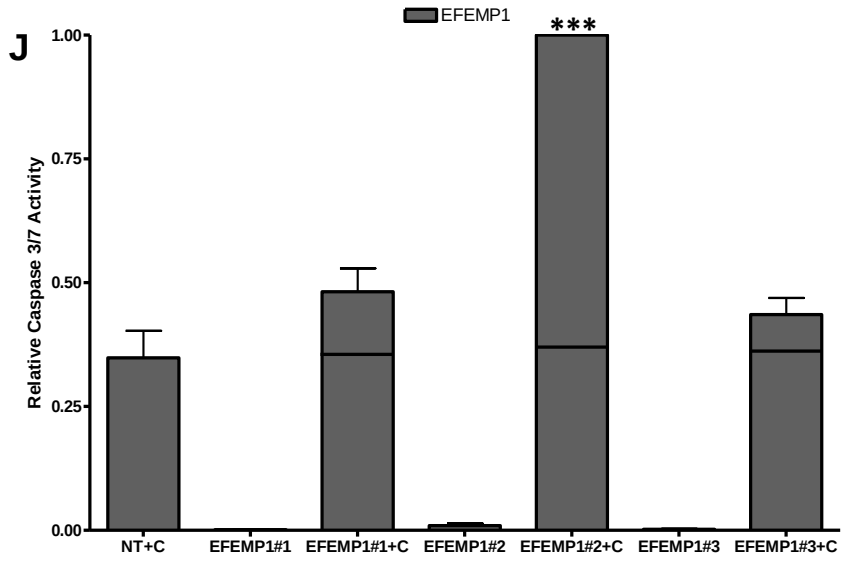


Figure 5.3: Relative caspase 3/7 assay in OVCAR-4 cell line following transfection with different siRNAs and treatment with carboplatin

OVCAR-4 cells were transfected with indicated individual siRNAs (100 nM) and after 24 hours treated with 50 μM carboplatin (denoted "C" in the axis labels). Caspase 3/7 activity was evaluated after a further 48 hours. The siRNA or carboplatin could significantly inhibit cell proliferation resulting in potential variation in cell number between each condition after treatment, therefore parallel samples were also subjected to the same treatment and relative cell number estimated by staining with SRB. The activity of caspase 3/7 was then normalized to the relative cell number measured in the SRB assay. Results (mean ± SD, n=3) are shown after subtraction of activity measured in cells transfected with NT-1 alone value and expressed as a fraction of the maximum caspase 3/7 activity obtained in each experiment and are significantly different from Bliss expected effect (shown as a horizontal bar) where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; paired *t*-test).

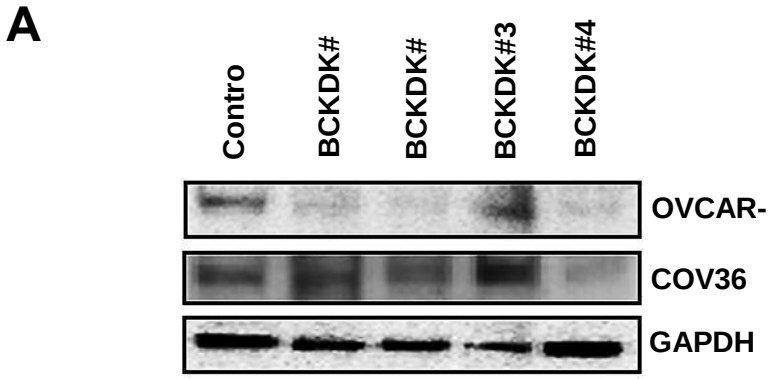
5.3.2. Candidate palitaxel-resistance gene

5.3.2.1 Knock-downs of the candidate palitaxel-resistance gene

Only one hit from the siRNA screen to identify genes that regulate sensitivity to paclitaxel met the criteria for confirmation experiments. This was *BCKDK*, the gene encoding branched-chain amino-acid dehydrogenase kinase (364).

Four different siRNAs were used to repress the expression of *BCKDK* in OVCAR-4 and COV362 and knock-down was analyzed by qPCR and western blotting. Immunoblotting studies demonstrated that although siRNA#3 failed to reduce BCKDK protein, BCKDK was reduced in both cell lines after transfection with siRNAs #2 and #4. Intriguingly, siRNA#1 reduced BCKDK in OVCAR-4 but not COV362 cells (figure 5.4 A and B).

In support of these observations, qPCR studies showed that BCKDK mRNA was reduced following transfection with siRNA#2 and #4 and also in OVCAR-4 cells following transfection with BCKDK#1.



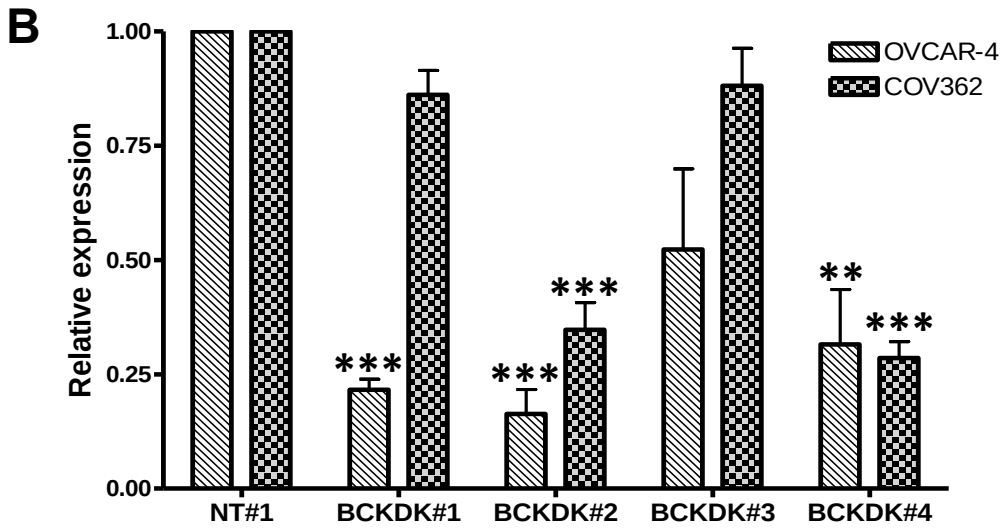


Figure 5.4: BCKDK expression in OVCAR-4 and COV362 cells following knock down with different individual BCKDK siRNAs.

A. The expression of BCKDK protein was determined by western blotting of protein lysates obtained from OVCAR-4 and COV362 cell lines 48 hours after transfection with the indicated BCKDK siRNA. B. Knock-down of BCKDK mRNA was also measured by Q-PCR. The results are expressed as a fraction of that measured in cells transfected with a non-targeting siRNA NT-1 and normalized to actin (n=3, mean± S.D.), and are significantly different from the expression measured in cells transfected with a non-targeting siRNA NT-1 where indicated (**, $P< 0.01$; *** $P< 0.001$; paired *t*-test).

5.3.2.2 Effect of BCKDK knock-down on the sensitivity to paclitaxel in cell growth assays

The impact on the sensitivity to paclitaxel after knocking-down the expression of BCKDK was explored in OVCAR-4 and COV362 cell lines by using cell growth assays. When compared to cells transfected with NT-1, knock-down of BCKDK with siRNA #1, #2 and #4 in OVCAR-4 increased sensitivity to paclitaxel (figures 5.5 and table 5.2). Similarly, in COV-362, siRNA #2 and #4 increased sensitivity to paclitaxel (figure 5.6, table 5.2). Reassuringly, siRNA#3, which failed to knock-down BCKDK expression, also failed to alter the sensitivity to paclitaxel.

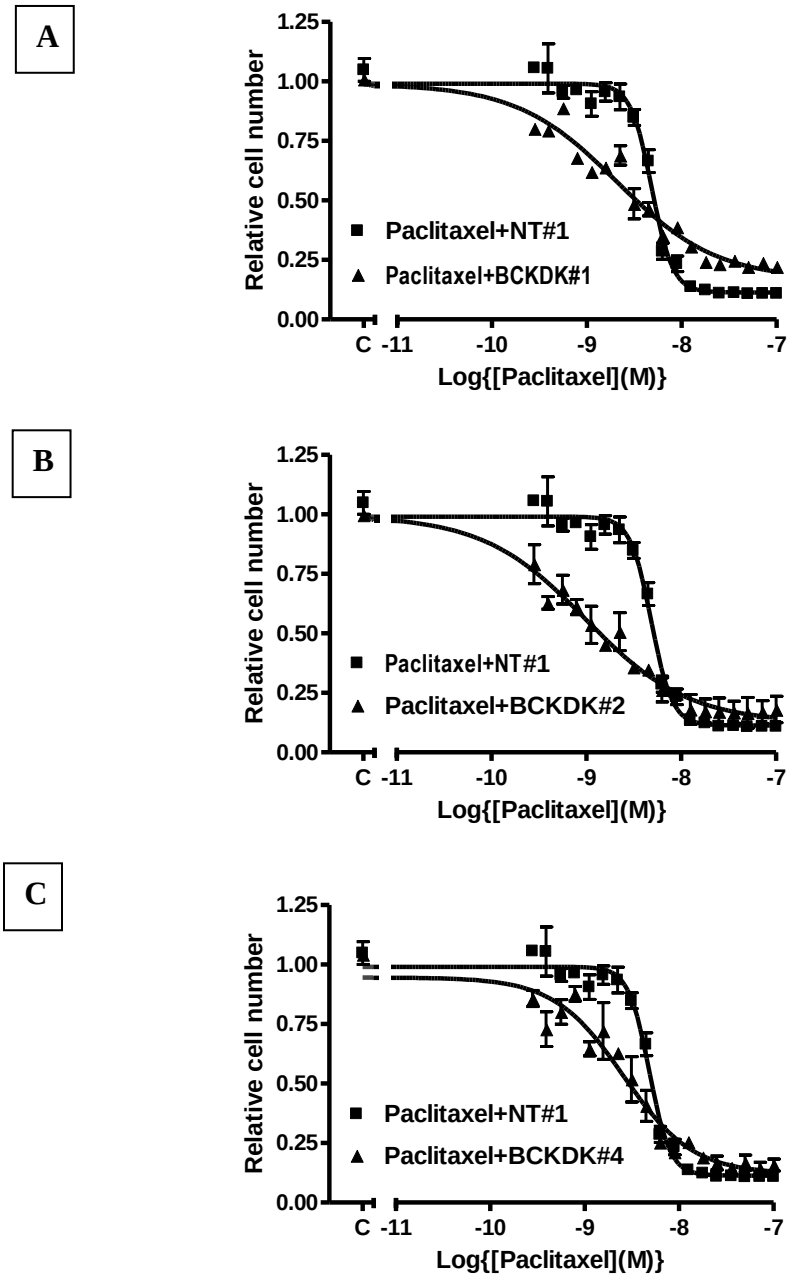


Figure 5.5: Effect of BCKDK knock-down on sensitivity to paclitaxel in OVCAR-4 cell growth assays.

OVCAR-4 cells were transfected with different individual BCKDK siRNA (100 nM, except 50 nM for BCKDK #2, as cell growth inhibition has been noticed by using more than this concentration) or NT-1 as indicated. The sensitivity of the cells to paclitaxel was subsequently measured in cell growth assays. The results (mean± S.D.; n=3) are expressed as a fraction of the relative cell number in the absence of paclitaxel (labelled "C").

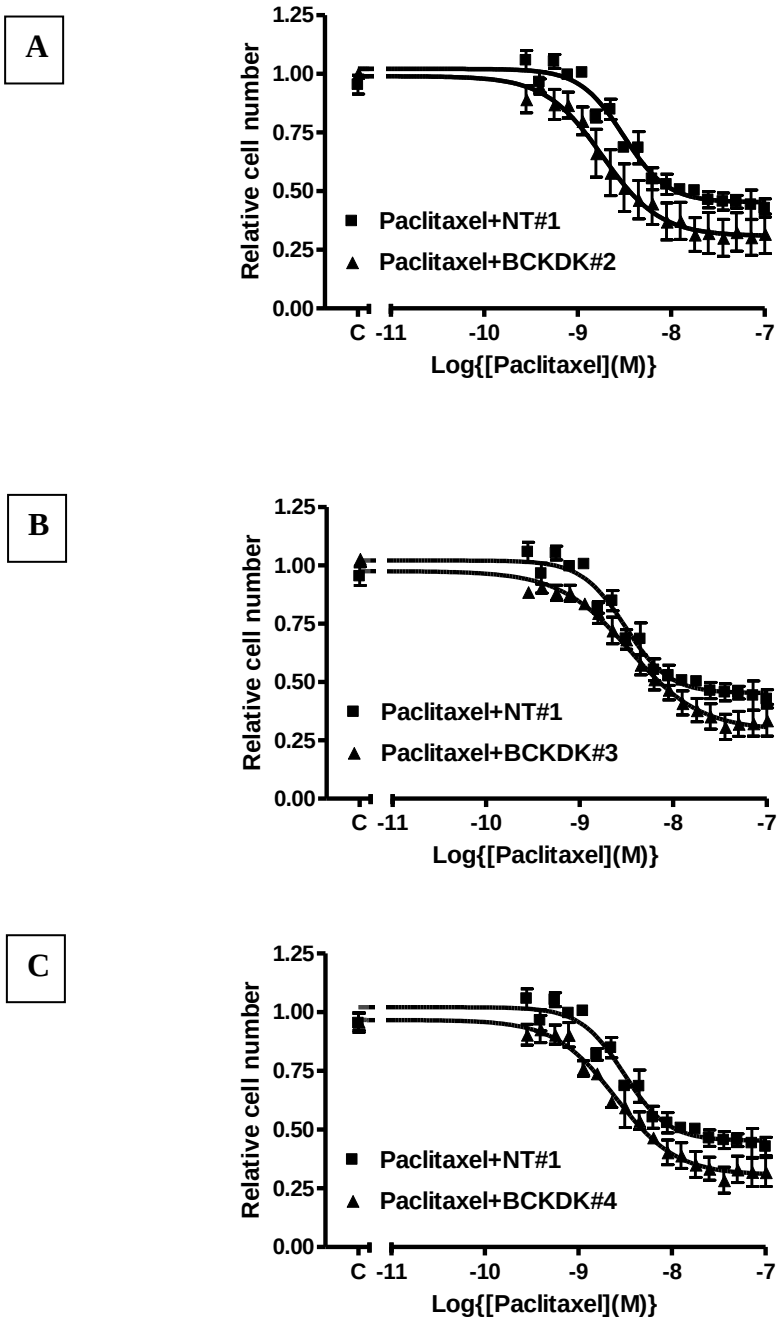


Figure 5.6: Effect of BCKDK knock-down on sensitivity to paclitaxel in COV362 cell growth assays.

COV362 cells were transfected with different individual BCKDK siRNA (100 nM) and NT-1 as indicated. The sensitivity of the cells to paclitaxel was subsequently measured in cell growth assays. The results (mean± S.D.; n=3) are expressed as a fraction of the relative cell number in the absence of paclitaxel (labelled "C").

Table 5.2: Potency of paclitaxel in cells in which BCKDK expression was repressed.

OVCAR-4		**COV362**	
siRNA	**Paclitaxel IC_{50} (nM)**	**siRNA**	**Paclitaxel IC_{50} (nM)**
NT#1	4.8±0.3	**NT#1**	3.2±0.2
BCKDK#1	1.9±0.3***	**BCKDK#2**	1.9±0.5*
BCKDK#2	0.96±0.2***	**BCKDK#3**	3.2±0.2
BCKDK#4	2.5±0.8**	**BCKDK#4**	2.2±0.2**

The change in potencies of paclitaxel following knock-down of *BCKDK* by different siRNA is reported as the IC_{50} in these experiments (mean± S.D.; n=3) and are significantly different from the potency measured in cells transfected with NT-1 where indicated (*, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$; paired *t*-test).

5.3.3.3 Caspase 3/7 assay

To confirm the results obtained in cell growth assays, caspase 3/7 activity was assessed following knock-down of BCKDK expression and exposure to paclitaxel. OVCAR-4 cells transfected with BCKDK siRNA did not show a significant increase in caspase 3/7 activity. Although paclitaxel increased caspase-3/7, this was augmented in cells transfected with BCKDK siRNA#1, #2 and #4 and was significantly higher than the effect estimated from the Bliss independence criterion if the siRNA and paclitaxel had an additive effect (figure 5.7).

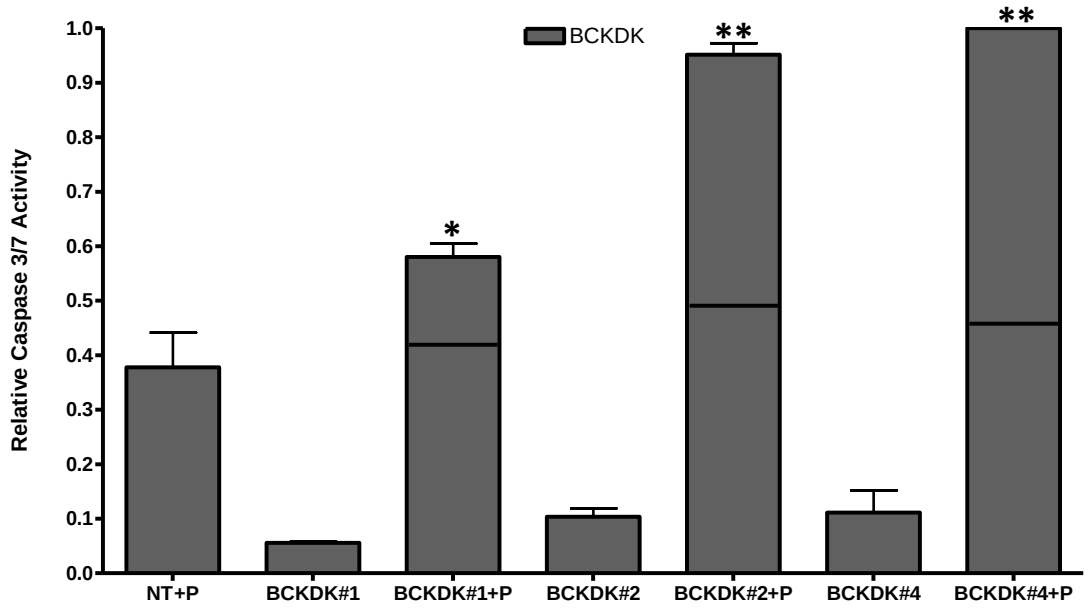


Figure 5.7: Relative caspase 3/7 assay in OVCAR-4 cell line after transfection with different individual BCKDK siRNAs and treatment with paclitaxel.

OVCAR-4 cells were transfected with indicated individual siRNAs (100 nM; except BCKDK#2 which was transfected with 50 nM and after 24 hours treated with 12 nM paclitaxel. Caspase 3/7 activity was evaluated after a further 48 hours. The siRNA or paclitaxel could potentially significantly inhibit cell proliferation resulting in potential variation in cell number between each condition after treatment, therefore, parallel samples were also subjected to the same treatment and relative cell number estimated by staining with SRB. The activity of caspase 3/7 was then normalized to the relative cell number measured in the SRB assay. Results (mean ± SD, n=3) are shown after subtraction of activity measured in cells transfected with NT-1 alone value and expressed as a fraction of the maximum caspase 3/7 activity obtained in each experiment and are significantly different from Bliss expected effect (shown as a horizontal bar) where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; paired *t*-test).

5.4 Discussion

RNA interference is increasingly being used to conduct high-throughput screens to unveiling potential molecular mechanisms for numerous diseases, and to identify novel targets for developing effective and safe treatments (369). The work presented in this chapter explored the hypothesis that silencing of specific genes, previously shown to be over-expressed in recurrent ovarian cancer, may increase the sensitivity of ovarian cancer cells to chemotherapy. The genes evaluated were hits from a high-throughput functional screen previously conducted in our lab for identification of genes that confer resistance to chemotherapy. Although several factors had been taken into account to establish a reliable screen, including specific consideration about timing of the addition of chemotherapeutic agents, the concentrations of these agents and the choice of cell lines the hits required confirmation before further work was warranted.

Fourteen potential candidate resistance genes ("hits") from the screen implicated in resistance to both carboplatin or paclitaxel were evaluated. The variable extent of knock-down shown with *CFHL* and *ENTPD* (figure 5.1 A and C) necessitate further experiments to identify suitable siRNA, and so they are excluded from the experiments that have investigated the impact of knock-down on the sensitivity to carboplatin measured in cell growth assays. In the case of *PLA2R1*, an siRNA which caused knock-down were not identified, instead an apparent modest increase in the expression of gene, (which was statistically not significant) was noticed and this led to exclusion of this gene from further investigations too.

The remaining 11 candidate genes were chosen for further investigations to validate them. These genes include: *BCKDK*, *CYR61*, *FABP4*, *FBLN5*, *FOXC1*, *TAGLN*, *MAPK*, *FBLN1*, *FOXO1A*, *LAMA2* and *EFEMP1*. The hits that have been confirmed in this research include *CYR61* and *TAGLN*, which were shown to be a potential candidate gene for carboplatin resistance, in addition to *BCKDK*, that has been shown to be implicated in paclitaxel

resistance. The confidence in the data obtained with these genes is reinforced because all of the different siRNA that repressed gene expression also changed drug sensitivity. Additionally, potentiation of the activity of chemotherapy by the siRNA targeted against these genes was confirmed in a separate assay (caspase 3/7). For *FOXC1*, *FOXO1A*, *FBLN5* and *EFEMP1*, some siRNA significantly increased sensitivity to carboplatin and some failed to do so, therefore this needs to be confirmed by further work. For *MAPK* and *LAMA2*, only one siRNA increased the potency of carboplatin. However, only two siRNAs were used for these genes and exploring additional siRNA is essential to draw a final decision about the validity of these hits.

One of the gene products identified in the screen was *CYR61*, Cysteine-rich angiogenic inducer 61 also known as CCN family member 1 (CCN1). The encoded protein is part of the extracellular matrix, and produced by different cells including endothelial cells, smooth muscle cells and fibroblasts. The CCN family (1,2), includes CCN1/Cyr61; connective tissue growth factor (CCN2/CTGF); nephroblastoma (CCN3/NOV); and Wnt inducible secreted proteins (CCN4-6/WISP1-3) (368). CYR61 binds to multiple integrins in different types of cells and has shown to be implicated in regulation of cell survival, proliferation, differentiation and apoptosis in addition to their role in inflammatory process, cardiovascular development, leukocyte migration, angiogenesis and carcinogenesis (370). The expression of *CYR61* is regulated by growth factors and cytokines. However, CYR61 can directly control migratory processes and cell adhesion and modulate the formation of other chemokines and cytokines through autocrine and paracrine feedback loops (371). It has been reported that CYR61 is involved during the course of the breast cancer progression to an invasive phenotype, where high level of CYR61 promotes breast cancer cell growth and invasion and therefore, it could be a potential target for therapeutic treatment of this disease and may suggest an approach to inhibit IGF-1 mediated CYR61 induced breast cancer (372,373). It has

also been shown that CYR61 works as a key regulator in cancer progression and considered as a prostate tumour-promoting factor. Over-expression of CYR61 is a clinically beneficial biomarker in prostate cancer patients (374). Furthermore, overproduction of CYR61 in ovarian carcinomas has been proposed as an independent and prognositc marker (375).

Work in this chapter has shown that knock-down of CYR61 increased the sensitivity of cells to carboplatin. This finding is in agreement with Lee and his colleagues who found that over-expression of CYR61 contributes to drug resistance, inhibiting apoptosis induced by carboplatin by increasing the levels of NF-κB, Bcl-2, Bcl-xL and Mcl-1 in addition to decreasing Bax and p53 expression. (376). This is particularly important because it gives confidence that the original screen conducted by our group identified *bona fide* drug resistance genes. Additional work is needed with CYR61 to explore if levels of CYR 61 are elevated in any of the cell lines used in this study.

TAGLN, which encodes transgelin was identified as hit in the screen and the validation evidence presented here strongly supports this as being an authentic hit. Inhibition of the expression of TAGLN in OVCAR-4 cell line using three individual siRNAs, and in each case this augmented the activity of carboplatin in both cell growth and caspase-3/7 assays. TAGLN, also known as SM22α, is an actin-linking protein that promotes normal cell motility and has shown to play a role in differentiation, migration, invasion and tumourigenesis (377). Increases or decreases in transgelin expression has reciprocal consequences on cancer behaviour, where higher expression might promote metastasis (378). Although the exact role of TAGLN in cancer is controversial, some studies have shown that high levels correlate with poor prognosis, aggressive tumour behaviour and advanced stage (42,43). It has also been found to be upregulated in gastric and colon cancers (381,382). Transgelin expression has an impact on metastatic potential in colorectal cancer cell lines, and this may happen in part

because of the altered level of downstream target genes that influence cell motility. Some studies propose that TAGLN is a potential biomarker for predicting the prognosis of lung adenocarcinoma patients and therefore, can be an attractive therapeutic target (383). A recent report has suggested that the overexpression of transgelin in tumour cells might predispose to chemoresistance, because *TAGLN* was found to be overexpressed in chemotherapy resistant human breast cancer and that suppression of it restored the sensitivity of cells to chemotherapy and significantly inhibited cancer cells proliferation, differentiation, metastasis, and invasion (384). Controversially, the function of TAGLN is usually linked with increased apoptosis in prostate cancer cells and glomerular epithelial cells and also with increased senescence in fibroblasts (385,386). TAGLN may suppresses MMP9, a known element of invasion, and therefore prevents the migration of colon and prostate cancer cells (387,388). The research presented here represents the first study to show an increased sensitivity of ovarian cancer cells to carboplatin when TAGLN function is impaired.

Several studies have suggested the significant contribution of actin binding proteins to apoptosis pathway and that actin dynamics play essential role in regulation of apoptosis. Malignant transformation cause changes in the organization of the actin cytoskeleton and it is postulated that remodeling of actin cytoskeleton by upregulation of actin-linking proteins is likely to enable cancer cells to evade apoptosis and develop resistance to chemotherapy. Inhibition of actin cytoskeletal remodeling by targeting the proteins that regulate actin polymerization is a potential strategy to suppress resistance to chemotherapy (389–391). These findings make TAGLN as an important potential target for sensitizing cancer cells to the chemotherapy.

The *FOXC1* gene that encodes FOXC1 protein belongs to the forkhead box (FOX) family proteins. These are a group of transcription factors that have important role in cell proliferation, differentiation, survival and death, in addition to the development and

progression of tumours (392–394). FOXC1 has been reported to play essential role in embryogenesis, ocular development, in addition to its participation in the development and maintenance of the mesenchymal niches for progenitor and haematopoietic stem cells (395). FOXC1 has been proposed as a diagnostic marker and therapeutic targets for different types of cancer (392–394). Recently, it has been demonstrated that FOXC1 plays a crucial role in cancer development and metastasis, where elevated levels of FOXC1 are potentially associated with poor prognosis in basal-like breast cancer (396). A novel signalling axis including EGFR - NF-κB - FOXC1 has been demonstrated to be critical for basal-like breast cancer cell function, endorsing the idea that targeting the FOXC1 pathway could provide potential modalities for the treatment of basal-like breast cancer (397).

The critical role of FOXC1 in tumour development and progression is not only limited to breast cancer, as it has also been reported to be involved in many other tumours. For example, over-expression of FOXC1 is linked with poor prognosis in pancreatic ductal adenocarcinoma (398), hepatocellular carcinoma (399), cervical carcinoma (400), and gastric cancer (401). However, the exact role of this gene in ovarian cancer has not yet been determined. In one recent study, a significant correlation has been observed between FOXC1 protein level and the pathological subtype of ovarian cancer as well as FIGO staging, and this suggests that high FOXC1 protein expression may provide a biological marker for benign serous ovarian carcinomas and imply a trend towards good prognosis (402). The research in this chapter provides the first evidence that downregulation of FOXC1 may increase the sensitivity of ovarian cancer cells to carboplatin.

Similarly, the mammalian forkhead box, O subclass (FOXO) family includes FOXO1A, FOXO3A, FOXO4, and FOXO6, serve as transcription factors and are involved in the control of several cellular processes such as differentiation, proliferation, metabolism, apoptosis, as well as responses to DNA damage (403). FOXO1A and other FOXO family proteins integrate

together to support signals from multiple pathways to regulate gene expression (404). Therefore, as a well-established negative regulator of the PI3K/Akt signalling pathway, FOXO1A downregulation in cancer might impair the therapeutic potential of PI3K/AKT inhibitors in the treatment of cancer (405). Increased FOXO1A phosphorylation has been implicated in different cancers including gastric carcinoma and therefore, it could serve as an important prognostic variable in gastric cancer and may predispose to the development of targeted therapy (406). On the other hand it has been shown trastuzumab has the ability to inhibit the HER2 overexpressing breast cancer cells proliferation by re-activating FOXO1A via blocking of the PI3K/Akt pathway (407). However, its potential role in ovarian cancer has not been studied to date. The research presented here suggests that inhibiting FOXO1A may increase the sensitivity of ovarian cancer cells to carboplatin. Considering FOXO1A, like FOXC1, is a transcription factor, it is not immediately clear how best to develop drugs to inhibit its function. It may be possible to inhibit the pathways that regulate these transcription factors and further work is desirable to explore this possibility.

Transcription factors have the potential to regulate multiple genes and so may contribute to drug resistance through several different pathways (408,409). Interestingly, the work here has revealed two hits from the forkhead family that serve as transcription factors, and this may offer a mechanism for inhibiting multiple drug-resistance pathways simultaneously and reduce chemoresistance in ovarian cancer.

Another hit from the screen that has been performed in this research was *EFEMP1* (also called fibulin-3), a gene that encodes epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (410). Fibulins have been demonstrated to modulate cell morphology, adhesion, growth and motility, some of the key hallmarks of cancer (411). EFEMP1 is a pivotal extracellular matrix protein and it has been noted that de-regulated *EFEMP1* gene expression in some forms of solid tumours has a wide range of conflicting

roles (412). It is interesting that several hits are extracellular matrix proteins, and this confirms the work that the screen was based on, which showed over-expressed proteins were enriched in ECM proteins (363).

EFEMP1 plays an important role in cancer initiation and progression, via both oncogenic and tumour-suppressive effects in different tumours. This paradoxical role of EFEMP1 is closely linked to tissue-specific expression (413,414). A potential tumour-suppressing role of EFEMP1 was demonstrated in a study of sporadic breast cancer, lung cancer and glioblastoma multiform, where EFEMP1 was associated with reduced cell migration and invasion (415–417). In contrast, cervical cancer and pancreatic cancer demonstrate high expression of EFEMP1, which plays an important role in development and metastasis and this may link it to poor prognosis (418,419). Likewise, *FBLN5* is an extracellular matrix protein that belongs to fibulin family. It is a multifunctional signalling protein that regulates cell proliferation, migration and invasion of normal and cancerous cells (420). In this research, using different individual EFEMP1 and FBLN5 siRNAs has resulted in potentiation of carboplatin activity, which was significant using two separate siRNA for each gene. Accordingly, *EFEMP1* and *FBLN5* may be valid hits, however, further investigations are desireable

Arguably, one of the most promising hits validating by the work presented here was *BCKDK*, which encodes the branched chain alpha-ketoacid dehydrogenase kinase. Several different siRNAs were used and each shown to increase sensitivity to paclitaxel in both cell growth assays and in experiments measuring caspase 3/7 activity. These results were initially observed in OVCAR-4 cells and subsequently extended to COV-362 cells. Although this gene was identified as hit in the screen conducted by our group for genes conferring resistance to paclitaxel, it did not have any impact in the screen on the activity of carboplatin.

The promising results obtained from BCKDK knock-down urged us to further pursue this in greater details. The choice of BCKDK for further investigations was strengthened because BCKDK is an enzyme, so it is easier to develop drugs to inhibit its activity. TAGLN is, in contrast, an ECM protein therefore, it is not clear how it can best be inhibited and a protein-protein interaction blocker may be required. Likewise, CYR61 is an ECM protein and has been identified by others as a resistance gene. On the other hand, FOXO1A and FOXC1 are transcription factors, making it challenging to design drugs to inhibit their function.

In conclusion, there are several hits from the screen that appear to be valid hits, including TAGLN, CYR61, BCKDK, FOXC1, FOXO1A, EFEMP1 and FBLN5, where more than one siRNA worked in knock-down, cell growth and caspase 3/7 assays however, additional work is required to sort out the inconsistencies of some hits. Accordingly, this finding suggests around half of the evaluated genes were valid hits. TAGLN and CYR61 clearly showed reproducible effects and almost all the siRNA targeting these genes worked in all assays that were performed. The same is true for BCKDK but the easy of "drugging" this target lead us to prioritize it for further investigations.

Chapter 6

Inhibition of the branched chain α-keto acid dehydrogenase kinase increases the sensitivity of ovarian cancer cells to paclitaxel

6.1 Introduction

Following the successful validation of several hits identified by the siRNA screen to discover novel regulators of paclitaxel sensitivity, branched-chain α-ketoacid dehydrogenase kinase (BCKDK) was selected for further investigation to understand its role as a determinant of the sensitivity of ovarian cancer cells to paclitaxel.

6.2 Overview of branched-chain amino acids metabolism

The branched chain amino acids (BCAA) leucine, isoleucine, and valine (figure 6.1) are essential amino acids that have key role in several physiological and biochemical processes. They are considered essential because they must be supplied from exogenous sources as the body cannot synthesize them. This makes them an important component of diet (421,422). The homeostasis of these compounds is largely regulated by catabolic pathways (423). The branched-chain amino acids share a common structural features of a branched-side chain and common initial steps of metabolism (424). Branched chain amino acids have a crucial role not only as essential amino acid ingredients for protein synthesis (accounting for 35% of the essential amino acids in muscle), but also as vital signals to activate mammalian target of rapamycin (mTOR) signalling pathway (discussed below) for inhibition of autophagy, cell growth, proliferation, migration in cancer cells as well as to stimulate angiogenesis, glucose consumption and neurotransmitter synthesis which are collectively provide the perfect environment for cancer (425,426).

Branched-chain amino acids catabolism is regulated by a network of enzymes, and a key step involves the branched-chain α-keto acid dehydrogenase complex (BCKDH) (427). BCKDH belongs to a family of the highly conserved mitochondrial α-keto acid dehydrogenases complex, which share a similar structure and function and in addition to it include α-ketoglutarate dehydrogenase, pyruvate dehydrogenase (428,429). BCKDH is found in all mammalian cells and its activity varies with the type of tissue and the presence of regulatory

enzymes within that tissue (430). Changes in the activity of BCKDH occur in response to the type of diet, exercise, some hormones and the BCAAs concentration (431). Low level of BCKDH activity leads to maple syrup urine disease, which is a rare inborn error of BCAAs metabolism that is characterized by accumulation of branched-chain α-keto acids and can cause brain oedema, neurological derangement, mental retardation, seizures, coma, and if not treated with lifelong restriction of BCAAs intake it may end with death (432).

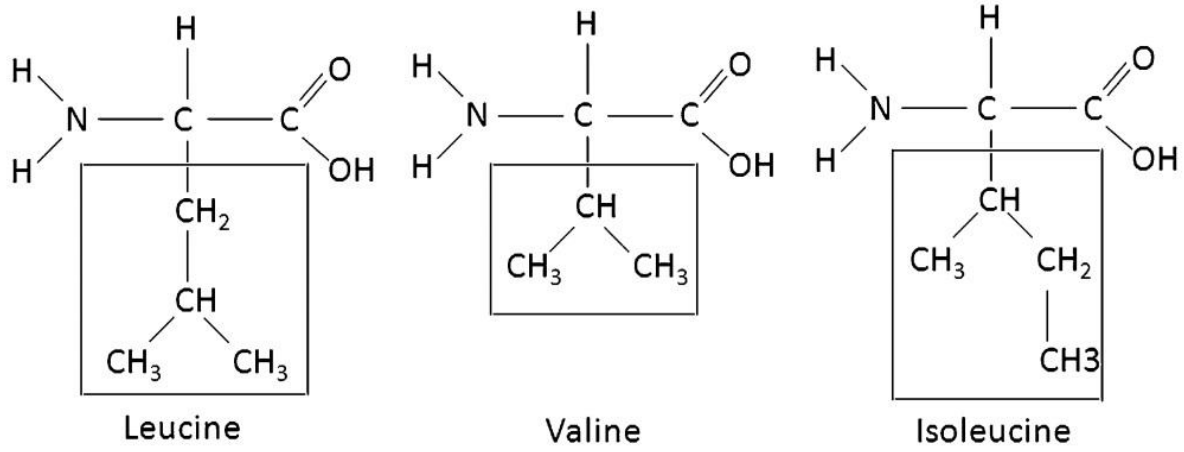


Figure 6.1: The chemical structure of BCAAs (leucine, valine and isoleucine)

The difference in the structure of the three BCAAs (shown as square outlines) gives rise for their different molecular weights (117.15 for valine and 131.17 for leucine and isoleucine) (433).

6.3 Composition of the branched-chain α-keto acid dehydrogenase complex

The branched-chain α-keto acid dehydrogenase complex consists of three catalytic components. A heterotetrameric $\alpha_2\beta_2$ branched chain α-keto acid decarboxylase (E_1), forms a set of 12 tetramers per complex decorated around a central core. The core comprises a homo-24 meric complex of dihydrolipoyl-transacylase (E_2). Lastly there are six homodimers of the dihydrolipoamide dehydrogenase (E_3) (434,435). Branched-chain α-keto acid dehydrogenase complex is similar to the pyruvate dehydrogenase complex (PDC) in many ways, both are located in the mitochondrial matrix space and made-up of a large multi-enzyme complexes (436). The E_1 and E_2 subunits of BCKDH and PDC catalyse similar reactions and need similar co-enzymes. The same E_3 catalytic component is present in both complexes, and is regulated by the same mechanisms, which include covalent modification (437).

6.4 Branched-chain amino acids catabolism

The catabolic pathway of BCAAs plays an important role in regulating physiological levels of these essential amino acids, which are required for cell metabolism, growth and survival (438). The first two steps of BCAAs catabolism starts by using the same enzymes. The first step is a transamination step and uses one of two phosphate-dependent, cytosolic or mitocondrial branched-chain amino acid transaminases (BCATc or BCATm) and ends with transfer of amino group to α-ketoglutarate to form a set of corresponding branched chain α-keto acids (BCKAs) and glutamate (439). The reaction is reversible, and can be used to generate BCAA from the BCKAs (440). The second step of catabolism is catalyzed by BCKDH which causes oxidative decarboxylation of the BCKAs resulting in the formation of the corresponding branched chain acyl-CoA esters. This reaction is irreversible, so it results in degradation of the BCAAs. Therefore, BCKDH is regarded as a crucial step in BCAA catabolism and regulation of its activity is crucial for conserving controlled systemic level of both BCAAs and BCKAs (441). Branched chain amino acid catabolism generates NADH,

CoA esters and $FADH_2$ which are used for ATP production (442). These products of metabolism control the activity of BCKDH by directly inhibiting the enzyme (figure 6.2) (443). In addition, two regulatory enzymes are associated with BCKDH through ionic interactions and control its activity via reversible phosphorylation/dephosphorylation (444,445). Phosphorylation of BCKDH inhibits its catalytic activity and this is catalyzed by branched-chain α-keto acid dehydrogenase kinase (BCKDK). Dephophorylation is catalyzed by branched-chain α-keto acid dehydrogenase phosphatase (BCKDP) (446). Branched-chain α-keto acid dehydrogenase kinase is one of the mitochondrial protein kinases family that is expressed widely, although in variable amounts in human tissues. This enzyme is encoded by the *BCKDK* gene on chromosome 16 (447–449). BCKDK is active when bound to BCKDH and catalyses the phosphorylation of two serine residues (Ser293 and Ser303) of the BCKDH E1α subunit, and completely inactivates the E_1 component, thereby restricting BCAAs breakdown. The extent of kinase binding to the complex increases the degree to which the complex is inactivated (450,451). Inhibition of BCKDH results in increased plasma BCAAs and BCKAs levels, a case that happens when the BCAAs becoming limited for protein synthesis, for example during starvation (432,452–454). In contrast, if Ser293 is not phosphorylated, the activity of BCKDH is unchecked and the BCAAs levels decrease dramatically (455).

Although inhibition of BCKDK prevents BCKDH phosphorylation, dephosphorylation is dependent on BCKDP and consequently the activity of BCKDH depends on the relative activities of BCKDP and BCKDK (456,457). BCKDP is still not well characterized in terms of regulation and its relationship to other phosphatases. Its regulation is an enigma and appropriate tools are needed for its study (458–461).

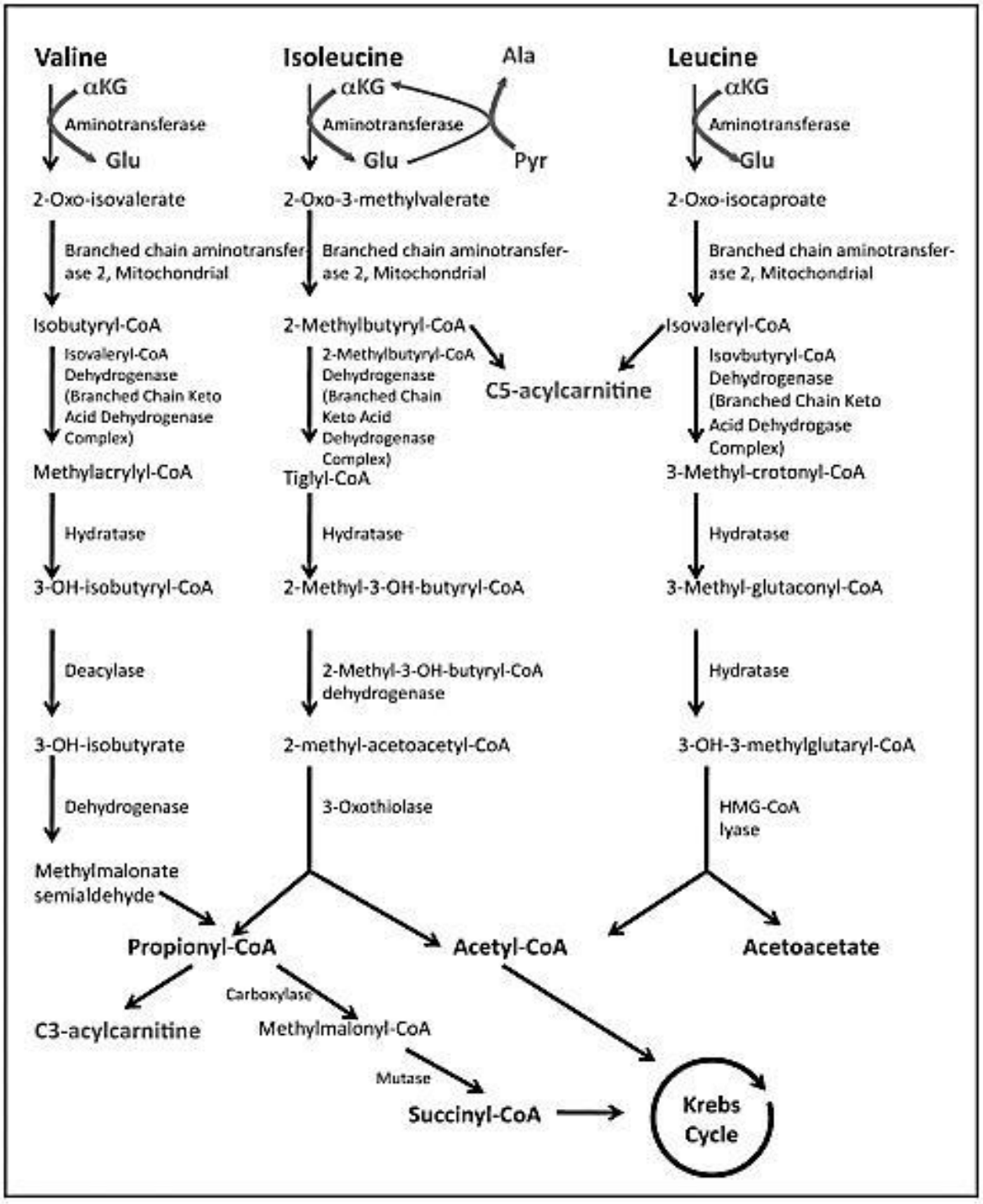


Figure 6.2: Schematic overview of BCAA metabolism

Reversible transamination of amino acids, resulting in the formation of correspoinding ketoacids, is catalysed by the cytosolic or mitochondrial branched chain amino acid transaminase (BCAT). The activity of the branched chain keto acid dehydrogenase complex (BCKDH) in the mitochondria results in generation of CO_2 from the 1-carbon of the keto-acids including α-ketoisocaproate (KIC). Subsequently, further intra-mitochondrial metabolism produces different acyl-coenzyme A (R-CoA) esters that leads to the formation of acylcarnitines (not displayed) (462).

Conversely, when BCAAs are abundant, one of BCAA metabolites, α-ketoisocaproate (KIC, generated by transamination of leucine) inhibits BCKDK, reducing its availability for binding with BCKDH, promoting activation of BCKDH and BCKA metabolism (463,464).

The regulation of BCKDH by BCKDK comprises both long and short term mechanisms. Short-term regulation is provided by phosphorylation as previously described. Long term regulation involves control of BCKDK expression by hormonal signalling and nutritional status. Hormonal signalling from insulin, thyroid hormones, sex hormones and glucocorticoid regulate BCKDK expression and activity in variable extent (465,466). Additionally, thiamine diphosphate, BCKAs, and other factors cause allosteric regulation of the activity of BCKDK (438). It is apparent that such regulation provide a key role in maintaining BCAA homeostasis under different growth, physiological and disease conditions (467). Additionally, the level of protein in diet can increase expression of BCKDK in the liver of rats fed with low protein diet (468).

After the oxidative decarboxylation of the three BCAAs (figure 6.3), the catabolic pathway diverges into different pathways depending on the individual BCAA and the products may feed into the citric acid cycle or mevalonate pathway (469).

