Investigation of the BH3-mimetics navitoclax and obatoclax as potential therapeutics for ovarian cancer

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ABSTRACT

Ovarian cancer is treated in most cases with a combination of surgery and chemotherapy. However, despite the overall improvement in survival rates, over the last 30 years there has not been a break-through therapeutic development. Following first-line treatment, the majority of patients experience periods of relapse characterised by resistance to anticancer drugs. Factors which contribute to ovarian cancer chemoresistance have been identified as potential targets for drug discovery, such as the Bcl-2 family of proteins, which regulates apoptosis. The strategy of targeting the anti-apoptotic members of the Bcl-2 family that are overexpressed in ovarian cancer has led to the discovery of BH3-mimetics. ABT-737, a selective BH3-mimetic, has been found to be synergistic with chemotherapy in ovarian cancer. In this study the activity of navitoclax, an orally available analogue of ABT-737 and obatoclax, a pan-Bcl-2 inhibitor, were evaluated in a series of ovarian cancer models. Navitoclax demonstrated synergy with both carboplatin and paclitaxel. Obatoclax potently inhibited the growth of ovarian cancer cell cultures. In part this was due to the anticipated induction of apoptosis, but in other cell lines an additional mechanism of cell death was implicated. Surprisingly, obatoclax was neither synergistic with carboplatin nor paclitaxel. These observations may be used to inform the design of clinical trials of these agents in ovarian cancer.

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ABBREVIATIONS

ATCC	American Type Culture Collection
Bax	Bcl-2-associated X protein
Bak	Bcl-2 antagonist killer 1
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
cDNA	complementary DNA (deoxyribose nucleic acid)
CI	Combination Index
CO_2	Carbon dioxide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial mesenchymal transition
EOC	Epithelial ovarian cancer
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
HRP	Horse radish peroxidase
mRNA	Messenger Ribonucleic acid
OSE	Ovarian surface epithelium
PBS	Phosphate buffer saline

PCOCC	Primary cultures of ovarian cancer cells
PCR	Polymerase chain reaction
qRTPCR	Quantitative RT-PCR
RNA	Ribonucleic acid
SEM	Standard Error of the mean
siRNA	Small interfering RNA
TGCA	The Cancer Genome Atlas

PUBLICATIONS

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Ithaca

When you set out for Ithaka

ask that your way be long,

full of adventure, full of instruction.

The Laistrygonians and the Cyclops,

angry Poseidon - do not fear them:

such as these you will never find

as long as your thought is lofty, as long as a rare

emotion touch your spirit and your body.

The Laistrygonians and the Cyclops,

angry Poseidon - you will not meet them

unless you carry them in your soul,

unless your soul raise them up before you.

Ask that your way be long.

At many a Summer dawn to enter

with what gratitude, what joy -

ports seen for the first time;

to stop at Phoenician trading centres,

and to buy good merchandise,

mother of pearl and coral, amber and ebony,

and sensuous perfumes of every kind,

sensuous perfumes as lavishly as you can;

to visit many Egyptian cities,

to gather stores of knowledge from the learned.

Have Ithaka always in your mind.

Your arrival there is what you are destined for.

But don't in the least hurry the journey.

Better it last for years,

so that when you reach the island you are old,

rich with all you have gained on the way,

not expecting Ithaka to give you wealth.

Ithaka gave you a splendid journey.

Without her you would not have set out.

She hasn't anything else to give you.

And if you find her poor, Ithaka hasn't deceived you.

So wise you have become, of such experience,

that already you'll have understood what these Ithakas mean.

Constantine P. Cavafy

Chapter 1: Introduction to ovarian cancer

1.1 The clinical facts of ovarian cancer

1.1.1 What is ovarian cancer?

The term “ovarian cancer” describes a number of malignant diseases involving the tissues of the ovaries [1]. Ovarian cancer spreads predominantly by dissemination to the peritoneum and direct extension to the neighbouring organs and there is some evidence to suggest additional hematogenous metastasis to the omentum or via the lymphatic system to the pelvic nodes [2], [3],[4]. Metastases can be found in the peritoneum, the pelvic and abdominal viscera, the diaphragm, the liver and the pulmonary tract or pleural surfaces [5], [6]. The progression of ovarian cancer can lead to the development of ascites in the peritoneal cavity in 45-75% of the patients, depending on the histotype of the tumour [7].