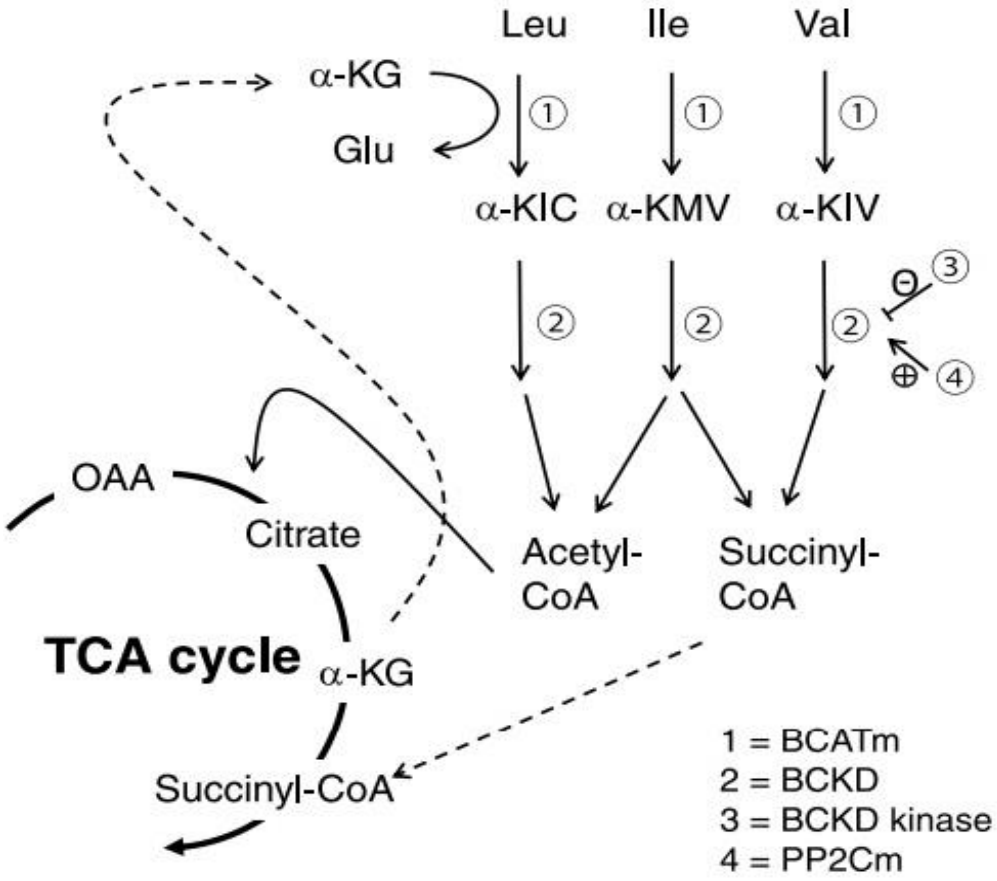


Figure 6.3: Effect of BCKDK and BCKDP on BCKDH

BCATm: mitochondrial branched-chain aminotransferase. BCAA: branched-chain amino acid. BCKD: branched-chain α-ketoacid dehydrogenase. BCKD kinase, branched-chain α-ketoacid dehydrogenase kinase. Ile: isoleucine. Leu: leucine. Val: valine. Glu:glutamate. α-KG: α-ketoglutarate. OAA: oxaloacetate. TCA cycle, tricarboxylic acid cycle. PP2Cm: BCKD phosphatase. (470). BCAT also donates an amino group to an acceptor molecule, typically α-KG, to form the amino acid glutamate.

6.5 Branched chain amino acids and energy metabolism

The main by-product from valine catabolism is propionyl-CoA, which is a glucogenic precursor of succinyl-CoA, whereas isoleucine catabolism ends with generation of propionyl-CoA and acetyl-CoA; therefore, isoleucine is considered as both ketogenic and glucogenic. Leucine catabolism produces acetyl-CoA and acetoacetyl-CoA which is then completely

disposed as CO_2, and thus it is strictly regarded as ketogenic (471). Anaplerotic reactions of BCAA result in production acetyl-CoA in addition to several carbon products that are then entered into the TCA cycle (472). The TCA cycle itself is not able to oxidase all these carbon intermediates completely; therefore, it is essential to eliminate such products by cataplerosis. Therefore it is evident that both anaplerotic and cataplerotic reactions are coordinated and work in equilibrium (473).

Remarkably, during exercise, branched chain amino acids are oxidized to be used for energy production. The rate of the TCA cycle becomes more intense; and the level of the other TCA products (particularly malate) are increased. The concentration of intramuscular α-ketoglutarate reduces, so it is assumed that exercise provokes efficient disposal of α-ketoglutarate from the TCA cycle (474). It has been shown that prevention of BCAA metabolism by deletion of the gene encoding BCATm makes mice intolerant to exercise, confirming that catabolism of BCAA is required for exercise performance (475). Acute exercise causes activation of the BCKDH by decreasing the activity of BCKDK. Consequently, in the course of exercise, BCAA are metabolized thus replenishing the cycle for energy production (476–478).

6.6 Roles of branched chain amino acids

Recently, it has been reported that BCAA participate in lipogenesis, lipolysis, glucose transport and metabolism. They also work as evolutionary preserved modulators of lifespan, in addition to enhancing functional capacities and prolongation of health in different organisms (462,479,480). Furthermore, high serum levels of the BCAAs can be considered as a biomarker or predictor for the early diagnosis of some chronic diseases in humans (figure 6.4) (481–483). Branched-chain amino acids were reported to increase insulin secretion (484). However, high level of BCAAs results in insulin resistance and type 2 diabetes mellitus

possibly due to continued activation of mTOR which causes uncoupling of the insulin receptor substrate for insulin receptor. Another probable mechanism is the abnormal metabolism of BCAAs may accumulate toxic BCAA metabolites and this might result in mitochondrial dysfunction associated with insulin resistance (482,485). Genetic defects in BCAAs catabolism (e.g. genetic ablation of the PP2Cm gene) cause several metabolic syndromes, including maple syrup urine disease which is characterized by excess of plasma BCAA and can be deleterious if untreated (458,486).

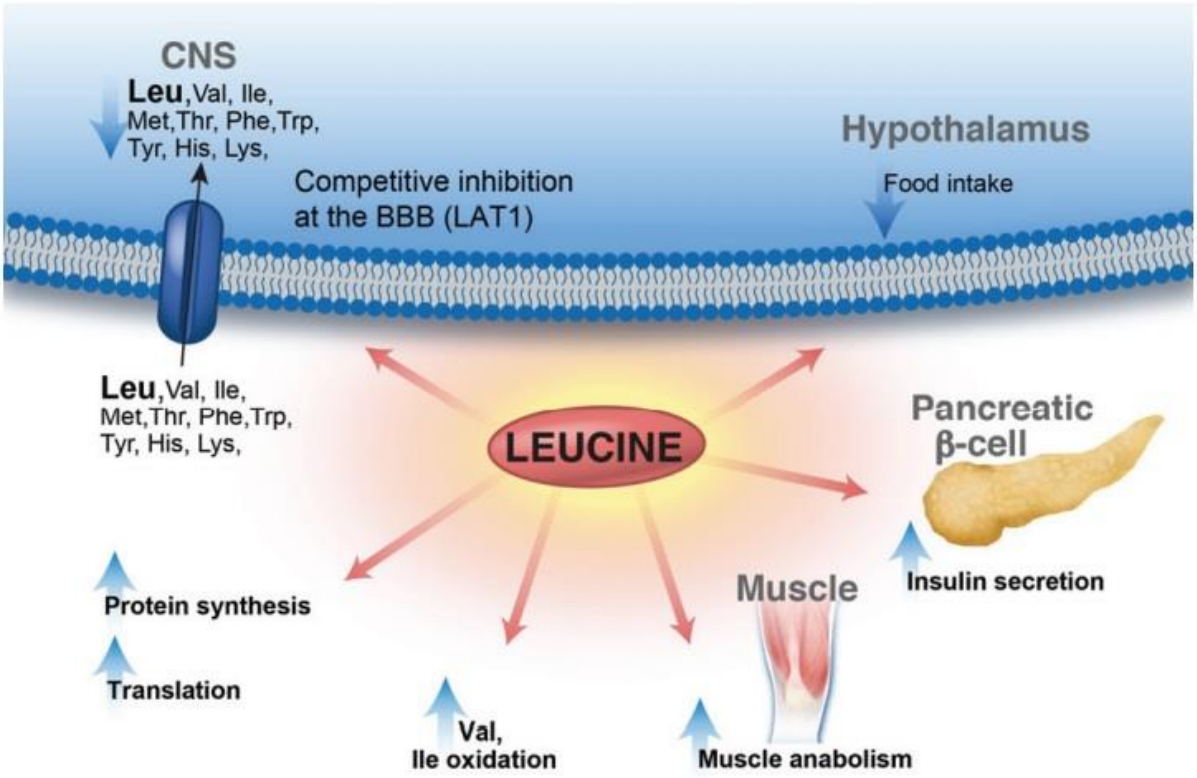


Figure 6.4: Metabolic effects of BCAA in multiple organ systems

Leucine, in particular, displays multiple effects in different organs: it stimulates the secretion of insulin and plays an important role in CNS food intake regulation in addition to feeding behaviour, BCAA in general enhances protein synthesis and inhibits protein breakdown especially in muscles. Leucine is transported through the blood–brain barrier via neutral amino acid transporter LAT1, affecting neurotransmitter biosynthesis. Furthermore, α-ketoisocaproate (which is derived from leucine) is a potent inhibitor of the BCKDK leading to activation of BCKDH and increase the oxidation of BCAA (valine and isoleucine) (487)

In contrast, low plasma BCAA concentration is also unhealthy, and can happen as a result of unrestrained catabolism or malnutrition. BCAA supplementation is essential in many disease conditions (488–490). Recently, a number of studies have linked some common metabolic, neurologic and cardiovascular diseases with abnormal BCAA levels. The observations from these studies have implicated a faulty BCAA catabolic pathway as a contributing factor to both common and rare human diseases (438,491–493).

6.7 BCAA and protein synthesis

The initiation step of mRNA translation can be inhibited if tRNA is deacylated (494). Branched chain amino acids enhance the initiation step of protein synthesis through the production of aminoacyl tRNA derivatives that stimulate the initiation step by reduction of tRNA deacylation. BCAAs metabolites may also contribute to the regulation of protein synthesis (495). BCAAs (particularly leucine) can increase the rate of protein synthesis and also limit the rate of protein degradation (496). This effect is enriched by a transient and small increase in serum insulin level. The sensitivity of protein synthesis to insulin can be enhanced by infusion of leucine and increasing the concentration of both leucine and insulin is pivotal for stimulation of protein synthesis. This mode of regulation of protein synthesis is rational as BCAAs provide the building blocks while insulin provides the signal generating energy for protein synthesis (497). However, high levels of leucine could be toxic and a suitable dose of leucine to deal with cachexia in cancer patients needs to be identified (452,498).

Leucine in particular has a critical role in stimulation of the protein synthesis via the mTOR signalling pathway (499–501). mTOR is a downstream effector of phosphatidylinositol (3,4,5)-trisphosphate kinase (PI3K/AKT) and can be found in two different multiprotein complexes, mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2). As a key regulator of protein synthesis, the mTOR pathway provides close monitoring of the levels of BCAAs and, crucially, the mTORC1

signalling pathway is shut down upon BCAAs depletion (502). Conversely, deregulation of mTOR can also result in neurological or developmental problems (503). mTOR can be activated by various stimuli, such as energy, nutrients, growth factors and stress signals via MAPK, PI3K or AMPK to link metabolic pathways with cell growth, proliferation and survival (504,505). In case of cancer, the uncontrollable activation of mTOR signalling pathway is accompanied by oxidative stress as well as stimulation of proinflammatory cytokines release, mediated via the activation of the nuclear transcription factor-κB (NF-κB) and this afford a selective advantage to cancer cells by contribution to all of the malignant transformation changes, which include unresponsiveness to growth-inhibitory signals, self-sufficiency in signals of growth, evasion of apoptosis, persistent angiogenesis, unlimited replication and tissue invasion in addition to metastasis (506–508). Consequently, it is not surprising that high mTORC1 signalling is usually apparent to a significant level in the most common forms of human cancers (509). mTORC1 activates p70 S6 kinase, which then phosphorylates ribosomal S6 protein, resulting in increased protein synthesis. Activated mTORC1 also stimulates production of the protein synthesis initiation complex by phosphorylation of eIF4E-binding protein 1 (4E-BP1) (figure 6.5) (510).

On the other hand, mTORC2, is insensitive to rapamycin, nutrients, stress and energy signals. However, it responds to growth factors like insulin. Insulin activates mTORC2 resulting in activation of protein kinase B (PKB)/AKT (511). The activated PKB/AKT stimulates some of the metabolic functions of insulin such as increasing glucose transport and activation of mTORC1 signalling pathway to drive protein synthesis accompanied by cell growth and proliferation (512). Deregulation of different components of the mTOR signalling pathway, including, PI3K, PKB/AKT, Rheb, S6K1, 4E-BP1, eIF4E, LKB1, TSC1/TSC2 and PTEN, are present in several types of cancers (513).

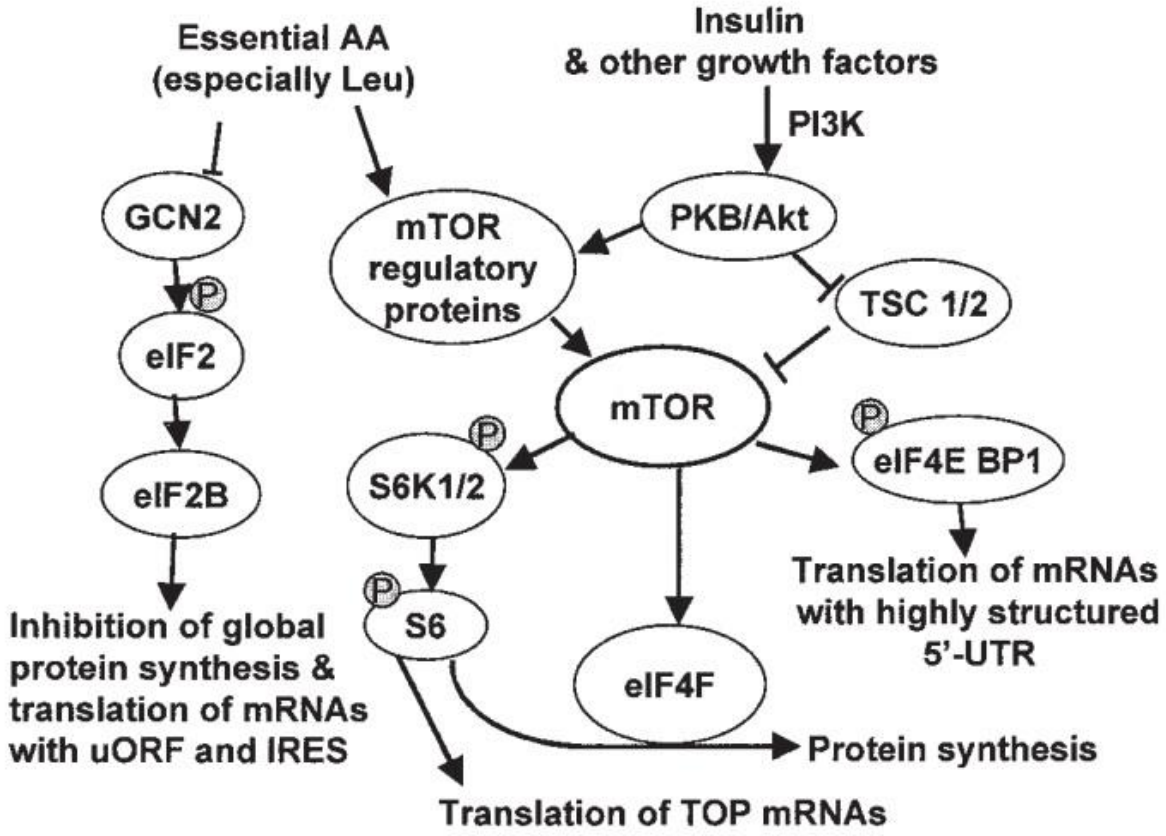


Figure 6.5: Essential amino acids regulation of protein synthesis via mTOR pathway

BCAA can activate protein synthesis via mTOR mediated phosphorylation of p70-S6 kinase 1 and 2 (S6K1/2) and eukaryotic initiation factor 4E binding protein 1 (eIF4E BP1). Increased translation is promoted by mRNA transcripts with highly structured 5'-untranslated regions (UTRs) or translation of terminal oligopyrimidine (TOP) tracts close to the 5'- cap structure. Insulin can also stimulates protein synthesis via modulation of the inhibitory effect of tuberous sclerosis complexes 1 and 2 (TSC1/2) and protein kinase B (PKB/Akt). Phosphorylation of eIF2 (by general control nonderepressing kinase-2 (GCN2)) can regulate which mRNA is translated into proteins. Signalling through this pathway causes selective translation of mRNA transcripts with specific internal ribosome entry site (IRES) or an upstream open reading frames (uORFs). From (514).

6.8 Branched chain amino acids and cancer

As a result of altered metabolism and increased growth, cancer cells need more nutrients and energy supply compared to non-malignant cells (515). In cancer cells, the causes of altered metabolism is multifactorial, including an intrinsic requirements for increased biosynthetic intermediates, such as nucleic acids for RNA and DNA syntheses, amino acids for protein synthesis and fatty acids for membrane synthesis (516). A low glucose level and hypoxia in the tumour microenvironment could also alter cancer cell metabolism (517). Branched chain amino acids and glutamine can serve as building blocks and energy substrates for cancer cells and their abundance may contribute to growth, proliferation and survival of these cells in different tissues (518,519). Additionally, cancer patients usually suffer from cachexia, characterized by loss of skeletal muscle mass due to high protein breakdown (520,521). Many tumours are deadly at a very low tumour burden (for example, about 1–2% of total body weight). Nonetheless, the relatively small tumour mass may become the main site for of capturing amino acids and this results in depletion of amino acid pools at other sites of the body (522). In this condition, supplementation with BCAAs might be regarded as an essential therapeutic intervention to improve outcomes by providing the building blocks to stimulate protein synthesis in skeletal muscle. However, this intervention conflicts with potentially, and inadvertently, supporting the extensive need of the tumour cells for BCAAs to fuel their growth and proliferation. This poses a dilemma to optimize BCAA supplementation because it is necessary to balance the demands of the host body with stimulating the growth and proliferative need of the tumour (433,523). Isoleucine and leucine have been reported to augment neoplasms in rats caused by N-butyl-N-(4-hydroxybutyl) nitrosamine. Positron emission tomography (PET) using 11C–leucine pointed to the high tumours uptake of amino acid. However, it has not yet been shown that BCAAs can be tumourigenic in the absence of oncogenic stimuli. However, BCAA supplementation in cancer patients resulted in increased

morbidity (524). These features have driven research in two opposite directions, one to investigate BCAA deprivation, in order to reduce tumour protein synthesis, growth and proliferation, and the other for BCAA supplementation, with an aim to help maintenance of host normal tissue. (331). It has been shown that supplementation of pancreatic cancer mice with leucine resulted in enhancement of tumour growth , whereas leucine-free diet inhibited the growth of tumour and stimulated apoptosis, suggesting caution against the idea of leucine supplementation to enhance protein synthesis of skeletal muscle in cachectic patients (525,526). Also, investigation of the tumour uptake of labelled leucine in children with glioma and its relation to glucose metabolism has shown increased leucine uptake in the region of angioma revealed a high demand for leucine to support protein synthesis, growth and proliferation in tumour more than the normal tissue (527).

6.8.1 Changes in amino acid uptake, metabolism and protein synthesis in tumours

Little is known about the tumour utilization of BCAA but, it may be assumed that the primary histological type, oncogenic properties of tumours and tumour burden have an impact on the relative use of BCAA by cancer cells. The primary histological type could affect the tumour uptake, metabolism and protein synthesis. Comparison of FDG uptake by primary non-small cell lung cancer (NSCLC) has shown that there are significant differences in FDG uptake and utilization in different histologic NSCLC subtypes (528). Additionally, BCAT may regulate BCAAs through differing expression levels in different tissue types. Consequently of the extent of differentiation of a tumour and its tissue of origin may affect the capacity for BCAA oxidation (440).

6.8.2 Contribution of BCAA to oncogenesis

There is growing body of evidence that BCAA metabolism is implicated in oncogenesis. High level of BCAA is an early marker for pancreatic cancer (529). BCKDK is over-expressed in colorectal cancer and this correlates with worse patient survival and the reduction of BCKDK expression slows the *in vitro* growth of colorectal cancer cells and supports survival of mice implanted with cancer xenografts. Mechanistically, in addition to affecting BCAA metabolism, BCKDK promotes colorectal cancer by enhancing the MAPK signaling pathway through direct MEK phosphorylation and anchorage independent growth, where the process above could be inhibited by a BCKDK inhibitors (530). In CML, BCAT1 upregulation promotes BCAA production in leukaemia cells by aminating the branched-chain keto acids. *In vitro* and *in vivo* blocking BCAT1 enzymatic activity or gene expression promote cellular differentiation and impairs the propagation of blast crisis CML. Supplementation with BCAAs ameliorates the defects caused by BCAT1 knockdown, indicating that BCAT1 exerts its oncogenic function through BCAA production in blast crisis CML cells (531). The level of BCAA in plasma and tissue is increased in patients with breast cancer, which is often accompanied by over-expression of BCAA metabolizing enzymes, and interfering with catabolism of BCAA by knocking down of BCAT1 enzyme inhibits the growth of breast cancer cells by inhibiting the mTOR-mediated mitochondrial protein synthesis (532). Likewise, BCAT1 is overexpressed in ovarian tumors and knockdown of the BCAT1 expression in ovarian cancer cells led to reduction of cell proliferation, migration and invasion and inhibited cell cycle progression. Moreover, BCAT1 suppression resulted in downregulation of numerous genes implicated in lipid production and protein synthesis and resulted in significantly prolonged survival time in xenograft model of ovarian cancer, suggesting its importance in controlling ovarian cancer metabolism (533). Finally, inhibition

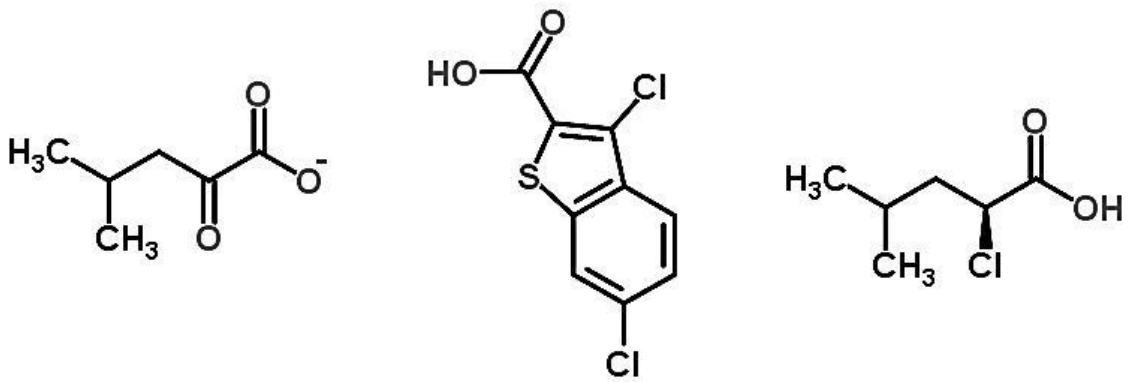
of BCAT also inhibits the growth of NSCLC tumour allografts in mice and increases survival in a murine model of CML (534).

Depending on the role of BCAAs in triggering protein biosynthesis and cell motility via its effect on activation of mTORC1, it is anticipated that high levels of BCAAs may promote the development or maintenance of cancer, inhibition of autophagy and augment invasiveness of tumour (535). In addition to its role in boosting of pancreatic cancer growth, high level of BCAAs overcome the activity of antineoplastic agents via over-activation of the mTOR pathway regulating protein synthesis, and this suggests caution against the supplementation of cancer patients with BCAA to resolve cachexia (536). Thus, proper control of BCAA concentration and mTORC1 activity is necessary for maintaining homeostatic control over the anabolic pathways that support tumour cells growth and proliferation (537). These data suggest that BCAA metabolism contributs to oncogenesis and accordingly an improved understanding of this may aid in the development of new therapeutic agents (538,539). Combined with discovery of BCKDK in our RNAi screen, make it desirable to explore the role of BCKDK in cancer, where inhibition of BCKDK is one potential therapeutic strategy to enhance BCAA metabolism by activating BCKDH (540,541).

6.9 BCKDK inhibitors

Inhibition of BCKDK by small molecules, like KIC, encouraged the design and development of several KIC analogues that are inhibitors of BCKDK, including clofibric acid, α-chloroisocaproate (CIC), thiamine pyrophosphate (TPP), S-α-choloro- phenylproprionate ((S)-CPP), phenylpyruvate and phenylbutyrate (542). Clofibric acid (the active metabolite of clofibrate) is hypolipidemic agent, Most of its activity in this setting is presumed to be mediated by potential activation of the nuclear receptor peroxisome proliferator-activated

receptor α (PPARα), which is a transcription factor that regulates genes implicated in lipid metabolism. However, clofibric acid, as well as phenylpyruvic acid, are able to lower BCAA levels via inhibition of BCKDK (543–545). α-chloroisocaproate (2-chloro-4-methylvaleric acid; CMVA) is also a KIC analogue (figure 6.6) that has been shown to inhibit the phosphorylation and inactivation BCKDH through inhibition of BCKDK (546). TPP is another BCKDK inhibitor able activate BCKDH and enhances the catabolism of BCAA (547). 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid (DCBC; figure 6.6) belongs benzothiophene derivatives family. However, DCBC exhibits significantly higher stability and improved potency than these compounds (548). Although BCKDK is a kinase, BCKDK inhibitors work through a mechanism distinct from many previously described kinase inhibitors. Instead of binding to the site normally occupied by ATP, these compounds bind to an allosteric site in the N-terminal domain and trigger helix movements. Such changes in the conformation prevent binding to the BCKDH core (542,546). This results in the release of BCKDK from the BCKDH core and the subsequent degradation of BCKDK. However, benzothiophene carboxylates, including DCBC, can also inhibit of Mcl-1, raising concerns about the specificity when used as a research tool (548,549)



KIC DCBC CMVA

Figure 6.6: Chemical structures of some BCKDK inhibitors

(542,548)

6.10 Aims

The aim of the work in this chapter was to investigate the contribution of BCKDK to the sensitivity of ovarian cancer cells to paclitaxel by using siRNA and compounds that inhibit BCKDK. This information could help to evaluate whether the development of BCKDK inhibitors is warranted for subsequent evaluation in clinical trials.

6.11 Results

6.11.1 Determination of BCKDK and BCKDH expression in cancer cell lines

Prior to evaluating the effectiveness of paclitaxel and the BCKDK inhibitors combinations, the expression of BCKDK and BCKDH in cancer cell lines were first determined. The expression of BCKDK and BCKDH were measured in 9 cell lines including normal human ovarian epithelial cells (HOE) and 8 ovarian cancer cell lines. This included OVCAR-4 and COV362 cells, the two cell lines in which augmentation of paclitaxel activity was observed when BCKDK was knocked-down by siRNA (Chapter 5).

BCKDK was expressed in all 9 cell lines in varying abundance with the highest levels being observed in COV362, FUOV-1, Igrov-1 and OVCAR-8, and the lowest levels in COV318 and OVCAR-3 cell lines. Although BCKDH was detectable in all cell lines, it was notably prominent in COV318, OVCAR-4 and FUOV-1 with very low expression in COV362 cell line (figure 6.7 A and B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization in each of these experiments.

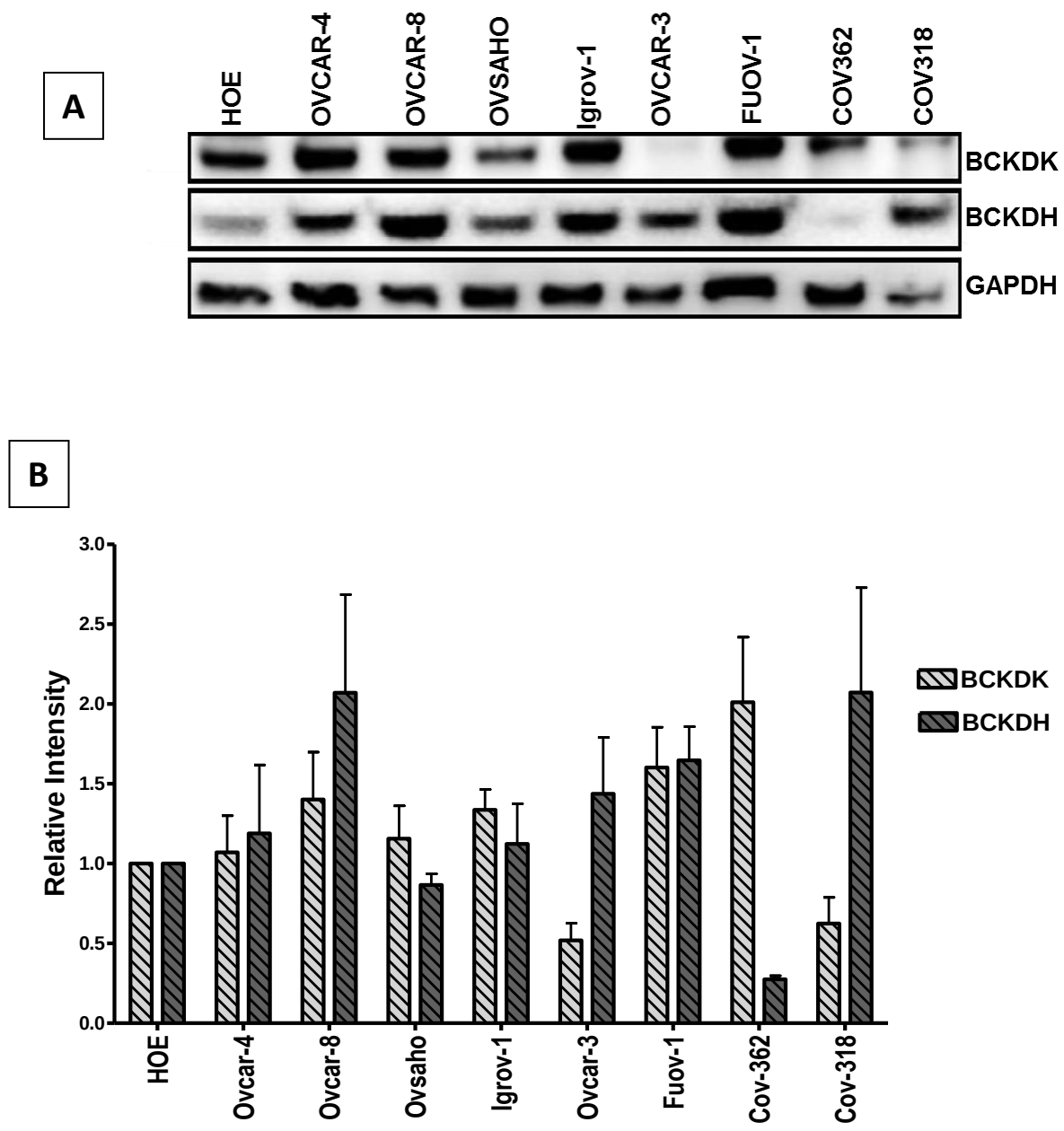


Figure 6.7: Expression of BCKDK and BCKDH in different cell lines

A. Protein lysates were prepared from different cell lines and the expression of BCKDK and BCKDH proteins determined by western blotting. B. The western blot results shown in figure 6.7 A were quantified by normalizing the intensity of the signal for each band to that of the GAPDH band in the indicated cell line and in the same western blot (results are expressed as mean ± S.D., number of experiments = 3).

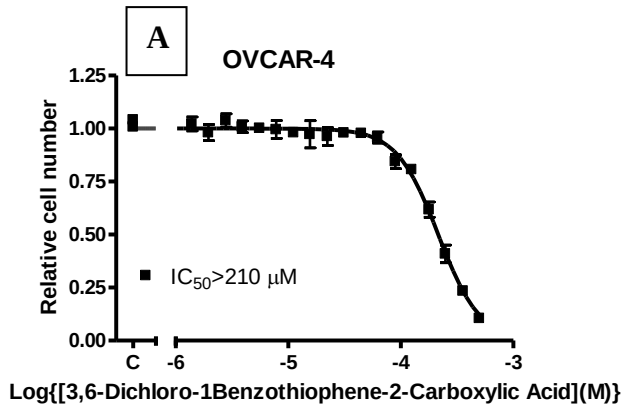
6.11.2 Single agent activity

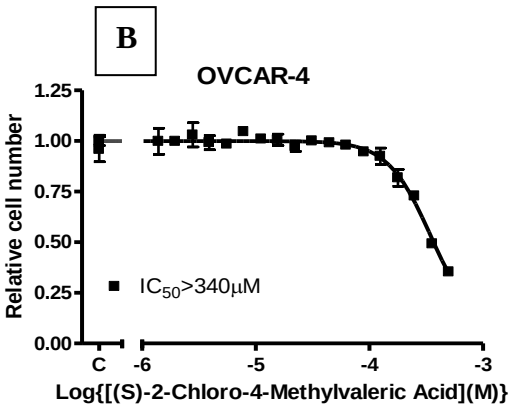
To support the siRNA studies which showed that knock-down of BCKDK increased sensitivity to paclitaxel, the BCKDK inhibitors (3,6-Dichloro-1-Benzothiophene-2-Carboxylic acid (DCBC) and (S)-2-Chloro-4-methylvaleric acid (CMVA) were used as an alternative to inhibit BCKDK. Prior to evaluating the effect of the drug combinations, the potency of the BCKDK inhibitors as single agents was assessed in cell growth assays using OVCAR-4, COV362, COV318 and FUOV1 cells. The IC_{50} values derived from the cell growth assays (figure 6.8 and table 6.1) reflect minimal inhibition of cell growth at inhibitor concentrations up to 100 µM.

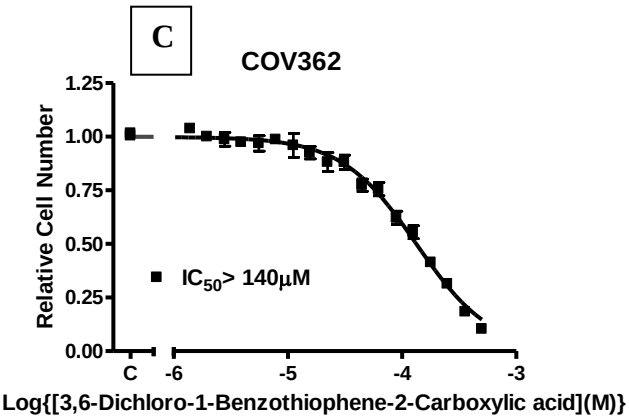
Table 6.1: IC_{50} values of DCBC and CMVA in different ovarian cancer cell lines

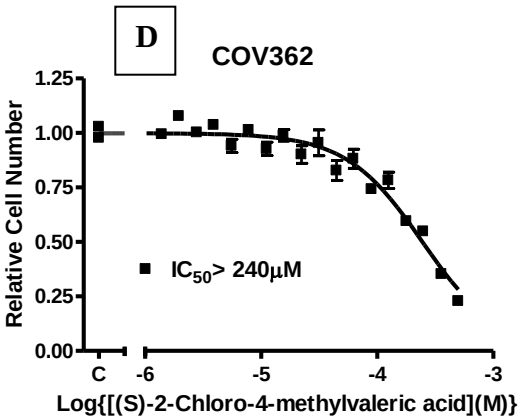
Cell line	**DCBC**	**CMVA**
	IC50 (µM)	**IC50 (µM)**
OVCAR-4	>210	>340
COV362	>140	>240
COV318	>225	>280
FUOV-1	>200	>280

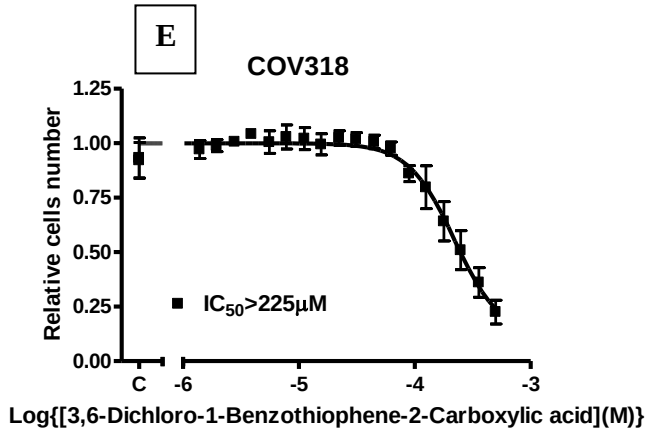
The activity of DCBC and CMVA was examined in four different ovarian cancer cell lines by using the SRB assay technique. The results are expressed as mean ± S.D from 4 different experiments.

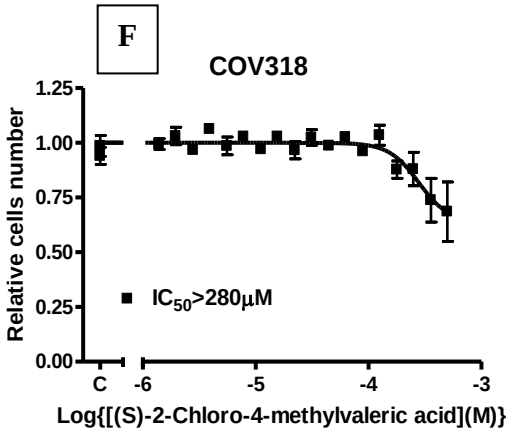

A
OVCAR-4
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
IC50>210 μM
C
-6
-5
-4
-3
Log{[3,6-Dichloro-1Benzothiophene-2-Carboxylic Acid](M)}


B
OVCAR-4
Relative cell number
1.25
1.00
0.75
0.50
0.25
0.00
IC50>340μM
C
-6
-5
-4
-3
Log{[(S)-2-Chloro-4-Methylvaleric Acid](M)}


C
COV362
Relative Cell Number
1.25
1.00
0.75
0.50
0.25
0.00
IC50> 140μM
C
-6
-5
-4
-3
Log{[3,6-Dichloro-1-Benzothiophene-2-Carboxylic acid](M)}


D
COV362
Relative Cell Number
1.25
1.00
0.75
0.50
0.25
0.00
IC50> 240μM
C
-6
-5
-4
-3
Log{[(S)-2-Chloro-4-methylvaleric acid](M)}


E
COV318
Relative cells number
1.25
1.00
0.75
0.50
0.25
0.00
IC50>225μM
C
-6
-5
-4
-3
Log{[3,6-Dichloro-1-Benzothiophene-2-Carboxylic acid](M)}


F
COV318
Relative cells number
1.25
1.00
0.75
0.50
0.25
0.00
IC50>280μM
C
-6
-5
-4
-3
Log{[(S)-2-Chloro-4-methylvaleric acid](M)}

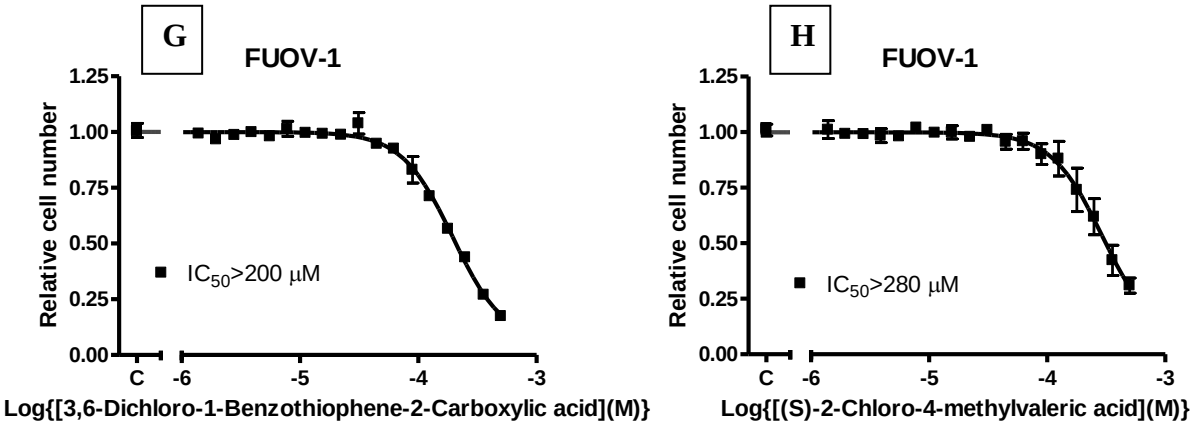


Figure 6.8: Activity of DCBC and CMVA in OVCAR-4 cell growth assays

The analysis of DCBC and CMVA was conducted in OVCAR-4, COV362, COV318 and FUOV-1 cell lines. A, C, E and G represent the activity of DCBC in OVCAR-4, COV362, COV318 and FUOV-1 cells, while (B, D, F and H) represents the activity of CMVA in the same cells. The figures show mean ± S.D. work for four experiments listed in table 6.1. "C" in X-axis indicates control cells (cells were treated with drugs solvent). The activity of drugs was measured by SRB assay.

6.11.3 Analysis of drug combinations

In combination studies paclitaxel was added simultaneously with the BCKDK inhibitors and the combination evaluated in cell growth assays. Four cell lines, each known to be representative of high-grade serous ovarian cancer (321), were selected for these experiments, OVCAR-4, COV362, COV318 and FUOV-1. Serial dilution of paclitaxel combined with the BCKDK inhibitors, which were used at either 30 or 100 μM fixed concentrations. This design was selected because the BCKDK inhibitors on their own had minimal effect when used at these concentrations and so any increase in the potency of paclitaxel when the inhibitors were

present would clearly be indicative of a synergistic interaction. To formally evaluate synergy, the effect of the drug combination was evaluated by calculating the combination index (CI). The combined drugs are considered to have a synergistic effect if their CI value is less than 1, while values that are around 1 or more than 1 indicate additivity or antagonism respectively between the two drugs. Synergy was observed when paclitaxel was combined with both DCBC and CMVA in cell growth assays using OVCAR-4, COV318 and FUOV-1 cell lines, while additivity was observed when paclitaxel was combined with DCBC and CMVA in COV362 (figures 6.9, 6.10, 6.11, and 6.12).

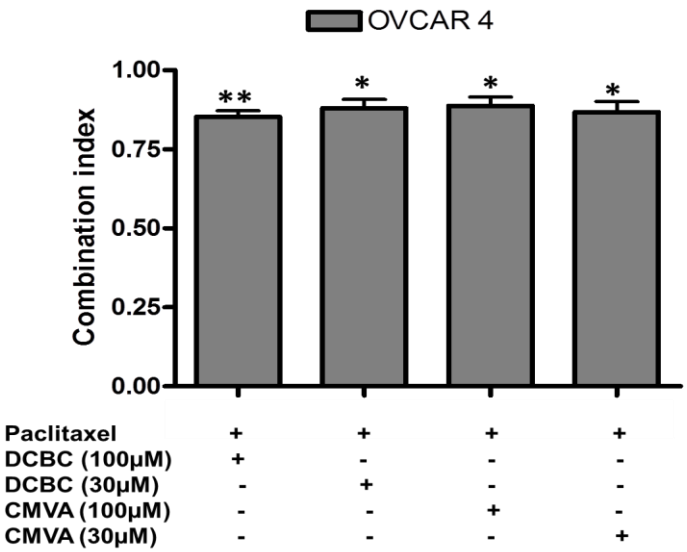


Figure 6.9: Activity of DCBC and CMVA in OVCAR-4 cell growth assay

The values of CI were calculated depending on Chou-Talalay method (mentioned in materials and methods). The CI values were statistically significant from unity where shown (*, $P \leq 0.05$, **, $P \leq 0.01$, paired t-test)

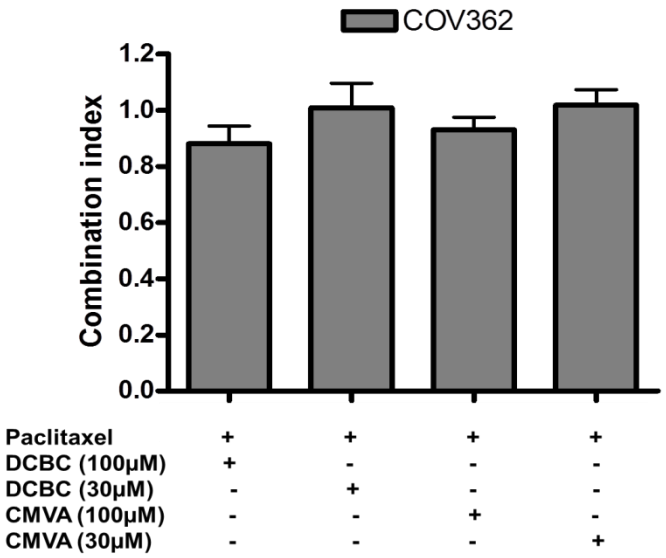


Figure 6.10: Activity of DCBC and CMVA in COV362 cell growth assay

The values of CI were calculated depending on Chou-Talalay method (mentioned in materials and methods). The results did not show statistically significant deviation from additivity.

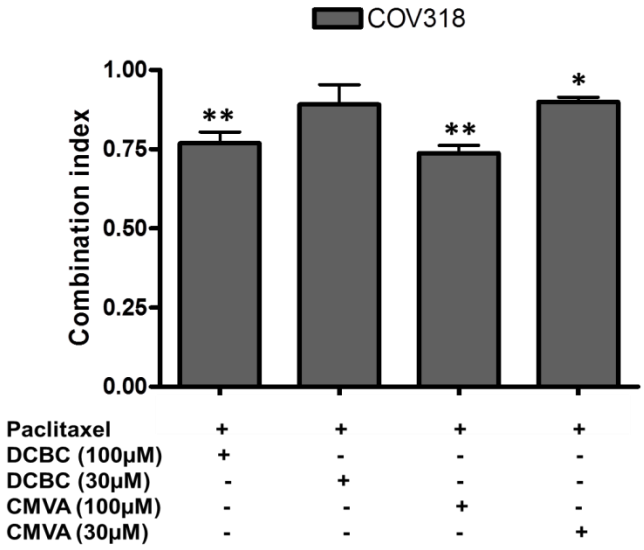


Figure 6.11: Activity of DCBC and CMVA in COV318 cell growth assay

The values of CI were calculated depending on Chou-Talalay method (mentioned in materials and methods). The CI values were statistically significant from unity where shown (*, $P \leq 0.05$, **, $P \leq 0.01$, paired t-test)

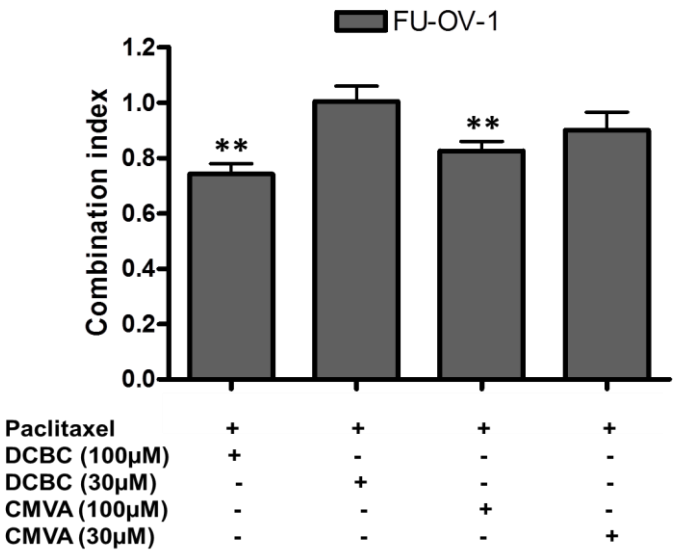


Figure 6.12: Activity of DCBC and CMVA in FUOV-1 cell growth assay

The values of CI were calculated depending on Chou-Talalay method (mentioned in materials and methods). The CI values were statistically significant from unity where shown (*, $P \leq 0.05$, **, $P \leq 0.01$, paired t-test)

6.11.4 Cell viability assay

To evaluate whether the synergy in the cell growth assays reflecting potentiation of cell death, rather than inhibition of proliferation, the viability of ovarian cancer cells was measured after 48 hours of treatment with paclitaxel, BCKDK inhibitors and paclitaxel combined with BCKDK inhibitors by using the trypan blue assay technique. The results revealed that the combination of paclitaxel with either DCBC or CMVA caused significant increase in the percentage of dead cells in OVCAR-4 and COV318 and this increase was higher than that expected from an additive interaction and calculated from the Bliss independence criterion (figure 6.13 and 6.15) respectively. This suggests the drugs caused a synergistic increase in cell death. However, consistent with cell growth assays and the low level of BCKDH in these

cells, the combination of paclitaxel with 100 µM of either DCBC or CMVA in COV362 did not result in significantly higher effect than that expected from the calculated Bliss independence criterion as shown in figure 6.14.

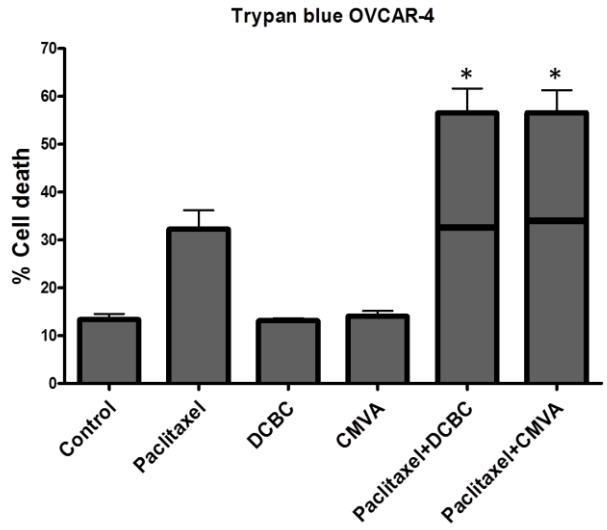


Figure 6.13: The effect of drug combinations on cell viability in OVCAR-4

Cells were exposed to combinations of paclitaxel (12 nM) and DCBC (100 µM) or CMVA (100 µM) and the number of viable cells determined by staining with trypan blue. The data are expressed as mean ± SD (n = 3). The horizontal bar indicates the expected effect if the drugs interacted additively and calculated from the Bliss independent criterion. Experimental results are significantly different from the expected additive effect where shown (paired t-test, *, $P \leq 0.05$).

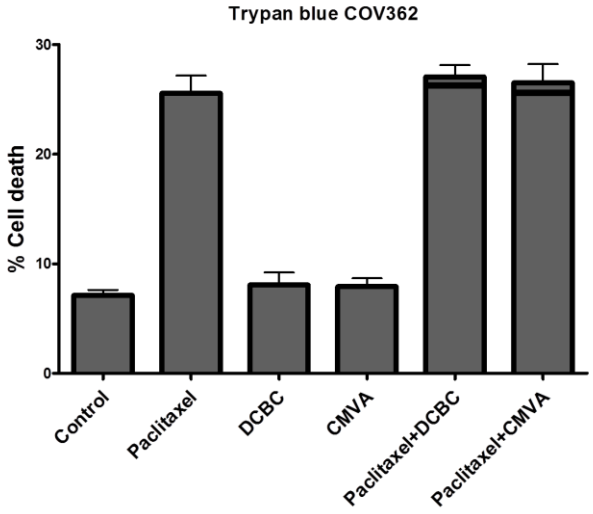


Figure 6.14: The effect of drug combinations on cell viability in COV362

Cells were exposed to combinations of paclitaxel (12 nM) and DCBC (100 µM) or CMVA (100 µM) and the number of viable cells determined by staining with trypan blue. The data are expressed as mean ± SD (n = 3). The horizontal bar indicates the expected effect if the drugs interacted additively and calculated from the Bliss independent criterion. Experimental results show no significant difference from the expected additive effect.

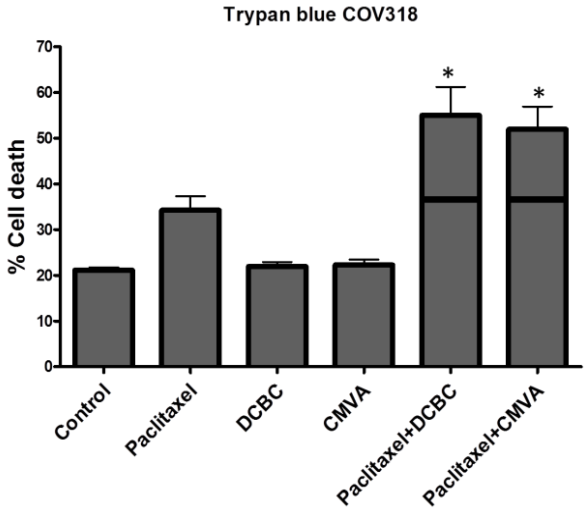


Figure 6.15: The effect of drug combinations on cell viability in COV318

Cells were exposed to combinations of paclitaxel (12 nM) and DCBC (100 µM) or CMVA (100 µM) and the number of viable cells determined by staining with trypan blue. The data are expressed as mean ± SD (n = 3). The horizontal bar indicates the expected effect if the drugs interacted additively and calculated from the Bliss independent criterion. Experimental results are significantly different from the expected additive effect where shown (paired t-test, *, $P \leq 0.05$).

6.11.5 Confirmation of cell death by apoptosis.

6.11.5.1 Western blotting

During apoptosis, Poly ADP ribose polymerase (PARP) undergoes cleavage to inactivate it. To evaluate whether the cell death caused by paclitaxel and BCKDK inhibitors was due to apoptosis, PARP cleavage was measured by western blotting using OVCAR-4, COV318 and COV362 cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control for protein normalization. After 48 hours of exposure to DCBC, CMVA and paclitaxel either alone or in combination, PARP cleavage was higher in cells treated with paclitaxel in combination with DCBC or CMVA than that in cells treated with single agents in OVCAR-4 and COV318 (figure 6.16). In keeping with the previous studies, in COV362 cells the drug combinations did not show significantly more cleavage than that in cells exposed to the single agent.

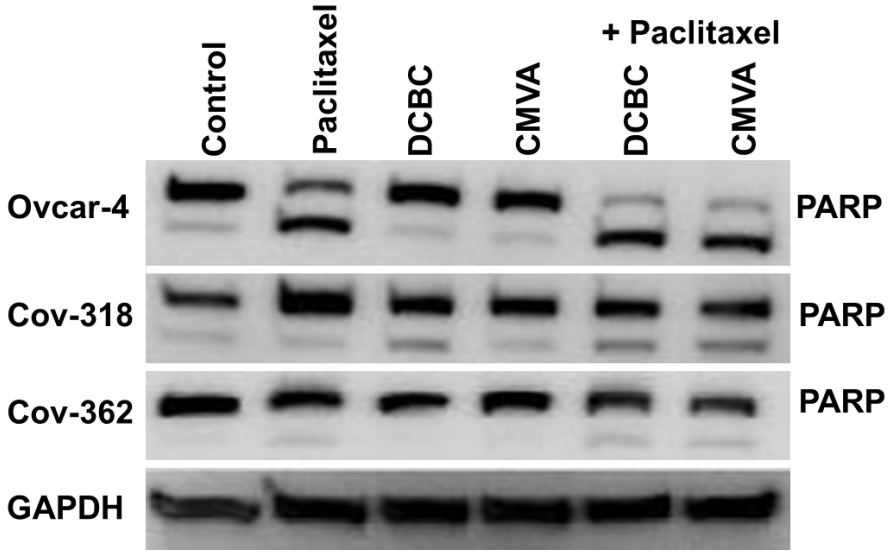


Figure 6.16: PARP-cleavage in some ovarian cancer cell lines

Cells were exposed to paclitaxel (12 nM) or either 100 µM DCBC or CMVA and after 48 hours cells were lysed and PARP-cleavage was assessed by western blotting of OVCAR-4, COV318 and COV362 cell lines. The results are representative of three experiments. PARP, Poly (ADP-ribose) polymerase. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

6.11.5.2 Caspase 3/7 assay

The cysteine-aspartic proteases (caspase) family of enzymes play an important role in programmed cell death. Therefore, the activity of the effector caspases 3 and 7, which catalyse PARP cleavage, was assayed in cells following 48 hours of exposure to paclitaxel, either alone or in combination with DCBC or CMVA. The BCKDK inhibitors on their own did not cause a significant increase in caspase 3/7 activity. However, combinations of paclitaxel with DCBC or CMVA resulted in an increase in caspase-3/7 activity which was significantly greater than that measured with paclitaxel alone (figure 6.17).

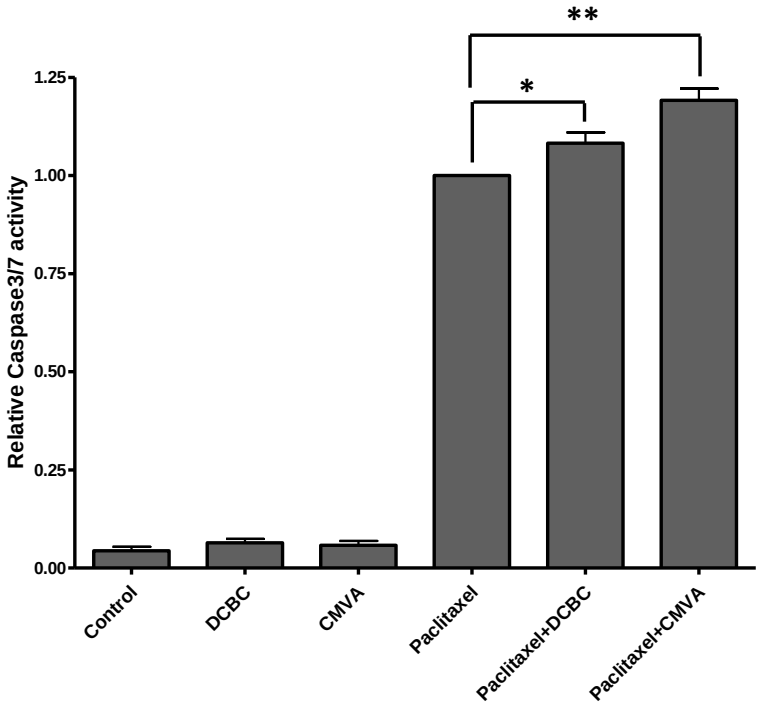
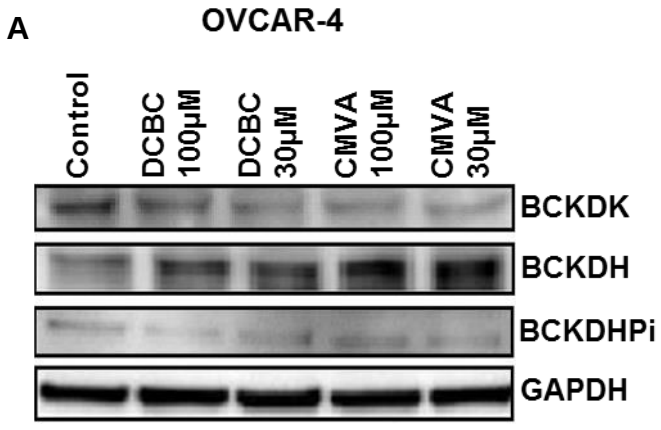


Figure 6.17: Relative caspase 3/7 activity in OVCAR-4 cell line

Ovcar-4 cells were exposed to paclitaxel (12 nM) or either 100 µM DCBC or CMVA and after 48 hours caspase 3/7 activity was measured. To control for potential effects of the drugs on cell number after 48 hours, caspase 3/7 activity was normalized to the surviving cell number estimated by staining with SRB at the same time and the results of caspase 3/7 assay. The results are expressed as a fraction of that measured in cells treated with paclitaxel alone (mean ± SD, n=3). The results were significantly different from those in cells treated with paclitaxel alone where indicated (*, P< 0.05, **, P<0.01paired t-test).

6.11.6 Evaluation of the effect of BCKDK inhibitors on of BCAA metabolizing enzymes

In order to confirm that the BCKDK inhibitors did indeed inhibit BCKDK, levels of BCKDK, BCKDH and BCKDH phosphorylation were assessed by western blotting. The results showed that, consistent with previous reports, the levels of BCKDK were reduced and this was associated with a decrease in BCKDH phosphorylation in OVCAR-4 and COV318 cells. This was also accompanied by an increase in the abundance of BCKDH (figures 6.18 A and B). Quantification of BCKDK, BCKDH and BCKDH phosphorylation protein level confirmed these results (figures 6.19). In contrast, in COV362 (the cell line in which synergy between paclitaxel and DCBC or CMVA was not observed) there was little effect of the BCKDK inhibitors, and the analysis of BCKDK, BCKDH and phosphorylated BCKDH by western blot and protein quantification (as shown in figure 6.18 C and figure 6.19) revealed no decrease in the abundance of BCKDK, explaining the lack of synergy observed in COV362 in the drug combination assays.



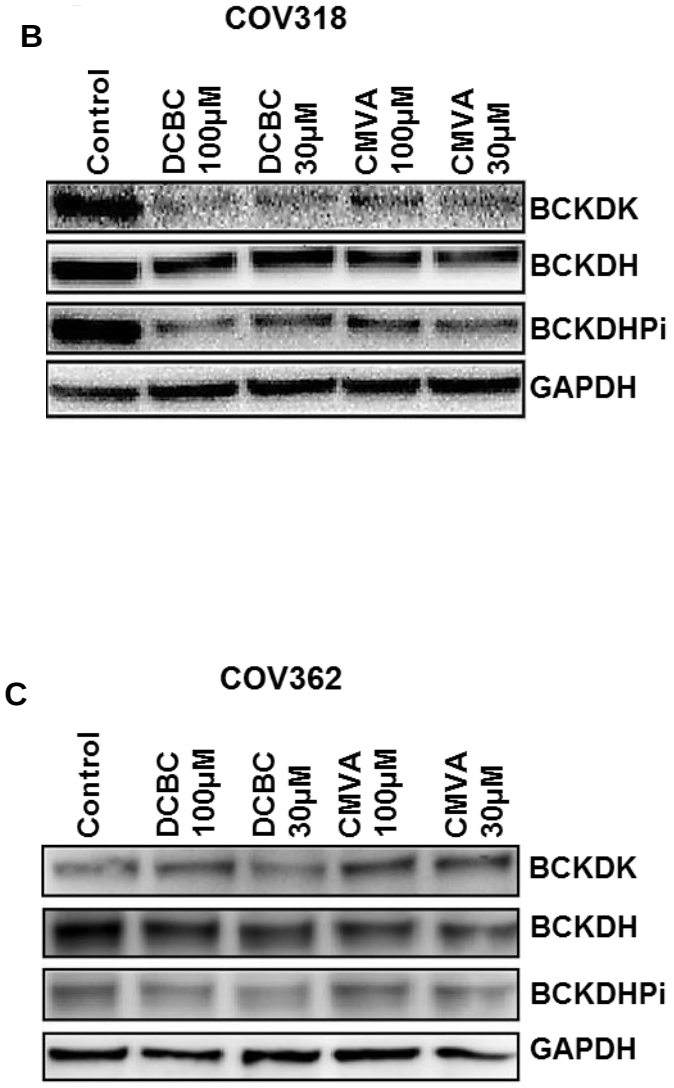


Figure 6.18: Expression of BCKDK, BCKDH and BCKDHPi in OVCAR-4, COV318 and COV362 cells after treatment with BCKDK inhibitors

Lysates were prepared from A. OVCAR-4, B. COV318 and C. COV362 which had been exposed to 100 µM of BCKDK inhibitors for 48 hours and the expression of BCKDK, BCKDH and BCKDHPi proteins determined by immunoblotting. The blot shown is representative of 3 experiments.

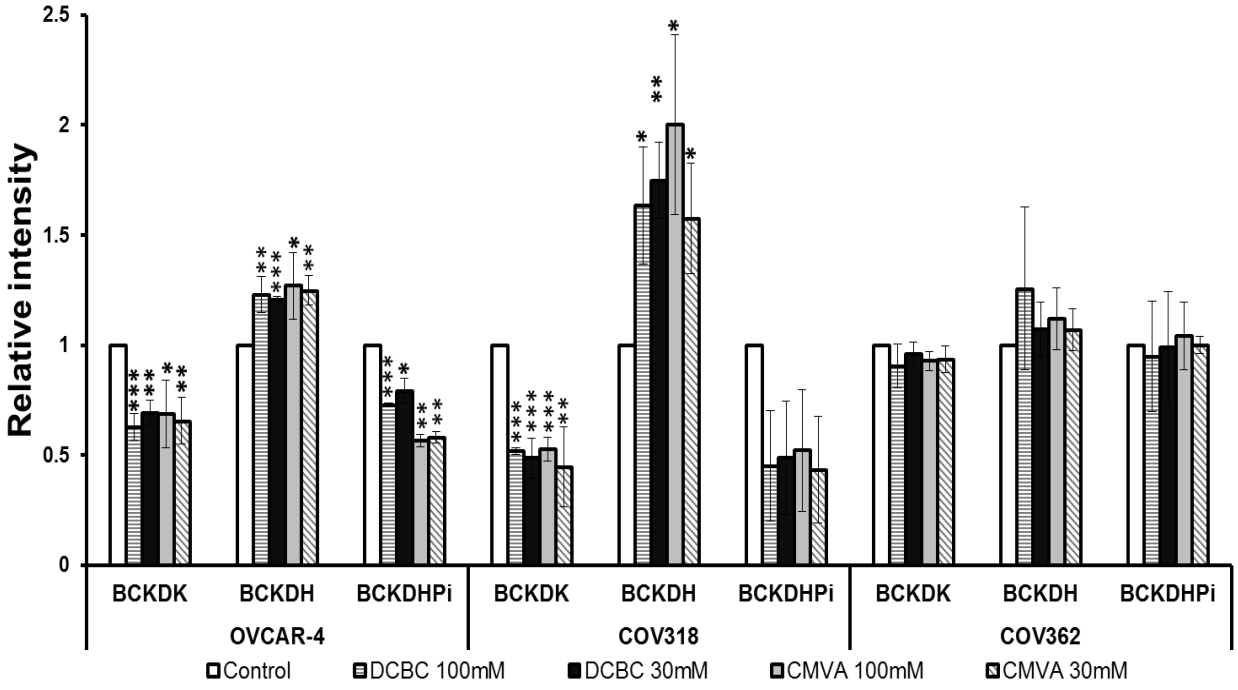


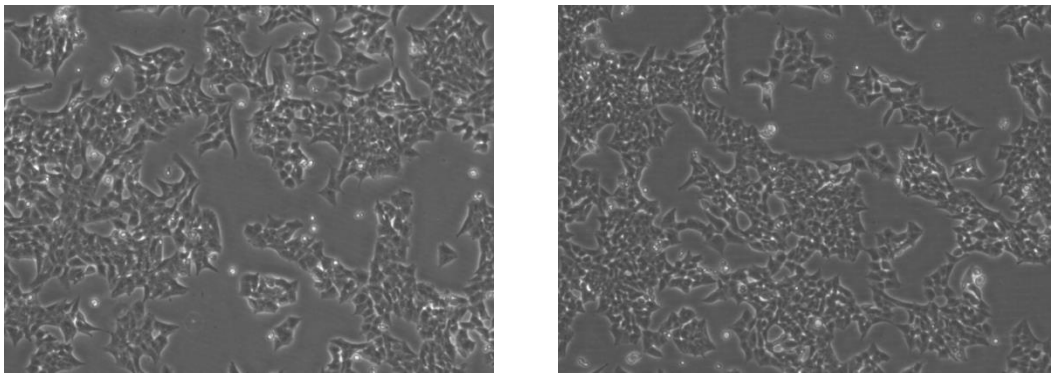
Figure 6.19: Quantification of BCKDK, BCKDH and BCKDHPi proteins in OVCAR-4, COV318 and COV362 cells after treatment with BCKDK inhibitors

The western blots shown in figures 6.18 A, B and C were quantified by measuring the relative intensity of each band and normalizing this intensity to the signal intensity of GAPDH in OVCAR-4, COV318 and COV362 cell lines and in the same blot. The results (mean ± S.D., n = 3) were normalized to the results obtained with cells exposed to vehicle ("control").

6.11.7 BCKDK gene editing and its impact on paclitaxel sensitivity

Gene-edited or "knock-out" cells are cells that are genetically engineered to lack one or more copies of a particular gene. They provide a useful tool to confirm the specificity of drugs because cells lacking the drug target might be expected to behave similarly to cells treated with the drug. BCKDK inhibitors and siRNA were synergistic with paclitaxel, leading to the hypothesis that cell gene-edited to lack BCKDK would be more sensitive to paclitaxel than the parental cells. Therefore, the activity of paclitaxel was evaluated in commercially available HAP-1 cells lacking the gene encoding BCKDK.

HAP-1 is an adherent human (male) chronic myeloid leukemia cell line (KBM-7) which have a fibroblast-like morphology (figure 6.20). The majority of commonly used cell lines are diploid or even have multiple copies of any particular allele. The haploid cell line has the advantage of having only a single copy of each human chromosome, facilitating elimination of all functional alleles by CRISPR technology.



HAP-1 cells BDK cells

Figure 6.20: Phase contrast microscopy images of HAP-1 and BDK cells

The morphological appearance of HAP-1 and BCKDK knock-out cells viewed under light microscopy.

6.11.7.1. Cell growth assay in HAP-1 and BDK cells

HAP-1 parental cells and BCKDK knockout cells ("BDK cells") were evaluated for their sensitivity to paclitaxel in cell growth assays. Unexpectedly, the BDK cells appeared, if anything, modestly less sensitive to paclitaxel (table 6.2, figure 6.21) than the HAP-1 parental cells. Although this difference was not statistically significant, this was surprising as it was not consistent with the earlier studies which suggested that inhibition of BCKDK increased sensitivity to paclitaxel.

Table 6.2: IC50 and Hill slope values of paclitaxel in HAP-1 and BDK cell lines

Cell line	Paclitaxel	
	IC50 (nM)	**Hill slope**
HAP-1	3.9±1.1	-5.1±0.1
BDK	4.8±1.1	-3.2±0.3

The activity of paclitaxel was examined in cell growth assays using HAP-1 and BDK cells (which lack the gene encoding BCKDK) by using the SRB assay. The results are expressed as mean ± S.D. from eight different experiments.

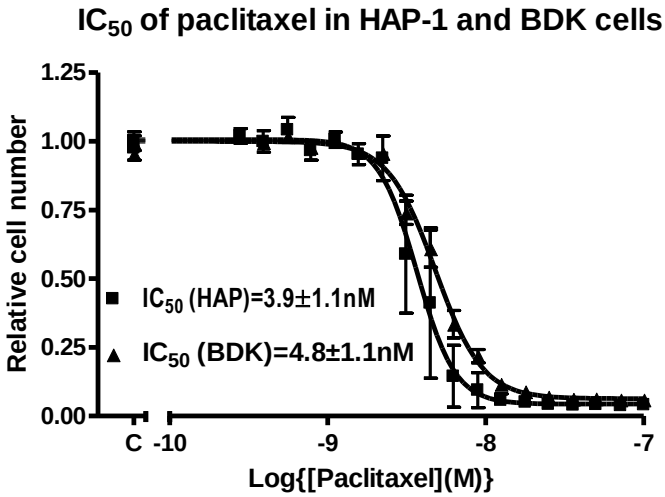


Figure 6.21: Activity of paclitaxel in HAP-1 and BDK cell growth assays

Paclitaxel was evaluated in cell growth assays using HAP-1 and BDK cell lines. The figure shows mean ± SD for eight experiments listed in table 6.2. "C" in X-axis indicates control cells treated with drug vehicle.

6.11.7.2 Cell death and apoptosis confirmation assays of BDK cells exposed to paclitaxel

To confirm the activity of paclitaxel observed in HAP1 and BDK cells, the activity of the drug combination was also assessed in trypan blue exclusion, caspase 3/7 activity and PARP cleavage assays. In cell viability studies (figure 6.22), consistent with the apparent decreased sensitivity of BDK cells to paclitaxel observed in cell growth assays, paclitaxel killed significantly more HAP-1 cells than the gene edited BDK cells. Similarly, the activation of caspase-3/7 by paclitaxel in HAP-1 cells was significantly more than that in BDK cell line (figure 6.23). Immunoblotting of cell lysates to determine PARP cleavage in both cell lines after treatment with paclitaxel revealed that most of PARP was cleaved in HAP-1 cells in comparison to BDK cells, which still retained some uncleaved PARP (figure 6.24). Thus, all three assays showed exposure to paclitaxel resulted in less cell death in BDK cells compared to the parental HAP-1 cells.

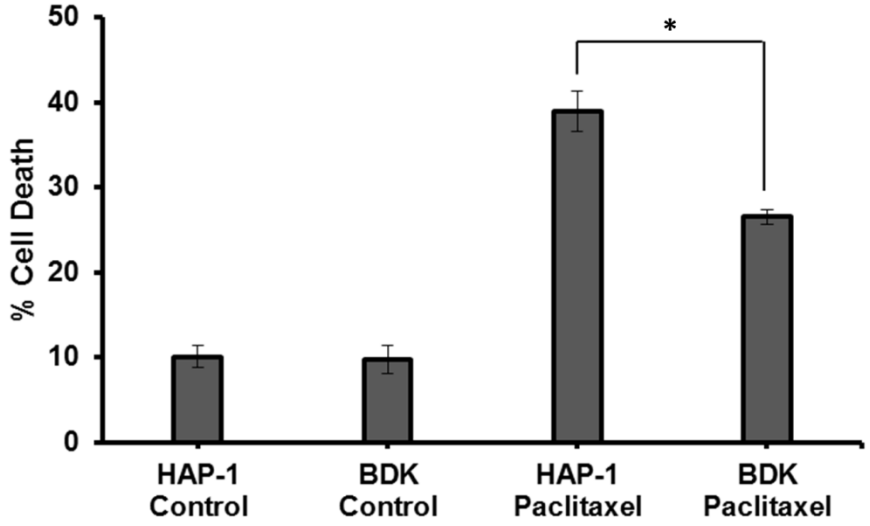


Figure 6.22: The effect of paclitaxel on cell viability in HAP-1 and BDK cell lines

HAP-1 and BDK cells were exposed to 5 nM paclitaxel for 24 hours and the percentage of viable cells (mean ± S.D., n=3) was determined by staining with trypan blue. There was significantly less cell death in the BDK cells compared to the HAP-1 cells (paired t- test, *, $P < 0.05$).

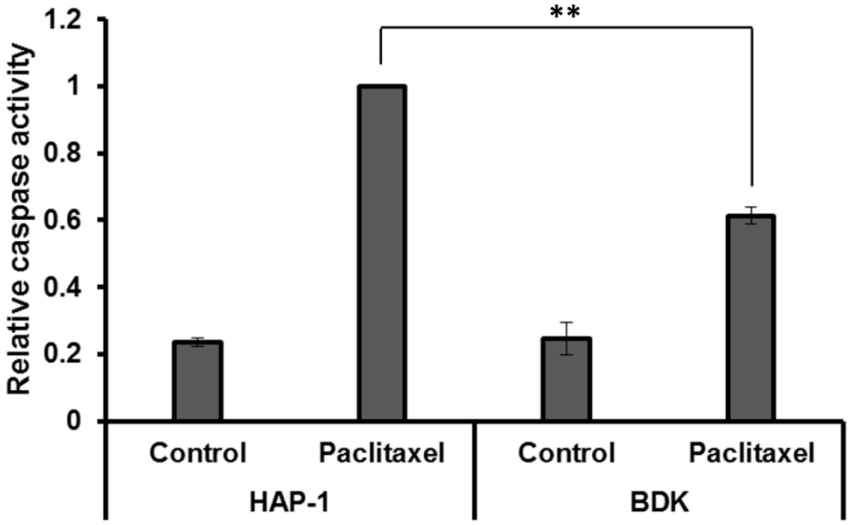


Figure 6.23: Relative caspase 3/7 activity in HAP-1 and BDK cell lines

HAP1 and BDK cells were exposed to 5 nM paclitaxel for 24 hours and caspase 3/7 activity determined. To normalize the caspase activity for any change in cell number which may occur as a result of the effect of the drug, a comparable experiment was performed in parallel and the relative cell number determined by SRB staining. The results (mean ±S.D., n = 3) are expressed as caspase activity measured in HAP-1 cells treated with paclitaxel and normalized to the relative cell number in each sample. The was significantly less caspase 3/7 activity where indicated (**, $P<0.01$; paired t-test).

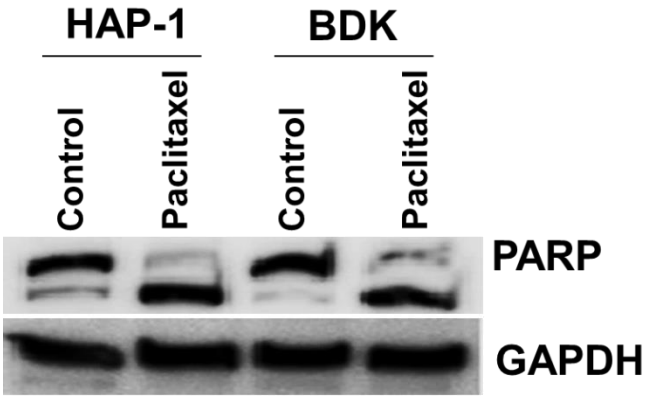


Figure 6.24: PARP-cleavage in HAP-1 and BDK cell lines

HAP-1 and BDK cells were exposed to paclitaxel (5 nM) for 24 hours and PARP-cleavage was assessed by western blotting immunoassay. A representative blot is shown (n=3). PARP, Poly (ADP-ribose) polymerase. GAPDH,Glyceraldehyde 3-phosphate dehydrogenase.

6.11.7.3 Determination of BCAA metabolizing enzymes in HAP-1 and BDK cells

To explain the results obtained in the previous experiments, which were in apparent contradiction to the foregoing studies which suggested that inhibition of BCKDK increased sensitivity to paclitaxel, western blotting of BCKDK, BCKDH and BCKDH phosphorylation was performed in untreated HAP-1 and BDK cells. The results (figure 6.25) revealed that, as expected, BCKDK was not detectable in BCK cells. However, both BCKDH and phosphorylated BCKDH were also both significantly reduced, suggesting that the deletion of BCKDK from the cells caused alteration in the expression of BCKDH to compensate for the loss of inhibitory regulation of BCKDH by BCKDK and presumably an increase in BCAA metabolism.

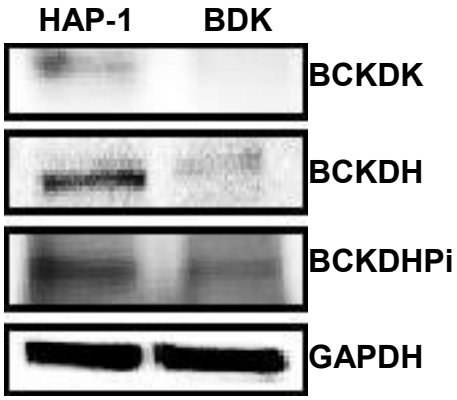


Figure 6.25: Expression of BCKDK, BCKDH and BCKDHPi in HAP-1 and BDK cell lines

Protein lysates were prepared from HAP-1 and BDK cells and the expression of BCKDK, BCKDH and phosphorylated BCKDH proteins determined by western blotting. GAPDH= Glyceraldehyde 3-phosphate dehydrogenase. Representative blots are shonw (n=3).

6.13.8 Over-expression of BCKDK in ovarian cancer cell lines

To complement the results in the previous section, over-expression of BCKDK was explored. BCKDK was originally identified in the screen conducted to identify genes which confer drug resistance (364) by virtue of its increased expression in chemoresistant compared to chemosensitive ovarian cancer (363). Consequently, it was hypothesized that over-expression of BCKDK would decrease sensitivity to paclitaxel in cell growth assays.

First, conditions for transfection of two ovarian cancer cell lines (COV318 and FUOV-1) were optimized. These cells were chosen because they are representative of high grade serous ovarian cancer and synergy between paclitaxel and BCKDK inhibitors in cell growth assays had already been observed in these cells. OVCAR-4 cell line was excluded as it was refractory to transfection in previous experiments in our laboratory.

The cell lines (COV318 and FUOV-1) were transfected with the DsRed plasmid encoding a red fluorescent protein and transfection efficiency checked by confocal microscopy. A range of cell numbers, lipofectamine 2000 and DNA concentrations were evaluated. The results showed that COV318 cells were transfected with higher efficiency than FUOV-1 cells (figures 6.26 A, B and C and 6.27 A, B and C). A ratio of 2:1 DNA: lipfectamine 2000 was found to provide the highest efficiency in COV318 cells, while in FUOV-1 the best ratio of transfection was 1:3.

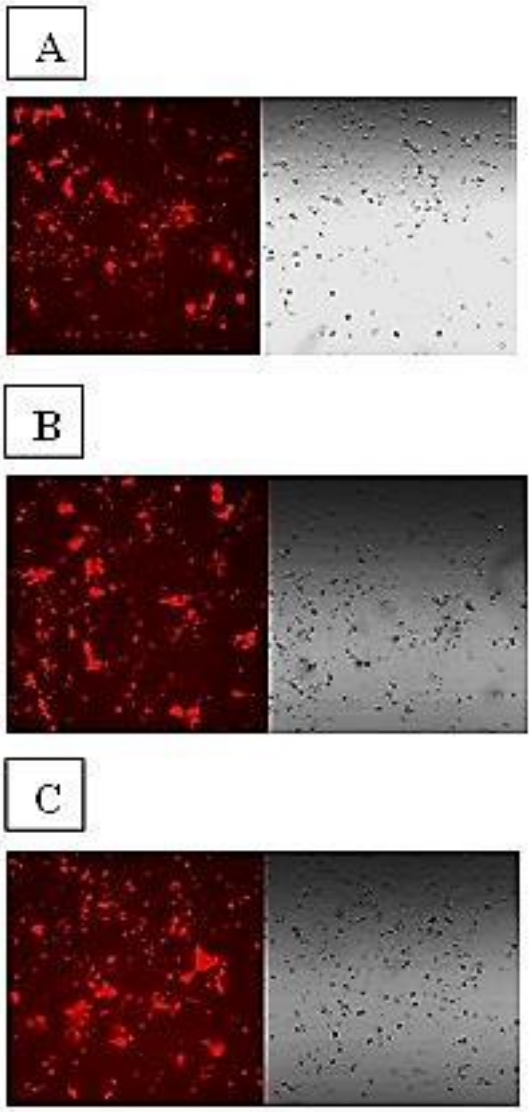


Figure 6.26: Immunofluorescence microscopy for detection of transient expression of DsRed in COV318 cells

Cells were seeded in 96 well plates at different densities (3000, 4500 or 6000 cell/well), then analyzed for DsRed expression as an indication of transfection efficiency. The toxicity of lipofectamine 2000 has been checked by using different ratios of DNA: lipofectamine 2000 and then analyzed by SRB cell growth assay. A. Represents seeding of 3000 cell and transfected with 0.2 µg DNA/0.1 µl lipofectamine 2000 (2:1) ratio. B. Represents seeding of 4500 cell and transfected with 0.2 µg DNA/0.1 µl lipofectamine 2000 (2:1) ratio. C. Represents seeding of 6000 cell and transfected with 0.2 µg DNA/0.1 µl lipofectamine 2000 (2:1) ratio. Photos taken at 60X power using confocal microscope.

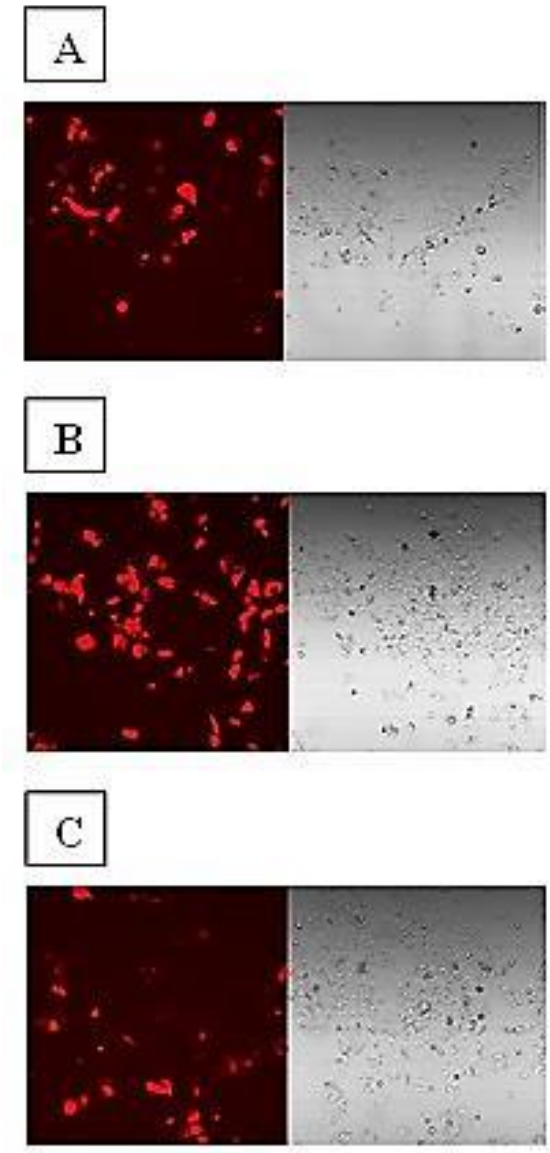
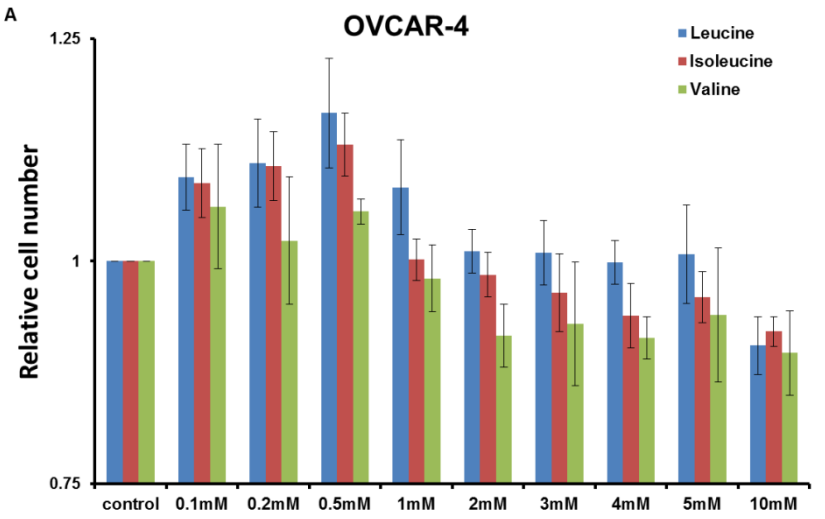


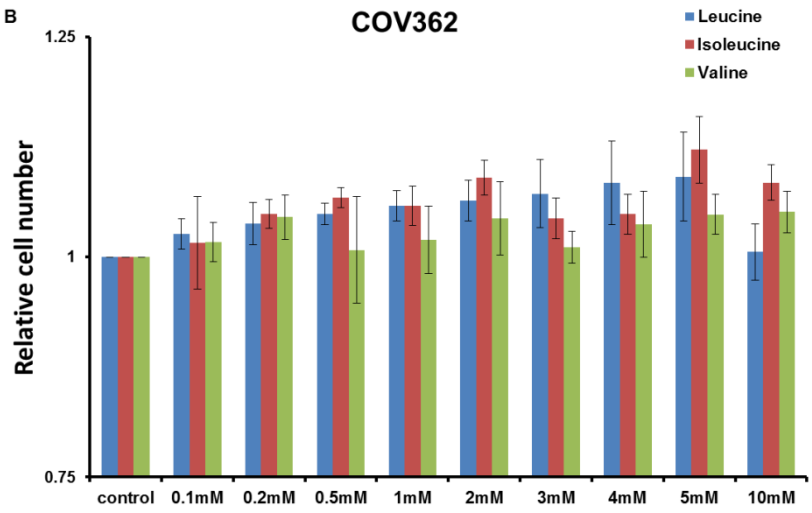
Figure 6.27: Immunofluorescence microscopy for detection of transient expression of DsRed in FUOV-1 cells

Cells were seeded in 96 well plates at different densities (3000, 4500 or 6000 cell/well), then analyzed for DsRed expression as an indication of transfection efficiency. The toxicity of lipofectamine 2000 has been checked by using different ratios of DNA: lipofectamine 2000 and then analyzed by SRB cell growth assay. A. Represents seeding of 3000 cell and transfected with 0.1 µg DNA/0.3 µl lipofectamine 2000 (1:3) ratio. B. Represents seeding of 4500 cell and transfected with 0.1 µg DNA/0.3 µl lipofectamine 2000 (1:3) ratio. C. Represents seeding of 6000 cell and transfected with 0.1 µg DNA/0.3 µl lipofectamine 2000 (1:3) ratio. Photos taken at 60X power using confocal microscope.

6.11.9 Evidence that BCKDK inhibitors have an “on target” mechanism of action

Inhibition of BCKDK is predicted to increase the activity of BCKDH and hence the catabolism of BCAA. This suggests that if the BCKDK inhibitors sensitized cells to paclitaxel by stimulating BCAA catabolism, addition of exogenous BCAA would reduce the synergy between the inhibitors and paclitaxel. However, little is known about the impact of altering the BCAA concentration on the growth of ovarian cancer cells. Therefore, the effect of various BCAA (leucine, isoleucine and valine) concentrations added to the culture medium was first determined. Cell culture medium (which contains 1 mM BCAA already) was supplemented with nine different concentrations of each BCAA, from 0.1-10 mM, and the effect measured in cell growth assays (figure 6.28 A-E). The results indicated that, in most cell lines, the cells could be supplemented with an additional 1 mM BCAA without any deleterious effects.


A
OVCAR-4
Leucine
Isoleucine
Valine
Relative cell number
1.25
1
0.75
control
0.1mM
0.2mM
0.5mM
1mM
2mM
3mM
4mM
5mM
10mM


B
COV362
Leucine
Isoleucine
Valine
Relative cell number
1.25
1
0.75
control
0.1mM
0.2mM
0.5mM
1mM
2mM
3mM
4mM
5mM
10mM

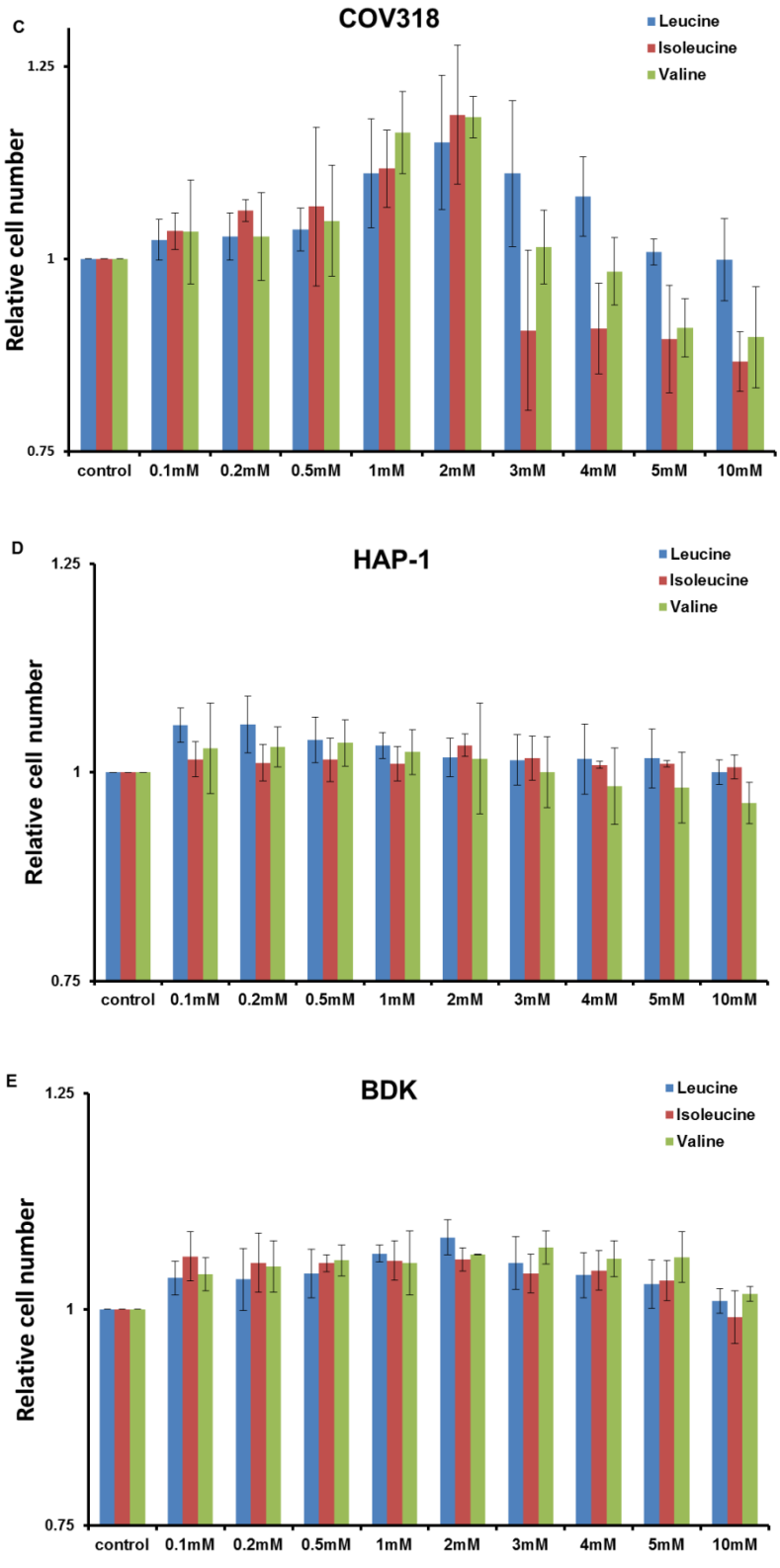


Figure 6.28: Effect of BCAA on cell growth in different cell lines

The effect of a range of concentrations of BCAA on the growth was assessed in the indicated cell lines using the SRB assay. The results (mean ± SD, n=3) were expressed as a fraction of A570 measured in cells grown in unsupplemented medium.