The symptoms of ovarian cancer are abdominal discomfort, bloating, feeling full quickly after eating, and increased urinary frequency/urgency. Studies evaluating screening tests for ovarian cancer have shown that only a small proportion of patients are symptomatic of the disease before their diagnosis and they often describe symptoms which overlap with those of many more common conditions of the gastro intestinal and urogenital tract [8],[9], [10]. The duration of these symptoms prior to a definitive diagnosis is not clear and only 21,2% - 32,2% of women are reported to present with symptoms within three months from the diagnosis of ovarian cancer [11] .

1.1.2 Types of ovarian cancer

The ovaries consist of three types of tissue; epithelial cells cover the ovary, germ cells produce the ova (eggs) inside the ovary and stromal cells hold the ovary together and produce most of the hormones (Figure 1).

Depending on the cell of origin, three types of ovarian cancer can be distinguished [12]:

- epithelial ovarian cancer (EOC)
- germ cell tumours
- sex cord stromal tumours.

Although the ovarian surface epithelium (OSE) cells have traditionally been considered the origin of ovarian carcinomas, newer evidence suggests that the majority of ovarian tumours arise from non-ovarian tissue [13],[14].

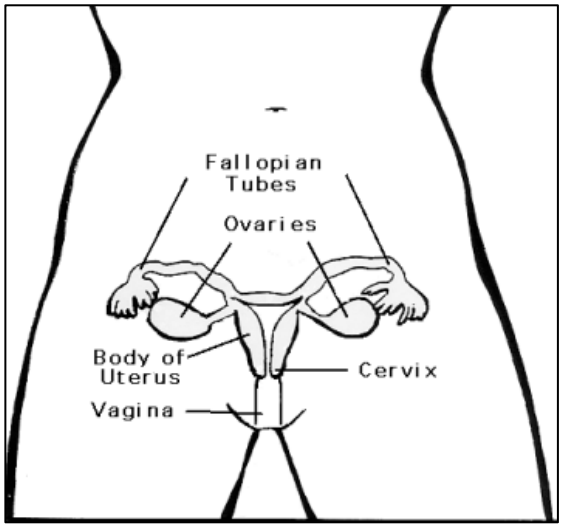

Figure 1: Anatomy of the female genital tract, reproduced from American Cancer Society website.

1.1.2.1 Subtypes of EOC

Embryologically, the OSE cells have the same mesodermal origin [15] as the fallopian tube, the uterus and the endocervix. There are five different subtypes of EOC (serous, endometrioid, mucinous, clear cell and undifferentiated subtype), which exhibit distinct histological features, similar to those of the differentiated Mullerian-duct-derived tissues (Table 1).

The subtypes of EOC			
Subtype	**Characteristics**	**Incidence (%)**	**5-year survival**
Serous	Fallopian tube-like	70%	20-35%
Endometrioid	Endometrium-like	10-20%	40-63%
Mucinous	Endocervix-like	5%	40-69%
Clear cell	Mesonephros-like	3-10%	35-50%
Undifferentiated	unknown	1%	11-29%

Table 1: The histological features, incidence rates and survival of EOC subtypes, reproduced with changes from [16],[17], [18].

1.1.2.2 Subtypes of germ cell tumours

Germ cell tumours comprise only 5% of ovarian cancers. They are diagnosed in younger women and represent 80% of pre-adolescent ovarian cancers [12]. The histologic subtypes of germ cell ovarian tumours are listed in Table 2.

The subtypes of germ cell ovarian tumours
Dysgerminoma
Teratoma (Immature/ Mature/ Monodermal highly specialized)
Endodermal sinus tumour
Embryonal carcinoma
Polyembryoma
Choriocarcinoma
Mixed forms
Granulosa cell tumours (Adult type or Juvenile type)

Table 2: The classification of germ cell tumours. [12]

1.1.2.3 Subtypes of sex cord stromal tumours

Sex cord stromal tumours represent only 3-5% of ovarian cancers and they are diagnosed in adult women [12]. Their histologic subtypes are listed in Table 3.

The subtypes of sex cord stromal tumours
Granulosa-stromal cell tumours
Tumours in the thecoma-fibroma group (Thecoma or Fibroma-fibrosarcoma or Sclerosing stromal tumour)
Sertoli-Leydig cell tumours (androblastomas)
Gynandroblastoma
Sex cord tumour with anular tubules
Unclassified

Table 3: The classification of sex cord stromal tumours. [12]

1.1.3 The origin of ovarian cancer

Although EOC is usually considered to arise from OSE cells, in reality the cell of origin is unknown. The models describing the origin of EOC can be divided into two groups. The first group supports the notion that the OSE is the site at which EOC originates. The second group represents a more recent view that EOC originates in Mullerian-duct-derived tissue.