6.11.9.1 Effect of BCAAs on the combination of paclitaxel and BCKDK inhibitors in OVCAR-4 cell line

To confirm that CMVA exerted its synergistic effect with paclitaxel in a manner that depended on BCAA, synergy between paclitaxel and CMVA was again assessed in cell growth assays, but this time using unsupplemented media or media supplemented with an additional 1 mM of each BCAA. The drug combination assays were performed using serial dilutions of paclitaxel in combination with solvent or 100 µM (fixed concentration) CMVA. In the unsupplemented media, synergy was again observed (C.I. < 1.0). However, in media supplemented with the BCAA, an additive interaction was observed (figure 6.29 A). This suggests that CMVA potentiated the activity of paclitaxel in a manner dependant on BCAAs and so is consistent with CMVA having an "on-target" mechanism of action.

To confirm this, the effect of the drug combination was again measured in cells with unsupplemented media and in media containing 1 mM BCAA and the effect of the drug combination on PARP cleavage was assessed. Paclitaxel alone induced PARP cleavage and this was augmented by the addition of CMVA in cells grown in unsupplemented media. However, in the presence of additional BCAA, PARP cleavage was reduced (figure 6.29 B).

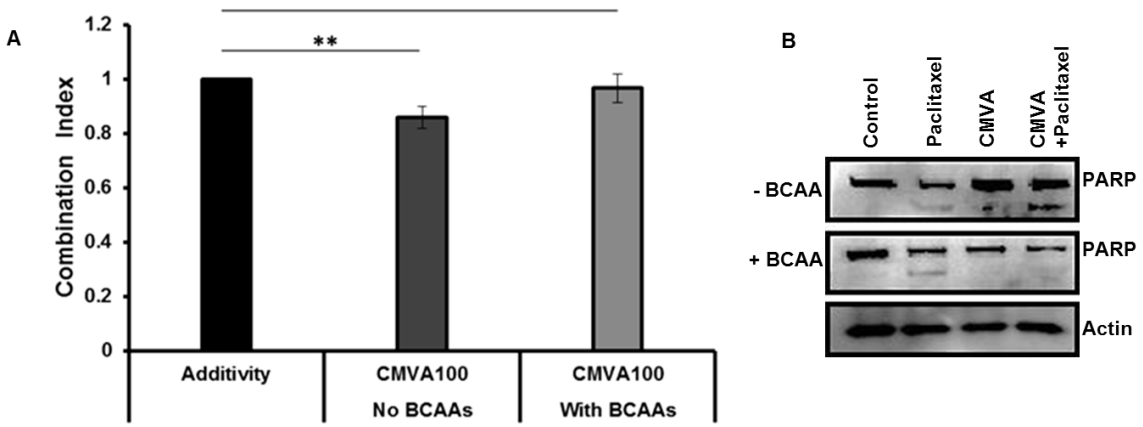


Figure 6.29: Effect of BCAAs on the combination of paclitaxel and CMVA

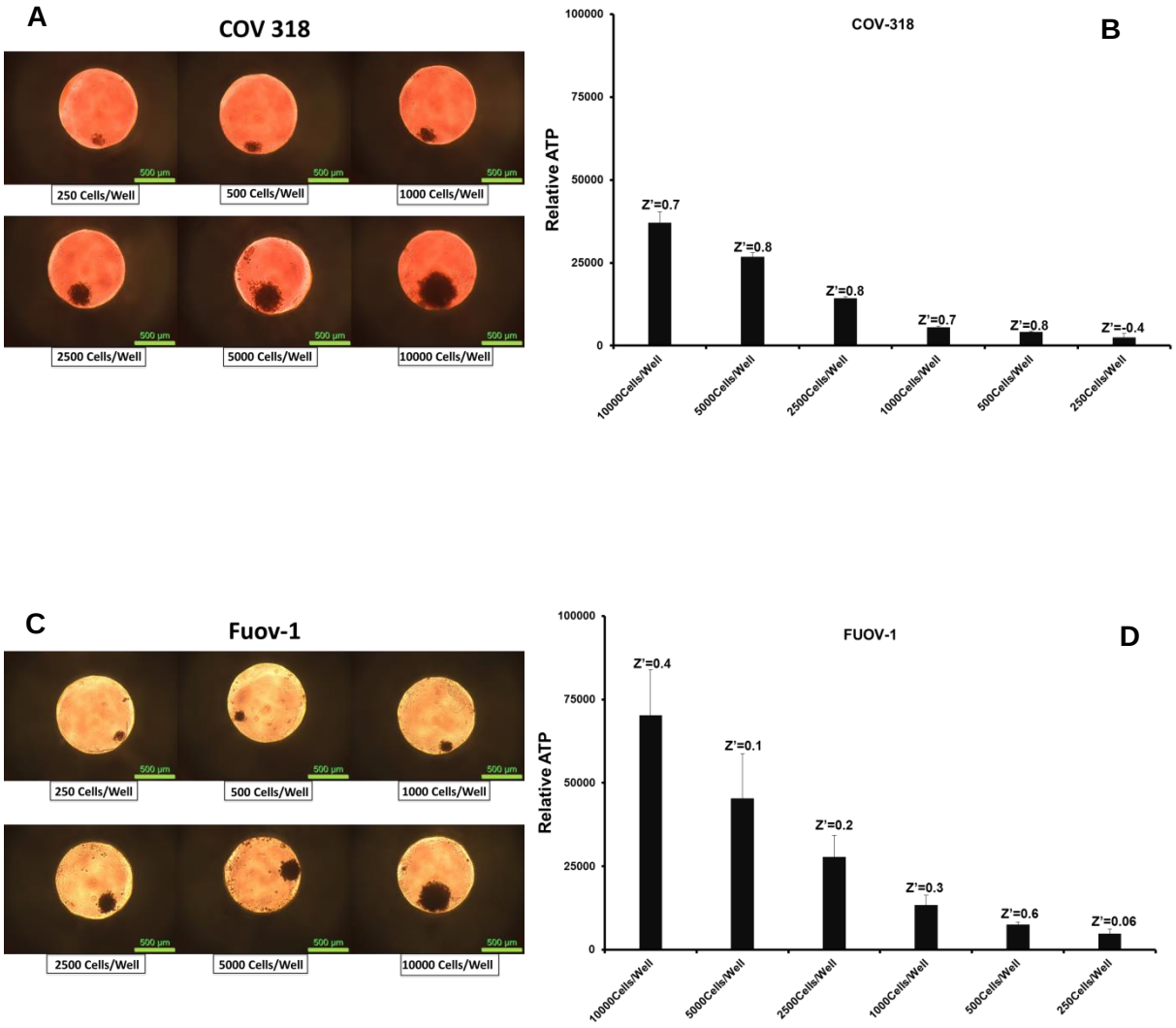
A. Evaluation of CMVA and paclitaxel combinations in SRB cell growth assays. OVCAR-4 cells were treated with combinations of serial dilution of paclitaxel started from 100 nM with 100 µM CMVA. After 72 hours, the viable cell number was determined by SRB staining. Combination indices (mean ± S.D., n = 3) were significantly different from additivity as indicated in the figure in cells that are treated with paclitaxel and CMVA in the absence of any added BCAAs (paired t-test **, $P \leq 0.01$). The addition 1 mM of each BCAA to the growth medium resulted in additivity. **B.** Representative western blots (n=3) of PARP cleavage in OVCAR-4 cell line. Cells were exposed to paclitaxel (12 nM) alone or in combination with CMVA (100 µM) for 48 hours. PARP= Poly (ADP-ribose) polymerase. Actin was used as a loading control.

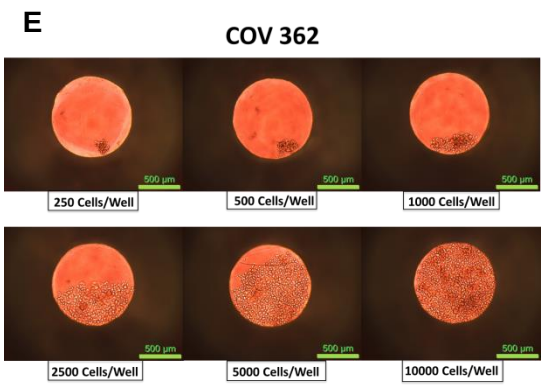
6.11.10 Evaluation of BCKDK inhibitors in spheroid cultures

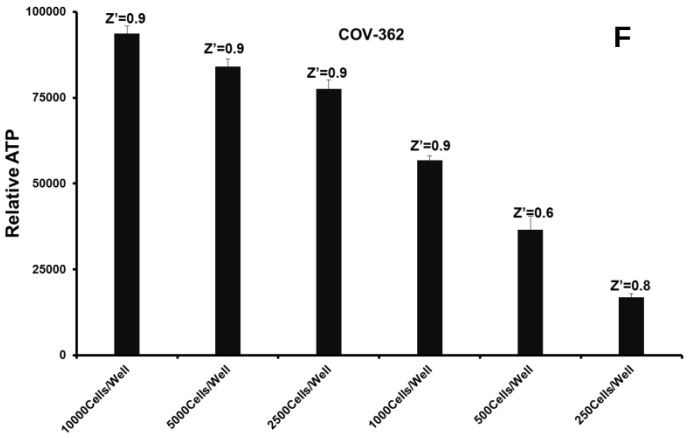
Three dimensional cell cultures can be used as a method to mimic in part the architecture of an authentic tumour and potentially improve the clinical relevance of laboratory in vitro models. The combination of BCKDK inhibitors and paclitaxel was therefore evaluated using cultures of spheroids. Each ovarian cancer cell line was first evaluated to determine if it can form a three dimensional spheroid by seeding a variable number of cells in an ultra low attachment 96 well plate. The relative cell number was assessed by intracellular ATP. All the cell lines tested were able to produce a spheroid like structure after a few days of culture

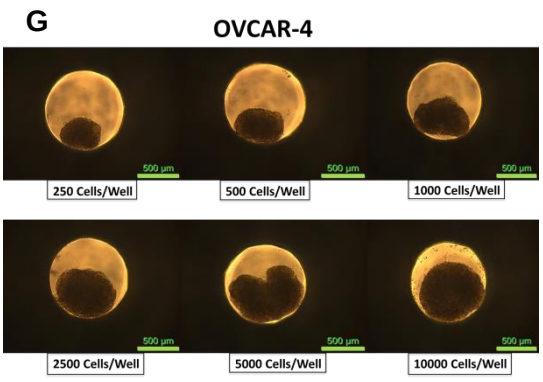
except COV362 which was unable to form an aggregate of cells. Therefore, this cell line should be excluded from further studies (figure 6.30).

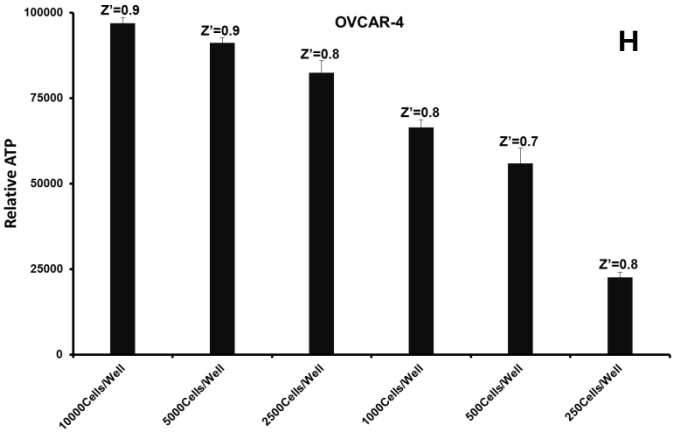
HAP-1 and BDK spheroids were subsequently exposed to paclitaxel. Measurement of ATP revealed that BDK cells were significantly less sensitive than HAP-1 cells to paclitaxel (figure 6.31), a result which was consistent with that of two dimensional culture assays. Experiments with combining the BCKDK inhibitors with paclitaxel were attempted in spheroids from other cell lines. However, the BCKDK inhibitors quenched the luminescence that is produced from the ATP CellTiterGlo assay, making it impossible to obtain reliable results with the other cell lines.

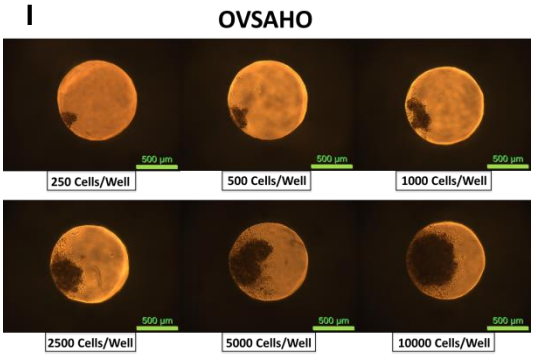


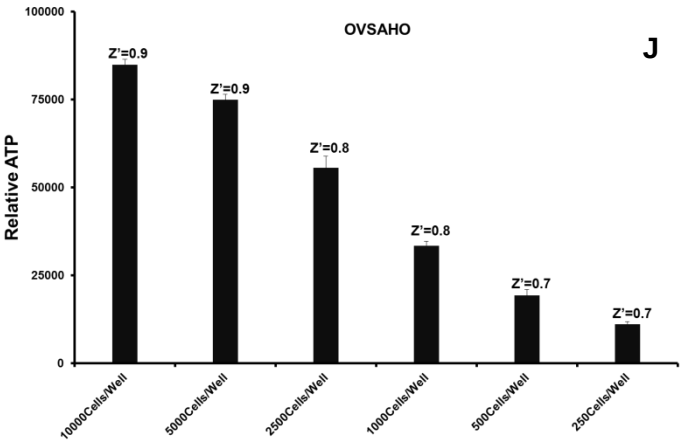

E
COV 362
250 Cells/Well
500 Cells/Well
1000 Cells/Well
2500 Cells/Well
5000 Cells/Well
10000 Cells/Well


F
COV-362
Relative ATP
100000
75000
50000
25000
0
Z'=0.9
Z'=0.9
Z'=0.9
Z'=0.9
Z'=0.6
Z'=0.8
10000Cells/Well
5000Cells/Well
2500Cells/Well
1000Cells/Well
500Cells/Well
250Cells/Well


G
OVCAR-4
250 Cells/Well
500 Cells/Well
1000 Cells/Well
2500 Cells/Well
5000 Cells/Well
10000 Cells/Well


H
OVCAR-4
Relative ATP
100000
75000
50000
25000
0
Z'=0.9
Z'=0.9
Z'=0.8
Z'=0.8
Z'=0.7
Z'=0.8
10000Cells/Well
5000Cells/Well
2500Cells/Well
1000Cells/Well
500Cells/Well
250Cells/Well


I
OVSAHO
250 Cells/Well
500 Cells/Well
1000 Cells/Well
2500 Cells/Well
5000 Cells/Well
10000 Cells/Well


J
OVSAHO
Relative ATP
100000
75000
50000
25000
0
Z'=0.9
Z'=0.9
Z'=0.8
Z'=0.8
Z'=0.7
Z'=0.7
10000Cells/Well
5000Cells/Well
2500Cells/Well
1000Cells/Well
500Cells/Well
250Cells/Well

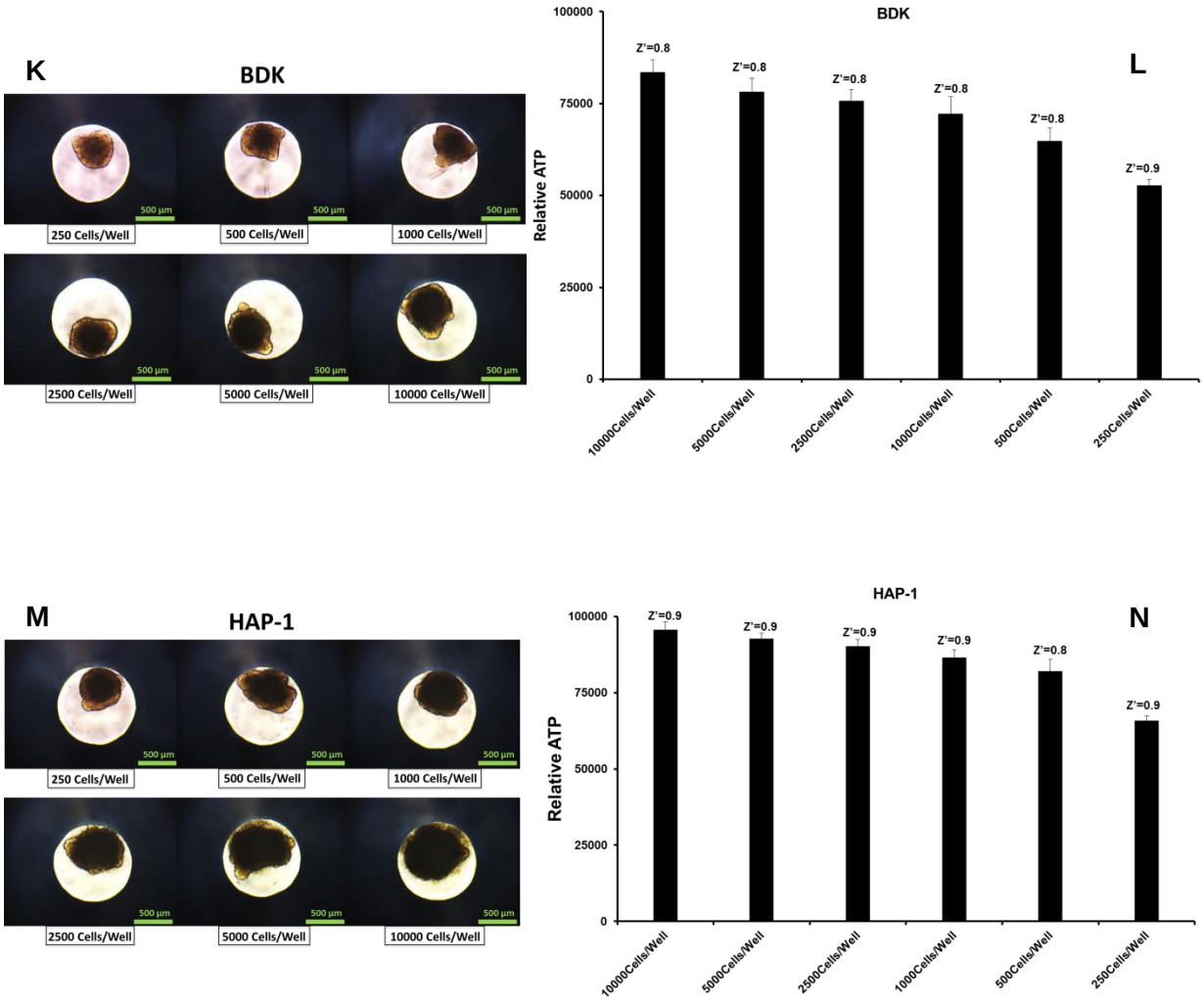


Figure 6.30: Three dimensional cell culture

The microscopic appearance of different cell lines after seven days of seeding in ULA-plates showing the ability of cells to produce three dimensional structure (A: COV318, C:FUOV-1, E:COV362, G:OVCAR-4, I:OVSAHO, K:BDK, M:HAP-1). Cells were seeded in 70 µl of growth medium at different initial seeding densities (250, 500, 1000, 2500, 5000 or 10000 cell/well). On day 7, CellTiterGlo reagent was used to check the viability of cells by measuring ATP. The bar graphs on the right hand show the mean ± SD results of three experiments of mean ATP luminescence and Z'-factors for ATP-based assay at various densities of cells for each cell line (B: COV318, D:FUOV-1, F:COV362, H:OVCAR-4, J:OVSAHO, L:BDK, N:HAP-1). The values of Z'-factor range from negative to positive values, < 0 is considered an unacceptable assay, > 0 an acceptable assay, and > 0.5 as a good assay.

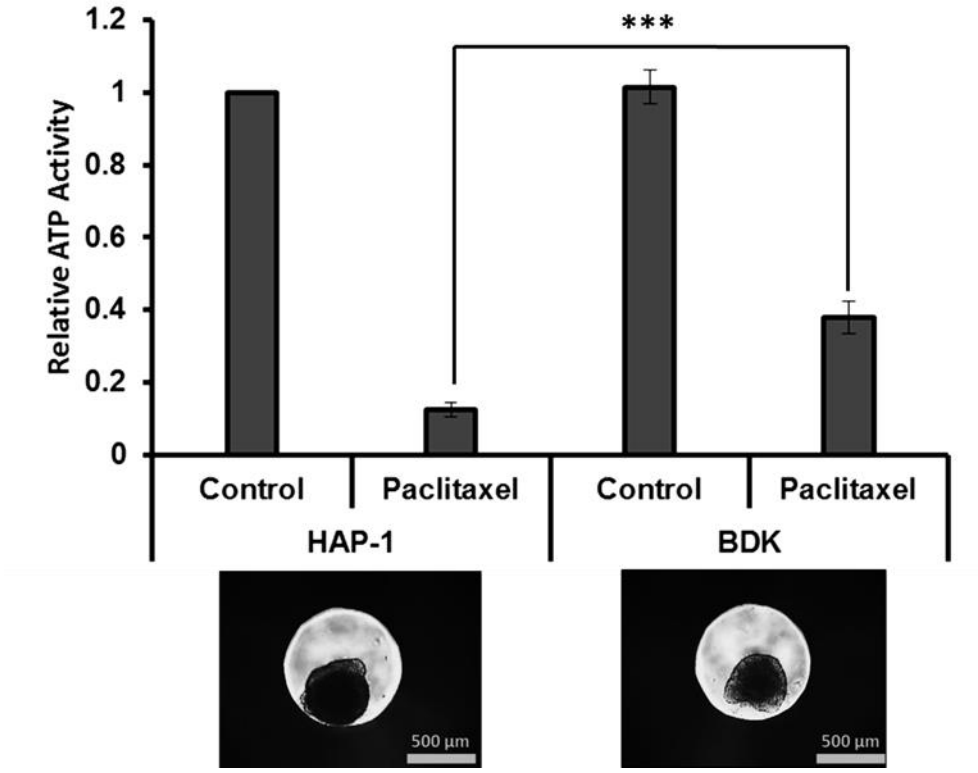


Figure 6.31: Relative ATP in paclitaxel treated HAP-1 and BDK cells

The effect of paclitaxel (30 nM, 72 h) was measured in HAP-1 and BDK cells. Spheroids were assessed by measurement of intracellular ATP (mean ± S.D., n = 3). The relative ATP activity was significantly higher in BDK spheroids compared to HAP-1 cell line (***, paired t-test, $P<0.001$). The "Control" was HAP-1 and BDK spheroids were exposed to drug vehicle.

6.11.11 The involvement of autophagy to cell death induced by Paclitaxel and BCKDK inhibitors

The previous results have identified apoptosis as one possible mechanism for cell death induced by a combination of paclitaxel and BCKDK inhibitors in different ovarian cancer cell lines. However the contribution of BCAA to mTOR activation, together with the role of mTOR as a regulator of autophagy, raised the hypothesis that autophagy also might contribute to cell death induced by the drug combination. To evaluate this, autophagy was assessed by measurement of LC3-II which is markedly increased when autophagy is stimulated. p62 was also evaluated as another marker of autophagy and this protein is degraded during autophagy. OVCAR-4 cells were exposed to 12 nM paclitaxel, 100 μM of DCBC, 100 μM of CMVA or a combination of paclitaxel with DCBC or CMVA for 48 hours.

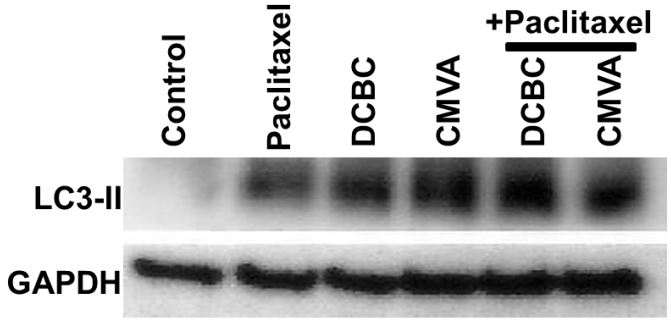


Figure 6.32: The effects of BCKDK inhibitors on LC3-II

OVCAR-4 cell line was exposed to 12 nM paclitaxel, 100 μM DCBC, 100 μM CMVA and combination of the given dose of paclitaxel in combination with BCKDK inhibitors for 48 hours. The expression of LC3-II was determined by western blotting assay. GAPDH was used as a loading control.

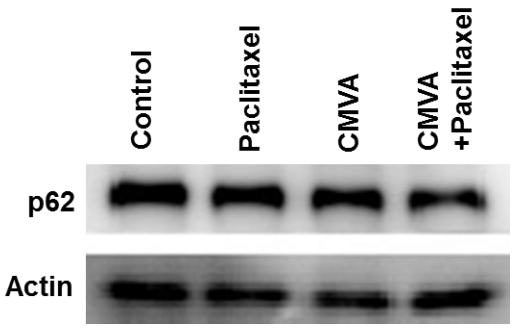


Figure 6.33: The effects of BCKDK inhibitors on p62

OVCAR-4 cell line was exposed to 12 nM paclitaxel, 100 μM CMVA and combination of the given dose of paclitaxel in combination with CMVA for 48 hours. The expression of p62 was determined by western blotting assay. Actin was used as a loading control.

Treatment with BCKDK inhibitors alone and in combination with paclitaxel resulted in an increase in LC3-II consistent with an activation of autophagy (figure 6.32). However, the level of LC3-II can also be increased when there is inhibition of autophagy and the turnover of LC3-II is prevented. A very modest decrease in p62 was also observed following treatment with CMVA or a combination of CMVA and paclitaxel in comparison to paclitaxel alone treated cells (figure 6.33) suggesting that autophagy stimulation was more likely. Further work is needed to confirm this.

6.12 Discussion

More than 75% of ovarian cancer patients that respond to chemotherapy ultimately relapse typically within 18-36 months after the end of the first treatment with chemotherapy. Therefore, it is desirable to develop novel targeted therapies that can re-sensitize the tumour to chemotherapy (550,551). To identify appropriate drug targets, genes that are over-expressed in the drug-resistant form of ovarian cancer were evaluated in the previous chapter by assessing the effect of their knock-down on the sensitivity to carboplatin or paclitaxel. One of the validated hits from siRNA screen that identified genes which can regulate the sensitivity of ovarian cancer cells to chemotherapy was BCKDK. The work described in this chapter confirmed that BCKDK regulates sensitivity of ovarian cancer cells to paclitaxel. BCKDK is a kinase enzyme involved in the regulation of BCAA catabolism. It is responsible for inactivation of BCKDH, which is the rate limiting enzyme in BCAA turnover. Branched chain amino acids are regarded as key nutrient molecules that play important role in normal function, growth and survival, as well as in providing the main building blocks for synthesis of proteins. Furthermore, BCAA possess an effective signalling function that control growth and metabolism (321).

Prior to further work on this gene, the level of BCKDK and BCKDH was determined at basal level in eight ovarian cancer cell lines, of which 5 cell lines (OVCAR-4, OVSAHO, FUOV-1, COV318 and COV362) closely resemble the profile of high grade serous ovarian cancer (321), in addition to OVCAR-8, OVAR-3 and Igrov-1 and the normal human epithelial cells. It was found that both of these enzymes are expressed in all cell lines at varying levels, with lower expression of BCKDK and BCKDH in OVCAR-3 and COV362 respectively. The human protein atlas shows that most malignant cells including breast, head and nech, thyroid, endometrial, prostate and ovarian cancers display variable expression of BCKDK. Therefore,

studies of clinical samples, particularly from patients with drug resistant disease, are desireable to assess this.

Since BCKDK knock-down resulted in sensitization of ovarian cancer cell lines to paclitaxel, it was speculated that inhibition of BCKDK by chemical compounds could also enhance the response of ovarian cancer cells to paclitaxel. For this purpose, two previously described BCKDK inhibitors were selected, (S)-2-Chloro-4-methylvaleric acid (CMVA) and 3,6-Dichloro-1-Benzothiophene-2-Carboxylic acid (DCBC). The IC_{50}s of these compounds were determined in cell growth assays using OVCAR-4 and COV362, the cell lines in which the activity of paclitaxel was potentiated when BCKDK was repressed by siRNA (chapter 5). In addition, COV318 and FUOV-1 cells were also used alongside these cell lines. The compounds when used as single agents did not potently induce cell death in any of the cell lines. This suggests that in the absence of cytotoxic stimuli, inhibition of BCKDK, and presumably stimulation of BCKDH is not cytotoxic under the conditions tested. Our work is in consistence with that of Tso and his colleagues who showed that DCBC significantly increases residual BCKDH activity in cultured cells as well as primary hepatocytes from a mouse model and patients of maple syrup urine disease with minimal toxicity. Administration of BCKDK inhibitors for 1 week resulted in nearly complete dephosphorylation of BCKDH in kidneys, heart, liver and muscle with reduction in plasma BCAA concentrations with no obvious toxicity. Thus, BCKDK inhibitors can provide useful treatment of metabolic disorders caused by elevated BCAA concentrations (548).

Both compounds were synergistic with paclitaxel in all cell lines with the exception of COV362 where additivity was observed using several different assays to confirm the synergy including cell growth assay, activation of caspase 3/7, cell death measurement assay, and PARP cleavage. These results are consistent with those obtained from the gene knock-down experiments reported in chapter 5. In COV362 cells, synergy was not observed in any of the

assays used to assess the effects of the drug combination. One explanation for this is that the compounds only inhibit BCKDK when it is bound to BCKDH (542). In contrast to OVCAR4, COV318 and FUOV-1, immunoblotting studies showed that BCKDH was not abundant in COV362 and consequently there is unlikely to be a significant pool of BCKDK bound to BCKDH. It is, therefore, not suprising that the BCKDK inhibitors were not synergistic with paclitaxel in this cell line.

In an effort to further confirm that the synergy observed between paclitaxel and inhibition of BCKDK, the activity of paclitaxel was evaluated in cells lacking BCKDK. HAP-1 cells have been genetically engineered to lack BCKDK, and were correspondingly anticipated to show increased sensitivity to paclitaxel. In cell growth assays, there was not significant changes in sensitivity to paclitaxel in the cells lacking BCKDK. However, in several additional assays, including trypan blue exclusion, PARP cleavage and caspase 3/7 activation the BDK cells lacking BCKDK showed reduced sensitivity to paclitaxel. This finding was also confirmed using 3D cultures. To explain these results, the basal level of BCAA metabolizing enzymes was measured. Unexpectedly, BCKDH was shown to be down-regulated in BDK cells. This is consistent with the cells having adapted to the loss of BCKDK. Loss of BCKDK is anticipated to prevent the inhibitory regulation of BCKDH by BCKDK and promote BCAA metabolism. A decrease in BCKDH may be an adaptive response to restrict the anticipated increase in BCAA metabolism in the BCKDK knock-out cells which may lead to deprivation of BCAA. Therefore, inactivation of BCKDH provides a mechanism to decrease BCAA metabolism and to promote survival. It would be desireable to measure BCAAs in cell cultures treated with BCKDK inhibitors to confirm this hypothesis. However, the apparently adaptive response of the cells in the BCAA catabolic pathway was associated with a change in sensitivity to paclitaxel and this further reinforces the argument that the BCAA metabolic pathway contributes to the sensitivity of cells to paclitaxel.

The BCKDK inhibitors reduced phosphorylation of BCKDH, in all the cells lines tested except COV362, confirming that the compounds inhibited BCKDK. However, this does not on its own rule out the possibility that synergy between paclitaxel and the BCKDK inhibitors results from the inhibitors acting through another target. However, several lines of evidence support the BCKDK inhibitors acting synergistically with paclitaxel by an "on target" mechanism. Firstly, the lack of inhibition of BCKDK in COV362 by the inhibitors and the lack of synergy in these cells, contrasts with synergy observed in cells expressing BCKDK in which the inhibitors successfully inhibited BCKDK. This supports the argument that the drugs work by inhibiting BCKDK, rather than having an off-target effect. Secondly, phosphorylation of BCKDH by BCKDK leads to its inactivation. This suggests that the BCKDK inhibitors activated the BCKDH complex which would be anticipated to lead to an increase in BCAA catabolism. When the cell culture media was supplemented with BCAA, the synergistic interaction between the BCKDK inhibitors and paclitaxel was lost. This suggests that synergy depends on the concentration of BCAA and is consistent with the BCKDK inhibitors modulating the BCAA metabolic pathway. Thirdly, synergy with paclitaxel was observed using two chemically distint BCKDK inhibitors which are unlikely to share a common target other than BCKDK which contributes to paclitaxel sensitivity. Forthly, cells engineered to lack BCKDK appeared to undergo a compensatory change in the expression of BCKDH and this resulted in altered sensitivity to paclitaxel. This also supports BCKDK regulating sensitivity to paclitaxel. Lastly, three different siRNA inhibiting BCKDK expression were also synergistic with paclitaxel (chapter 5). It is unlikely that these would all share off-target effects which sensitize cells to paclitaxel. Taken together, these data provide strong evidence that inhibition of BCKDK sensitizes cells to paclitaxel.

The cellular autophagy pathway recycles damaged cellular components and contributes to survival during unfavourable growth conditions. BCAA activate mTOR, a regulator of the

autophagy pathway, and result in inhibition of autophagy (552,553). On the other hand, BCAA starvation stimulates autophagy. Additionally, mTORC1 and autophagy pathways play essential roles in protein synthesis and inhibition of protein degradation depending on BCAA concentration (554). This led to the hypothesis that BCKDK inhibitors would reduce BCAA levels and activate autophagy. To assess this, the autophagy markers LC3-II and p62 were evaluated in OVCAR-4 cell line following treatment with BCKDK inhibitors and paclitaxel. LC3 markedly changed following treatment with BCKDK inhibitors alone and in combination with paclitaxel, which may reflect a contribution of autophagy to cell death mechanism. However, p62 was not altered significantly, therefore further work is needed to define the effect of BCKDK inhibitors on the autophagy pathway. These could include using a novel Atg5-Atg12 interaction assay recently developed in our laboratory. Alternatively, the use of bafilomycin (an autophagy inhibitor) to measure the level of LC3-II turnover following treatment with BCKDK inhibitors may be helpful to distinguish between inhibition and stimulation of the autophagy pathway, both of which can lead to accumulation of LC-3II.

The BCKDK inhibitors used in this study are likely to be insufficiently potent for clinical use and new inhibitors are required. Over 26 kinases inhibitors have now been approved by the FDA for the treatment of cancer (555,556). However, BCKDK lacks a classical kinase domain that is present in most protein kinases and it is considered as an atypical protein kinase with a crystal structure closely resembling bacterial histidine kinases. Correspondingly, the inhibitors which were used in our research (DCBC and CMVA) have a mode of action distinct from the ATP competitive kinase inhibitors. (S)-2-Chloro-4-methylvaleric acid (α-chloroisocaproate; CMVA) is an analog of the natural KIC with a chlorine atom has exchanged the α-carbonyl group in KIC, while DCBC was synthesized based on the structure of BCKDK enzyme to produce a potent BCKDK inhibitor. Both compounds work by targeting an unique allosteric site in the enzyme, causing deformation of the enzyme's helix

framework and accelerated breakdown of the kinase due to detachment from BCKDH (546,548). The atypical kinase structure of BCKDK is important in the development of novel inhibitors because it suggests that it will not be productive to screen libraries of known kinase inhibitor motifs that bind the "classical" kinase domain present in most serine/threonine or tyrosine kinases. However, it does suggest the prospect that compounds that work as allosteric modulators may not inhibit other enzymes of the classical kinase family, that constitutes more that 500 enzymes, and therefore these compounds may be more selective. Work is underway in our laboratory to design improved BCKDK inhibitors.

In summary, the findings of this research offer a rationale to target BCKDK to re-sensitize the ovarian cancer cells to paclitaxel and to overcome resistance to chemotherapy in ovarian cancers and probably other forms of cancer by targeting the metabolic pathway of the three essential amino acids (leucine, isoleucine and valine). Substantial further work is required to develop this initial finding that BCKDK is a regulator of paclitaxel into a novel therapeutic, and that is discussed in the final chapter.

Chapter 7

General discussion, conclusion and future work

Ovarian cancer accounts for about 240,000 new cases with 152,000 deaths each year worldwide. Europe exhibits the highest rates of new cases (11.4 per 100,000) and mortality (6.0 per 100,000) (9,557). The prognosis for patients with advanced ovarian cancer is very poor (558). Although patients respond well to debulking surgery followed by platinum-taxane based chemotherapy the majority of them relapse, leading to the stubbornly low 5-year survival rates (559,560). Therefore, new strategies for more effective treatment of women with ovarian cancer are needed to re-sensitize tumours to chemotherapy. In general, chemotherapy in ovarian cancer has two principal challenges, one is the adverse effects of the cytotoxic agents and the other is the emergence of resistance of the tumour cells towards these antineoplastic agents which impedes the effectiveness of therapy (561). The emergence of drug resistance, particularly to the standard chemotherapeutic agents like platinum-based compounds, is a complex multi-factorial molecular issue (562). Understanding the molecular mechanisms of resistance has the potential to help clinicians in selecting patients that will benefit from chemotherapy. Such knowledge would spare patients from unnecessary cytotoxicity and adverse effects of ineffective treatment. The work in this thesis explored two strategies to address this problem. Firstly, to exploit recently developed drugs which sensitize cells to apoptosis and which could correspondingly increase the effectiveness of chemotherapy in drug-resistant cancer. Secondly, to identify new drivers of drug resistance with the intention that these could subsequently be used to develop new chemosensitizing agents.

A major hallmark of cancer is a defect in apoptosis. There are several causes for developing it including upregulation of pro-survival members of Bcl-2 family proteins, which also predispose to chemoresistance (563). This has made the apoptosis pathway an attractive therapeutic target for the development of new therapies to treat this disease (564).

Previous work in our laboratory has explored relatively non-selective BH3 mimetics such as navitoclax and ABT-737 and demonstrated that these drugs are synergisitic with chemotherapy *in vitro* and in animal studies (294). Since that work was conducted, two new BH3 mimetics, venetoclax and WEHI-539, that selectively inhibit the anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-X_L respectively, have been reported (293,297,565). However, work presented here, and reported by others (300,318,566) have shown that Bcl-X_L is the most prominent anti-apoptotic Bcl-2 family protein expressed in ovarian cancer suggesting that, amongst Bcl-2 family members, its inhibition is crucial to sensitize ovarian cancer cells to chemotherapy. Correspondingly, both ABT-737 and WEHI-539 (selective Bcl-X_L inhibitor) were synergistic with carboplatin in the studies presented here. In contrast, the low expression of Bcl-2 protein in ovarian cancer predicted that the selective Bcl-2 inhibitors venetoclax would be ineffective, and this was found to be the case. Therefore, inhibiting Bcl-X_L remains the most promising strategy for the use of BH3 mimetics as chemosenitizing agents in ovarian cancer. Unfortunately, inhibition of Bcl-X_L has been associated with thrombocytopenia, and this led to the halt of the clinical development of navitoclax (ABT-263). This questions whether inhibition of the Bcl-2 family of proteins is a viable strategy for the treatment of ovarian cancer unless strategies are developed to minimize the thrombocytopenia.

It remains possible that a small proportion of ovarian cancers which are not driven by Bcl-X_L but rather by Bcl-2 and may benefit from this strategy. Performing proteomic profiling may identify ovarian cancer patients whose tumours possess apoptotic vulnerabilities which could be targeted in combination with anti-cancer agents to induce cell death. Dissecting the balance between the anti-apoptotic and pro-apoptotic protein regulators using BH3 profiling can help in identifying drug response predictors. Measuring the expression of known predictors of drug response (such as high Bim, Bcl-X_L and caspase-3) and resistance (e.g. high Mcl-1, XIAP)

(567), possibly in circulating tumour cells, may help to select those patients that may respond to BH3-mimetics, and those who most likely to benefit from the addition of Mcl-1 inhibitors to the regimen. Mcl-1 is another important anti-apoptotic member of Bcl-2 family proteins, and cases with over-expression of Mcl-1 and lower ratio of Bim–Mcl-1 are associated with resistance to BH3 mimetics that do not inhibit this protein (568). Although ovarian tumours are predominantly addicted to Bcl-X_L, inhibiting the anti-apoptotic function of Mcl-1 may reduce the "anti-apoptotic" capacity of a cells, sensitize tumours to Bcl-X_L targeting strategies and potentially minimize thrombocytopenia. This might also allow BH3 mimetics to be used without the addition of chemotherapy. Although in the work presented in this thesis showed that inhibitors of Bcl-X_L were able to potentiate carboplatin activity, carboplatin can indirectly inhibit Mcl-1 (566,569) in several human ovarian cancer cell lines. It is possible that carboplatin could be replaced by another Mcl-1 inhibitor. This could be medically exploitable, as numerous currently available cyclin-dependent kinase (CDK) inhibitors such as sorafenib and seliciclib can, indirectly, downregulate the levels of Mcl-1 and therefore, can be effectively combined with BH3-mimetics (570–572). Patients could be potentially selected for therapy by testing the efficacy of compounds on cultures of tissues collected from patients during surgery. However, repeated debulking surgery in patients who have developed drug-resistant disease is not common practice in the UK, limiting the supply of appropriate tissue. The identification of new biomarkers, for example in a liquid biopsy, that can predict response to a BH3 mimetic is probably preferable.

The critical role for Bim as a mediator of targeted therapy-induced cell death in different types of cancer (573), makes it important predictor for response to BH3-mimetics. Therefore, it is suggested that pre-treatment detection of the Bim expression in ovarian cancer patients may indicate the likelihood of response to BH3- mimetics as well as other targeted therapies. Additionally, in patients with low Bim levels, it is recommended to use agents that target

epigenetic regulators in an aim to increase Bim expression levels and overcome resistance to apoptosis in these cancers.

Limitations of the study were the absence of a full description of the expression of all Bcl-2 family protein members in the studied cell lines, in addition to the absence of animal models to further confirm the results. Although our group has previously reported synergy due to the combination of ABT-737 and carboplatin in xengraft models of ovarian cancer (294), it is helpful to identify the potency of WEHI-539 or venetoclax in combination with carboplatin in these models. As Bim, Bak, Bax and other proapoptotic proteins are essential for driving of mitochondrial outer membrane permeability, it would be useful to show if high levels of these proteins were responsible for variation in response that have been reported between different cell lines. Additionally, it should be taken into account that there is no *in vivo* cancer model or single cell culture method that definitely predict the efficacy of cytotoxic agents in human clinical trials. Approaches that are used in this study provide the advantage of human cancer-derived cell lines, but cannot mimic the heterogeneity and complexity of the interaction between cancer cells and the co-evolving microenvironment in the real tumour.

The second aim of the work presented here was to develop a new strategy to sensitize ovarian cancer to chemotherapy. The identification of genes that are over-expressed in drug resistant cancers may provide new targets to senstizie cell to chemostherapy. Additionally, It offers further insight into the mechanism of action of chemotherapeutic agents (574). The work presented in chapters 5 and 6 identified BCKDK as a potential new target to sensitize cells to chemotherapy. However, considerable work is required to achieve this and the mechanistic basis behind this synergy warrants further exploration. One possibility is through the mTORC1 pathway which is regulated by BCAA. mTORC1 regulates aurora phosphophorylation (T288) and also that of Polo-like kinase 1 (plk1) (T210). These are essential mitotic regulators. Considering that paclitaxel causing mitotic arrest as a result of its

effect on microtubules, this may potentially explain the mechanism of synergy between BCKDK inhibitors and paclitaxel.

Simplistic colourimetric assays are planned for further future studies to better show the localization of BCKDK following inhibition in addition to further illustrate the mechanism of action of inhibitor. Kinases and phosphatases participate in a variety of cellular processes like metabolism, protein-protein interactions, apoptosis as well as gene transcription and translation, through protein phosphorylation and dephosphorylation (575). Accordingly, several approaches, including different colorimetric assays, are available to detect these enzymes, their functions and activity (576). In general, many analytical techniques have been reported for the quantitative determination of enzymes activity, including ion-sensitive field-effect transistor devices, electrochemistry, colorimetry, Gold nanoparticles (GNP)-aggregation-based assay and fluorometry (576–580). Among these, optical sensing techniques have aroused an interest due to their intrinsic simplicity, accuracy and high sensitivity. Many fluorescent probes, including carbon based-nanomaterials, noble metal nanoclusters, quantum dots and semiconducting polymer dots have been utilized for the detection of enzymatic activity and these may be tried to show the mechanistic effect of BCKDK inhibition and the mechanism of action of the inhibitors (581,582). Gold nanoparticles are proven to provide a promising colorimetric probe since they can detect enzymatic activity by monitoring the color change by naked eye or using UV–vis spectroscopy (583). Although these nanomaterial-based approaches displayed good sensitivity and selectivity, the majority of them show some drawbacks, such as time-consuming and labor-intensive sample pretreatment. Recently, a proposed a method has been reported to quantitatively detect enzymatic activity with the help of UV–vis spectroscopy. This method is highly sensitive with a low detection limit. Also it is inexpensive and fairly simple by excluding the use of complicated nanomaterials. Moreover, it constructs a useful platform for inhibitors screening (584).

Generally, the tumour-bearing condition features extensive BCAA breakdown and altered BCAA availability, which is accompanied by suppression of protein synthesis and activation proteolysis in skeletal muscle and eventually results in skeletal muscle atrophy, erosion of lean body mass and involuntary weight loss. Dietary supplementation with BCAA for cancer patients is suggested on the basis of these symptoms in attempt to satisfy whole body requirements for BCAAs. However, consideration should be made of the demand and supply of BCAA to tumours for its invasive and proliferative activities. In general, most of the current researches focus of supplementation of patients with BCAAs to reverse cachexia, but the need to decide this supplementation should be balanced by the need to avoid providing building blocks for cancer growth. It will be interesting to investigate if manipulating BCAA metabolism, independent of chemotherapy, can affect the balance between the tumour and host in a way that favors the patients. Ovarian cancer may require adequate amounts of BCAA as building materials to generate new cells, and this raises the potential for other enzymes in the BCAA metabolic pathway to be used as drug targets. Currently, there are relatively few medications in clinical trials that particularly target amino acid metabolic pathways in tumour cells (585).

Our work provided valuable insights on the potential impact of metabolic control of BCAA in ovarian cancer and has opened the way to add a novel drug target that could be utilized in clinical trials to specifically target BCAA metabolism in cancer cells. However, the potential use of BCKDK inhibitors as anti-cancer drugs in combination with paclitaxel in ovarian and other cancer patients still needs to be validated in animal studies. In addition, further evidence is required that tumour cells will be sensitized to the combination of BCKDK inhibitors and paclitaxel more than non-tumour tissue.

Cancer genetics, tissue of origin and tumor microenvironment can all affect the tumor's metabolic dependencies (586). Both the genetic nature (intrinsic factors) and nurturing environment (extrinsic factors) define tumors that arise from normal tissues. Additionally, changes in metabolism may contribute to the first differences described between normal and tumour tissues and it is possible that BCKDK may play a role in this. However, the increased expression of BCKDK in drug-resistant versus chemosensitive tumours suggests its predominant role may be later in oncogenesis.

Understanding the precise contributions of metabolic changes of BCAAs in ovarian cancer holds the best hope for identifying patients that may respond to BCKDK inhibitors. It is possible that altering BCAA metabolism may be beneficial in several cancer types, not just ovarian cancer. To do so, identifying biomarkers that predict response to these agents seems to be essential. BCKDH may serve as the most promising biomarker considering the work presented in this thesis that showed better responses to BCKDK inhibitors in cells expressing BCKDH. Likewise, BCAT could also serve as a biomarker to predict patients that may respond to BCKDK inhibitors as it has shown to be over-expressed in many types of cancer. Also, the presence of reasonable level of BCKDP to reactivate BCKDH following the inhibition of BCKDK should be evaluated prior to the use of BCKDK inhibitors. It is reasonable to predict that low levels of BCKDH and/or BCKDP may lead to limited response to these agents. Additionally, it is still plausible that elevated plasma level of BCAAs in ovarian cancer patients could predict response to BCKDK inhibition, an issue that needs to be explored in clinical studies. However, the identification of high level of BCAAs in the plasma of patients with early stage of pancreatic cancer encourage measuring these amino acids in ovarian cancer (587). NSCLC tumors show increased uptake of BCAAs compared with normal lung tissue (534), could also support this hypothesis. The increased requirement for amino acid (particularly BCAA) uptake for cancer development and the reprogramming of

metabolic pathways to supply tumour with BCAA precedes the diagnosis of cancer by upto 5 five years (588). This encourages exploring the level of BCAAs uptake by the ovaries to identify patients that are predicted to respond to a combination of paclitaxel and BCKDK inhibitors.

Increasing evidence shows that leucine and other BCAAs do not only support biosynthetic demands by providing the essential building materials, but also has a nutrient signal regulating effect via the activation of mTOR pathway which can lead to hypertrophy as well as proliferation and migration of cancer cells (589,590). By controlling cell growth, proliferation, translation of protein, and autophagy, this pathway may be as an important regulator of cellular function (591). Furthermore, the upregulation of mTOR signalling pathway in different types of cancer has made it as an important target for the treatment of various types of cancer. Accordingly, it would be interesting to combine the BCKDK inhibitors with mTOR inhibitors as a novel targeted combination therapy in patients with ovarian cancer.

The transition from the traditional model of cytotoxic agents that depends one-size-fits-all strategy to the most recent personalized medicine approach that instead depends on the development of targeted therapy, requires understanding the particular genetic addictions of a tumour to predict drug efficacy and hopefully overcome the resistance to chemotherapy. Research on innovative trial designs is in progress to optimize the clinical usefulness of the targeted agents and hopefully in the next few years BH3-mimetics and BCKDK inhibitors may be exploited clinically. However, although targeted therapeutics produced clinically significant responses, this can also be followed by the development of drug resistance, which is likely to occur with BH3-mimetics and BCKDK inhibitors as well. One way to avoid this is to use combinations of these two targeted therapies. It is interesting to speculate on the potential for combining BCKDK inhibitors with Bcl-2 inhibitors considering the previous

successful combination of BH3 mimetics and kinase inhibitors (592,593). The finding that Mcl-1 can be inhibited by existing BCKDK benzothiophene carboxylate inhibitors, raises a possible rationale for this combination which may be useful in patients that are resistant to navitoclax due to high expression of Mcl-1 (548,549). Furthermore, the level of anti-apoptotic Bcl-2 family protein could be explored following treatment with BCKDK inhibitors to check if these agents have the potential to block these protein to supplement the effect to the BH3-mimetics. Also adding BCKDK inhibitors to the combination of carboplatin, Bcl-2 inhibitors and paclitaxel could have several potential advantages. The platelet sparing activity of paclitaxel may make the use of carboplatin and navitoclax feasible and the inclusion of a BCKDK inhibitor may potentiate the efficacy of paclitaxel. However, the use of several drugs raises concerns about potential toxicity and proper manipulation of doses is likely to be needed. Additionally, identifying ovarian cancer patients who might respond to such a combination is essential.

References

1. Toss A, De Matteis E, Rossi E, Casa L, Iannone A, Federico M, et al. Ovarian Cancer: Can Proteomics Give New Insights for Therapy and Diagnosis? Int J Mol Sci [Internet]. 2013 Apr 15;14(4):8271–90. Available from: http://www.mdpi.com/1422-0067/14/4/8271/

2. Chien J, Kuang R, Landen C, Shridhar V. Platinum-Sensitive Recurrence in Ovarian Cancer: The Role of Tumor Microenvironment. Front Oncol [Internet]. 2013;3(September):1–6. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2013.00251/abstract

3. Rooth C. Ovarian cancer: risk factors, treatment and management. Br J Nurs [Internet]. 2013 Sep 26;22(Sup17):S23–30. Available from: http://linkinghub.elsevier.com/retrieve/pii/B9781437703726000190

4. Krupa M, Byun K. Leptomeningeal carcinomatosis and bilateral internal auditory canal metastases from ovarian carcinoma. Radiol Case Reports [Internet]. 2017 Jun;12(2):386–90. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1930043316303569

5. Cozzi GD, Levinson RT, Toole H, Snyder M, Deng A, Crispens MA, et al. Blood type, ABO genetic variants, and ovarian cancer survival. Prokunina-Olsson L, editor. PLoS One [Internet]. 2017 Apr 27;12(4):e0175119. Available from: https://doi.org/10.1371/journal.pone.0175119 April

6. Seibaek L, Petersen LK, Blaakaer J, Hounsgaard L. Symptom interpretation and health care seeking in ovarian cancer. BMC Womens Health [Internet]. 2011 Dec 23;11(1):31. Available from: http://www.biomedcentral.com/1472-6874/11/31

7. Birmann BM, Barnard ME, Bertrand KA, Bao Y, Crous-Bou M, Wolpin BM, et al. Nurses' Health Study Contributions on the Epidemiology of Less Common Cancers: Endometrial, Ovarian, Pancreatic, and Hematologic. Am J Public Health [Internet]. 2016 Sep;106(9):1608–15. Available from: http://ajph.aphapublications.org/doi/10.2105/AJPH.2016.303337

8. Oseledchyk A, Zivanovic O. Intraoperative Hyperthermic Intraperitoneal Chemotherapy in Patients With Advanced Ovarian Cancer. Oncology (Williston Park) [Internet]. 2015 Sep;29(9):695–701. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26384807

9. Brett M. R, Jennifer B. P, Thomas A. S, Brett M. R, Jennifer B. P, Thomas A. S. Epidemiology of ovarian cancer: a review. Cancer Biol Med [Internet]. 2017;14(1):9–32. Available from: http://www.cancerbiomed.org/index.php/cocr/article/view/1004

10. Lukanova A, Kaaks R. Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol biomarkers Prev [Internet]. 2005 Jan;14(1):98–107. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15668482

11. Foroughi F, Lam AK-Y, Lim MS., Saremi N, Ahmadvand A. "Googling" for Cancer: An Infodemiological Assessment of Online Search Interests in Australia, Canada, New Zealand, the United Kingdom, and the United States. JMIR Cancer [Internet]. 2016 May 4;2(1):e5. Available from: http://cancer.jmir.org/2016/1/e5/

12. Lambert P, Galloway K, Altman A, Nachtigal MW, Turner D. Ovarian cancer in

Manitoba : trends in incidence and survival , 1992 – 2011. 2017;24(2):78–84. Available from: https://www.ncbi.nlm.nih.gov/pubmed/28490929

13. Khan MMH, Khan A, Nojima M, Suzuki S, Fujino Y, Tokudome S, et al. Ovarian cancer mortality among women aged 40-79 years in relation to reproductive factors and body mass index: latest evidence from the Japan Collaborative Cohort study. J Gynecol Oncol [Internet]. 2013;24(3):249. Available from: https://synapse.koreamed.org/DOIx.php?id=10.3802/jgo.2013.24.3.249

14. Baldwin L, Chen Q, Tucker T, White C, Ore R, Huang B. Ovarian Cancer Incidence Corrected for Oophorectomy. Diagnostics [Internet]. 2017 Apr 1;7(2):19. Available from: http://www.mdpi.com/2075-4418/7/2/19

15. National Cancer Intelligence Network. Overview of Ovarian Cancer in England : Incidence , Mortality and Survival. 2012;(November):2–59. Available from: http://www.ncin.org.uk/cancer_type_and_topic_specific_work/cancer_type_specific_work/gynaecological_cancer/gynaecological_cancer_hub/resources/ovarian_cancer

16. Kroeger PT, Drapkin R. Pathogenesis and heterogeneity of ovarian cancer. Curr Opin Obstet Gynecol [Internet]. 2017 Feb;29(1):26–34. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00001703-201702000-00006

17. Lu Y, Cuellar-Partida G, Painter JN, Nyholt DR, Morris AP, Fasching PA, et al. Shared genetics underlying epidemiological association between endometriosis and ovarian cancer. Hum Mol Genet [Internet]. 2015 Oct 15;24(20):5955–64. Available from: https://academic.oup.com/hmg/article-lookup/doi/10.1093/hmg/ddv306

18. McLemore MR, Miaskowski C, Aouizerat BE, Chen L, Dodd MJ. Epidemiological and Genetic Factors Associated With Ovarian Cancer. Cancer Nurs [Internet]. 2009 Jul;32(4):281–8. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00002820-200907000-00005

19. Tomao F, Lo Russo G, Spinelli G, Stati V, Prete A, Prinzi N, et al. Fertility drugs, reproductive strategies and ovarian cancer risk. J Ovarian Res [Internet]. 2014;7(1):51. Available from: http://ovarianresearch.biomedcentral.com/articles/10.1186/1757-2215-7-51

20. Pearce CL, Chung K, Pike MC, Wu AH. Increased ovarian cancer risk associated with menopausal estrogen therapy is reduced by adding a progestin. Cancer [Internet]. 2009 Feb 1;115(3):531–9. Available from: http://doi.wiley.com/10.1002/cncr.23956

21. Gayther SA, Pharoah PD. The inherited genetics of ovarian and endometrial cancer. Curr Opin Genet Dev [Internet]. 2010 Jun;20(3):231–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0959437X10000341

22. Pruthi S, Gostout BS, Lindor NM. Identification and Management of Women With BRCA Mutations or Hereditary Predisposition for Breast and Ovarian Cancer. Mayo Clin Proc [Internet]. 2010 Dec;85(12):1111–20. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0025619611603650

23. Sowter HM. BRCA1 and BRCA2 as ovarian cancer susceptibility genes.

Carcinogenesis [Internet]. 2005 May 19;26(10):1651–6. Available from: https://academic.oup.com/carcin/article-lookup/doi/10.1093/carcin/bgi136

24. Kauff ND, Mitra N, Robson ME, Hurley KE, Chuai S, Goldfrank D, et al. Risk of Ovarian Cancer in BRCA1 and BRCA2 Mutation-Negative Hereditary Breast Cancer Families. JNCI J Natl Cancer Inst [Internet]. 2005 Sep 21;97(18):1382–4. Available from: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/dji281

25. Olaitan A, Doufekas K. Clinical epidemiology of epithelial ovarian cancer in the UK. Int J Womens Health [Internet]. 2014 May;6(1):537. Available from: http://www.dovepress.com/clinical-epidemiology-of-epithelial-ovarian-cancer-in-the-uk-peer-reviewed-article-IJWH

26. Fathalla MF. INCESSANT OVULATION—A FACTOR IN OVARIAN NEOPLASIA ? Lancet [Internet]. 1971 Jul;298(7716):163. Available from: http://linkinghub.elsevier.com/retrieve/pii/S014067367192335X

27. Fathalla MF. Incessant ovulation and ovarian cancer - a hypothesis re-visited. Facts, views Vis ObGyn [Internet]. 2013;5(4):292–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24753957%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3987381

28. Murdoch WJ, Townsend RS, McDonnel AC. Ovulation-Induced DNA Damage in Ovarian Surface Epithelial Cells of Ewes: Prospective Regulatory Mechanisms of Repair/Survival and Apoptosis. Biol Reprod. 2001;65(5):389–94.

29. Mok SC, Kwong J, Welch WR, Samimi G, Ozbun L, Bonome T, et al. Etiology and Pathogenesis of Epithelial Ovarian Cancer. Dis Markers [Internet]. 2007;23(5–6):367–76. Available from: http://www.hindawi.com/journals/dm/2007/474320/abs/

30. Choi J-H, Wong AST, Huang H-F, Leung PCK. Gonadotropins and Ovarian Cancer. Endocr Rev [Internet]. 2007 Jun;28(4):440–61. Available from: https://academic.oup.com/edrv/article-lookup/doi/10.1210/er.2006-0036

31. Rampersad AC, Wang Y, Smith ER, Xu X. Menopause and ovarian cancer risk : mechanisms and experimental support. 2015;2(1):14–23.

32. Smith ER, Xu X-X. Ovarian ageing, follicle depletion, and cancer: a hypothesis for the aetiology of epithelial ovarian cancer involving follicle depletion. Lancet Oncol [Internet]. 2008 Nov;9(11):1108–11. Available from: http://linkinghub.elsevier.com/retrieve/pii/S147020450870281X

33. Hannon PR, Flaws JA. The Effects of Phthalates on the Ovary. Front Endocrinol (Lausanne) [Internet]. 2015 Feb 2;6(FEB):1–19. Available from: http://journal.frontiersin.org/Article/10.3389/fendo.2015.00008/abstract

34. Han KH, Kim M-K, Kim HS, Chung HH, Song YS. Protective Effect of Progesterone during Pregnancy against Ovarian Cancer. J Cancer Prev [Internet]. 2013 Jun 30;18(2):113–22. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4189458/

35. Hillier SG. Nonovarian origins of ovarian cancer. Proc Natl Acad Sci [Internet]. 2012 Mar 6;109(10):3608–9. Available from: http://www.pnas.org/content/109/10/3608.full

36. Saad AF, Hu W, Sood AK. Microenvironment and Pathogenesis of Epithelial Ovarian Cancer. Horm Cancer [Internet]. 2010 Dec 30;1(6):277–90. Available from: http://link.springer.com/10.1007/s12672-010-0054-2

37. Clendenen T V., Lundin E, Zeleniuch-Jacquotte A, Koenig KL, Berrino F, Lukanova A, et al. Circulating Inflammation Markers and Risk of Epithelial Ovarian Cancer. Cancer Epidemiol Biomarkers Prev [Internet]. 2011 May 1;20(5):799–810. Available from: http://cebp.aacrjournals.org/cgi/doi/10.1158/1055-9965.EPI-10-1180

38. George SHL, Shaw P. BRCA and Early Events in the Development of Serous Ovarian Cancer. Front Oncol [Internet]. 2014;4(January):1–8. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2014.00005/abstract

39. Kumar N, Drabu S, Mondal SC. NSAID's and selectively COX-2 inhibitors as potential chemoprotective agents against cancer. Arab J Chem [Internet]. 2013 Jan;6(1):1–23. Available from: http://dx.doi.org/10.1016/j.arabjc.2011.07.020

40. Bowtell DD, Böhm S, Ahmed AA, Aspuria P-J, Bast RC, Beral V, et al. Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer. Nat Rev Cancer [Internet]. 2015 Oct 23;15(11):668–79. Available from: http://www.nature.com/doifinder/10.1038/nrc4019

41. Diniz PM, Carvalho JP, Baracat EC, Carvalho FM. Fallopian tube origin of supposed ovarian high-grade serous carcinomas. Clinics [Internet]. 2011;66(1):73–6. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1807-59322011000100013&lng=en&nrm=iso&tlng=en

42. Reade CJ, McVey RM, Tone AA, Finlayson SJ, McAlpine JN, Fung-Kee-Fung M, et al. The Fallopian Tube as the Origin of High Grade Serous Ovarian Cancer: Review of a Paradigm Shift. J Obstet Gynaecol Canada [Internet]. 2014 Feb;36(2):133–40. Available from: http://dx.doi.org/10.1016/S1701-2163(15)30659-9

43. Kim J, Coffey DM, Creighton CJ, Yu Z, Hawkins SM, Matzuk MM. High-grade serous ovarian cancer arises from fallopian tube in a mouse model. Proc Natl Acad Sci [Internet]. 2012 Mar 6;109(10):3921–6. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1117135109

44. Corzo C, Iniesta MD, Patrono MG, Lu KH, Ramirez PT. Role of Fallopian Tubes in the Development of Ovarian Cancer. J Minim Invasive Gynecol [Internet]. 2017 Feb;24(2):230–4. Available from: http://dx.doi.org/10.1016/j.jmig.2016.12.007

45. Cho KR, Shih I-M. Ovarian Cancer. Annu Rev Pathol Mech Dis [Internet]. 2009 Feb;4(1):287–313. Available from: http://www.annualreviews.org/doi/10.1146/annurev.pathol.4.110807.092246

46. Koshiyama M, Matsumura N, Konishi I. Recent Concepts of Ovarian Carcinogenesis: Type I and Type II. Biomed Res Int [Internet]. 2014;2014:1–11. Available from: http://www.hindawi.com/journals/bmri/2014/934261/

47. Low JJH, Ilancheran A, Ng JS. Malignant ovarian germ-cell tumours. Best Pract Res Clin Obstet Gynaecol [Internet]. 2012 Jun;26(3):347–55. Available from: http://dx.doi.org/10.1016/j.bpobgyn.2012.01.002

48. Ledermann JA, Luvero D, Shafer A, O'Connor D, Mangili G, Friedlander M, et al. Gynecologic Cancer InterGroup (GCIG) Consensus Review for Mucinous Ovarian Carcinoma. Int J Gynecol Cancer [Internet]. 2014 Nov;24(November):S14–9. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00009577-201411003-00005

49. Vargas AN. Natural history of ovarian cancer. Ecancermedicalscience [Internet]. 2014;8:465. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4176445&tool=pmcentrez&rendertype=abstract

50. LaRock CN, Nizet V. Cationic antimicrobial peptide resistance mechanisms of streptococcal pathogens. Biochim Biophys Acta - Biomembr [Internet]. 2015 Nov;1848(11):3047–54. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0005273615000504

51. Rathore R, Arora D, Agarwal S, Sharma S. Correlation of foxl2 with inhibin and calretinin in the diagnosis of ovarian sex cord stromal tumors. Turkish J Pathol [Internet]. 2016;121–8. Available from: http://www.turkjpath.org/doi.php?doi=10.5146/tjpath.2016.01382

52. Horta M, Cunha TM. Sex cord-stromal tumors of the ovary: a comprehensive review and update for radiologists. Diagnostic Interv Radiol [Internet]. 2015 Jul 3;21(4):277–86. Available from: http://www.dirjournal.org/sayilar/77/buyuk/277-286.pdf

53. Kurman RJ, Shih I-M. The Origin and Pathogenesis of Epithelial Ovarian Cancer: A Proposed Unifying Theory. Am J Surg Pathol [Internet]. 2010 Mar;34(3):433–43. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2841791/

54. Rosen DG, Yang G, Liu G, Mercado-Uribe I, Chang B, Xiao XS, et al. Ovarian cancer: pathology, biology, and disease models. Front Biosci [Internet]. 2009 Jan 1;14:2089–102. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2858969&tool=pmcentrez&rendertype=abstract

55. Imai A. Transitional cell carcinoma of the ovary (Review). Oncol Lett [Internet]. 2011 Oct 21;3(1):3–6. Available from: http://www.spandidos-publications.com/10.3892/ol.2011.453

56. Karst AM, Drapkin R. Ovarian Cancer Pathogenesis: A Model in Evolution. J Oncol [Internet]. 2010;2010:1–13. Available from: http://www.hindawi.com/journals/jo/2010/932371/

57. George SHL, Garcia R, Slomovitz BM. Ovarian Cancer: The Fallopian Tube as the Site of Origin and Opportunities for Prevention. Front Oncol [Internet]. 2016 May 2;6(May). Available from: http://journal.frontiersin.org/Article/10.3389/fonc.2016.00108/abstract

58. Tone AA, Salvador S, Finlayson SJ, Tinker A V., Kwon JS, Lee C-H, et al. The role of the fallopian tube in ovarian cancer. Clin Adv Hematol Oncol [Internet]. 2012 May;10(5):296–306. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22706539

59. Erickson BK, Conner MG, Landen CN. The role of the fallopian tube in the origin of ovarian cancer. Am J Obstet Gynecol [Internet]. 2013 Nov;209(5):409–14. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0002937813003827

60. Herzog TJ, Dinkelspiel HE. Fallopian tube removal: "STIC-ing" it to ovarian cancer: what is the utility of prophylactic tubal removal? Curr Oncol [Internet]. 2013 Jun 5;20(3):148. Available from: http://www.current-oncology.com/index.php/oncology/article/view/1548

61. Grant DJ, Moorman PG, Akushevich L, Palmieri RT, Bentley RC, Schildkraut JM. Primary peritoneal and ovarian cancers: an epidemiological comparative analysis. Cancer Causes Control [Internet]. 2010 Jul 23;21(7):991–8. Available from: http://link.springer.com/10.1007/s10552-010-9525-6

62. Keng SL, Wahab SBA, Chiu LB, Yusuf A. Awareness of Ovarian Cancer Risk Factors among Women in Malaysia: A Preliminary Study. Asian Pacific J Cancer Prev [Internet]. 2015 Feb 25;16(2):537–40. Available from: http://koreascience.or.kr/journal/view.jsp?kj=POCPA9&py=2015&vnc=v16n2&sp=537

63. Goff BA. Frequency of Symptoms of Ovarian Cancer in Women Presenting to Primary Care Clinics. JAMA [Internet]. 2004 Jun 9;291(22):2705. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.291.22.2705

64. Bankhead CR, Collins C, Stokes-Lampard H, Rose P, Wilson S, Clements A, et al. Identifying symptoms of ovarian cancer: A qualitative and quantitative study. BJOG An Int J Obstet Gynaecol. 2008;115(8):1008–14.

65. Hamilton W, Peters TJ, Bankhead C, Sharp D. Risk of ovarian cancer in women with symptoms in primary care: population based case-control study. BMJ [Internet]. 2009 Aug 25;339(aug25 2):b2998–b2998. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19706933

66. Ahmed N, Stenvers KL. Getting to Know Ovarian Cancer Ascites: Opportunities for Targeted Therapy-Based Translational Research. Front Oncol [Internet]. 2013;3(September):1–12. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2013.00256/abstract

67. Brasseur K, Gévry N, Asselin E. Chemoresistance and targeted therapies in ovarian and endometrial cancers. Oncotarget [Internet]. 2017 Jan 16;8(3):4008–42. Available from: http://www.oncotarget.com/abstract/14021

68. Jelovac D, Armstrong DK. Recent progress in the diagnosis and treatment of ovarian cancer. CA Cancer J Clin [Internet]. 2011 May;61(3):183–203. Available from: http://onlinelibrary.wiley.com/doi/10.3322/caac.20113/full%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3576854&tool=pmcentrez&rendertype=abstract

69. Kim HS, Song YS. International Federation of Gynecology and Obstetrics (FIGO) staging system revised: what should be considered critically for gynecologic cancer? J Gynecol Oncol [Internet]. 2009;20(3):135. Available from: https://synapse.koreamed.org/DOIx.php?id=10.3802/jgo.2009.20.3.135

70. Rauh-Hain J a, Krivak TC, Del Carmen MG, Olawaiye AB. Ovarian cancer screening

and early detection in the general population. Rev Obstet Gynecol [Internet]. 2011;4(1):15–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21629494

71. Twickler DM, Moschos E. Ultrasound and Assessment of Ovarian Cancer Risk. Am J Roentgenol [Internet]. 2010 Feb;194(2):322–9. Available from: http://www.ajronline.org/doi/10.2214/AJR.09.3562

72. Pepin K, Carmen M del, Brown A, Dizon DS. CA 125 and Epithelial Ovarian Cancer: Role in Screening, Diagnosis, and Surveillance. Am J Hematol / Oncol [Internet]. 2014;10(6):22–9. Available from: http://www.gotoper.com/publications/ajho/2014/2014dec/ca-125-and-epithelial-ovarian-cancer-role-in-screening-diagnosis-and-surveillance

73. Pignata S, Cannella L, Leopardo D, Bruni GS, Facchini G, Pisano C. Follow-up with CA125 after primary therapy of advanced ovarian cancer: in favor of continuing to prescribe CA125 during follow-up. Ann Oncol [Internet]. 2011 Dec 1;22(Suppl 8):viii40-viii44. Available from: https://academic.oup.com/annonc/article-lookup/doi/10.1093/annonc/mdr470

74. Mapelli P, Incerti E, Fallanca F, Gianolli L, Picchio M. Imaging biomarkers in ovarian cancer: the role of ^{18}F-FDG PET/CT. Q J Nucl Med Mol imaging [Internet]. 2016 Jun;60(2):93–102. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26859083

75. Nakamura K, Sawada K, Yoshimura A, Kinose Y, Nakatsuka E, Kimura T. Clinical relevance of circulating cell-free microRNAs in ovarian cancer. Mol Cancer [Internet]. 2016 Dec 24;15(1):48. Available from: http://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-016-0536-0

76. Soung Y, Ford S, Zhang V, Chung J. Exosomes in Cancer Diagnostics. Cancers (Basel) [Internet]. 2017 Jan 12;9(1):8. Available from: http://www.mdpi.com/2072-6694/9/1/8

77. Hiss D. Optimizing Molecular-Targeted Therapies in Ovarian Cancer: The Renewed Surge of Interest in Ovarian Cancer Biomarkers and Cell Signaling Pathways. J Oncol [Internet]. 2012;2012:1–23. Available from: http://www.hindawi.com/journals/jo/2012/737981/

78. Hope JM, Pothuri B. The Role of Palliative Surgery in Gynecologic Cancer Cases. Oncologist [Internet]. 2013 Jan 1;18(1):73–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3556259&tool=pmcentrez&rendertype=abstract

79. Deng J, Wang L, Chen H, Hao J, Ni J, Chang L, et al. Targeting epithelial-mesenchymal transition and cancer stem cells for chemoresistant ovarian cancer. Oncotarget [Internet]. 2016 Aug 22;7(34):55771–88. Available from: http://www.oncotarget.com/abstract/9908

80. Schorge JO, McCann C, Del Carmen MG. Surgical debulking of ovarian cancer: what difference does it make? Rev Obstet Gynecol [Internet]. 2010;3(3):111–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21364862

81. Erekson EA, Martin DK, Ratner ES. Oophorectomy. Menopause J North Am Menopause Soc [Internet]. 2013 Jan;20(1):110–4. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3514564&tool=pmcentrez

&rendertype=abstract

82. Oliver KE, McGuire WP. Ovarian cancer and antiangiogenic therapy: caveat emptor. J Clin Oncol [Internet]. 2014;32(30):3353–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25225422

83. Trimbos B, Timmers P, Pecorelli S, Coens C, Ven K, van der Burg M, et al. Surgical Staging and Treatment of Early Ovarian Cancer: Long-term Analysis From a Randomized Trial. JNCI J Natl Cancer Inst [Internet]. 2010 Jul 7;102(13):982–7. Available from: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/djq149

84. Luvero D, Milani A, Ledermann JA. Treatment options in recurrent ovarian cancer: latest evidence and clinical potential. Ther Adv Med Oncol [Internet]. 2014 Sep;6(5):229–39. Available from: http://journals.sagepub.com/doi/10.1177/1758834014544121

85. Galanis E, Atherton PJ, Maurer MJ, Knutson KL, Dowdy SC, Cliby WA, et al. Oncolytic Measles Virus Expressing the Sodium Iodide Symporter to Treat Drug-Resistant Ovarian Cancer. Cancer Res [Internet]. 2015 Jan 1;75(1):22–30. Available from: http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-14-2533

86. Amptoulach S, Tsavaris N. Neurotoxicity Caused by the Treatment with Platinum Analogues. Chemother Res Pract [Internet]. 2011;2011:1–5. Available from: http://www.hindawi.com/journals/cherp/2011/843019/

87. Dasari S, Bernard Tchounwou P. Cisplatin in cancer therapy: Molecular mechanisms of action. Eur J Pharmacol [Internet]. 2014 Oct;740:364–78. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0014299914005627

88. McWhinney SR, Goldberg RM, McLeod HL. Platinum neurotoxicity pharmacogenetics. Mol Cancer Ther [Internet]. 2009 Jan 1;8(1):10–6. Available from: http://mct.aacrjournals.org/cgi/doi/10.1158/1535-7163.MCT-08-0840

89. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer [Internet]. 2007 Aug 12;7(8):573–84. Available from: http://www.nature.com/doifinder/10.1038/nrc2167

90. Staudacher L, Cottu PH, Dieras V, Vincent-Salomon A, Guilhaume MN, Escalup L, et al. Platinum-based chemotherapy in metastatic triple-negative breast cancer: the Institut Curie experience. Ann Oncol [Internet]. 2011 Apr 1;22(4):848–56. Available from: https://academic.oup.com/annonc/article-lookup/doi/10.1093/annonc/mdq461

91. Johnstone TC, Park GY, Lippard SJ. Understanding and improving platinum anticancer drugs - Phenanthriplatin. Anticancer Res. 2014;34(1):471–6.

92. Eckstein N. Platinum resistance in breast and ovarian cancer cell lines. J Exp Clin Cancer Res [Internet]. 2011;30(1):91. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3197542&tool=pmcentrez&rendertype=abstract

93. Horio M, Kato T, Mii S, Enomoto A, Asai M, Asai N, et al. Expression of RET finger protein predicts chemoresistance in epithelial ovarian cancer. Cancer Med [Internet]. 2012 Oct;1(2):218–29. Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3544444&tool=pmcentrez&rendertype=abstract

94. Stornetta A, Zimmermann M, Cimino GD, Henderson PT, Sturla SJ. DNA Adducts from Anticancer Drugs as Candidate Predictive Markers for Precision Medicine. Chem Res Toxicol [Internet]. 2017 Jan 17;30(1):388–409. Available from: http://pubs.acs.org/doi/10.1021/acs.chemrestox.6b00380

95. Sousa GF de, Wlodarczyk SR, Monteiro G. Carboplatin: molecular mechanisms of action associated with chemoresistance. Brazilian J Pharm Sci [Internet]. 2014 Dec;50(4):693–701. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1984-82502014000400693&lng=en&tlng=en

96. Cazzaniga ME, Mustacchi G, Pronzato P, De Matteis A, Di Costanzo F, Floriani I. Adjuvant treatment of early breast cancer: do the St Gallen recommendations influence clinical practice? Results from the NORA study. Ann Oncol [Internet]. 2007 Dec 1;18(12):1976–80. Available from: https://academic.oup.com/annonc/article-lookup/doi/10.1093/annonc/mdm365

97. Fukada I, Araki K, Kobayashi K, Kobayashi T, Horii R, Akiyama F, et al. Therapeutic effect of taxanes on metastatic breast cancer of various immunohistochemical subtypes. Oncol Lett [Internet]. 2016 May 25;12(1):663–9. Available from: http://www.spandidos-publications.com/10.3892/ol.2016.4627

98. Crown J. Docetaxel and Paclitaxel in the Treatment of Breast Cancer: A Review of Clinical Experience. Oncologist [Internet]. 2004 Jun 2;9(suppl_2):24–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15161988

99. Tanaka Y, Yoshida K, Tanahashi T, Okumura N, Matsuhashi N, Yamaguchi K. Phase II trial of neoadjuvant chemotherapy with docetaxel, nedaplatin, and S1 for advanced esophageal squamous cell carcinoma. Cancer Sci [Internet]. 2016 Jun;107(6):764–72. Available from: http://doi.wiley.com/10.1111/cas.12943

100. Mang J, Merkle K, Heller M, Schüler J, Tolstov Y, Li J, et al. Molecular complexity of taxane-induced cytotoxicity in prostate cancer cells. Urol Oncol Semin Orig Investig [Internet]. 2017 Jan;35(1):32.e9-32.e16. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1078143916302034

101. Kellogg EH, Hejab NMA, Howes S, Northcote P, Miller JH, Díaz JF, et al. Insights into the Distinct Mechanisms of Action of Taxane and Non-Taxane Microtubule Stabilizers from Cryo-EM Structures. J Mol Biol [Internet]. 2017 Mar;429(5):633–46. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022283617300153

102. Fauzee NJS. Taxanes: promising anti-cancer drugs. Asian Pac J Cancer Prev [Internet]. 2011;12(4):837–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21790213

103. Cimino GD, Pan C, Henderson PT. Personalized medicine for targeted and platinum-based chemotherapy of lung and bladder cancer. Bioanalysis [Internet]. 2013 Feb;5(3):369–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23394702%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3644565%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3644565&tool=pmcentrez&rendertype=abstract

104. Wang X, Lin Y. Tumor necrosis factor and cancer, buddies or foes? Acta Pharmacol Sin [Internet]. 2008 Nov;29(11):1275–88. Available from: http://www.nature.com/doifinder/10.1111/j.1745-7254.2008.00889.x

105. Sprowl JA, Reed K, Armstrong SR, Lanner C, Guo B, Kalatskaya I, et al. Alterations in tumor necrosis factor signaling pathways are associated with cytotoxicity and resistance to taxanes: a study in isogenic resistant tumor cells. Breast Cancer Res [Internet]. 2012 Feb 6;14(1):R2. Available from: http://breast-cancer-research.biomedcentral.com/articles/10.1186/bcr3083

106. Bocci G, Di Paolo A, Danesi R. The pharmacological bases of the antiangiogenic activity of paclitaxel. Angiogenesis [Internet]. 2013 Jul 7;16(3):481–92. Available from: http://link.springer.com/10.1007/s10456-013-9334-0

107. Sarafraz M, Ahmadi K. Paraclinical evaluation of side-effects of Taxanes on auditory system. Acta Otorhinolaryngol Ital [Internet]. 2008 Oct;28(5):239–42. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2689535&tool=pmcentrez&rendertype=abstract

108. Donati A, Castro LGM. Cutaneous adverse reactions to chemotherapy with taxanes . The dermatologist â€™ s point of view. An Bras Dermatol [Internet]. 2011;86(4):755–8. Available from: http://www.scielo.br/pdf/abd/v86n4/en_v86n4a20.pdf

109. Cella D, Peterman A, Hudgens S, Webster K, Socinski MA. Measuring the side effects of taxane therapy in oncology. Cancer [Internet]. 2003 Aug 15;98(4):822–31. Available from: http://doi.wiley.com/10.1002/cncr.11578

110. Minisini AM. Taxane-induced nail changes: incidence, clinical presentation and outcome. Ann Oncol [Internet]. 2003 Feb 1;14(2):333–7. Available from: https://academic.oup.com/annonc/article-lookup/doi/10.1093/annonc/mdg050

111. Foo J, Michor F. Evolution of acquired resistance to anti-cancer therapy. J Theor Biol [Internet]. 2014 Aug;355(10):10–20. Available from: http://pubs.acs.org/doi/abs/10.1021/nl061786n

112. Zeller C, Brown R. Therapeutic modulation of epigenetic drivers of drug resistance in ovarian cancer. Ther Adv Med Oncol [Internet]. 2010 Sep 1;2(5):319–29. Available from: http://tam.sagepub.com/cgi/doi/10.1177/1758834010375759

113. Büsselberg D, Florea A-M. Targeting Intracellular Calcium Signaling ([Ca2+]i) to Overcome Acquired Multidrug Resistance of Cancer Cells: A Mini-Overview. Cancers (Basel) [Internet]. 2017 May 9;9(5):48. Available from: http://www.mdpi.com/2072-6694/9/5/48

114. Alfarouk KO, Stock C-M, Taylor S, Walsh M, Muddathir AK, Verduzco D, et al. Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp. Cancer Cell Int [Internet]. 2015 Dec 15;15(1):71. Available from: http://www.cancerci.com/content/15/1/71

115. Stewart DJ. Tumor and host factors that may limit efficacy of chemotherapy in non-small cell and small cell lung cancer. Crit Rev Oncol Hematol [Internet]. 2010 Sep;75(3):173–234. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2888634/

116. El-Awady R, Saleh E, Hashim A, Soliman N, Dallah A, Elrasheed A, et al. The Role of Eukaryotic and Prokaryotic ABC Transporter Family in Failure of Chemotherapy. Front Pharmacol [Internet]. 2017 Jan 10;7(JAN):1–15. Available from: http://journal.frontiersin.org/article/10.3389/fphar.2016.00535/full

117. Abouzeid AH, Patel NR, Torchilin VP. Polyethylene glycol-phosphatidylethanolamine (PEG-PE)/vitamin E micelles for co-delivery of paclitaxel and curcumin to overcome multi-drug resistance in ovarian cancer. Int J Pharm [Internet]. 2014 Apr 10;464(1–2):178–84. Available from: http://www.annualreviews.org/doi/10.1146/annurev-cellbio-092910-154240

118. Chang A. Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. Lung Cancer [Internet]. 2011 Jan;71(1):3–10. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0169500210004307

119. Abdullah LN, Chow EK-H. Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med [Internet]. 2013;2(1):3. Available from: http://clintransmed.springeropen.com/articles/10.1186/2001-1326-2-3

120. Eid SY, El-Readi MZ, Fatani SH, Mohamed Nour Eldin EE, Wink M. Natural Products Modulate the Multifactorial Multidrug Resistance of Cancer. Pharmacol & Pharm [Internet]. 2015;6(3):146–76. Available from: http://www.scirp.org/journal/PaperDownload.aspx?DOI=10.4236/pp.2015.63017

121. Pang J, Shi Q, Liu Z, He J, Liu H, Lin P, et al. Resistin induces multidrug resistance in myeloma by inhibiting cell death and upregulating ABC transporter expression. Haematologica [Internet]. 2017 Mar 30;haematol.2016.154062. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28360146%0Ahttp://www.haematologica.org/lookup/doi/10.3324/haematol.2016.154062

122. Noack A, Noack S, Hoffmann A, Maalouf K, Buettner M, Couraud P-O, et al. Drug-Induced Trafficking of P-Glycoprotein in Human Brain Capillary Endothelial Cells as Demonstrated by Exposure to Mitomycin C. Szakacs G, editor. PLoS One [Internet]. 2014 Feb 4;9(2):e88154. Available from: http://dx.plos.org/10.1371/journal.pone.0088154

123. Gameiro M, Silva R, Rocha-Pereira C, Carmo H, Carvalho F, Bastos M, et al. Cellular Models and In Vitro Assays for the Screening of modulators of P-gp, MRP1 and BCRP. Molecules [Internet]. 2017 Apr 8;22(4):600. Available from: http://www.mdpi.com/1420-3049/22/4/600

124. Zeller C, Dai W, Steele NL, Siddiq A, Walley AJ, Wilhelm-Benartzi CSM, et al. Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer identified by methylome and expression profiling. Oncogene [Internet]. 2012 Oct 18;31(42):4567–76. Available from: http://www.nature.com/doifinder/10.1038/onc.2011.611

125. Han X, Wang J, Sun Y. Circulating Tumor DNA as Biomarkers for Cancer Detection. Genomics Proteomics Bioinformatics [Internet]. 2017 Apr;15(2):59–72. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1672022917300487

126. Qin Z, Ljubimov VA, Zhou C, Tong Y, Liang J. Cell-free circulating tumor DNA in cancer. Chin J Cancer [Internet]. 2016 Dec 7;35(1):36. Available from:

http://cjcjournal.biomedcentral.com/articles/10.1186/s40880-016-0092-4

127. YIN F, LIU X, LI D, WANG Q, ZHANG W, LI L. Tumor suppressor genes associated with drug resistance in ovarian cancer (Review). Oncol Rep [Internet]. 2013 Jul 9;30(1):3–10. Available from: http://www.spandidos-publications.com/10.3892/or.2013.2446

128. Sun W, Yang J. Functional Mechanisms for Human Tumor Suppressors. J Cancer [Internet]. 2010;1(1):136–40. Available from: http://www.jcancer.org/v01p0136.htm

129. Lee EYHP, Muller WJ. Oncogenes and Tumor Suppressor Genes. Cold Spring Harb Perspect Biol [Internet]. 2010 Oct 1;2(10):a003236–a003236. Available from: http://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a003236

130. Velez AA, Howard M. Tumor-suppressor genes, cell cycle regulatory checkpoints, and the skin. N Am J Med Sci [Internet]. 2015;7(5):176. Available from: http://www.najms.org/text.asp?2015/7/5/176/157476

131. Davidson B, Tropé CG, Reich R. Epithelial–Mesenchymal Transition in Ovarian Carcinoma. Front Oncol [Internet]. 2012;2(April):33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22655269%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3356037

132. Fang D, Chen H, Zhu JY, Wang W, Teng Y, Ding H-F, et al. Epithelial–mesenchymal transition of ovarian cancer cells is sustained by Rac1 through simultaneous activation of MEK1/2 and Src signaling pathways. Oncogene [Internet]. 2017 Mar 16;36(11):1546–58. Available from: http://www.annualreviews.org/doi/10.1146/annurev-immunol-032713-120240

133. Haslehurst AM, Koti M, Dharsee M, Nuin P, Evans K, Geraci J, et al. EMT transcription factors snail and slug directly contribute to cisplatin resistance in ovarian cancer. BMC Cancer [Internet]. 2012 Dec 19;12(1):91. Available from: http://bmccancer.biomedcentral.com/articles/10.1186/1471-2407-12-91

134. Gavande NS, VanderVere-Carozza PS, Hinshaw HD, Jalal SI, Sears CR, Pawelczak KS, et al. DNA repair targeted therapy: The past or future of cancer treatment? Pharmacol Ther [Internet]. 2016 Apr;160:65–83. Available from: http://dx.doi.org/10.1016/j.pharmthera.2016.02.003

135. Iyama T, Wilson DM. DNA repair mechanisms in dividing and non-dividing cells. DNA Repair (Amst) [Internet]. 2013 Aug;12(8):620–36. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1568786413000967

136. Davis JD. DNA damage and breast cancer. World J Clin Oncol [Internet]. 2011;2(9):329. Available from: http://www.wjgnet.com/2218-4333/full/v2/i9/329.htm

137. Hosoya N, Miyagawa K. Targeting DNA damage response in cancer therapy. Cancer Sci [Internet]. 2014 Apr;105(4):370–88. Available from: http://doi.wiley.com/10.1111/cas.12366

138. Velic D, Couturier A, Ferreira M, Rodrigue A, Poirier G, Fleury F, et al. DNA Damage Signalling and Repair Inhibitors: The Long-Sought-After Achilles' Heel of Cancer. Biomolecules [Internet]. 2015 Nov 20;5(4):3204–59. Available from:

http://www.mdpi.com/2218-273X/5/4/3204

139. Jasin M, Rothstein R. Repair of Strand Breaks by Homologous Recombination. Cold Spring Harb Perspect Biol [Internet]. 2013 Nov 1;5(11):a012740–a012740. Available from: http://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a012740

140. Chen Y, Liu H, Zhang H, Sun C, Hu Z, Tian Q, et al. And-1 coordinates with CtIP for efficient homologous recombination and DNA damage checkpoint maintenance. Nucleic Acids Res [Internet]. 2017 Mar 17;45(5):2516–30. Available from: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gkw1212

141. von Nicolai C, Ehlén Å, Martin C, Zhang X, Carreira A. A second DNA binding site in human BRCA2 promotes homologous recombination. Nat Commun [Internet]. 2016 Sep 15;7:12813. Available from: http://www.nature.com/doifinder/10.1038/ncomms12813

142. Jensen RB, Carreira A, Kowalczykowski SC. Purified human BRCA2 stimulates RAD51-mediated recombination. Nature [Internet]. 2010 Oct 7;467(7316):678–83. Available from: http://www.nature.com/articles/nature09399

143. Stolz A, Ertych N, Bastians H. Loss of the tumour-suppressor genes CHK2 and BRCA1 results in chromosomal instability. Biochem Soc Trans [Internet]. 2010 Dec 1;38(6):1704–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21118151

144. Li Y, Luo K, Yin Y, Wu C, Deng M, Li L, et al. USP13 regulates the RAP80-BRCA1 complex dependent DNA damage response. Nat Commun [Internet]. 2017 Jun 1;8:15752. Available from: http://dx.doi.org/10.1038/ncomms15752

145. Basourakos SP, Li L, Aparicio AM, Corn PG, Kim J, Thompson TC. Combination Platinum-based and DNA Damage Response-targeting Cancer Therapy: Evolution and Future Directions. Curr Med Chem [Internet]. 2017 Jun 30;24(15):84–91. Available from: http://www.nature.com/articles/nature13478

146. Legerski RJ. Repair of DNA interstrand cross-links during S phase of the mammalian cell cycle. Environ Mol Mutagen [Internet]. 2010 Oct 10;145(2):NA-NA. Available from: http://link.springer.com/10.1007/s10955-011-0269-9

147. Clauson C, Scharer OD, Niedernhofer L. Advances in Understanding the Complex Mechanisms of DNA Interstrand Cross-Link Repair. Cold Spring Harb Perspect Biol [Internet]. 2013 Oct 1;5(10):a012732–a012732. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24086043%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4123742

148. Deans AJ, West SC. DNA interstrand crosslink repair and cancer. Nat Rev Cancer [Internet]. 2011 Jul 1;11(7):467–80. Available from: http://www.nature.com/articles/nrc3088

149. Liu G, Yang D, Sun Y, Shmulevich I, Xue F, Sood AK, et al. Differing clinical impact of BRCA1 and BRCA2 mutations in serous ovarian cancer. Pharmacogenomics [Internet]. 2012 Oct;13(13):1523–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23057551%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3603383

150. Norquist B, Wurz KA, Pennil CC, Garcia R, Gross J, Sakai W, et al. Secondary Somatic Mutations Restoring BRCA1/2 Predict Chemotherapy Resistance in Hereditary Ovarian Carcinomas. J Clin Oncol [Internet]. 2011 Aug;29(22):3008–15. Available from: http://ascopubs.org/doi/10.1200/JCO.2010.34.2980

151. Dziadkowiec KN, Gąsiorowska E, Nowak-Markwitz E, Jankowska A. PARP inhibitors: review of mechanisms of action and BRCA1/2 mutation targeting. Menopausal Rev [Internet]. 2016;4(4):215–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28250726%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5327624

152. Konecny GE, Kristeleit RS. PARP inhibitors for BRCA1/2-mutated and sporadic ovarian cancer: current practice and future directions. Br J Cancer [Internet]. 2016 Nov 13;115(10):1157–73. Available from: http://dx.doi.org/10.1038/bjc.2016.311

153. Helleday T. The underlying mechanism for the PARP and BRCA synthetic lethality: Clearing up the misunderstandings. Mol Oncol [Internet]. 2011 Aug;5(4):387–93. Available from: http://dx.doi.org/10.1016/j.molonc.2011.07.001

154. Ba X, Garg NJ. Signaling Mechanism of Poly(ADP-Ribose) Polymerase-1 (PARP-1) in Inflammatory Diseases. Am J Pathol [Internet]. 2011 Mar;178(3):946–55. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0002944010002142

155. Neff RT, Senter L, Salani R. BRCA mutation in ovarian cancer: testing, implications and treatment considerations. Ther Adv Med Oncol [Internet]. 2017 Aug 19;9(8):519–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28086035

156. Kim Y, Kim A, Sharip A, Sharip A, Jiang J, Yang Q, et al. Reverse the Resistance to PARP Inhibitors. Int J Biol Sci [Internet]. 2017;13(2):198–208. Available from: http://www.ijbs.com/v13p0198.htm

157. Broustas CG, Lieberman HB. DNA Damage Response Genes and the Development of Cancer Metastasis. Radiat Res [Internet]. 2014 Feb;181(2):111–30. Available from: http://www.bioone.org/doi/10.1667/RR13515.1

158. Dedes KJ, Wilkerson PM, Wetterskog D, Weigelt B, Ashworth A, Reis-Filho JS. Synthetic lethality of PARP inhibition in cancers lacking BRCA1 and BRCA2 mutations. Cell Cycle [Internet]. 2011 Apr 15;10(8):1192–9. Available from: http://www.tandfonline.com/doi/abs/10.4161/cc.10.8.15273

159. Shapiro AM, Miller-Pinsler L, Wells PG. Breast cancer 1 (BRCA1)-deficient embryos develop normally but are more susceptible to ethanol-initiated DNA damage and embryopathies. Redox Biol [Internet]. 2016 Apr;7:30–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2213231715300069

160. Bunting SF, Callén E, Wong N, Chen H, Polato F, Gunn A, et al. 53BP1 Inhibits Homologous Recombination in Brca1-Deficient Cells by Blocking Resection of DNA Breaks. Cell [Internet]. 2010 Apr;141(2):243–54. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0092867410002850

161. Aly A, Ganesan S. BRCA1, PARP, and 53BP1: Conditional synthetic lethality and synthetic viability. J Mol Cell Biol. 2011;3(1):66–74.