1.1.3.1 Evidence that EOC arises from OSE cells

A number of theories have been developed which propose how EOC arises from the OSE cells. The incessant ovulation theory proposes that the ovulation-related inflammation and cell proliferation leads to ovarian carcinogenesis [19]. This hypothesis is supported by epidemiological data showing that birth control, increased number of pregnancies, breastfeeding and the use of oral contraceptives reduce the risk of ovarian cancer [20].

The epithelial mesenchymal transition (EMT) hypothesis suggests that epithelial cells undergo metaplasia towards a mesenchymal type with characteristics of the Mullerian-duct derived tissue. The transformed cells demonstrate different proliferation and motility patterns necessary for the ovarian surface remodelling during ovulation [21], [22]. Evidence to support the EMT are the expression in EOC of vimentin, N-cadherin and smooth muscle alpha-actin, the production of proteolytic enzymes and type III collagen, all of which are characteristic of mesenchymal cells [15]. It has been hypothesized that

EMT is necessary but not sufficient for the genesis of EOC.

The ovarian surface contains inclusion cysts, lined with OSE cells, which are believed to be the site of origin of many tumours and express markers of Mullerian-duct derived tissue including CA125 and CA19-9 and are 2-3 times more metaplastic in EOC patients [23].The hypothesis that EOC arises from inclusion cysts is complementary to the incessant ovulation theory, as higher ovulatory activity results in higher risk for developing inclusion cysts and creating favourable conditions for carcinogenesis [24]. *In vitro* data from animal studies where the persistent culturing of ovarian surface cells resulted in spontaneous cancer transformation, support this hypothesis [25], [26], [27].

The gonadotropins hypothesis offers an alternative view where the high levels of these hormones after the menopause stimulate the surface epithelium and generate the production of inclusion cysts from where cancer can arise [28], [29], [30].

A further observation, which supports the hypothesis that OSE cells are the cell of origin of ovarian cancer and also explains their ability to differentiate into different histological subtypes, is that OSE demonstrate features of cancer stem cells. Gene expression profile studies [31] concluded that OSE cells have features in common with stem cells.

1.1.3.2 Evidence that EOC arises from Mullerian-duct-derived tissue

It has been argued that the transition of epithelial cells to Mullerian-duct like as part of the carcinogenesis process contradicts the fundamental concept that cancer cells are less differentiated from their original counterparts [32]. This has led to the hypothesis that EOC arises from Mullerian-duct derived tissue which migrates to the ovary [33]. The evidence to support this can be summarised as follows:

I. There are common histological features between EOC cells and Mullerian-duct derived cells.

II. There is a correlation of the gene expression of the various cancer histotypes with their morphological counterparts in normal fallopian tissue [34].

III. Cells clinically identical to ovarian cancer cells have been found in the peritoneal cavity years after the removal of the ovaries for non-cancerous reasons [35], [36], [37].

IV. Mutations in p53 and BRCA genes are very common in ovarian carcinomas [38],[39] . Pre-neoplastic changes and mutations of p53 have been discovered in the fimbriae of the fallopian tubes of carriers of mutated *BRCA* [40], [41],[42], [43]. In one group of *BRCA* mutation carriers, p53 mutations were identified in the fimbriae of 38% of the population but not in any of the cells of the inclusion cysts of the ovarian surface [44].

V. Cells from the hilum (the junction between OSE and the fallopian epithelium) express stem cell markers and are readily transformed after inactivation of *TP53* and Rb [45]

VI. Mutations of the tumour-suppressor gene PTEN have been reported in a significant proportion of ovarian cancer cases [46].Deletion of *Dicer* and *PTEN* in mice leads to the development of fallopian tube tumours which subsequently spread to the ovary prior to metastasis throughout the abdominal cavity [47].

It is interesting that one study identified a group of patients who carried p53 mutations in the fimbriae of the fallopian tubes and also exhibited risk factors of the incessant ovulation theory such as increased maternal age at first pregnancy and lower parity, therefore “marrying” the two different models for the cell of origin [48]. It is possible that the process of ovulation creates an appropriate environment that allows the cells to migrate from the fallopian tube to the ovary [49].