162. Chen J-Q, Russo J. ERα-negative and triple negative breast cancer: Molecular features and potential therapeutic approaches. Biochim Biophys Acta - Rev Cancer [Internet]. 2009 Dec 10;1796(2):162–75. Available from: http://www.ajnr.org/cgi/doi/10.3174/ajnr.A1256

163. Liu J, Cristea MC, Frankel P, Neuhausen SL, Steele L, Engelstaedter V, et al. Clinical characteristics and outcomes of BRCA-associated ovarian cancer: genotype and survival. Cancer Genet [Internet]. 2012 Jan;205(1–2):34–41. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2210776212000373

164. Dalton HJ, Coleman RL. New Biologic Frontiers in Ovarian Cancer: Olaparib and PARP Inhibition. Am J Hematol / Oncol [Internet]. 2016;12(11):14–22. Available from: http://www.gotoper.com/publications/ajho/2015/2015may/new-biologic-frontiers-in-ovarian-cancer-olaparib-and-parp-inhibition

165. Mouw KW, Goldberg MS, Konstantinopoulos PA, D'Andrea AD. DNA Damage and Repair Biomarkers of Immunotherapy Response. Cancer Discov [Internet]. 2017 Jul;7(7):675–93. Available from: http://cancerdiscovery.aacrjournals.org/lookup/doi/10.1158/2159-8290.CD-17-0226

166. Johnson SF, Cruz C, Greifenberg AK, Dust S, Stover DG, Chi D, et al. CDK12 Inhibition Reverses De Novo and Acquired PARP Inhibitor Resistance in BRCA Wild-Type and Mutated Models of Triple-Negative Breast Cancer. Cell Rep [Internet]. 2016 Nov;17(9):2367–81. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2211124716315121

167. Johnson N, Johnson SF, Yao W, Li Y-C, Choi Y-E, Bernhardy AJ, et al. Stabilization of mutant BRCA1 protein confers PARP inhibitor and platinum resistance. Proc Natl Acad Sci [Internet]. 2013 Oct 15;110(42):17041–6. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1305170110

168. Gu Y, Bouwman P, Greco D, Saarela J, Yadav B, Jonkers J, et al. Suppression of BRCA1 sensitizes cells to proteasome inhibitors. Cell Death Dis [Internet]. 2014 Dec 18;5(12):e1580–e1580. Available from: http://www.nature.com/articles/cddis2014537

169. Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer [Internet]. 2003 Jul;3(7):502–16. Available from: http://www.nature.com/doifinder/10.1038/nrc1123

170. Wright AA, Cronin A, Milne DE, Bookman MA, Burger RA, Cohn DE, et al. Use and Effectiveness of Intraperitoneal Chemotherapy for Treatment of Ovarian Cancer. J Clin Oncol [Internet]. 2015 Sep 10;33(26):2841–7. Available from: http://ascopubs.org/doi/10.1200/JCO.2015.61.4776

171. Markman M. Intraperitoneal chemotherapy in the management of ovarian cancer: focus on carboplatin. Ther Clin Risk Manag [Internet]. 2009 Feb;5(1):161. Available from: http://www.dovepress.com/intraperitoneal-chemotherapy-in-the-management-of-ovarian-cancer-focus-peer-reviewed-article-TCRM

172. Konner JA, Grabon DM, Gerst SR, Iasonos A, Thaler H, Pezzulli SD, et al. Phase II Study of Intraperitoneal Paclitaxel Plus Cisplatin and Intravenous Paclitaxel Plus Bevacizumab As Adjuvant Treatment of Optimal Stage II/III Epithelial Ovarian Cancer. J Clin Oncol [Internet]. 2011 Dec 10;29(35):4662–8. Available from:

http://ascopubs.org/doi/10.1200/JCO.2011.36.1352

173. Armstrong DK, Bundy B, Wenzel L, Huang HQ, Baergen R, Lele S, et al. Intraperitoneal Cisplatin and Paclitaxel in Ovarian Cancer. N Engl J Med [Internet]. 2006 Jan 5;354(1):34–43. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa052985

174. Mackay HJ, Provencheur D, Heywood M, Tu D, Eisenhauer E a, Oza a M, et al. CURRENT IP STUDY IN OVARIAN CANCER Phase ii / iii study of intraperitoneal chemotherapy after neoadjuvant chemotherapy for ovarian cancer : Cancer Res. 2011;18(2):84–90.

175. Sun V, Otis-Green S, Morgan R, Wakabayashi M, Hakim A, Callado ME, et al. Toxicities, complications, and clinical encounters during intraperitoneal chemotherapy in 17 women with ovarian cancer. Eur J Oncol Nurs [Internet]. 2013 Jun;17(3):375–80. Available from: http://dx.doi.org/10.1016/j.ejon.2012.10.005

176. Rowan K. Intraperitoneal Therapy for Ovarian Cancer: Why Has It Not Become Standard? JNCI J Natl Cancer Inst [Internet]. 2009 Jun 2;101(11):775–7. Available from: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/djp151

177. Azzi S, Hebda JK, Gavard J. Vascular Permeability and Drug Delivery in Cancers. Front Oncol [Internet]. 2013;3(August):1–14. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2013.00211/abstract

178. Ma P. Paclitaxel Nano-Delivery Systems: A Comprehensive Review. J Nanomed Nanotechnol [Internet]. 2013;4(2):1–35. Available from: https://www.omicsonline.org/paclitaxel-nano-delivery-systems-a-comprehensive-review-2157-7439.1000164.php?aid=11467

179. Deshpande PP, Biswas S, Torchilin VP. Current trends in the use of liposomes for tumor targeting. Nanomedicine [Internet]. 2013 Sep;8(9):1509–28. Available from: http://www.futuremedicine.com/doi/10.2217/nnm.13.118

180. Mokhtari RB, Homayouni TS, Baluch N, Morgatskaya E, Kumar S, Das B, et al. Combination therapy in combating cancer. Oncotarget [Internet]. 2015 Jul 18;8(23):38022–43. Available from: http://www.oncotarget.com/abstract/16723

181. Meng J, Guo F, Xu H, Liang W, Wang C, Yang X-D. Combination Therapy using Co-encapsulated Resveratrol and Paclitaxel in Liposomes for Drug Resistance Reversal in Breast Cancer Cells in vivo. Sci Rep [Internet]. 2016 Apr 7;6(1):22390. Available from: http://www.nature.com/articles/srep22390

182. Staropoli N, Ciliberto D, Botta C, Fiorillo L, Grimaldi A, Lama S, et al. Pegylated liposomal doxorubicin in the management of ovarian cancer. Cancer Biol Ther [Internet]. 2014 Jun 21;15(6):707–20. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4049787&tool=pmcentrez&rendertype=abstract

183. Akin J, Waddell J, Solimando, Jr D. Paclitaxel and Carboplatin (TC) Regimen for Ovarian Cancer. Hosp Pharm [Internet]. 2014 May;49(5):425–31. Available from: http://archive.hospital-pharmacy.com/doi/abs/10.1310/hpj4905-425

184. Decaudin D, Le Tourneau C. Combinations of targeted therapies in human cancers. Aging (Albany NY) [Internet]. 2016 Oct 26;8(10):2258–9. Available from: www.thelancet.com/oncology

185. Yan L, Rosen N, Arteaga C. Targeted cancer therapies. Chin J Cancer [Internet]. 2011 Jan 5;30(1):1–4. Available from: http://www.cjcsysu.cn/abstract.asp?idno=17537

186. Yap TA, Omlin A, de Bono JS. Development of Therapeutic Combinations Targeting Major Cancer Signaling Pathways. J Clin Oncol [Internet]. 2013 Apr 20;31(12):1592–605. Available from: http://ascopubs.org/doi/10.1200/JCO.2011.37.6418

187. Nikanjam M, Liu S, Kurzrock R. Dosing targeted and cytotoxic two-drug combinations: Lessons learned from analysis of 24,326 patients reported 2010 through 2013. Int J Cancer [Internet]. 2016 Nov 1;139(9):2135–41. Available from: http://doi.wiley.com/10.1002/ijc.30262

188. Fotopoulou C. Limitations to the use of carboplatin-based therapy in advanced ovarian cancer. Eur J Cancer Suppl [Internet]. 2014 Dec;12(2):13–6. Available from: http://dx.doi.org/10.1016/S1359-6349(15)70005-4

189. Høberg-Vetti H, Bjorvatn C, Fiane BE, Aas T, Woie K, Espelid H, et al. BRCA1/2 testing in newly diagnosed breast and ovarian cancer patients without prior genetic counselling: the DNA-BONus study. Eur J Hum Genet [Internet]. 2016 Jun 9;24(6):881–8. Available from: http://www.nature.com/articles/ejhg2015196

190. Karakasis K, Burnier J V., Bowering V, Oza AM, Lheureux S. Ovarian Cancer and BRCA1/2 Testing: Opportunities to Improve Clinical Care and Disease Prevention. Front Oncol [Internet]. 2016 May 11;6(May):1–10. Available from: http://journal.frontiersin.org/Article/10.3389/fonc.2016.00119/abstract

191. Burrell RA, Swanton C. Tumour heterogeneity and the evolution of polyclonal drug resistance. Mol Oncol [Internet]. 2014 Sep;8(6):1095–111. Available from: http://dx.doi.org/10.1016/j.molonc.2014.06.005

192. Diaz-Cano SJ. Tumor Heterogeneity: Mechanisms and Bases for a Reliable Application of Molecular Marker Design. Int J Mol Sci [Internet]. 2012 Feb 13;13(12):1951–2011. Available from: http://www.mdpi.com/1422-0067/13/2/1951

193. Hu Z, Sun R, Curtis C. A population genetics perspective on the determinants of intra-tumor heterogeneity. Biochim Biophys Acta - Rev Cancer [Internet]. 2017 Apr;1867(2):109–26. Available from: http://dx.doi.org/10.1016/j.bbcan.2017.03.001

194. Tellez-Gabriel M, Ory B, Lamoureux F, Heymann M-F, Heymann D. Tumour Heterogeneity: The Key Advantages of Single-Cell Analysis. Int J Mol Sci [Internet]. 2016 Dec 20;17(12):2142. Available from: http://www.mdpi.com/1422-0067/17/12/2142

195. Begicevic R-R, Falasca M. ABC Transporters in Cancer Stem Cells: Beyond Chemoresistance. Int J Mol Sci [Internet]. 2017 Nov 8;18(11):2362. Available from: http://www.mdpi.com/1422-0067/18/11/2362

196. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. Nature [Internet]. 2013 Sep 19;501(7467):328–37. Available from:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4521623/

197. Easwaran H, Tsai H-C, Baylin SB. Cancer Epigenetics: Tumor Heterogeneity, Plasticity of Stem-like States, and Drug Resistance. Mol Cell [Internet]. 2014 Jun;54(5):716–27. Available from: http://pubs.acs.org/doi/abs/10.1021/nl061786n

198. Magee JA, Piskounova E, Morrison SJ. Cancer Stem Cells: Impact, Heterogeneity, and Uncertainty. Cancer Cell [Internet]. 2012 Mar;21(3):283–96. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1535610812000864

199. Lee J-Y, Yoon J-K, Kim B, Kim S, Kim MA, Lim H, et al. Tumor evolution and intratumor heterogeneity of an epithelial ovarian cancer investigated using next-generation sequencing. BMC Cancer [Internet]. 2015 Dec 26;15(1):85. Available from: http://bmccancer.biomedcentral.com/articles/10.1186/s12885-015-1077-4

200. Hansen E, Woods RJ, Read AF. How to Use a Chemotherapeutic Agent When Resistance to It Threatens the Patient. Schneider D, editor. PLOS Biol [Internet]. 2017 Feb 9;15(2):e2001110. Available from: http://dx.plos.org/10.1371/journal.pbio.2001110

201. Pritchard JR, Lauffenburger DA, Hemann MT. Understanding resistance to combination chemotherapy. Drug Resist Updat [Internet]. 2012 Oct;15(5–6):249–57. Available from: http://linkinghub.elsevier.com/retrieve/pii/S136876461200057X

202. De Souza R, Zahedi P, Badame RM, Allen C, Piquette-Miller M. Chemotherapy Dosing Schedule Influences Drug Resistance Development in Ovarian Cancer. Mol Cancer Ther [Internet]. 2011 Jul 1;10(7):1289–99. Available from: http://mct.aacrjournals.org/cgi/doi/10.1158/1535-7163.MCT-11-0058

203. Bonilla L, Ben-Aharon I, Vidal L, Gafter-Gvili A, Leibovici L, Stemmer SM. Dose-Dense Chemotherapy in Nonmetastatic Breast Cancer: A Systematic Review and Meta-analysis of Randomized Controlled Trials. JNCI J Natl Cancer Inst [Internet]. 2010 Dec 15;102(24):1845–54. Available from: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/djq409

204. Chan T, Ho, Turcan. Epigenetic therapy: use of agents targeting deacetylation and methylation in cancer management. Onco Targets Ther [Internet]. 2013 Mar;6(March 2013):223. Available from: http://www.dovepress.com/epigenetic-therapy-use-of-agents-targeting-deacetylation-and-methylati-peer-reviewed-article-OTT

205. Kelly TK, De Carvalho DD, Jones P a. Epigenetic modifications as therapeutic targets. Nat Biotechnol [Internet]. 2010 Oct 13;28(10):1069–78. Available from: http://www.nature.com/doifinder/10.1038/nbt.1678

206. Popovic R, Shah MY, Licht JD. Epigenetic therapy of hematological malignancies: where are we now? Ther Adv Hematol [Internet]. 2013 Apr 21;4(2):81–91. Available from: http://journals.sagepub.com/doi/10.1177/2040620712466864

207. Rius M, Lyko F. Epigenetic cancer therapy: rationales, targets and drugs. Oncogene [Internet]. 2012 Sep 27;31(39):4257–65. Available from: http://dx.doi.org/10.1038/onc.2011.601

208. Valdespino-Gomez VM, Valdespino PM. Potential of epigenetic therapies in the

management of solid tumors. Cancer Manag Res [Internet]. 2015 Jul;7:241. Available from: http://www.dovepress.com/potential-of-epigenetic-therapies-in-the-management-of-solid-tumors-peer-reviewed-article-CMAR

209. Stordal B, Timms K, Farrelly A, Gallagher D, Busschots S, Renaud M, et al. BRCA1/2 mutation analysis in 41 ovarian cell lines reveals only one functionally deleterious BRCA1 mutation. Mol Oncol [Internet]. 2013 Jun;7(3):567–79. Available from: http://doi.wiley.com/10.1016/j.molonc.2012.12.007

210. Hamilt T, Young R, Ozols R. Experimental model systems of ovarian cancer: applications to the design and evaluation of new treatment approaches. Semin Oncol [Internet]. 1984;11(3):295–8. Available from: https://www.ncbi.nlm.nih.gov/pubmed/6385258

211. Bénard J, Silva J Da, Blois M De, Bã J, Boyer P, Duvillard P, et al. Characterization of a Human Ovarian Adenocarcinoma Line , IGROV1 , in Tissue Culture and in Nude Mice Characterization of a Human Ovarian Adenocarcinoma Line , IGROV1 , in Tissue Culture and in Nude Mice1. Cancer R. 1985;45(October):4970–9.

212. Hamilton TC, Young RC, Mckoy WM, Grotzinger KR, Green JA, Chu EW, et al. Characterization of a Human Ovarian Carcinoma Cell Line (NIH : OVCAR-3) 1 with Androgen and Estrogen Receptors. 1983;(NOVEMBER):5379–89.

213. Yanagibashi T, Gorai I, Nakazawa T, Miyagi E, Hirahara F, Kitamura H, et al. Complexity of expression of the intermediate filaments of six new human ovarian carcinoma cell lines: new expression of cytokeratin 20. Br J Cancer. 1997;76(7):829–35.

214. van den Berg-Bakker CAM, Hagemeijer A, Franken-Postma EM, Smit VTHBM, Kuppen PJK, Claasen HHVR, et al. Establishment and characterization of 7 ovarian carcinoma cell lines and one granulosa tumor cell line: Growth features and cytogenetics. Int J Cancer [Internet]. 1993 Feb 20;53(4):613–20. Available from: http://doi.wiley.com/10.1002/ijc.2910530415

215. Zhang J-H. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen [Internet]. 1999 Apr 1;4(2):67–73. Available from: http://jbx.sagepub.com/cgi/doi/10.1177/108705719900400206%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/10838414%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/10838414%5Cnhttp://jbx.sagepub.com/content/4/2/67.full.pdf

216. Chou T-C. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. Cancer Res [Internet]. 2010 Jan 15;70(2):440–6. Available from: http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-09-1947

217. CHOU T-C, TALALAY P. Generalized Equations for the Analysis of Inhibitions of Michaelis-Menten and Higher-Order Kinetic Systems with Two or More Mutually Exclusive and Nonexclusive Inhibitors. Eur J Biochem [Internet]. 2005 Mar 3;115(1):207–16. Available from: http://doi.wiley.com/10.1111/j.1432-1033.1981.tb06218.x

218. Al-Jassim ZG, Al-Abbassi MG, Yaseen NY. The Cytotoxic Effect of 2-Deoxy-D-Glucose Combination with 5- Fluorourasil and NO-Aspirin on Mammary

Adenocacinoma Cell Line. J Nat Sci Res www. 2014;4(19):2225–921.

219. Tran S-L, Puhar A, Ngo-Camus M, Ramarao N. Trypan Blue Dye Enters Viable Cells Incubated with the Pore-Forming Toxin HlyII of Bacillus cereus. Gorvel J-P, editor. PLoS One [Internet]. 2011 Sep 6;6(9):e22876. Available from: http://dx.plos.org/10.1371/journal.pone.0022876

220. Yeh PJ, Hegreness MJ, Aiden AP, Kishony R. Drug interactions and the evolution of antibiotic resistance. Nat Rev Microbiol [Internet]. 2009 Jun;7(6):460–6. Available from: http://apps.webofknowledge.com.globalproxy.cvt.dk/full_record.do?product=UA&search_mode=GeneralSearch&qid=3&SID=X1qEsyvCLMyKoKq5bMM&page=1&doc=5

221. Plati J, Bucur O, Khosravi-Far R. Apoptotic cell signaling in cancer progression and therapy. Integr Biol [Internet]. 2011;3(4):279. Available from: http://xlink.rsc.org/?DOI=c0ib00144a

222. Elkholi R, Floros K V., Chipuk JE. The Role of BH3-Only Proteins in Tumor Cell Development, Signaling, and Treatment. Genes Cancer [Internet]. 2011 May 1;2(5):523–37. Available from: http://gan.sagepub.com/lookup/doi/10.1177/1947601911417177

223. Finlay D, Teriete P, Vamos M, Cosford NDP, Vuori K. Inducing death in tumor cells: roles of the inhibitor of apoptosis proteins. F1000Research [Internet]. 2017;6:587. Available from: https://f1000research.com/articles/6-587/v1

224. Wong RS. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res [Internet]. 2011;30(1):87. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3197541&tool=pmcentrez&rendertype=abstract

225. Kasibhatla S, Tseng B. Why Target Apoptosis in Cancer Treatment ? Minireview Why Target Apoptosis in Cancer Treatment ? Mol Cancer Ther. 2003;2:573–80.

226. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer [Internet]. 1972 Aug;26(4):239–57. Available from: http://doi.wiley.com/10.1111/j.1365-2796.2005.01570.x

227. Bottone M, Santin G, Aredia F, Bernocchi G, Pellicciari C, Scovassi A. Morphological Features of Organelles during Apoptosis: An Overview. Cells [Internet]. 2013 May 8;2(2):294–305. Available from: http://www.mdpi.com/2073-4409/2/2/294/

228. Mariño G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol [Internet]. 2014 Jan 8;15(2):81–94. Available from: http://www.nature.com/doifinder/10.1038/nrm3735

229. Rock KL, Kono H. The Inflammatory Response to Cell Death. Annu Rev Pathol Mech Dis [Internet]. 2008 Feb;3(1):99–126. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3094097&tool=pmcentrez&rendertype=abstract

230. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol [Internet]. 2007;35(4):495–516. Available from:

http://journals.sagepub.com/doi/10.1080/01926230701320337

231. Gross A. BCL-2 family proteins as regulators of mitochondria metabolism. Biochim Biophys Acta - Bioenerg [Internet]. 2016 Aug;1857(8):1243–6. Available from: http://dx.doi.org/10.1016/j.bbabio.2016.01.017

232. Van Cruchten S, Van den Broeck W. Morphological and Biochemical Aspects of Apoptosis, Oncosis and Necrosis. Anat Histol Embryol J Vet Med Ser C [Internet]. 2002 Aug;31(4):214–23. Available from: http://doi.wiley.com/10.1046/j.1439-0264.2002.00398.x

233. Ouyang L, Shi Z, Zhao S, Wang F-T, Zhou T-T, Liu B, et al. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif [Internet]. 2012 Dec;45(6):487–98. Available from: http://doi.wiley.com/10.1111/j.1365-2184.2012.00845.x

234. Koff J, Ramachandiran S, Bernal-Mizrachi L. A Time to Kill: Targeting Apoptosis in Cancer. Int J Mol Sci [Internet]. 2015 Jan 28;16(2):2942–55. Available from: http://www.mdpi.com/1422-0067/16/2/2942/

235. Cuda CM, Pope RM, Perlman H. The inflammatory role of phagocyte apoptotic pathways in rheumatic diseases. Nat Rev Rheumatol [Internet]. 2016 Aug 23;12(9):543–58. Available from: http://www.nature.com/doifinder/10.1038/nrrheum.2016.132

236. Tomita T. Apoptosis of pancreatic β-cells in Type 1 diabetes. Bosn J Basic Med Sci [Internet]. 2017 Apr 3;(January):1–11. Available from: http://www.bjbms.org/ojs/index.php/bjbms/article/view/1961

237. Pileczki V, Cojocneanu-Petric R, Maralani M, Berindan Neagoe I, Sandulescu R. MicroRNAs as regulators of apoptosis mechanisms in cancer. Clujul Med [Internet]. 2016 Feb 2;89(1):50. Available from: http://www.clujulmedical.umfcluj.ro/index.php/cjmed/article/view/512

238. Lawton JS. A fate worse than death. J Thorac Cardiovasc Surg [Internet]. 2016 Jul;152(1):97–8. Available from: http://dx.doi.org/10.1038/nrc.2016.58

239. You Y, Cheng A-C, Wang M-S, Jia R-Y, Sun K-F, Yang Q, et al. The suppression of apoptosis by α-herpesvirus. Cell Death Dis [Internet]. 2017 Apr 13;8(4):e2749. Available from: http://www.nature.com/doifinder/10.1038/cddis.2017.139

240. Bratton SB, Salvesen GS. Regulation of the Apaf-1-caspase-9 apoptosome. J Cell Sci [Internet]. 2010 Oct 1;123(19):3209–14. Available from: http://jcs.biologists.org/cgi/doi/10.1242/jcs.073643

241. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol [Internet]. 2013 Dec 20;15(1):49–63. Available from: http://www.nature.com/doifinder/10.1038/nrm3722

242. Ryder C, McColl K, Zhong F, Distelhorst CW. Acidosis Promotes Bcl-2 Family-mediated Evasion of Apoptosis: INVOLVEMENT OF ACID-SENSING G PROTEIN-COUPLED RECEPTOR GPR65 SIGNALING TO MEK/ERK. J Biol Chem [Internet].

2012 Aug 10;287(33):27863–75. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M112.384685

243. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 Family Reunion. Mol Cell [Internet]. 2010 Feb;37(3):299–310. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1097276510000791

244. Shamas-Din A, Kale J, Leber B, Andrews DW. Mechanisms of Action of Bcl-2 Family Proteins. Cold Spring Harb Perspect Biol [Internet]. 2013 Apr 1;5(4):a008714–a008714. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23545417%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3683897%0Ahttp://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a008714

245. Delbridge ARD, Strasser A. The BCL-2 protein family, BH3-mimetics and cancer therapy. Cell Death Differ [Internet]. 2015 Jul 8;22(7):1071–80. Available from: http://www.nature.com/doifinder/10.1038/cdd.2015.50

246. Lopez J, Tait SWG. Mitochondrial apoptosis: killing cancer using the enemy within. Br J Cancer [Internet]. 2015 Mar 5;112(6):957–62. Available from: http://www.nature.com/doifinder/10.1038/bjc.2015.85

247. Tahir SK, Smith ML, Hessler P, Rapp LR, Idler KB, Park CH, et al. Potential mechanisms of resistance to venetoclax and strategies to circumvent it. BMC Cancer [Internet]. 2017 Dec 2;17(1):399. Available from: http://bmccancer.biomedcentral.com/articles/10.1186/s12885-017-3383-5%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/28570130%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/28578655%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5457565

248. Pécot J, Maillet L, Le Pen J, Vuillier C, Trécesson S de C, Fétiveau A, et al. Tight Sequestration of BH3 Proteins by BCL-xL at Subcellular Membranes Contributes to Apoptotic Resistance. Cell Rep [Internet]. 2016 Dec;17(12):3347–58. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2211124716316382

249. Llambi F, Wang Y-M, Victor B, Yang M, Schneider DM, Gingras S, et al. BOK Is a Non-canonical BCL-2 Family Effector of Apoptosis Regulated by ER-Associated Degradation. Cell [Internet]. 2016 Apr;165(2):421–33. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0092867416301283

250. Indran IR, Tufo G, Pervaiz S, Brenner C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochim Biophys Acta - Bioenerg [Internet]. 2011 Jun;1807(6):735–45. Available from: http://dx.doi.org/10.1016/j.bbabio.2011.03.010

251. Lomonosova E, Chinnadurai G. BH3-only proteins in apoptosis and beyond: an overview. Oncogene [Internet]. 2008 Dec;27(Suppl 1):S2–19. Available from: http://www.nature.com/doifinder/10.1038/onc.2009.39

252. Tzifi F, Economopoulou C, Gourgiotis D, Ardavanis A, Papageorgiou S, Scorilas A. The Role of BCL2 Family of Apoptosis Regulator Proteins in Acute and Chronic Leukemias. Adv Hematol [Internet]. 2012;2012:1–15. Available from: http://www.hindawi.com/journals/ah/2012/524308/

253. Dai H, Meng W, Kaufmann S. BCL2 Family, Mitochondrial Apoptosis, and Beyond. Cancer Transl Med [Internet]. 2016;2(1):7. Available from: http://www.cancertm.com/text.asp?2016/2/1/7/177558

254. Hardwick JM, Soane L. Multiple Functions of BCL-2 Family Proteins. Cold Spring Harb Perspect Biol [Internet]. 2013 Feb 1;5(2):a008722–a008722. Available from: http://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a008722

255. Kuwana T, Newmeyer DD. Bcl-2-family proteins and the role of mitochondria in apoptosis. Curr Opin Cell Biol [Internet]. 2003 Dec;15(6):691–9. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0955067403001315

256. Greenberg EF, Lavik AR, Distelhorst CW. Bcl-2 regulation of the inositol 1,4,5-trisphosphate receptor and calcium signaling in normal and malignant lymphocytes: Potential new target for cancer treatment. Biochim Biophys Acta - Mol Cell Res [Internet]. 2014 Oct;1843(10):2205–10. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0167488914000925

257. Choudhary GS, Al-harbi S, Mazumder S, Hill BT, Smith MR, Bodo J, et al. MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies. Cell Death Dis [Internet]. 2015 Jan 15;6(1):e1593. Available from: http://www.nature.com/doifinder/10.1038/cddis.2014.525

258. Juin P, Geneste O, Gautier F, Depil S, Campone M. Decoding and unlocking the BCL-2 dependency of cancer cells. Nat Rev Cancer [Internet]. 2013 Jun 20;13(7):455–65. Available from: http://www.nature.com/doifinder/10.1038/nrc3538

259. Akl H, Vervloessem T, Kiviluoto S, Bittremieux M, Parys JB, De Smedt H, et al. A dual role for the anti-apoptotic Bcl-2 protein in cancer: Mitochondria versus endoplasmic reticulum. Biochim Biophys Acta - Mol Cell Res [Internet]. 2014 Oct;1843(10):2240–52. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0167488914001359

260. Liu Z, Ding Y, Ye N, Wild C, Chen H, Zhou J. Direct Activation of Bax Protein for Cancer Therapy. Med Res Rev [Internet]. 2016 Mar;36(2):313–41. Available from: http://doi.wiley.com/10.1002/nbm.3369

261. Vervliet T, Parys JB, Bultynck G. Bcl-2 proteins and calcium signaling: complexity beneath the surface. Oncogene [Internet]. 2016 Sep 29;35(39):5079–92. Available from: http://www.nature.com/doifinder/10.1038/onc.2016.31

262. George NM, Targy N, Evans JJD, Zhang L, Luo X. Bax Contains Two Functional Mitochondrial Targeting Sequences and Translocates to Mitochondria in a Conformational Change- and Homo-oligomerization-driven Process. J Biol Chem [Internet]. 2010 Jan 8;285(2):1384–92. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M109.049924

263. Anilkumar U, Prehn JHM. Anti-apoptotic BCL-2 family proteins in acute neural injury. Front Cell Neurosci [Internet]. 2014 Sep 30;8(September):1–6. Available from: http://journal.frontiersin.org/article/10.3389/fncel.2014.00281/abstract

264. Marquez RT, Xu L. Bcl-2:Beclin 1 complex: multiple, mechanisms regulating

autophagy/apoptosis toggle switch. Am J Cancer Res [Internet]. 2012;2(2):214–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22485198%5Cnhttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC3304572/pdf/ajcr0002-0214.pdf

265. Lindqvist LM, Heinlein M, Huang DCS, Vaux DL. Prosurvival Bcl-2 family members affect autophagy only indirectly, by inhibiting Bax and Bak. Proc Natl Acad Sci [Internet]. 2014 Jun 10;111(23):8512–7. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1406425111

266. Westphal D, Dewson G, Czabotar PE, Kluck RM. Molecular biology of Bax and Bak activation and action. Biochim Biophys Acta - Mol Cell Res [Internet]. 2011 Apr;1813(4):521–31. Available from: http://dx.doi.org/10.1016/j.bbamcr.2010.12.019

267. Tait SWG, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol [Internet]. 2010 Sep 4;11(9):621–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20683470

268. Law J, Yu VC, Baksh S. Modulator of Apoptosis 1: A Highly Regulated RASSF1A-Interacting BH3-Like Protein. Mol Biol Int [Internet]. 2012;2012:1–10. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3382356&tool=pmcentrez&rendertype=abstract

269. Fulda S. Evasion of Apoptosis as a Cellular Stress Response in Cancer. Int J Cell Biol [Internet]. 2010;2010:1–6. Available from: http://www.hindawi.com/journals/ijcb/2010/370835/

270. Wang R-A, Li Q-L, Li Z-S, Zheng P-J, Zhang H-Z, Huang X-F, et al. Apoptosis drives cancer cells proliferate and metastasize. J Cell Mol Med [Internet]. 2013 Jan;17(1):205–11. Available from: http://doi.wiley.com/10.1111/j.1582-4934.2012.01663.x

271. Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and Molecular Targeting Therapy in Cancer. Biomed Res Int [Internet]. 2014;2014:1–23. Available from: http://www.hindawi.com/journals/bmri/2014/150845/

272. Zong W-X. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev [Internet]. 2001 Jun 15;15(12):1481–6. Available from: http://www.genesdev.org/cgi/doi/10.1101/gad.897601

273. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta - Mol Cell Res [Internet]. 2013 Dec;1833(12):3448–59. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0167488913002243

274. Yaacoub K, Pedeux R, Tarte K, Guillaudeux T. Role of the tumor microenvironment in regulating apoptosis and cancer progression. Cancer Lett [Internet]. 2016 Aug;378(2):150–9. Available from: http://dx.doi.org/10.1016/j.canlet.2016.05.012

275. Safa AR. Resistance to Cell Death and Its Modulation in Cancer Stem Cells. Crit Rev Oncog [Internet]. 2016;21(3–4):203–19. Available from:

http://www.dl.begellhouse.com/journals/439f422d0783386a,5c1116ad47548349,320db83e0fe2d1a2.html

276. Abed MN, Abdullah MI, Richardson A. Antagonism of Bcl-XL is necessary for synergy between carboplatin and BH3 mimetics in ovarian cancer cells. J Ovarian Res [Internet]. 2016 Dec 14;9(1):25. Available from: http://ovarianresearch.biomedcentral.com/articles/10.1186/s13048-016-0234-y

277. Siddiqui WA, Ahad A, Ahsan H. The mystery of BCL2 family: Bcl-2 proteins and apoptosis: an update. Arch Toxicol [Internet]. 2015 Mar 25;89(3):289–317. Available from: http://link.springer.com/10.1007/s00204-014-1448-7

278. Brotin E, Meryet-Figuière M, Simonin K, Duval RE, Villedieu M, Leroy-Dudal J, et al. Bcl-x L and MCL-1 constitute pertinent targets in ovarian carcinoma and their concomitant inhibition is sufficient to induce apoptosis. Int J Cancer [Internet]. 2009;126(4):NA-NA. Available from: http://doi.wiley.com/10.1002/ijc.24787

279. Williams J, Lucas PC, Griffith KA, Choi M, Fogoros S, Hu YY, et al. Expression of Bcl-xL in ovarian carcinoma is associated with chemoresistance and recurrent disease. Gynecol Oncol [Internet]. 2005 Feb;96(2):287–95. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0090825804008285

280. Certo M, Moore VDG, Nishino M, Wei G, Korsmeyer S, Armstrong SA, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell [Internet]. 2006 May;9(5):351–65. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1535610806001139

281. Kazi A, Sun J, Doi K, Sung S-S, Takahashi Y, Yin H, et al. The BH3 -Helical Mimic BH3-M6 Disrupts Bcl-XL, Bcl-2, and MCL-1 Protein-Protein Interactions with Bax, Bak, Bad, or Bim and Induces Apoptosis in a Bax- and Bim-dependent Manner. J Biol Chem [Internet]. 2011 Mar 18;286(11):9382–92. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M110.203638

282. Adams JM. Therapeutic potential of a peptide targeting BCL-2 cell guardians in cancer. J Clin Invest [Internet]. 2012 Jun 1;122(6):1965–7. Available from: http://www.jci.org/articles/view/64120

283. Al-harbi S, Hill BT, Mazumder S, Singh K, DeVecchio J, Choudhary G, et al. An antiapoptotic BCL-2 family expression index predicts the response of chronic lymphocytic leukemia to ABT-737. Blood [Internet]. 2011 Sep 29;118(13):3579–90. Available from: http://www.bloodjournal.org/content/118/13/3579.abstract

284. Mazumder S, Choudhary GS, Al-harbi S, Almasan A. Mcl-1 Phosphorylation Defines ABT-737 Resistance That Can Be Overcome by Increased NOXA Expression in Leukemic B cells. Cancer Res [Internet]. 2012 Jun 15;72(12):3069–79. Available from: http://pubs.acs.org/doi/abs/10.1021/nl061786n

285. Ludwig LM, Nassin ML, Hadji A, LaBelle JL. Killing Two Cells with One Stone: Pharmacologic BCL-2 Family Targeting for Cancer Cell Death and Immune Modulation. Front Pediatr [Internet]. 2016 Dec 21;4(December). Available from: http://journal.frontiersin.org/article/10.3389/fped.2016.00135/full

286. Kim EY, Jung JY, Kim A, Chang YS, Kim SK. ABT-737 Synergizes with Cisplatin

Bypassing Aberration of Apoptotic Pathway in Non-small Cell Lung Cancer. Neoplasia [Internet]. 2017 Apr;19(4):354–63. Available from: http://dx.doi.org/10.1016/j.neo.2017.02.008

287. Alford SE, Kothari A, Loeff FC, Eichhorn JM, Sakurikar N, Goselink HM, et al. BH3 Inhibitor Sensitivity and Bcl-2 Dependence in Primary Acute Lymphoblastic Leukemia Cells. Filizola M, editor. Cancer Res [Internet]. 2015 Apr 1;75(7):1366–75. Available from: http://link.springer.com/10.1007/978-1-4939-2914-6

288. High LM, Szymanska B, Wilczynska-Kalak U, Barber N, O'Brien R, Khaw SL, et al. The Bcl-2 Homology Domain 3 Mimetic ABT-737 Targets the Apoptotic Machinery in Acute Lymphoblastic Leukemia Resulting in Synergistic in Vitro and in Vivo Interactions with Established Drugs. Mol Pharmacol [Internet]. 2010 Mar 1;77(3):483–94. Available from: http://molpharm.aspetjournals.org/cgi/doi/10.1124/mol.109.060780

289. Ackler S, Xiao Y, Mitten MJ, Foster K, Oleksijew A, Refici M, et al. ABT-263 and rapamycin act cooperatively to kill lymphoma cells in vitro and in vivo. Mol Cancer Ther [Internet]. 2008 Oct 1;7(10):3265–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18852130

290. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, et al. ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. Cancer Res [Internet]. 2008 May 1;68(9):3421–8. Available from: http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-07-5836

291. Vo T-T, Letai A. BH3-Only Proteins and Their Effects on Cancer. In: Advances in Experimental Medicine and Biology [Internet]. 2010. p. 49–63. Available from: http://link.springer.com/10.1007/978-1-4419-6706-0_3

292. Renault TT, Elkholi R, Bharti A, Chipuk JE. B Cell Lymphoma-2 (BCL-2) Homology Domain 3 (BH3) Mimetics Demonstrate Differential Activities Dependent upon the Functional Repertoire of Pro- and Anti-apoptotic BCL-2 Family Proteins. J Biol Chem [Internet]. 2014 Sep 19;289(38):26481–91. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.M114.569632

293. Stamelos VA, Robinson E, Redman CW, Richardson A. Navitoclax augments the activity of carboplatin and paclitaxel combinations in ovarian cancer cells. Gynecol Oncol [Internet]. 2013 Feb;128(2):377–82. Available from: http://dx.doi.org/10.1016/j.ygyno.2012.11.019

294. Witham J, Valenti MR, De-Haven-Brandon AK, Vidot S, Eccles SA, Kaye SB, et al. The Bcl-2/Bcl-XL Family Inhibitor ABT-737 Sensitizes Ovarian Cancer Cells to Carboplatin. Clin Cancer Res [Internet]. 2007 Dec 1;13(23):7191–8. Available from: http://clincancerres.aacrjournals.org/cgi/doi/10.1158/1078-0432.CCR-07-0362

295. Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. Blood [Internet]. 2010 Apr 22;115(16):3304–13. Available from: http://www.bloodjournal.org/content/115/16/3304.long

296. Boiani M, Daniel C, Liu X, Hogarty MD, Marnett LJ. The Stress Protein BAG3 Stabilizes Mcl-1 Protein and Promotes Survival of Cancer Cells and Resistance to Antagonist ABT-737. J Biol Chem [Internet]. 2013 Mar 8;288(10):6980–90. Available

from: http://www.jbc.org/cgi/doi/10.1074/jbc.M112.414177

297. Stamelos VA, Redman CW, Richardson A. Understanding sensitivity to BH3 mimetics: ABT-737 as a case study to foresee the complexities of personalized medicine. J Mol Signal [Internet]. 2012 Aug 16;7:12. Available from: http://www.jmolecularsignaling.com/articles/10.1186/1750-2187-7-12/

298. Wang C, Huang S-B, Yang M-C, Lin Y-T, Chu I-H, Shen Y-N, et al. Combining Paclitaxel with ABT-263 Has a Synergistic Effect on Paclitaxel Resistant Prostate Cancer Cells. Villunger A, editor. PLoS One [Internet]. 2015 Mar 26;10(3):e0120913. Available from: http://dx.plos.org/10.1371/journal.pone.0120913

299. Lieber J, Eicher C, Wenz J, Kirchner B, Warmann SW, Fuchs J, et al. The BH3 mimetic ABT-737 increases treatment efficiency of paclitaxel against hepatoblastoma. BMC Cancer [Internet]. 2011 Dec 19;11(1):362. Available from: http://bmccancer.biomedcentral.com/articles/10.1186/1471-2407-11-362

300. Yokoyama T, Kohn EC, Brill E, Lee J-M. Apoptosis is augmented in high-grade serous ovarian cancer by the combined inhibition of Bcl-2/Bcl-xL and PARP. Int J Oncol [Internet]. 2017 Apr 15;50(4):1064–74. Available from: http://www.spandidos-publications.com/10.3892/ijo.2017.3914

301. Delbridge ARD, Grabow S, Strasser A, Vaux DL. Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. Nat Rev Cancer [Internet]. 2016 Jan 29;16(2):99–109. Available from: http://www.nature.com/doifinder/10.1038/nrc.2015.17%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/26822577

302. Cleary JM, Lima CMSR, Hurwitz HI, Montero AJ, Franklin C, Yang J, et al. A phase I clinical trial of navitoclax, a targeted high-affinity Bcl-2 family inhibitor, in combination with gemcitabine in patients with solid tumors. Invest New Drugs [Internet]. 2014 Oct 11;32(5):937–45. Available from: http://link.springer.com/10.1007/s10637-014-0110-9

303. Schoenwaelder SM, Jarman KE, Gardiner EE, Hua M, Qiao J, White MJ, et al. Bcl-xL-inhibitory BH3 mimetics can induce a transient thrombocytopathy that undermines the hemostatic function of platelets. Blood [Internet]. 2011 Aug 11;118(6):1663–74. Available from: http://www.bloodjournal.org/cgi/doi/10.1182/blood-2011-04-347849

304. Cang S, Iragavarapu C, Savooji J, Song Y, Liu D. ABT-199 (venetoclax) and BCL-2 inhibitors in clinical development. J Hematol Oncol [Internet]. 2015 Dec 20;8(1):129. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4654800&tool=pmcentrez&rendertype=abstract

305. Peirs S, Matthijssens F, Goossens S, Van de Walle I, Ruggero K, de Bock CE, et al. ABT-199 mediated inhibition of BCL-2 as a novel therapeutic strategy in T-cell acute lymphoblastic leukemia. Blood [Internet]. 2014 Dec 11;124(25):3738–47. Available from: http://www.bloodjournal.org/cgi/doi/10.1182/blood-2014-05-574566

306. Touzeau C, Dousset C, Le Gouill S, Sampath D, Leverson JD, Souers a J, et al. The Bcl-2 specific BH3 mimetic ABT-199: a promising targeted therapy for t(11;14) multiple myeloma. Leukemia [Internet]. 2014 Jan 17;28(1):210–2. Available from:

http://dx.doi.org/10.1038/leu.2013.216

307. Vogler M, Dinsdale D, Dyer MJS, Cohen GM. ABT-199 selectively inhibits BCL2 but not BCL2L1 and efficiently induces apoptosis of chronic lymphocytic leukaemic cells but not platelets. Br J Haematol [Internet]. 2013 Oct;163(1):139–42. Available from: http://doi.wiley.com/10.1111/bjh.12457

308. Vogler M, Dinsdale D, Dyer MJS, Cohen GM. Acute myeloid leukemia cells harboring MLL fusion genes or with the acute promyelocytic leukemia phenotype are sensitive to the Bcl-2-selective inhibitor ABT-199. Br J Haematol [Internet]. 2013 Oct 17;163(1):139–42. Available from: http://doi.wiley.com/10.1111/bjh.12457

309. Vandenberg CJ, Cory S. ABT-199, a new Bcl-2-specific BH3 mimetic, has in vivo efficacy against aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia. Blood [Internet]. 2013 Mar 21;121(12):2285–8. Available from: http://www.bloodjournal.org/cgi/doi/10.1182/blood-2013-01-475855

310. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med [Internet]. 2013 Jan 6;19(2):202–8. Available from: http://www.nature.com/doifinder/10.1038/nm.3048

311. Itchaki G, Brown JR. The potential of venetoclax (ABT-199) in chronic lymphocytic leukemia. Ther Adv Hematol [Internet]. 2016 Oct 31;7(5):270–87. Available from: http://journals.sagepub.com/doi/10.1177/2040620716655350

312. Vandenberg CJ, Cory S, Dc W, Vandenberg CJ, Cory S. aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia ABT-199 , a new Bcl-2 – speci fi c BH3 mimetic , has in vivo ef fi cacy against aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia. 2013;121(12):2285–8.

313. Inoue-Yamauchi A, Jeng PS, Kim K, Chen H-C, Han S, Ganesan YT, et al. Targeting the differential addiction to anti-apoptotic BCL-2 family for cancer therapy. Nat Commun [Internet]. 2017 Jul 17;8(May):16078. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28714472%0Ahttp://www.nature.com/doifinder/10.1038/ncomms16078

314. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data: Figure 1. Cancer Discov [Internet]. 2012 May;2(5):401–4. Available from: http://cancerdiscovery.aacrjournals.org/lookup/doi/10.1158/2159-8290.CD-12-0095

315. Park H-A, Licznerski P, Mnatsakanyan N, Niu Y, Sacchetti S, Wu J, et al. Inhibition of Bcl-xL prevents pro-death actions of ΔN-Bcl-xL at the mitochondrial inner membrane during glutamate excitotoxicity. Cell Death Differ [Internet]. 2017 Aug 4;1–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28777375%0Ahttp://www.nature.com/doifinder/10.1038/cdd.2017.123

316. Besbes S, Mirshahi M, Pocard M, Billard C. New dimension in therapeutic targeting of BCL-2 family proteins. Oncotarget [Internet]. 2015;6(15):12862–71. Available from: http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=articl

e&op=view&path[]=3868%5Cnhttp://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path[]=3868&path[]=8360%5Cnhttp://www.ncbi.nlm.nih.gov/pub

317. Erlanson DA, Fesik SW, Hubbard RE, Jahnke W, Jhoti H. Twenty years on: the impact of fragments on drug discovery. Nat Rev Drug Discov [Internet]. 2016 Jul 15;15(9):605–19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27417849

318. Wong M, Tan N, Zha J, Peale F V., Yue P, Fairbrother WJ, et al. Navitoclax (ABT-263) Reduces Bcl-xL-Mediated Chemoresistance in Ovarian Cancer Models. Mol Cancer Ther [Internet]. 2012 Apr 1;11(4):1026–35. Available from: http://mct.aacrjournals.org/cgi/doi/10.1158/1535-7163.MCT-11-0693

319. Gao Y, Trivedi S, Ferris RL, Koide K. Regulation of HPV16 E6 and MCL1 by SF3B1 inhibitor in head and neck cancer cells. Sci Rep [Internet]. 2014;4(August):6098. Available from: http://www.nature.com/articles/srep06098

320. Lessene G, Czabotar PE, Sleebs BE, Zobel K, Lowes KN, Adams JM, et al. Structure-guided design of a selective BCL-XL inhibitor. Nat Chem Biol [Internet]. 2013 Apr 21;9(6):390–7. Available from: http://www.nature.com/doifinder/10.1038/nchembio.1246

321. Domcke S, Sinha R, Levine DA, Sander C, Schultz N. Evaluating cell lines as tumour models by comparison of genomic profiles. Nat Commun [Internet]. 2013 Jul 9;4:1–10. Available from: http://www.nature.com/doifinder/10.1038/ncomms3126

322. Matsuura K, Huang N-J, Cocce K, Zhang L, Kornbluth S. Downregulation of the proapoptotic protein MOAP-1 by the UBR5 ubiquitin ligase and its role in ovarian cancer resistance to cisplatin. Oncogene [Internet]. 2017 Mar 23;36(12):1698–706. Available from: https://www.nature.com/onc/journal/v36/n12/pdf/onc2016336a.pdf

323. Mani J, Vallo S, Rakel S, Antonietti P, Gessler F, Blaheta R, et al. Chemoresistance is associated with increased cytoprotective autophagy and diminished apoptosis in bladder cancer cells treated with the BH3 mimetic (−)-Gossypol (AT-101). BMC Cancer [Internet]. 2015 Dec 7;15(1):224. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4409725&tool=pmcentrez&rendertype=abstract

324. Pistritto G, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. Aging (Albany NY) [Internet]. 2016 Mar 27;8(4):603–19. Available from: http://www.aging-us.com/article/100934

325. Mohammad RM, Muqbil I, Lowe L, Yedjou C, Hsu H-Y, Lin L-T, et al. Broad targeting of resistance to apoptosis in cancer. Semin Cancer Biol [Internet]. 2015 Dec;35:S78–103. Available from: http://dx.doi.org/10.1016/j.semcancer.2015.03.001

326. Williams MM, Cook RS. Bcl-2 family proteins in breast development and cancer: could Mcl-1 targeting overcome therapeutic resistance? Oncotarget [Internet]. 2015 Feb 28;6(6):3519–30. Available from: http://www.oncotarget.com/fulltext/2792

327. Bray K, Chen H-Y, Karp CM, May M, Ganesan S, Karantza-Wadsworth V, et al. Bcl-2 Modulation to Activate Apoptosis in Prostate Cancer. Mol Cancer Res [Internet]. 2009

Sep 1;7(9):1487–96. Available from: http://mcr.aacrjournals.org/cgi/doi/10.1158/1541-7786.MCR-09-0166

328. Letai A. Cell Death and Cancer Therapy: Don't Forget to Kill the Cancer Cell! Clin Cancer Res [Internet]. 2015 Nov 15;21(22):5015–20. Available from: http://clincancerres.aacrjournals.org/cgi/doi/10.1158/1078-0432.CCR-15-1204

329. Leverson JD, Zhang H, Chen J, Tahir SK, Phillips DC, Xue J, et al. Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). Cell Death Dis [Internet]. 2015 Jan 15;6(1):e1590. Available from: http://www.nature.com/doifinder/10.1038/cddis.2014.561

330. Pan R, Hogdal LJ, Benito JM, Bucci D, Han L, Borthakur G, et al. Selective BCL-2 Inhibition by ABT-199 Causes On-Target Cell Death in Acute Myeloid Leukemia. Cancer Discov [Internet]. 2014 Mar 1;4(3):362–75. Available from: http://cancerdiscovery.aacrjournals.org/cgi/doi/10.1158/2159-8290.CD-13-0609

331. Li Y, Deutzmann A, Felsher DW. BIM-mediated apoptosis and oncogene addiction. Aging (Albany NY) [Internet]. 2016 Sep 29;8(9):1834–5. Available from: http://www.aging-us.com/article/101072

332. Del Gaizo Moore V, Letai A. BH3 profiling – Measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions. Cancer Lett [Internet]. 2013 May;332(2):202–5. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3770266&tool=pmcentrez&rendertype=abstract

333. Butterworth M, Pettitt A, Varadarajan S, Cohen GM. BH3 profiling and a toolkit of BH3-mimetic drugs predict anti-apoptotic dependence of cancer cells. Br J Cancer [Internet]. 2016 Mar 15;114(6):638–41. Available from: http://www.nature.com/doifinder/10.1038/bjc.2016.49

334. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest [Internet]. 2007 Jan 2;117(1):112–21. Available from: http://www.jci.org/cgi/doi/10.1172/JCI28281

335. Habata S, Iwasaki M, Sugio A, Suzuki M, Tamate M, Satohisa S, et al. BAG3-mediated Mcl-1 stabilization contributes to drug resistance via interaction with USP9X in ovarian cancer. Int J Oncol [Internet]. 2016 Apr 21;49(1):402–10. Available from: http://www.spandidos-publications.com/10.3892/ijo.2016.3494

336. Goncharenko-Khaider N, Matte I, Lane D, Rancourt C, Piché A. Ovarian cancer ascites increase Mcl-1 expression in tumor cells through ERK1/2-Elk-1 signaling to attenuate TRAIL-induced apoptosis. Mol Cancer [Internet]. 2012;11(1):84. Available from: http://molecular-cancer.biomedcentral.com/articles/10.1186/1476-4598-11-84

337. Rolland SG, Conradt B. New role of the BCL2 family of proteins in the regulation of mitochondrial dynamics. Curr Opin Cell Biol [Internet]. 2010 Dec;22(6):852–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0955067410001213

338. Schmid D, Jarvis GE, Fay F, Small DM, Greene MK, Majkut J, et al.

Nanoencapsulation of ABT-737 and camptothecin enhances their clinical potential through synergistic antitumor effects and reduction of systemic toxicity. Cell Death Dis [Internet]. 2014 Oct 9;5(10):e1454. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25299779%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4649518%5Cnhttp://www.nature.com/doifinder/10.1038/cddis.2014.413

339. Sato S, Itamochi H. Neoadjuvant chemotherapy in advanced ovarian cancer: latest results and place in therapy. Ther Adv Med Oncol [Internet]. 2014 Nov;6(6):293–304. Available from: http://tam.sagepub.com/cgi/doi/10.1177/1758834014544891

340. Zahreddine H, Borden KLB. Mechanisms and insights into drug resistance in cancer. Front Pharmacol [Internet]. 2013;4(March):1–8. Available from: http://journal.frontiersin.org/article/10.3389/fphar.2013.00028/abstract

341. Cheung-Ong K, Giaever G, Nislow C. DNA-Damaging Agents in Cancer Chemotherapy: Serendipity and Chemical Biology. Chem Biol [Internet]. 2013 May;20(5):648–59. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1074552113001312

342. Masui K, Gini B, Wykosky J, Zanca C, Mischel PS, Furnari FB, et al. A tale of two approaches: complementary mechanisms of cytotoxic and targeted therapy resistance may inform next-generation cancer treatments. Carcinogenesis [Internet]. 2013 Apr 1;34(4):725–38. Available from: https://academic.oup.com/carcin/article-lookup/doi/10.1093/carcin/bgt086

343. Januchowski R, Sterzy?ska K, Zaorska K, Sosi?ska P, Klejewski A, Br?zert M, et al. Analysis of MDR genes expression and cross-resistance in eight drug resistant ovarian cancer cell lines. J Ovarian Res [Internet]. 2016 Dec 18;9(1):65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27756418%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5069986%0Ahttps://www.ncbi.nlm.nih.gov/pubmed/27756418

344. Crow J, Atay S, Banskota S, Artale B, Schmitt S, Godwin AK. Exosomes as mediators of platinum resistance in ovarian cancer. Oncotarget [Internet]. 2017 Jan 2;8(7):11917–36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28060758%0Ahttp://www.oncotarget.com/abstract/14440

345. McGranahan N, Swanton C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. Cell [Internet]. 2017 Feb;168(4):613–28. Available from: http://dx.doi.org/10.1016/j.cell.2017.01.018

346. Chen C-C, Lee K-D, Pai M-Y, Chu P-Y, Hsu C-C, Chiu C-C, et al. Changes in DNA methylation are associated with the development of drug resistance in cervical cancer cells. Cancer Cell Int [Internet]. 2015 Dec 13;15(1):98. Available from: http://cancerci.biomedcentral.com/articles/10.1186/s12935-015-0248-3

347. Yan B, Yin F, Wang Q, Zhang W, Li L. Integration and bioinformatics analysis of DNA?methylated genes associated with drug resistance in ovarian cancer. Oncol Lett [Internet]. 2016 May 18;8(6):6847–58. Available from: http://www.spandidos-publications.com/10.3892/ol.2016.4608

348. Guo S, Zou J, Wang G. Advances in the proteomic discovery of novel therapeutic targets in cancer. Drug Des Devel Ther [Internet]. 2013 Oct;7:1259. Available from: http://www.dovepress.com/advances-in-the-proteomic-discovery-of-novel-therapeutic-targets-in-ca-peer-reviewed-article-DDDT

349. Kim HK, Choi IJ, Kim CG, Kim HS, Oshima A, Michalowski A, et al. A Gene Expression Signature of Acquired Chemoresistance to Cisplatin and Fluorouracil Combination Chemotherapy in Gastric Cancer Patients. Navarro A, editor. PLoS One [Internet]. 2011 Feb 18;6(2):e16694. Available from: http://dx.plos.org/10.1371/journal.pone.0016694

350. Wrzeszczynski KO, Varadan V, Byrnes J, Lum E, Kamalakaran S, Levine DA, et al. Identification of Tumor Suppressors and Oncogenes from Genomic and Epigenetic Features in Ovarian Cancer. Guan X, editor. PLoS One [Internet]. 2011 Dec 8;6(12):e28503. Available from: http://dx.plos.org/10.1371/journal.pone.0028503

351. Han K, Jeng EE, Hess GT, Morgens DW, Li A, Bassik MC. Synergistic drug combinations for cancer identified in a CRISPR screen for pairwise genetic interactions. Nat Biotechnol [Internet]. 2017 Mar 20;35(5):463–74. Available from: http://www.nature.com/doifinder/10.1038/nbt.3834

352. Alvarez-Calderon F, Gregory MA, DeGregori J. Using functional genomics to overcome therapeutic resistance in hematological malignancies. Immunol Res [Internet]. 2013 Mar 1;55(1–3):100–15. Available from: http://link.springer.com/10.1007/s12026-012-8353-z

353. Vasey PA. Resistance to chemotherapy in advanced ovarian cancer: mechanisms and current strategies. Br J Cancer [Internet]. 2003 Dec;89:S23–8. Available from: http://www.nature.com/doifinder/10.1038/sj.bjc.6601497

354. Qian Y, Chen X. Senescence Regulation by the p53 Protein Family. In: Galluzzi L, Vitale I, Kepp O, Kroemer G, editors. Methods Mol Biol [Internet]. Totowa, NJ: Humana Press; 2013. p. 37–61. (Methods in Molecular Biology; vol. 965). Available from: http://link.springer.com/10.1007/978-1-62703-239-1

355. Mohell N, Alfredsson J, Fransson Å, Uustalu M, Byström S, Gullbo J, et al. APR-246 overcomes resistance to cisplatin and doxorubicin in ovarian cancer cells. Cell Death Dis [Internet]. 2015 Jun 18;6(6):e1794. Available from: http://www.nature.com/doifinder/10.1038/cddis.2015.143

356. Perego P, Romanelli S, Carenini N, Magnani I, Leone R, Bonetti A, et al. Ovarian cancer cisplatin-resistant cell lines: Multiple changes including collateral sensitivity to Taxol. Ann Oncol [Internet]. 1998 Apr;9(4):423–30. Available from: http://academic.oup.com/annonc/article/9/4/423/136424/Ovarian-cancer-cisplatinresistant-cell-lines

357. Busschots S, O'Toole S, O'Leary JJ, Stordal B. Carboplatin and taxol resistance develops more rapidly in functional BRCA1 compared to dysfunctional BRCA1 ovarian cancer cells. Exp Cell Res [Internet]. 2015 Aug;336(1):1–14. Available from: http://dx.doi.org/10.1016/j.yexcr.2014.12.001

358. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer [Internet]. 2013 Sep 24;13(10):714–26.

Available from: http://www.nature.com/doifinder/10.1038/nrc3599

359. Inaoka K, Inokawa Y, Nomoto S. Genomic-Wide Analysis with Microarrays in Human Oncology. Microarrays [Internet]. 2015;4(4):454–73. Available from: http://www.mdpi.com/2076-3905/4/4/454/

360. Sherman-Baust CA, Becker KG, Wood III WH, Zhang Y, Morin PJ. Gene expression and pathway analysis of ovarian cancer cells selected for resistance to cisplatin, paclitaxel, or doxorubicin. J Ovarian Res [Internet]. 2011;4(1):21. Available from: http://ovarianresearch.biomedcentral.com/articles/10.1186/1757-2215-4-21

361. Kinose Y, Sawada K, Nakamura K, Kimura T. The Role of MicroRNAs in Ovarian Cancer. Biomed Res Int [Internet]. 2014;2014:1–11. Available from: http://www.hindawi.com/journals/bmri/2014/249393/

362. Polyak K. On Using Functional Genetics to Understand Breast Cancer Biology. Cold Spring Harb Perspect Biol [Internet]. 2012 Jul 1;4(7):a013516–a013516. Available from: http://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a013516

363. Jazaeri AA. Gene Expression Profiles Associated with Response to Chemotherapy in Epithelial Ovarian Cancers. Clin Cancer Res [Internet]. 2005 Sep 1;11(17):6300–10. Available from: http://clincancerres.aacrjournals.org/cgi/doi/10.1158/1078-0432.CCR-04-2682

364. Vidot S, Witham J, Agarwal R, Greenhough S, Bamrah HS, Tigyi GJ, et al. Autotaxin delays apoptosis induced by carboplatin in ovarian cancer cells. Cell Signal [Internet]. 2010 Jun;22(6):926–35. Available from: http://dx.doi.org/10.1016/j.cellsig.2010.01.017

365. Liu S, Umezu-Goto M, Murph M, Lu Y, Liu W, Zhang F, et al. Expression of Autotaxin and Lysophosphatidic Acid Receptors Increases Mammary Tumorigenesis, Invasion, and Metastases. Cancer Cell [Internet]. 2009 Jun;15(6):539–50. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1535610809001159

366. Samadi N, Gaetano C, Goping IS, Brindley DN. Autotaxin protects MCF-7 breast cancer and MDA-MB-435 melanoma cells against Taxol-induced apoptosis. Oncogene [Internet]. 2009 Feb 19;28(7):1028–39. Available from: http://www.nature.com/doifinder/10.1038/onc.2008.442

367. Long X, Yu Y, Perlaky L, Man T-K, Redell MS. Stromal CYR61 Confers Resistance to Mitoxantrone via Spleen Tyrosine Kinase Activation in Human Acute Myeloid Leukaemia. Br J Haematol [Internet]. 2015 Sep;170(5):704–18. Available from: http://doi.wiley.com/10.1111/bjh.13492

368. Chen C, Lau L. CYR61 (cysteine-rich, angiogenic inducer, 61). Atlas Genet Cytogenet Oncol Haematol [Internet]. 2013 Jun;17(7):445–52. Available from: http://hdl.handle.net/2042/51039

369. Perwitasari O, Bakre A, Tompkins S, Tripp R. siRNA Genome Screening Approaches to Therapeutic Drug Repositioning. Pharmaceuticals [Internet]. 2013 Jan 28;6(2):124–60. Available from: http://www.mdpi.com/1424-8247/6/2/124/

370. Malik AR, Urbanska M, Gozdz A, Swiech LJ, Nagalski A, Perycz M, et al. Cyr61, a

Matricellular Protein, Is Needed for Dendritic Arborization of Hippocampal Neurons. J Biol Chem [Internet]. 2013 Mar 22;288(12):8544–59. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.M112.411629

371. Emre Y, Imhof BA. Matricellular protein CCN1/CYR61: a new player in inflammation and leukocyte trafficking. Semin Immunopathol [Internet]. 2014 Mar 18;36(2):253–9. Available from: http://link.springer.com/10.1007/s00281-014-0420-1

372. O'Kelly J, Chung A, Lemp N, Chumakova K, Yin D, Wang H-J, et al. Functional domains of CCN1 (Cyr61) regulate breast cancer progression. Int J Oncol [Internet]. 2008 Jul;33(1):59–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18575751

373. Sarkissyan S, Sarkissyan M, Wu Y, Cardenas J, Koeffler HP, Vadgama J V. IGF-1 Regulates Cyr61 Induced Breast Cancer Cell Proliferation and Invasion. Li J, editor. PLoS One [Internet]. 2014 Jul 25;9(7):e103534. Available from: http://dx.plos.org/10.1371/journal.pone.0103534

374. Liang C-Z, Lin C-M. Cyr61: a potential therapeutic target for prostate cancer. Asian J Androl [Internet]. 2014;16(5):788. Available from: http://www.ajandrology.com/text.asp?2014/16/5/788/128517

375. Shen H, Cai M, Zhao S, Wang H, Li M, Yao S, et al. CYR61 overexpression associated with the development and poor prognosis of ovarian carcinoma. Med Oncol [Internet]. 2014 Aug 22;31(8):117. Available from: http://link.springer.com/10.1007/s12032-014-0117-2

376. Lee K-B, Byun H-J, Park SH, Park C-Y, Lee S-H, Rho SB. CYR61 controls p53 and NF-κB expression through PI3K/Akt/mTOR pathways in carboplatin-induced ovarian cancer cells. Cancer Lett [Internet]. 2012 Feb;315(1):86–95. Available from: http://dx.doi.org/10.1016/j.canlet.2011.10.016

377. Yu B, Chen X, Li J, Qu Y, Su L, Peng Y, et al. Stromal fibroblasts in the microenvironment of gastric carcinomas promote tumor metastasis via upregulating TAGLN expression. BMC Cell Biol [Internet]. 2013;14(1):17. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3610155&tool=pmcentrez&rendertype=abstract

378. Zhou H, Fang Y, Weinberger PM, Ding L, Cowell JK, Hudson FZ, et al. Transgelin increases metastatic potential of colorectal cancer cells in vivo and alters expression of genes involved in cell motility. BMC Cancer [Internet]. 2016 Dec 4;16(1):55. Available from: http://bmccancer.biomedcentral.com/articles/10.1186/s12885-016-2105-8

379. Lee E-K, Han G-Y, Park HW, Song Y-J, Kim C-W. Transgelin Promotes Migration and Invasion of Cancer Stem Cells. J Proteome Res [Internet]. 2010 Oct;9(10):5108–17. Available from: http://pubs.acs.org/doi/abs/10.1021/pr100378z

380. Zhou L, Zhang R, Zhang L, Sun Y, Yao W, Zhao A, et al. Upregulation of transgelin is an independent factor predictive of poor prognosis in patients with advanced pancreatic cancer. Cancer Sci [Internet]. 2013 Apr;104(4):423–30. Available from: http://doi.wiley.com/10.1111/cas.12107

381. Lin Y, Buckhaults PJ, Lee JR, Xiong H, Farrell C, Podolsky RH, et al. Association of the Actin-Binding Protein Transgelin with Lymph Node Metastasis in Human Colorectal Cancer. Neoplasia [Internet]. 2009 Sep;11(9):864–IN5. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1476558609800390

382. Li N, Zhang J, Liang Y, Shao J, Peng F, Sun M, et al. A Controversial Tumor Marker: Is SM22 a Proper Biomarker for Gastric Cancer Cells? J Proteome Res [Internet]. 2007 Aug;6(8):3304–12. Available from: http://pubs.acs.org/doi/abs/10.1021/pr0702363

383. Wu X, Dong L, Zhang R, Ying K, Shen H. Transgelin overexpression in lung adenocarcinoma is associated with tumor progression. Int J Mol Med [Internet]. 2014 Jun 16;34(2):585–91. Available from: http://www.spandidos-publications.com/10.3892/ijmm.2014.1805

384. Meng T, Liu L, Hao R, Chen S, Dong Y. Transgelin-2: A potential oncogenic factor. Tumor Biol [Internet]. 2017 Jun 22;39(6):101042831770265. Available from: http://journals.sagepub.com/doi/10.1177/1010428317702650

385. Thompson O, Moghraby JS, Ayscough KR, Winder SJ. Depletion of the actin bundling protein SM22/transgelin increases actin dynamics and enhances the tumourigenic phenotypes of cells. BMC Cell Biol [Internet]. 2012;13(January):1. Available from: http://www.biomedcentral.com/1471-2121/13/1

386. Sayar N, Karahan G, Konu O, Bozkurt B, Bozdogan O, Yulug IG. Transgelin gene is frequently downregulated by promoter DNA hypermethylation in breast cancer. Clin Epigenetics [Internet]. 2015 Dec 28;7(1):104. Available from: http://dx.doi.org/10.1186/s13148-015-0138-5

387. Prasad PD, Stanton J-AL, Assinder SJ. Expression of the actin-associated protein transgelin (SM22) is decreased in prostate cancer. Cell Tissue Res [Internet]. 2010 Feb 11;339(2):337–47. Available from: http://link.springer.com/10.1007/s00441-009-0902-y

388. Yeo M, Park HJ, Kim D-K, Kim YB, Cheong JY, Lee KJ, et al. Loss of SM22 is a characteristic signature of colon carcinogenesis and its restoration suppresses colon tumorigenicity in vivo and in vitro. Cancer [Internet]. 2010;116(11):NA-NA. Available from: http://doi.wiley.com/10.1002/cncr.25003

389. Desouza M, Gunning PW, Stehn JR. The actin cytoskeleton as a sensor and mediator of apoptosis. Bioarchitecture [Internet]. 2012 May 20;2(3):75–87. Available from: http://www.tandfonline.com/doi/abs/10.4161/bioa.20975

390. Kim MH, Kim J, Hong H, Lee S-H, Lee J-K, Jung E, et al. Actin remodeling confers BRAF inhibitor resistance to melanoma cells through YAP/TAZ activation. EMBO J [Internet]. 2016 Mar 1;35(5):462–78. Available from: http://emboj.embopress.org/content/early/2015/12/14/embj.201592081.abstract

391. Po'uha ST, Shum MSY, Goebel A, Bernard O, Kavallaris M. LIM-kinase 2, a regulator of actin dynamics, is involved in mitotic spindle integrity and sensitivity to microtubule-destabilizing drugs. Oncogene [Internet]. 2010 Jan 28;29(4):597–607. Available from: http://www.nature.com/doifinder/10.1038/onc.2009.367

392. Han B, Qu Y, Jin Y, Yu Y, Deng N, Wawrowsky K, et al. FOXC1 Activates

Smoothened-Independent Hedgehog Signaling in Basal-like Breast Cancer. Cell Rep [Internet]. 2015 Nov;13(5):1046–58. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2211124715011055

393. Medina-Trillo C, Sánchez-Sánchez F, Aroca-Aguilar J-D, Ferre-Fernández J-J, Morales L, Méndez-Hernández C-D, et al. Hypo- and Hypermorphic FOXC1 Mutations in Dominant Glaucoma: Transactivation and Phenotypic Variability. Cai T, editor. PLoS One [Internet]. 2015 Mar 18;10(3):e0119272. Available from: http://dx.plos.org/10.1371/journal.pone.0119272

394. Lay K, Kume T, Fuchs E. FOXC1 maintains the hair follicle stem cell niche and governs stem cell quiescence to preserve long-term tissue-regenerating potential. Proc Natl Acad Sci [Internet]. 2016 Mar 15;113(11):E1506–15. Available from: http://www.pnas.org/content/113/11/E1506.abstract

395. Omatsu Y, Seike M, Sugiyama T, Kume T, Nagasawa T. Foxc1 is a critical regulator of haematopoietic stem/progenitor cell niche formation. Nature [Internet]. 2014 Mar 2;508(7497):536–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24590069

396. Han B, Bhowmick N, Qu Y, Chung S, Giuliano AE, Cui X. FOXC1: an emerging marker and therapeutic target for cancer. Oncogene [Internet]. 2017 Jul 13;36(28):3957–63. Available from: http://www.nature.com/doifinder/10.1038/onc.2017.48

397. Chung S, Jin Y, Han B, Qu Y, Gao B, Giuliano AE, et al. Identification of EGF-NF-κB-FOXC1 signaling axis in basal-like breast cancer. Cell Commun Signal [Internet]. 2017 Dec 19;15(1):22. Available from: http://biosignaling.biomedcentral.com/articles/10.1186/s12964-017-0180-3

398. Wang L, Gu F, Liu C-Y, Wang R-J, Li J, Xu J-Y. High level of FOXC1 expression is associated with poor prognosis in pancreatic ductal adenocarcinoma. Tumor Biol [Internet]. 2013 Apr 16;34(2):853–8. Available from: http://link.springer.com/10.1007/s13277-012-0617-7

399. Xia L, Huang W, Tian D, Zhu H, Qi X, Chen Z, et al. Overexpression of forkhead box C1 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. Hepatology [Internet]. 2013 Feb;57(2):610–24. Available from: http://doi.wiley.com/10.1002/hep.26029

400. Huang L, Huang Z, Fan Y, He L, Ye M, Shi K, et al. FOXC1 promotes proliferation and epithelial-mesenchymal transition in cervical carcinoma through the PI3K-Akt signal pathway. Am J Transl Res. 2017;9(3):1297–306.

401. Xu Y, Shao Q, Yao H, Jin Y, Ma Y, Jia L. Overexpression of FOXC1 correlates with poor prognosis in gastric cancer patients. Histopathology [Internet]. 2014 Jun;64(7):963–70. Available from: http://doi.wiley.com/10.1111/his.12347

402. Wang L, Li L, Yang Z. Correlation of FOXC1 protein with clinicopathological features in serous ovarian tumors. Oncol Lett [Internet]. 2015 Dec 3;11(2):933–8. Available from: http://www.spandidos-publications.com/10.3892/ol.2015.3996

403. Dallas PB, Egli S, Terry P a, Kees UR. Aberrant over-expression of a forkhead family member, FOXO1A, in a brain tumor cell line. BMC Cancer [Internet]. 2007 Dec

19;7(1):67. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1878494&tool=pmcentrez&rendertype=abstract

404. Arden KC. Multiple roles of FOXO transcription factors in mammalian cells point to multiple roles in cancer. Exp Gerontol. 2006;41(8):709–17.

405. Dong X-Y, Chen C, Sun X, Guo P, Vessella RL, Wang R-X, et al. FOXO1A Is a Candidate for the 13q14 Tumor Suppressor Gene Inhibiting Androgen Receptor Signaling in Prostate Cancer. Cancer Res [Internet]. 2006 Jul 15;66(14):6998–7006. Available from: http://cancerres.aacrjournals.org/lookup/doi/10.1158/0008-5472.CAN-06-0411

406. Kim JH, Kim MK, Lee HE, Cho SJ, Cho YJ, Lee BL, et al. Constitutive phosphorylation of the FOXO1A transcription factor as a prognostic variable in gastric cancer. Mod Pathol [Internet]. 2007 Aug 11;20(8):835–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17491598

407. Wu Y, Shang X, Sarkissyan M, Slamon D, Vadgama J V. FOXO1A Is a Target for HER2-Overexpressing Breast Tumors. Cancer Res [Internet]. 2010 Jul 1;70(13):5475–85. Available from: http://cancerres.aacrjournals.org/content/70/13/5475.short

408. Kohno K, Uchiumi T, Niina I, Wakasugi T, Igarashi T, Momii Y, et al. Transcription factors and drug resistance. Eur J Cancer [Internet]. 2005 Nov;41(16):2577–86. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0959804905007069

409. Li T, Wang H, Sun Y, Zhao L, Gang Y, Guo X, et al. Transcription Factor CUTL1 Is a Negative Regulator of Drug Resistance in Gastric Cancer. J Biol Chem [Internet]. 2013 Feb 8;288(6):4135–47. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.M112.345942

410. Fu L, Garland D, Yang Z, Shukla D, Rajendran A, Pearson E, et al. The R345W mutation in EFEMP1 is pathogenic and causes AMD-like deposits in mice. Hum Mol Genet [Internet]. 2007 Oct 15;16(20):2411–22. Available from: https://academic.oup.com/hmg/article-lookup/doi/10.1093/hmg/ddm198

411. Chen J, Zhang J, Liu X, Fang R, Zhao Y, Ma D. Overexpression of fibulin-4 is associated with tumor progression and poor prognosis in patients with cervical carcinoma. Oncol Rep [Internet]. 2014 Apr 16;31(6):2601–10. Available from: http://www.spandidos-publications.com/10.3892/or.2014.3139

412. Zhou Y-H, Hu Y, Yu L, Ke C, Vo C, Hsu H, et al. Weaponizing human EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) for 21st century cancer therapeutics. Oncoscience [Internet]. 2016 Jul 8;3(7–8). Available from: http://impactjournals.com/oncoscience/index.php?abs=306

413. Hu Y, Ke C, Ru N, Chen Y, Yu L, Siegel ER, et al. Cell context-dependent dual effects of EFEMP1 stabilizes subpopulation equilibrium in responding to changes of *in vivo* growth environment. Oncotarget [Internet]. 2015 Oct 13;6(31):30762–72. Available from: http://www.oncotarget.com/fulltext/5220

414. Lang Y, Meng J, Song X, Chen X. [EFEMP1 suppresses growth and invasion of lung cancer cells by downregulating matrix metalloproteinase-7 expression]. Zhongguo Fei

Ai Za Zhi [Internet]. 2015 Feb;18(2):92–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25676403

415. Hu Y, Pioli P, Siegel E, Zhang Q, Nelson J, Chaturbedi A, et al. EFEMP1 suppresses malignant glioma growth and exerts its action within the tumor extracellular compartment. Mol Cancer [Internet]. 2011;10(1):123. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3204287&tool=pmcentrez&rendertype=abstract

416. Yue W, Dacic S, Sun Q, Landreneau R, Guo M, Zhou W, et al. Frequent Inactivation of RAMP2, EFEMP1 and Dutt1 in Lung Cancer by Promoter Hypermethylation. Clin Cancer Res [Internet]. 2007 Aug 1;13(15):4336–44. Available from: http://clincancerres.aacrjournals.org/cgi/doi/10.1158/1078-0432.CCR-07-0015

417. Sadr-Nabavi A, Ramser J, Volkmann J, Naehrig J, Wiesmann F, Betz B, et al. Decreased expression of angiogenesis antagonist EFEMP1 in sporadic breast cancer is caused by aberrant promoter methylation and points to an impact of EFEMP1 as molecular biomarker. Int J Cancer [Internet]. 2009 Apr 1;124(7):1727–35. Available from: http://doi.wiley.com/10.1002/ijc.24108

418. Sandra D, Wenzel P, Szameitat M, Eser P, Paul MC, Seidler B, et al. Efemp1 and p27Kip1 modulate responsiveness of pancreatic cancer cells towards a dual PI3K/mTOR inhibitor in preclinical models. Oncotarget [Internet]. 2013 Feb 1;4(2):277–88. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3712573&tool=pmcentrez&rendertype=abstract

419. Song E, Hou Y, Yu S, Chen S, Huang J, Luo T, et al. EFEMP1 expression promotes angiogenesis and accelerates the growth of cervical cancer in vivo. Gynecol Oncol [Internet]. 2011 Apr;121(1):174–80. Available from: http://dx.doi.org/10.1016/j.ygyno.2010.11.004

420. Lee YH, Albig AR, Regner M, Schiemann BJ, Schiemann WP. Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-?? in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis. 2008;29(12):2243–51.

421. Mitsuhashi S. Current topics in the biotechnological production of essential amino acids, functional amino acids, and dipeptides. Curr Opin Biotechnol [Internet]. 2014 Apr;26:38–44. Available from: http://dx.doi.org/10.1016/j.copbio.2013.08.020

422. MARCHESINI G, BIANCHI G, MERLI M, AMODIO P, PANELLA C, LOGUERCIO C, et al. Nutritional Supplementation With Branched-Chain Amino Acids in Advanced Cirrhosis: A Double-Blind, Randomized Trial. Gastroenterology [Internet]. 2003;124:1792–801. Available from: http://www.gastrojournal.org/article/S0016-5085(03)00323-8/pdf

423. Szpetnar M, Matras P, Boguszewska-Czubara A, Kiełczykowska M, Rudzki S, Musik I. Is additional enrichment of diet in branched-chain amino acids or glutamine beneficial for patients receiving total parenteral nutrition after gastrointestinal cancer surgery? Adv Clin Exp Med [Internet]. 2014;23(3):423–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24979515

424. Crown SB, Marze N, Antoniewicz MR. Catabolism of Branched Chain Amino Acids Contributes Significantly to Synthesis of Odd-Chain and Even-Chain Fatty Acids in 3T3-L1 Adipocytes. Tochtrop GP, editor. PLoS One [Internet]. 2015 Dec 28;10(12):e0145850. Available from: http://dx.plos.org/10.1371/journal.pone.0145850

425. Zhang S, Zeng X, Ren M, Mao X, Qiao S. Novel metabolic and physiological functions of branched chain amino acids: a review. J Anim Sci Biotechnol [Internet]. 2017 Dec 23;8(1):10. Available from: http://jasbsci.biomedcentral.com/articles/10.1186/s40104-016-0139-z

426. Zhenyukh O, Civantos E, Ruiz-Ortega M, Sánchez MS, Vázquez C, Peiró C, et al. High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation. Free Radic Biol Med [Internet]. 2017 Mar;104:165–77. Available from: http://dx.doi.org/10.1016/j.freeradbiomed.2017.01.009

427. Muller EA, Danner DJ. Tissue-specific Translation of Murine Branched-chain α-Ketoacid Dehydrogenase Kinase mRNA Is Dependent upon an Upstream Open Reading Frame in the 5′-Untranslated Region. J Biol Chem [Internet]. 2004 Oct 22;279(43):44645–55. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.M406550200

428. Ævarsson A, Chuang JL, Max Wynn R, Turley S, Chuang DT, Hol WG. Crystal structure of human branched-chain α-ketoacid dehydrogenase and the molecular basis of multienzyme complex deficiency in maple syrup urine disease. Structure [Internet]. 2000 Mar;8(3):277–91. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0969212600001052

429. Soreze Y, Boutron A, Habarou F, Barnerias C, Nonnenmacher L, Delpech H, et al. Mutations in human lipoyltransferase gene LIPT1 cause a Leigh disease with secondary deficiency for pyruvate and alpha-ketoglutarate dehydrogenase. Orphanet J Rare Dis [Internet]. 2013;8(1):192. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3905285&tool=pmcentrez&rendertype=abstract

430. Peng C, Uygun S, Shiu S-H, Last RL. The Impact of the Branched-Chain Ketoacid Dehydrogenase Complex on Amino Acid Homeostasis in Arabidopsis. Plant Physiol [Internet]. 2015 May 18;169(3):pp.00461.2015. Available from: http://www.plantphysiol.org/content/169/3/1807.abstract

431. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose Tissue Branched Chain Amino Acid (BCAA) Metabolism Modulates Circulating BCAA Levels. J Biol Chem [Internet]. 2010 Apr 9;285(15):11348–56. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M109.075184

432. Sonnet DS, O'Leary MN, A. Gutierrez M, Nguyen SM, Mateen S, Hsu Y, et al. Metformin inhibits Branched Chain Amino Acid (BCAA) derived ketoacidosis and promotes metabolic homeostasis in MSUD. Sci Rep [Internet]. 2016 Sep 4;6(1):28775. Available from: http://www.nature.com/articles/srep28775

433. de Campos-Ferraz PL, Andrade I, das Neves W, Hangai I, Alves CRR, Lancha Jr AH. An overview of amines as nutritional supplements to counteract cancer cachexia. J Cachexia Sarcopenia Muscle [Internet]. 2014 Jun;5(2):105–10. Available from:

http://doi.wiley.com/10.1007/s13539-014-0138-x

434. Kumaran S, Patel M, Jordan F. Nuclear Magnetic Resonance Approaches in the Study of 2-Oxo Acid Dehydrogenase Multienzyme Complexes—A Literature Review. Molecules [Internet]. 2013 Sep 26;18(10):11873–903. Available from: http://www.mdpi.com/1420-3049/18/10/11873/

435. Jia F, Cui M, Than MT, Han M. Developmental Defects of Caenorhabditis elegans Lacking Branched-chain α-Ketoacid Dehydrogenase Are Mainly Caused by Monomethyl Branched-chain Fatty Acid Deficiency. J Biol Chem [Internet]. 2016 Feb 5;291(6):2967–73. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.M115.676650

436. Wynn RM, Li J, Brautigam CA, Chuang JL, Chuang DT. Structural and Biochemical Characterization of Human Mitochondrial Branched-chain -Ketoacid Dehydrogenase Phosphatase. J Biol Chem [Internet]. 2012 Mar 16;287(12):9178–92. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M111.314963

437. Brautigam CA, Wynn RM, Chuang JL, Naik MT, Young BB, Huang T -h., et al. Structural and Thermodynamic Basis for Weak Interactions between Dihydrolipoamide Dehydrogenase and Subunit-binding Domain of the Branched-chain -Ketoacid Dehydrogenase Complex. J Biol Chem [Internet]. 2011 Jul 1;286(26):23476–88. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M110.202960

438. Burrage LC, Nagamani SCS, Campeau PM, Lee BH. Branched-chain amino acid metabolism: from rare Mendelian diseases to more common disorders. Hum Mol Genet [Internet]. 2014 Sep 15;23(R1):R1–8. Available from: https://academic.oup.com/hmg/article-lookup/doi/10.1093/hmg/ddu123

439. Vianna D, Teodoro GFR, Torres-Leal FL, Tirapegui J. Protein synthesis regulation by leucine. Brazilian J Pharm Sci [Internet]. 2010 Mar;46(1):29–36. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1984-82502010000100004&lng=en&tlng=en

440. Sweatt AJ. unique segregation of pathway enzymes in organ systems and peripheral nerves. AJP Endocrinol Metab [Internet]. 2003 May 20;286(1):64E–76. Available from: http://ajpendo.physiology.org/cgi/doi/10.1152/ajpendo.00276.2003

441. John T. Brosnan and Margaret E. Brosnan. Branched-Chain Amino Acids: Enzyme and Substrate Regulation. J Nutr [Internet]. 2006;136:207S–2011S. Available from: http://jn.nutrition.org/content/136/1/207S.full.pdf+html

442. Shimomura Y, Honda T, Shiraki M, Murakami T, Sato J, Kobayashi H, et al. Branched-Chain Amino Acids : Metabolism , Physiological Function , and Application. J Nutr. 2006;136(3):250–3.

443. Lackey DE, Lynch CJ, Olson KC, Mostaedi R, Ali M, Smith WH, et al. Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. AJP Endocrinol Metab [Internet]. 2013 Jun 1;304(11):E1175–87. Available from: http://ajpendo.physiology.org/cgi/doi/10.1152/ajpendo.00630.2012

444. Chang C-F. Solution Structure and Dynamics of the Lipoic Acid-bearing Domain of

Human Mitochondrial Branched-chain alpha -Keto Acid Dehydrogenase Complex. J Biol Chem [Internet]. 2002 Apr 26;277(18):15865–73. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M110952200

445. Besant PG. Inhibition of Branched-Chain alpha -Keto Acid Dehydrogenase Kinase and Sln1 Yeast Histidine Kinase by the Antifungal Antibiotic Radicicol. Mol Pharmacol [Internet]. 2002 Aug 1;62(2):289–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12130680

446. Machius M, Chuang JL, Wynn RM, Tomchick DR, Chuang DT. Structure of rat BCKD kinase: Nucleotide-induced domain communication in a mitochondrial protein kinase. Proc Natl Acad Sci [Internet]. 2001 Sep 25;98(20):11218–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11562470

447. Oyarzabal A, Martínez-Pardo M, Merinero B, Navarrete R, Desviat LR, Ugarte M, et al. A Novel Regulatory Defect in the Branched-Chain α-Keto Acid Dehydrogenase Complex Due to a Mutation in the PPM 1 K Gene Causes a Mild Variant Phenotype of Maple Syrup Urine Disease. Hum Mutat [Internet]. 2013 Feb;34(2):355–62. Available from: http://doi.wiley.com/10.1002/humu.22242

448. Islam MM, Wallin R, Wynn RM, Conway M, Fujii H, Mobley JA, et al. A Novel Branched-chain Amino Acid Metabolon: PROTEIN-PROTEIN INTERACTIONS IN A SUPRAMOLECULAR COMPLEX. J Biol Chem [Internet]. 2007 Apr 20;282(16):11893–903. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M700198200

449. Gohara DW, Di Cera E. Molecular Mechanisms of Enzyme Activation by Monovalent Cations. J Biol Chem [Internet]. 2016 Sep 30;291(40):20840–8. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.R116.737833

450. Howarth KR, Burgomaster KA, Phillips SM, Gibala MJ. Exercise training increases branched-chain oxoacid dehydrogenase kinase content in human skeletal muscle. AJP Regul Integr Comp Physiol [Internet]. 2007 Jun 20;293(3):R1335–41. Available from: http://ajpregu.physiology.org/cgi/doi/10.1152/ajpregu.00115.2007

451. García-Cazorla A, Oyarzabal A, Fort J, Robles C, Castejón E, Ruiz-Sala P, et al. Two Novel Mutations in the BCKDK (Branched-Chain Keto-Acid Dehydrogenase Kinase) Gene Are Responsible for a Neurobehavioral Deficit in Two Pediatric Unrelated Patients. Hum Mutat [Internet]. 2014 Apr;35(4):470–7. Available from: http://doi.wiley.com/10.1002/humu.22513

452. Kimball SR, Jefferson LS. Signaling Pathways and Molecular Mechanisms through which Branched-Chain Amino Acids Mediate Translational Control of. 2006;(6):227–31.

453. Kitsy A, Carney S, Vivar JC, Knight MS, Pointer MA, Gwathmey JK, et al. Effects of Leucine Supplementation and Serum Withdrawal on Branched-Chain Amino Acid Pathway Gene and Protein Expression in Mouse Adipocytes. Chen X, editor. PLoS One [Internet]. 2014 Jul 22;9(7):e102615. Available from: http://dx.plos.org/10.1371/journal.pone.0102615

454. Li Z, Murakami T, Nakai N, Nagasaki M, Obayashi M, Xu M, et al. Modification of Hepatic by Exercise Training of Activity and Enzyme Expression Dehydrogenase

Complex in Diabetic Rats , Graduate School of Medicine and 5 Research Center of Health , In the catabolism of branched-chain amino acids (BCAA), the initial ste. 2001;345–50.

455. Zigler JS, Hodgkinson CA, Wright M, Klise A, Sundin O, Broman KW, et al. A Spontaneous Missense Mutation in Branched Chain Keto Acid Dehydrogenase Kinase in the Rat Affects Both the Central and Peripheral Nervous Systems. Nógrádi A, editor. PLoS One [Internet]. 2016 Jul 29;11(7):e0160447. Available from: http://dx.plos.org/10.1371/journal.pone.0160447

456. Wynn RM, Kato M, Machius M, Chuang JL, Li J, Tomchick DR, et al. Molecular Mechanism for Regulation of the Human Mitochondrial Branched-Chain α-Ketoacid Dehydrogenase Complex by Phosphorylation. Structure [Internet]. 2004 Dec;12(12):2185–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15576032

457. Novarino G, El-Fishawy P, Kayserili H, Meguid NA, Scott EM, Schroth J, et al. Mutations in BCKD-kinase Lead to a Potentially Treatable Form of Autism with Epilepsy. Science (80-) [Internet]. 2012 Oct 19;338(6105):394–7. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1224631

458. Lu G, Sun H, She P, Youn J, Warburton S, Ping P, et al. Protein phosphatase 2Cm is a critical regulator of branched-chain amino acid catabolism in mice and cultured cells. J Clin Invest [Internet]. 2009 Jun 1;119(6):1678–87. Available from: http://www.jci.org/articles/view/38151

459. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. AJP Endocrinol Metab [Internet]. 2007 Oct 23;293(6):E1552–63. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2767201&tool=pmcentrez&rendertype=abstract

460. AKITA K, FUJIMURA Y, BAJOTTO G, SHIMOMURA Y. Inhibition of Branched-Chain α-Ketoacid Dehydrogenase Kinase by Thiamine Pyrophosphate at Different Potassium Ionic Levels. Biosci Biotechnol Biochem [Internet]. 2009 May 23;73(5):1189–91. Available from: http://www.tandfonline.com/doi/full/10.1271/bbb.80749

461. Vogel KR, Kennedy AA, Whitehouse LA, Gibson KM. Therapeutic hepatocyte transplant for inherited metabolic disorders: functional considerations, recent outcomes and future prospects. J Inherit Metab Dis. 2014;37(2):165–76.

462. Newgard CB. Interplay between Lipids and Branched-Chain Amino Acids in Development of Insulin Resistance. Cell Metab [Internet]. 2012 May;15(5):606–14. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1550413112001039

463. Zhen H, Kitaura Y, Kadota Y, Ishikawa T, Kondo Y, Xu M, et al. mTORC1 is involved in the regulation of branched-chain amino acid catabolism in mouse heart. FEBS Open Bio [Internet]. 2016 Jan;6(1):43–9. Available from: http://doi.wiley.com/10.1002/2211-5463.12007

464. Hutson SM. The case for regulating indispensable amino acid metabolism: the branched-chain α-keto acid dehydrogenase kinase-knockout mouse. Biochem J

[Internet]. 2006 Nov 15;400(1):e1. Available from: http://www.biochemj.org/content/400/1/e1.abstract

465. Andrew C. Shin, Martin Fasshauer, Nika Filatova, Linus A. Grundell E, Zielinski, Jian-Ying Zhou, Thomas Scherer, Claudia Lindtner, Phillip J. White A, L. Lapworth, Olga Ilkayeva, Uwe Knippschild, Anna M. Wolf, Ludger Scheja KL, Grove, Richard D. Smith, Wei-Jun Qian, Christopher J. Lynch CB, Newgard and CB. Brain insulin lowers circulating BCAA levels by inducing hepatic BCAA catabolism. Cell Metab. 2014;20(4):898–909.

466. Mullur R, Liu Y-Y, Brent GA. Thyroid Hormone Regulation of Metabolism. Physiol Rev [Internet]. 2014 Apr 1;94(2):355–82. Available from: http://physrev.physiology.org/cgi/doi/10.1152/physrev.00030.2013

467. Su Y, Lam TKT, He W, Pocai A, Bryan J, Aguilar-Bryan L, et al. Hypothalamic Leucine Metabolism Regulates Liver Glucose Production. Diabetes [Internet]. 2012 Jan 1;61(1):85–93. Available from: http://diabetes.diabetesjournals.org/cgi/doi/10.2337/db11-0857

468. Harris RA, Kobayashi R, Murakami T, Shimomura Y. Regulation of Branched-Chain a-Keto Acid Dehydrogenase Kinase Expression in Rat Liver. J Nutr. 2001;131:841–5.

469. Li Z-L, Wang Y-H, Chu J, Zhuang Y-P, Zhang S-L. Effect of branched-chain amino acids, valine, isoleucine and leucine on the biosythesis of bitespiramycin 4"-O-acylspiramycins. Brazilian J Microbiol [Internet]. 2009 Dec;40(4):734–46. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822009000400003&lng=en&nrm=iso&tlng=en

470. Kainulainen H, Hulmi JJ, Kujala UM. Potential Role of Branched-Chain Amino Acid Catabolism in Regulating Fat Oxidation. Exerc Sport Sci Rev [Internet]. 2013;41(4):194–200. Available from: http://0-search.ebscohost.com.library.ucc.ie/login.aspx?direct=true&db=s3h&AN=90582224&site=ehost-live

471. Adeva MM, Calviño J, Souto G, Donapetry C. Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids [Internet]. 2012 Jul 8;43(1):171–81. Available from: http://link.springer.com/10.1007/s00726-011-1088-7

472. Cooper A, Jeitner T. Central Role of Glutamate Metabolism in the Maintenance of Nitrogen Homeostasis in Normal and Hyperammonemic Brain. Biomolecules [Internet]. 2016 Mar 26;6(2):16. Available from: http://www.mdpi.com/2218-273X/6/2/16

473. Newman JC, He W, Verdin E. Mitochondrial Protein Acylation and Intermediary Metabolism: Regulation by Sirtuins and Implications for Metabolic Disease. J Biol Chem [Internet]. 2012 Dec 14;287(51):42436–43. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.R112.404863

474. She P, Zhou Y, Zhang Z, Griffin K, Gowda K, Lynch CJ. Disruption of BCAA metabolism in mice impairs exercise metabolism and endurance. J Appl Physiol [Internet]. 2010 Apr 1;108(4):941–9. Available from: http://www.jappl.org/content/108/4/941.short%5Cnpapers3://publication/doi/10.1152/japplphysiol.01248.2009

475. Williams M. Dietary Supplements and Sports Performance: Amino Acids. J Int Soc Sports Nutr [Internet]. 2005;2(2):63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18500957%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2129148

476. Monteiro GA, Aoki MS, Santos CB, Monteiro AG, Russo A, Piçarro IC. Effects of branched-chain amino acid (BCAA) supplementation on endurance exercise performance of pregnant rats. Sci Sports [Internet]. 2009 Apr;24(2):102–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0765159708001184

477. Matsumoto K, Koba T, Hamada K, Tsujimoto H, Mitsuzono R. Branched-chain amino acid supplementation increases the lactate threshold during an incremental exercise test in trained individuals. J Nutr Sci Vitaminol (Tokyo) [Internet]. 2009 Feb;55(1):52–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19352063

478. Hatazawa Y, Tadaishi M, Nagaike Y, Morita A, Ogawa Y, Ezaki O, et al. PGC-1α-Mediated Branched-Chain Amino Acid Metabolism in the Skeletal Muscle. Csernoch L, editor. PLoS One [Internet]. 2014 Mar 17;9(3):e91006. Available from: http://dx.plos.org/10.1371/journal.pone.0091006

479. Higuchi N, Kato M, Miyazaki M, Tanaka M, Kohjima M, Ito T, et al. Potential role of branched-chain amino acids in glucose metabolism through the accelerated induction of the glucose-sensing apparatus in the liver. J Cell Biochem [Internet]. 2011 Jan;112(1):30–8. Available from: http://doi.wiley.com/10.1002/jcb.22688

480. Valerio A, D'Antona G, Nisoli E. Branched-chain amino acids, mitochondrial biogenesis, and healthspan: an evolutionary perspective. Aging (Albany NY) [Internet]. 2011 Apr 30;3(5):464–78. Available from: http://www.aging-us.com/article/100322

481. Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med [Internet]. 2014 Sep 28;20(10):1193–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25261994

482. Chen T, Ni Y, Ma X, Bao Y, Liu J, Huang F, et al. Branched-chain and aromatic amino acid profiles and diabetes risk in Chinese populations. Sci Rep [Internet]. 2016 Aug 5;6(1):20594. Available from: http://www.nature.com/srep/2016/160205/srep20594/full/srep20594.html

483. Kujala UM, Peltonen M, Laine MK, Kaprio J, Heinonen OJ, Sundvall J, et al. Branched-Chain Amino Acid Levels Are Related with Surrogates of Disturbed Lipid Metabolism among Older Men. Front Med [Internet]. 2016 Nov 25;3(November):57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27933294%5Cnhttp://journal.frontiersin.org/article/10.3389/fmed.2016.00057/full

484. van Loon LJC, Kruijshoop M, Menheere PPCA, Wagenmakers AJM, Saris WHM, Keizer HA. Amino Acid Ingestion Strongly Enhances Insulin Secretion in Patients With Long-Term Type 2 Diabetes. Diabetes Care [Internet]. 2003 Mar 1;26(3):625–30. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12610012

485. Lotta LA, Scott RA, Sharp SJ, Burgess S, Luan J, Tillin T, et al. Genetic Predisposition to an Impaired Metabolism of the Branched-Chain Amino Acids and Risk of Type 2 Diabetes: A Mendelian Randomisation Analysis. Minelli C, editor. PLOS Med [Internet]. 2016 Nov 29;13(11):e1002179. Available from: http://dx.plos.org/10.1371/journal.pmed.1002179

486. Chuang DT, Chuang JL, Wynn RM. Lessons from Genetic Disorders of Branched-Chain Amino. J Nutr. 2006;243–9.

487. Manoli I, Venditti CP. Disorders of branched chain amino acid metabolism. Transl Sci Rare Dis [Internet]. 2016 Nov 7;1(2):91–110. Available from: http://www.medra.org/servlet/aliasResolver?alias=iospress&doi=10.3233/TRD-160009

488. Hiroshige K. Oral supplementation of branched-chain amino acid improves nutritional status in elderly patients on chronic haemodialysis. Nephrol Dial Transplant [Internet]. 2001 Sep 1;16(9):1856–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11522870

489. Tajiri K. Branched-chain amino acids in liver diseases. World J Gastroenterol [Internet]. 2013;19(43):7620. Available from: http://www.wjgnet.com/1007-9327/full/v19/i43/7620.htm

490. Ishikawa T, Kitaura Y, Kadota Y, Morishita Y, Ota M, Yamanaka F, et al. Muscle-specific deletion of BDK amplifies loss of myofibrillar protein during protein undernutrition. Sci Rep [Internet]. 2017 Jan 4;7(January):39825. Available from: http://www.nature.com/articles/srep39825

491. De Simone R, Vissicchio F, Mingarelli C, De Nuccio C, Visentin S, Ajmone-Cat MA, et al. Branched-chain amino acids influence the immune properties of microglial cells and their responsiveness to pro-inflammatory signals. Biochim Biophys Acta - Mol Basis Dis [Internet]. 2013 May;1832(5):650–9. Available from: http://dx.doi.org/10.1016/j.bbadis.2013.02.001

492. Yang P, Hu W, Fu Z, Sun L, Zhou Y, Gong Y, et al. The positive association of branched-chain amino acids and metabolic dyslipidemia in Chinese Han population. Lipids Health Dis [Internet]. 2016 Dec 25;15(1):120. Available from: http://lipidworld.biomedcentral.com/articles/10.1186/s12944-016-0291-7

493. Ferguson JF, Wang TJ. Branched-Chain Amino Acids and Cardiovascular Disease: Does Diet Matter? Clin Chem [Internet]. 2016 Apr 1;62(4):545–7. Available from: http://www.clinchem.org/cgi/doi/10.1373/clinchem.2016.254318

494. Frank J, Gao H, Sengupta J, Gao N, Taylor DJ. The process of mRNA-tRNA translocation. Proc Natl Acad Sci [Internet]. 2007 Dec 11;104(50):19671–8. Available from: http://www.pnas.org/content/104/50/19671.long%5Cnhttp://www.pnas.org/cgi/doi/10.1073/pnas.0708517104

495. Rennie MJ, Bohe J, Smith K, Wackerhage H, Greenhaff P. Branched-Chain Amino Acids as Fuels and Anabolic Signals in Human. J Nutr. 2006;136:264–8.

496. Karlsson KR, Ko R, Blomstrand E. Branched-Chain Amino Acids Activate Key Enzymes in Protein Synthesis. J Nutr. 2006;136(15):269–73.

497. Garlick PJ. The role of leucine in the regulation of protein metabolism. J Nutr [Internet]. 2005 Jun;135(6 Suppl):1553S–6S. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15930468

498. Glynn EL, Fry CS, Drummond MJ, Timmerman KL, Dhanani S, Volpi E, et al. Excess Leucine Intake Enhances Muscle Anabolic Signaling but Not Net Protein Anabolism in Young Men and Women. J Nutr [Internet]. 2010 Nov 1;140(11):1970–6. Available from: http://jn.nutrition.org/cgi/doi/10.3945/jn.110.127647

499. Kato H, Suzuki H, Mimura M, Inoue Y, Sugita M, Suzuki K, et al. Leucine-enriched essential amino acids attenuate muscle soreness and improve muscle protein synthesis after eccentric contractions in rats. Amino Acids [Internet]. 2015 Jun 14;47(6):1193–201. Available from: http://dx.doi.org/10.1007/s00726-015-1946-9

500. Wang XJ, Yang X, Wang RX, Jiao HC, Zhao JP, Song ZG, et al. Leucine alleviates dexamethasone-induced suppression of muscle protein synthesis via synergy involvement of mTOR and AMPK pathways. Biosci Rep [Internet]. 2016 Jun 17;36(3):e00346–e00346. Available from: http://www.bioscirep.org/content/early/2016/04/28/BSR20160096%5Cnhttp://www.bioscirep.org/content/early/2016/04/28/BSR20160096.long%5Cnhttp://www.bioscirep.org/content/ppbioscirep/early/2016/04/28/BSR20160096.full.pdf%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed

501. Manjarín R, Columbus DA, Suryawan A, Nguyen H V., Hernandez-García AD, Hoang N-M, et al. Leucine supplementation of a chronically restricted protein and energy diet enhances mTOR pathway activation but not muscle protein synthesis in neonatal pigs. Amino Acids [Internet]. 2016 Jan 3;48(1):257–67. Available from: http://link.springer.com/10.1007/s00726-015-2078-y

502. Meijer AJ, Lorin S, Blommaart EF, Codogno P. Regulation of autophagy by amino acids and MTOR-dependent signal transduction. Amino Acids. 2014;47(10):2037–63.

503. Linares JF, Duran A, Reina-Campos M, Aza-Blanc P, Campos A, Moscat J, et al. Amino Acid Activation of mTORC1 by a PB1-Domain-Driven Kinase Complex Cascade. Cell Rep [Internet]. 2015 Aug;12(8):1339–52. Available from: http://dx.doi.org/10.1016/j.celrep.2015.07.045

504. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov [Internet]. 2009 Aug;8(8):627–44. Available from: http://www.nature.com/doifinder/10.1038/nrd2926

505. Deng H, Zheng A, Liu G, Chang W, Zhang S, Cai H. Activation of mammalian target of rapamycin signaling in skeletal muscle of neonatal chicks: Effects of dietary leucine and age. Poult Sci [Internet]. 2014 Jan 1;93(1):114–21. Available from: http://psoxfordjournals.wowweightlossing.com/cgi/content/full/93/1/114%5Cnhttp://ps.oxfordjournals.org/cgi/doi/10.3382/ps.2013-03287

506. Ahmad A, Biersack B, Li Y, Kong D, Bao B, Schobert R, et al. Targeted Regulation of PI3K/Akt/mTOR/NF-κB Signaling by Indole Compounds and their Derivatives: Mechanistic Details and Biological Implications for Cancer Therapy. Anticancer Agents Med Chem [Internet]. 2013 Jul 1;13(7):1002–13. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3901097&tool=pmcentrez&rendertype=abstract

507. Hanahan D, Weinberg RA. The Hallmarks of Cancer Review. Cell. 2000;100:57–70.

508. Lawrence MS, Stojanov P, Polak P, Kryukov G V, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature [Internet]. 2013 Jun 16;499(7457):214–8. Available from: http://dx.doi.org/10.1038/nature12213

509. Laplante M, Sabatini DM. mTOR Signaling in Growth Control and Disease. Cell [Internet]. 2012 Apr;149(2):274–93. Available from: http://dx.doi.org/10.1016/j.cell.2012.03.017

510. Ekim B, Magnuson B, Acosta-Jaquez HA, Keller JA, Feener EP, Fingar DC. mTOR Kinase Domain Phosphorylation Promotes mTORC1 Signaling, Cell Growth, and Cell Cycle Progression. Mol Cell Biol [Internet]. 2011 Jul 15;31(14):2787–801. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21576368%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3133410

511. Wang X, Proud CG. mTORC2 is a tyrosine kinase. Cell Res [Internet]. 2016 Jan 20;26(1):1–2. Available from: http://www.nature.com/doifinder/10.1038/cr.2015.134

512. Vadlakonda L, Dash A, Pasupuleti M, Anil Kumar K, Reddanna P. The Paradox of Akt-mTOR Interactions. Front Oncol [Internet]. 2013;3(June):165. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23802099%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3687210

513. Yoon M-S. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. Nutrients [Internet]. 2016 Jul 1;8(7):405. Available from: http://www.mdpi.com/2072-6643/8/7/405

514. K. Sreekumaran Nair and Kevin R. Short. Interrelationship between Physical Activity and Branched-Chain Amino Acids 1. 2005;1547S–1552S.

515. Zhang Y, Yang J-M. Altered energy metabolism in cancer. Cancer Biol Ther [Internet]. 2013 Feb 27;14(2):81–9. Available from: http://www.tandfonline.com/doi/abs/10.4161/cbt.22958

516. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. Sci Adv [Internet]. 2016 May 27;2(5):e1600200–e1600200. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27386546%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4928883

517. Eales KL, Hollinshead KER, Tennant DA. Hypoxia and metabolic adaptation of cancer cells. Oncogenesis [Internet]. 2016 Jan 25;5(1):e190. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4728679&tool=pmcentrez&rendertype=abstract

518. Yoshizawa F. New Therapeutic Strategy for Amino Acid Medicine: Notable Functions of Branched Chain Amino Acids as Biological Regulators. J Pharmacol Sci [Internet]. 2012;118(2):149–55. Available from: http://joi.jlc.jst.go.jp/JST.JSTAGE/jphs/11R05FM?from=CrossRef

519. Chen J, Lee H-J, Wu X, Huo L, Kim S-J, Xu L, et al. Gain of Glucose-Independent

Growth upon Metastasis of Breast Cancer Cells to the Brain. Cancer Res [Internet]. 2015 Feb 1;75(3):554–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25511375

520. Choudry HA, Pan M, Karinch AM, Souba WW. Branched-Chain Amino Acid-Enriched Nutritional Support in Surgical and Cancer Patients. J Nutr. 2006;136:314S–318S.

521. MacDonald AJ, Johns N, Stephens N, Greig C, Ross JA, Small AC, et al. Habitual myofibrillar protein synthesis is normalin patients with upper GI cancer cachexia. Clin Cancer Res. 2015;21(7):1734–40.

522. Baracos VE, Mackenzie ML. Branched-Chain Amino Acids : Metabolism , Physiological Function , and Application Investigations of Branched-Chain Amino Acids and Their Metabolites in. 2006;237–42.

523. O'Connell T. The Complex Role of Branched Chain Amino Acids in Diabetes and Cancer. Metabolites [Internet]. 2013;3(4):931–45. Available from: http://www.mdpi.com/2218-1989/3/4/931/

524. Bifari F, Nisoli E. Branched-chain amino acids differently modulate catabolic and anabolic states in mammals: a pharmacological point of view. Br J Pharmacol [Internet]. 2017 Jun;174(11):1366–77. Available from: http://doi.wiley.com/10.1111/bph.13624

525. Liu K a, Lashinger LM, Rasmussen AJ, Hursting SD. Leucine supplementation differentially enhances pancreatic cancer growth in lean and overweight mice. Cancer Metab [Internet]. 2014;2(1):6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24685128

526. Xiao F, Wang C, Yin H, Yu J, Chen S, Fang J. Leucine deprivation inhibits proliferation and induces apoptosis of human breast cancer cells via fatty acid synthase. 2016;7(39):1–11.

527. Alkonyi B, Chugani HT, Muzik O, Chugani DC, Sundaram SK, Kupsky WJ, et al. Increased L-[1-11C] Leucine Uptake in the Leptomeningeal Angioma of Sturge-Weber Syndrome: A PET Study. J Neuroimaging [Internet]. 2012 Apr;22(2):177–83. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3135745/

528. Vesselle H, Salskov A, Turcotte E, Wiens L, Schmidt R, Jordan CD, et al. Relationship between non-small cell lung cancer FDG uptake at PET, tumor histology, and Ki-67 proliferation index. J Thorac Oncol [Internet]. 2008;3(9):971–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18758298

529. Xue P, Zeng F, Duan Q, Xiao J, Liu L, Yuan P, et al. BCKDK of BCAA Catabolism Cross-talking With the MAPK Pathway Promotes Tumorigenesis of Colorectal Cancer. EBioMedicine [Internet]. 2017 Jun;20:50–60. Available from: http://www.sciencedirect.com/science/article/pii/S2352396417301925

530. Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med [Internet]. 2014 Sep 28;20(10):1193–8. Available from: http://www.nature.com/doifinder/10.1038/nm.3686

531. Hattori A, Tsunoda M, Konuma T, Kobayashi M, Nagy T, Glushka J, et al. Cancer progression by reprogrammed BCAA metabolism in myeloid leukaemia. Nature [Internet]. 2017 May 17;545(7655):500–4. Available from: http://www.nature.com/doifinder/10.1038/nature22314

532. Zhang L, Han J. Branched-chain amino acid transaminase 1 (BCAT1) promotes the growth of breast cancer cells through improving mTOR-mediated mitochondrial biogenesis and function. Biochem Biophys Res Commun [Internet]. 2017 Apr;486(2):224–31. Available from: http://dx.doi.org/10.1016/j.bbrc.2017.02.101

533. Wang Z-Q, Faddaoui A, Bachvarova M, Plante M, Gregoire J, Renaud M-C, et al. BCAT1 expression associates with ovarian cancer progression: possible implications in altered disease metabolism. Oncotarget [Internet]. 2015 Oct 13;6(31):31522–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26372729%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4741622

534. Mayers JR, Torrence ME, Danai L V., Papagiannakopoulos T, Davidson SM, Bauer MR, et al. Tissue of origin dictates branched-chain amino acid metabolism in mutant Kras-driven cancers. Science (80-) [Internet]. 2016 Sep 9;353(6304):1161–5. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.aaf5171

535. Martineau Y, Müller D, Pyronnet S. Targeting protein synthesis in cancer cells. Oncoscience [Internet]. 2014 Jul 4;1(7):484. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4278317&tool=pmcentrez&rendertype=abstract

536. Liu KA, Lashinger LM, Rasmussen AJ, Hursting SD. Leucine supplementation differentially enhances pancreatic cancer growth in lean and overweight mice. Cancer Metab [Internet]. 2014 Dec 31;2(1):27. Available from: http://cancerandmetabolism.biomedcentral.com/articles/10.1186/2049-3002-2-6

537. Altomare DA, Khaled AR. Homeostasis and the importance for a balance between AKT/mTOR activity and intracellular signaling. Curr Med Chem [Internet]. 2012;19(22):3748–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22680924%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3414727

538. Mersey BD. Human microRNA (miR29b) expression controls the amount of branched chain -ketoacid dehydrogenase complex in a cell. Hum Mol Genet [Internet]. 2005 Oct 12;14(22):3371–7. Available from: https://academic.oup.com/hmg/article-lookup/doi/10.1093/hmg/ddi368

539. Horstman AMH, Damink SWO, Schols AMWJ, van Loon LJC. Is cancer cachexia attributed to impairments in basal or postprandial muscle protein metabolism? Nutrients. 2016;8(8):4–9.

540. Lerin C, Goldfine AB, Boes T, Liu M, Kasif S, Dreyfuss JM, et al. Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism. Mol Metab [Internet]. 2016 Oct;5(10):926–36. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2212877816301156

541. Doering CB, Danner DJ. Amino acid deprivation induces translation of branched-chain

alpha-ketoacid dehydrogenase kinase. Am J Physiol Cell Physiol [Internet]. 2000 Nov;279(5):C1587-94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11029306

542. Tso S-C, Qi X, Gui W-J, Chuang JL, Morlock LK, Wallace AL, et al. Structure-based design and mechanisms of allosteric inhibitors for mitochondrial branched-chain -ketoacid dehydrogenase kinase. Proc Natl Acad Sci [Internet]. 2013 Jun 11;110(24):9728–33. Available from: http://www.pnas.org/cgi/pmidlookup?view=long&pmid=23716694

543. Pardridge WM, Casanello-Ertl D, Duducgian-Vartavarian L. Branched chain amino acid oxidation in isolated skeletal muscle cells: Selective inhibition by therapeutic levels of clofibric acid. Clin Res. 1980;28(1):88–93.

544. Danner DJ, Sewell ET, Elsas LJ. Clofibric Acid and Phenylpyruvic Acid as Biochemical Probes for Studying Soluble Bovine Liver Branched Chain Ketoacid Dehydrogenase. J Biol Chem. 1982;257(2):659–62.

545. Corcoran J, Winter MJ, Lange A, Cumming R, Owen SF, Tyler CR. Effects of the lipid regulating drug clofibric acid on PPARα-regulated gene transcript levels in common carp (Cyprinus carpio) at pharmacological and environmental exposure levels. Aquat Toxicol [Internet]. 2015 Apr;161:127–37. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0166445X15000417

546. Harris RA, Paxton R, Depaoli-roach AA. Inhibition of Branched Chain alpha-Ketoacid Dehydrogenase Kinase Activity by alpha-Chloroisocaproat. J Biol Chem [Internet]. 1982;257(23):13915–8. Available from: http://www.jbc.org/content/257/23/13915.full.pdf

547. Lau KS, Fatania HR, Randle PJ. Regulation of the branched chain 2-oxoacid dehydrogenase kinase reaction. FEBS Lett [Internet]. 1982 Jul 19;144(1):57–62. Available from: http://doi.wiley.com/10.1016/0014-5793%2882%2980568-1

548. Tso S-C, Gui W-J, Wu C-Y, Chuang JL, Qi X, Skvorak KJ, et al. Benzothiophene Carboxylate Derivatives as Novel Allosteric Inhibitors of Branched-chain α-Ketoacid Dehydrogenase Kinase. J Biol Chem [Internet]. 2014 Jul 25;289(30):20583–93. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.M114.569251

549. Friberg A, Vigil D, Zhao B, Daniels RN, Burke JP, Pedro M, et al. Discovery of potent myeloid cell leukemia 1 (Mcl 1) inhibitors using fragment based methods and structure based design. 2014;56(1):15–30.

550. Tassi RA, Todeschini P, Siegel ER, Calza S, Cappella P, Ardighieri L, et al. FOXM1 expression is significantly associated with chemotherapy resistance and adverse prognosis in non-serous epithelial ovarian cancer patients. J Exp Clin Cancer Res [Internet]. 2017 Dec 8;36(1):63. Available from: http://jeccr.biomedcentral.com/articles/10.1186/s13046-017-0536-y

551. Smith HJ, Straughn JM, Buchsbaum DJ, Arend RC. Epigenetic therapy for the treatment of epithelial ovarian cancer: A clinical review. Gynecol Oncol Reports [Internet]. 2017 May;20:81–6. Available from: http://dx.doi.org/10.1016/j.gore.2017.03.007

552. Zhenyukh O, Civantos E, Ruiz-Ortega M, Sánchez MS, Vázquez C, Peiró C, et al. High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation. Free Radic Biol Med [Internet]. 2017 Mar;104(January):165–77. Available from: http://dx.doi.org/10.1016/j.freeradbiomed.2017.01.009

553. Jung CH, Ro S-H, Cao J, Otto NM, Kim D-H. mTOR regulation of autophagy. FEBS Lett [Internet]. 2010 Apr 2;584(7):1287–95. Available from: http://doi.wiley.com/10.1016/j.febslet.2010.01.017

554. Nishida H, Ikegami A, Kaneko C, Kakuma H, Nishi H, Tanaka N, et al. Dexamethasone and BCAA Failed to Modulate Muscle Mass and mTOR Signaling in GH-Deficient Rats. Musaro A, editor. PLoS One [Internet]. 2015 Jun 18;10(6):e0128805. Available from: http://dx.plos.org/10.1371/journal.pone.0128805

555. Jeong W, Doroshow JH, Kummar S. United States Food and Drug Administration approved oral kinase inhibitors for the treatment of malignancies. Curr Probl Cancer [Internet]. 2013 May;37(3):110–44. Available from: http://doi.wiley.com/10.1002/ana.22528

556. Abramson HN. Kinase inhibitors as potential agents in the treatment of multiple myeloma. Oncotarget [Internet]. 2015 Jul 18;7(49):81926–68. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27655636

557. Grippo P, Maker A V. Targeting cancer with tumor-specific therapeutic strategies—metabolic reprogramming beyond the Warburg effect. Transl Cancer Res [Internet]. 2017 May 21;6(S3):S585–6. Available from: http://www.annualreviews.org/doi/10.1146/annurev-immunol-032713-120240

558. Marchetti C, Kristeleit R, McCormack M, Mould T, Olaitan A, Widschwendter M, et al. Outcome of patients with advanced ovarian cancer who do not undergo debulking surgery: A single institution retrospective review. Gynecol Oncol [Internet]. 2017 Jan;144(1):57–60. Available from: http://dx.doi.org/10.1016/j.ygyno.2016.11.001

559. Pepa C Della, Tonini G, Pisano C, Di Napoli M, Cecere SC, Tambaro R, et al. Ovarian cancer standard of care: are there real alternatives? Chin J Cancer [Internet]. 2015 Jan 5;34(1):17–27. Available from: http://www.cjcsysu.cn/abstract.asp?fr=doi&idno=21273

560. Gabra H. Introduction to managing patients with recurrent ovarian cancer. Eur J Cancer Suppl [Internet]. 2014 Dec;12(2):2–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1359634915700030

561. Hui L-M, Zhao G-D, Zhao J-J. δ-Cadinene inhibits the growth of ovarian cancer cells via caspase-dependent apoptosis and cell cycle arrest. Int J Clin Exp Pathol [Internet]. 2015;8(6):6046–56. Available from: www.ijcep.com

562. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, et al. Molecular mechanisms of cisplatin resistance. Oncogene [Internet]. 2012 Apr 12;31(15):1869–83. Available from: http://www.nature.com/doifinder/10.1038/onc.2011.384

563. Fouad YA, Aanei C. Revisiting the hallmarks of cancer. Am J Cancer Res [Internet]. 2017 Jan;7(5):1016–36. Available from:

http://linkinghub.elsevier.com/retrieve/pii/S1526952309004073

564. Wang Y, Compton C, Rankin GO, Cutler SJ, Rojanasakul Y, Tu Y, et al. 3-Hydroxyterphenyllin, a natural fungal metabolite, induces apoptosis and S phase arrest in human ovarian carcinoma cells. Int J Oncol [Internet]. 2017 Apr;50(4):1392–402. Available from: https://www.spandidos-publications.com/

565. Stamelos VA, Fisher N, Bamrah H, Voisey C, Price JC, Farrell WE, et al. The BH3 Mimetic Obatoclax Accumulates in Lysosomes and Causes Their Alkalinization. Villunger A, editor. PLoS One [Internet]. 2016 Mar 7;11(3):e0150696. Available from: http://dx.plos.org/10.1371/journal.pone.0150696

566. Lheureux S, N'Diaye M, Blanc-Fournier C, Dugué AE, Clarisse B, Dutoit S, et al. Identification of predictive factors of response to the BH3-mimetic molecule ABT-737: An ex vivo experiment in human serous ovarian carcinoma. Int J Cancer [Internet]. 2015 Mar 1;136(5):E340–50. Available from: http://doi.wiley.com/10.1002/ijc.29104

567. Zervantonakis IK, Iavarone C, Chen H-Y, Selfors LM, Palakurthi S, Liu JF, et al. Systems analysis of apoptotic priming in ovarian cancer identifies vulnerabilities and predictors of drug response. Nat Commun [Internet]. 2017 Dec 28;8(1):365. Available from: http://www.nature.com/articles/s41467-017-00263-7

568. Brahmbhatt H, Oppermann S, Osterlund EJ, Leber B, Andrews DW. Molecular Pathways: Leveraging the BCL-2 Interactome to Kill Cancer Cells--Mitochondrial Outer Membrane Permeabilization and Beyond. Clin Cancer Res [Internet]. 2015 Jun 15;21(12):2671–6. Available from: http://clincancerres.aacrjournals.org/cgi/doi/10.1158/1078-0432.CCR-14-0959

569. Seo SU, Cho HK, Min K, Woo SM, Kim S, Park J-W, et al. Thioridazine enhances sensitivity to carboplatin in human head and neck cancer cells through downregulation of c-FLIP and Mcl-1 expression. Cell Death Dis [Internet]. 2017 Feb 9;8(2):e2599. Available from: http://www.nature.com/doifinder/10.1038/cddis.2017.8

570. Pei X-Y, Dai Y, Felthousen J, Chen S, Takabatake Y, Zhou L, et al. Circumvention of Mcl-1-Dependent Drug Resistance by Simultaneous Chk1 and MEK1/2 Inhibition in Human Multiple Myeloma Cells. Gartel AL, editor. PLoS One [Internet]. 2014 Mar 4;9(3):e89064. Available from: http://dx.plos.org/10.1371/journal.pone.0089064

571. Bose P, Grant S. Mcl-1 as a therapeutic target in acute myelogenous leukemia (AML). Leuk Res Reports [Internet]. 2013;2(1):12–4. Available from: http://dx.doi.org/10.1016/j.lrr.2012.11.006

572. Choudhary GS, Tat TT, Misra S, Hill BT, Smith MR, Almasan A, et al. Cyclin E/Cdk2-dependent phosphorylation of Mcl-1 determines its stability and cellular sensitivity to BH3 mimetics. Oncotarget [Internet]. 2015 Jul 10;6(19):16912–25. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4627281&tool=pmcentrez&rendertype=abstract

573. Hata AN, Engelman JA, Faber AC. The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. Cancer Discov [Internet]. 2015 May 1;5(5):475–87. Available from: http://cancerdiscovery.aacrjournals.org/cgi/doi/10.1158/2159-8290.CD-15-0011

574. Emad A, Cairns J, Kalari KR, Wang L, Sinha S. Knowledge-guided gene prioritization reveals new insights into the mechanisms of chemoresistance. Genome Biol [Internet]. 2017 Dec 11;18(1):153. Available from: http://genomebiology.biomedcentral.com/articles/10.1186/s13059-017-1282-3

575. Bononi A, Agnoletto C, De Marchi E, Marchi S, Patergnani S, Bonora M, et al. Protein Kinases and Phosphatases in the Control of Cell Fate. Enzyme Res [Internet]. 2011;2011:1–26. Available from: http://www.hindawi.com/journals/er/2011/329098/

576. Wang G-L, Jin L-Y, Dong Y-M, Wu X-M, Li Z-J. Intrinsic enzyme mimicking activity of gold nanoclusters upon visible light triggering and its application for colorimetric trypsin detection. Biosens Bioelectron [Internet]. 2015 Feb;64:523–9. Available from: http://dx.doi.org/10.1016/j.bios.2014.09.071

577. Bisswanger H. Enzyme assays. Perspect Sci [Internet]. 2014 May;1(1–6):41–55. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2213020914000068

578. Gates AJ, Kemp GL, To CY, Mann J, Marritt SJ, Mayes AG, et al. The relationship between redox enzyme activity and electrochemical potential—cellular and mechanistic implications from protein film electrochemistry. Phys Chem Chem Phys [Internet]. 2011;13(17):7720. Available from: http://xlink.rsc.org/?DOI=c0cp02887h

579. Chang K-M, Chang C-T, Chan K-M. Development of an Ion Sensitive Field Effect Transistor Based Urea Biosensor with Solid State Reference Systems. Sensors [Internet]. 2010 Jun 21;10(12):6115–27. Available from: http://www.mdpi.com/1424-8220/10/6/6115

580. Oishi J, Asami Y, Mori T, Kang J-H, Niidome T, Katayama Y. Colorimetric Enzymatic Activity Assay Based on Noncrosslinking Aggregation of Gold Nanoparticles Induced by Adsorption of Substrate Peptides. Biomacromolecules [Internet]. 2008 Sep 8;9(9):2301–8. Available from: http://pubs.acs.org/doi/abs/10.1021/bm800192d

581. Hu Y, Geng X, Zhang L, Huang Z, Ge J, Li Z. Nitrogen-doped Carbon Dots Mediated Fluorescent on-off Assay for Rapid and Highly Sensitive Pyrophosphate and Alkaline Phosphatase Detection. Sci Rep [Internet]. 2017 Dec 19;7(1):5849. Available from: http://dx.doi.org/10.1038/s41598-017-06356-z

582. Wolfbeis OS. An overview of nanoparticles commonly used in fluorescent bioimaging. Chem Soc Rev [Internet]. 2015;44(14):4743–68. Available from: http://xlink.rsc.org/?DOI=C4CS00392F

583. Zhu S, Yang J, Zhao X, Kong R, Wang H, You J. Simple and fast determination of catecholamines in pharmaceutical samples using Ag + –3,3′,5,5′-tetramethylbenzidine as a colorimetric probe. Anal Methods [Internet]. 2015;7(16):6785–90. Available from: http://xlink.rsc.org/?DOI=C5AY01353D

584. Lei C, Zhao X-E, Sun J, Yan X, Gao Y, Gao H, et al. A simple and novel colorimetric assay for tyrosinase and inhibitor screening using 3,3′,5,5′-tetramethylbenzidine as a chromogenic probe. Talanta [Internet]. 2017 Dec;175(July):457–62. Available from: http://dx.doi.org/10.1016/j.talanta.2017.07.070

585. Ananieva E. Targeting amino acid metabolism in cancer growth and anti-tumor

immune response. World J Biol Chem [Internet]. 2015;6(4):281. Available from: http://www.wjgnet.com/1949-8454/full/v6/i4/281.htm

586. Mayers JR, Vander Heiden MG. Nature and Nurture: What Determines Tumor Metabolic Phenotypes? Cancer Res [Internet]. 2017 Jun 15;77(12):3131–4. Available from: http://cancerres.aacrjournals.org/lookup/doi/10.1158/0008-5472.CAN-17-0165

587. Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med [Internet]. 2014 Sep 28;20(10):1193–8. Available from: http://www.nature.com/doifinder/10.1038/nm.3686

588. Cohen R, Neuzillet C, Tijeras-Raballand A, Faivre S, de Gramont A, Raymond E. Targeting cancer cell metabolism in pancreatic adenocarcinoma. Oncotarget [Internet]. 2015 Jul 10;6(19):16832–47. Available from: http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path[]=4160&path[]=11560

589. Jackman SR, Witard OC, Philp A, Wallis GA, Baar K, Tipton KD. Branched-Chain Amino Acid Ingestion Stimulates Muscle Myofibrillar Protein Synthesis following Resistance Exercise in Humans. Front Physiol [Internet]. 2017 Jun 7;8(JUN). Available from: http://journal.frontiersin.org/article/10.3389/fphys.2017.00390/full

590. Wolfe RR. Branched-chain amino acids and muscle protein synthesis in humans: myth or reality? J Int Soc Sports Nutr [Internet]. 2017 Dec 22;14(1):30. Available from: http://jissn.biomedcentral.com/articles/10.1186/s12970-017-0184-9

591. Boroughs LK, DeBerardinis RJ. Metabolic pathways promoting cancer cell survival and growth. Nat Cell Biol [Internet]. 2015 Mar 16;17(4):351–9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4939711/pdf/nihms798862.pdf

592. Carter BZ, Mak PY, Mu H, Zhou H, Mak DH, Schober W, et al. Combined targeting of BCL-2 and BCR-ABL tyrosine kinase eradicates chronic myeloid leukemia stem cells. Sci Transl Med [Internet]. 2016 Sep 7;8(355):355ra117-355ra117. Available from: http://stm.sciencemag.org/cgi/doi/10.1126/scitranslmed.aag1180

593. Song JH, Kraft AS. Pim Kinase Inhibitors Sensitize Prostate Cancer Cells to Apoptosis Triggered by Bcl-2 Family Inhibitor ABT-737. Cancer Res [Internet]. 2012 Jan 1;72(1):294–303. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1086/498510

Appendix 1: Antibodies used for western blots

Antibody	Dilution	Supplier, code
Anti-Bcl-2 primary antibody	1:1000	Cell Signaling Technology, 2870
Anti-Bcl-X_L primary antibody	1:1000	Cell Signaling Technology, 2764
Anti-Mcl-1 primary antibody	1:1000	Cell Signaling Technology, 5453
Anti-Bcl-w primary antibody	1:1000	Cell Signaling Technology, 2724
Anti-LC3 primary antibody	1:2000	Nanotools, LC3-2G6
Anti-GAPDH primary antibody	1:5000	Millipore, MAB374
Anti-PARP primary antibody	1:1000	Cell Signaling Technology, 9542
Anti-α-Actinin primary antibody	1:1000	Cell Signaling Technology, 3134
Anti-BCKDH primary antibody	1:1000	Bethyl, A303-790A-M
Anti-BCKDH, phosphor (S293) primary antibody	1:1000	Bethyl, A304-672A-M
Anti-BCKDK primary antibody	1:2000	OriGene, TA500685
Anti-P62 primary antibody	1:1000	Abcam, ab56416
Anti-mouse IgG, HRP-linked secondary antibody	1:2000	Cell Signaling Technology, 7076
Anti-rabbit IgG, HRP-linked secondary antibody	1:2000	Cell Signaling Technology, 7074

The antibodies were diluted in tris buffer saline tween (TBST) containing 5% skimmed milk powder.

Appendix 2: siRNA transfection

siRNA buffer (Dharmacon) was used to reconstitute the individual siRNA (Dharmacon) to 20 μM stock solutions, and then used at 100nM final concentration. The details of siRNA target sequence are shown below.

Name	Target sequence
CYR61#2	GGGCAGACCCUGUGAAUAU
CYR61#4	GGCCAGAAAUGUAUUGUUC
CYR61#5	GGUCAAAGUUACCGGGCAG
CYR61#6	GCAGCAAGACCAAGAAAUC
FABP4#1	GUAGGUACCUGGAAACUUG
FABP4#2	GAAAUGGGAUGGAAAAUCA
FABP4#3	GAUGUGAUCACCAUUAAAU
FABP4#4	GAAAGUCAAGAGCACCAUA
FBLN5#1	GCAGACGUGCUACAAUUUA
FBLN5#2	GGACGAGUGUGCAACAGAU
FBLN5#3	GUGCAAACCUGCGUCAACA
FBLN5#4	UAACCAAAAUGGCGGGUAU
FOXC1#1	GAACAACUCUCCAGUGAAC
FOXC1#2	AAUAGUAGCUGUCAAAUGG
FOXC1#3	GUCCGGAGCUUUCGUCUAC
FOXC1#4	ACAAGAAGAUCACCCUGAA
TAGLN#2	AGAAAGCGCAGGAGCAUAA
TAGLN#3	CCAGACUGUUGACCUCUUU
TAGLN#4	CUCGGCAGAUCAUCAGUUA
TAGLN#5	CCAAAAUCGAGAAGAAGUA
MAPK1#1	CCAAAGCUCUGGACUUAUU
MAPK1#3	AAACAGAUCUUUACAAGCU
MAPK1#4	CAAGAGGAUUGAAGUAGAA
MAPK1#9	GUACAGGGCUCCAGAAAUU
FBLN1#1	GACAAUAGCUGCAAAGAUA
FBLN1#2	CAAGAGUGGCUUUAUACAA
FBLN1#3	GCGAAUGCAAGACGGGUUA
FBLN1#4	AAUGAGUGUUUGAGUAUCA
FOXO1A#5	CCAGGCAUCUCAUAACAAA
FOXO1A#6	CCAGAUGCCUAUACAAACA
FOXO1A#7	GGAGGUAUGAGUCAGUAUA
FOXO1A#8	GAGGUAUGAGUCAGUAUAA
LAMA2#1	UGACAGUGCUUGCGAAUUU
LAMA2#2	CGACACAGCUAAAGAUGUA
LAMA2#3	GAAGUUAGCAAAUGAUGUA
LAMA2#4	UCACACAGGUGGCCCAUAU
EFEMP1#1	CAAGCUACCUGUGUCAAUA
EFEMP1#2	CCAGAGAACCGAUGUGUUU

EFEMP1#3	CACCAAAGAUGCGUGAAUA
EFEMP1#4	CAACUAUACCUGCGUAGAU
CFHL1#1	CGAAUAACAUGUAGAAAUG
CFHL1#2	GAGAGUACGUUAUCAAUGU
CFHL1#3	CCAAACAGAAGCUUUAUUU
CFHL1#4	CAGCUGAAUUUGUGUGUAA
PLA2R1#1	GCAAGGAGGUACGCUGUUA
PLA2R1#2	GAGCAGCCAUUAAGCUUAU
PLA2R1#3	UAAAAGCGCUAUCCAAGUA
PLA2R1#4	GCAAAUAUGACUUGGUAUG
ENTPD1#1	GUAGAAGAAUGCAGGGUUA
ENTPD1#2	AGAAGUACCUGAGUGAAUA
ENTPD1#3	GUAAUGAAAUUCUCAGGGA
ENTPD1#4	ACACAGGCGUGGUGCAUCA
BCKDK#1	GAAAGGAGCAAGACGAUGA
BCKDK#2	GCACGUGCAUGAGCUAUAU
BCKDK#3	GCAGAGGGCCUACGUGAGA
BCKDK#4	GUACUCGUCUCUCACCAAA

Appendix 3: Primer sequences

Primers were obtained from Sigma-Aldrich. DNase/RNase-free distilled water used to reconstitute the primers to 100 μM stock concentration, then diluted to 100 nM as a final concentration for each primer in the reaction.

Primer	Forward (5' → 3')	Reverse (5' → 3')
CYR61	AAGAAACCCGGATTTGTGAG	TCTTGGTCTTGCTGCATTTC
FABP4	TGGAAATTGTCTCCAGTGAA	CATGCCAGCCACTTTCCT
FBLN5	CGCTGTGAGGAGCCTTATCT	CAAGATGGTAAAGGGCTGGT
FOXC1	GACCCGGACTCCTACAACAT	TGTCCTTCTCCTCCTTGTCC
TAGLN	GCTGAGGACTATGGGGTCAT	ACTAGGGTCCTCTGCACTG
MAPK1	TGCTAGATTCCAGCCAGGAT	GAACACCGATGTCTGAGCAC
FBLN1	GAGACCGGAGATTTGGATGT	CAGCGGTCATTCAGATATGG
FOXO1A	GGATAAGGGTGACAGCAACA	GTTCCTTCATTCTGCACACG
LAMA2	AGGGATGTGCATCTGCTATG	ATCGCCACATGTGTTATGCT
EFEMP1	GGATCCTTTGCATGTCAGTG	TCTTTGGTGGCAATATGGAG
CFHL1	ACAATGGTCAGAACCACCAA	CTGTTTGGCTGTCCACCTTA
PLA2R1	CTGTCCAGATGGTTTGGTTG	CCAGTTTGACTGGTCTGTGG
ENTPD1	TCAACTATCTGCTGGGCAAA	AAGCTCCAAAGGTTTCCTGA
BCKDK	AATGGCGGTGGACTGTTC	AGGATGCTGACAGGCTCA