1.1.4 The epidemiology and risk factors of ovarian cancer

Ovarian cancer is currently the 5th most common cancer in women in the UK [50]and the gynaecological cancer with the highest mortality, with more deaths than all the other gynaecological cancers combined[51]. It is the 2nd most common gynaecological cancer after endometrial cancer and it was estimated in 2008 that there were over 225,000 new cases worldwide [52]. The risk for developing EOC for any woman in the general population is 1,3-2% [53],[54], although this increases significantly in less developed countries. In comparison, the yearly adjusted incidence rate for germ cell tumours and stromal tumours is 0.00037 % and 0.00022 % respectively [12]. The incidence rate of ovarian cancer in the developed countries has been slowly rising over the past two decades [55],[51],[56].

The recognised risk factors for developing ovarian cancer are as follows:

1) Increasing age: the vast majority of epithelial ovarian cancer cases are diagnosed in postmenopausal women. In the UK from 2008 to 2010 53% of cases were diagnosed in women above 65 years of age and 75% in women aged 55 and over [50].

2) Family history: up to 15% of ovarian cancers are familial [55]. The risk of developing ovarian cancer is tripled in the presence of a family history of ovarian or breast cancer. The life-time risk estimate is 2.5% for the sister and 7% for the mother of an ovarian cancer patient [57]. Additionally, the same risk is increased by 39-46% for BRCA1 mutation carries and by 12-20% for BRCA2 mutation carriers [58]. The

women of this group are usually offered a prophylactic bilateral salpingo-oophorectomy.

3) Factors which prolong ovulation: nulliparity and infertility appear to increase the risk of a woman for ovarian cancer [59], [19].

4) Environmental carcinogens: the use of talc and the escorting chronic inflammation has been associated with the development of ovarian cancer [60], [61].

The use of an algorithm, which includes seven risk factors for ovarian cancer (age over 45, long term genital talc use, family history of ovarian or breast cancer, Jewish ethnicity, no use of oral contraceptives, no live births, no breastfeeding, no tubal ligation) showed that women with 6 or 7 positive factors were 6 times more likely to develop ovarian cancer [62].

Some prophylactic factors have also been acknowledged that could be protective against ovarian cancer. Factors that interrupt ovulation such as pregnancy, breastfeeding and using oral contraceptives are amongst them [59]. The use of oral contraceptives offers some protection against ovarian cancer as there is a 50% reduction in the incidence for women using oral contraceptives for more than 10 years [63]. Undergoing a prophylactic bilateral salpingo-oophorectomy where both fallopian tubes and ovaries are removed appears to be a protective and cost-effective approach for high-risk patients; however the alternative of a salpingectomy and delayed oophorectomy remains a valid option with improved quality of life [64]. For BRCA positive patients a large study showed an 85% reduction of gynaecological cancers among women who underwent a prophylactic salpingo-oophorectomy compared to women who chose to be monitored regularly [65]. It is interesting that

there is still a 0.2% risk of ovarian cancer for women who have undergone an oophorectomy [41], as it supports the theory for the origin of EOC being outside the ovaries.

1.1.5 Screening for ovarian cancer

Ovarian cancer has been described as ‘’the silent killer’’ because of its late clinical presentation. Only 20-25% of women are diagnosed with disease limited to the ovaries (FIGO disease stage I-II) [66] while 75-80% of the patients present with evidence of metastatic spread (FIGO disease stage III-IV) [67],[68],[5]. The 5-year survival of ovarian cancer patients increases significantly when the disease is diagnosed early. It is possible that the establishment of an effective screening strategy could be a cornerstone for reducing the ovarian cancer mortality. Such a screening test needs to be characterised by very high sensitivity for early-stage disease (>75%) and very high specificity (99.6%) to secure a predictive value of at least 10% [69].

The development of such a screening tool is hindered by the following facts:

1) The criteria established by the WHO for designing screening methods are not fulfilled; the natural history of the disease from early to advanced stages is not fully known and there increasing evidence suggests that the precursor lesion is not the OSE cells as previously thought, but fallopian tube cells [69].

2) Genetic and biomarker profiling has shown that each EOC subtype has a distinct “molecular signature” and the Cancer Genome Atlas project further validated this observation [70]. A recent immunohistochemical study found that in a group of 20 candidate biomarkers, each subtype showed a different pattern of expression and when the analysis was made subtype specific then two thirds of the markers had no prognostic

value [71]. Thus, each subtype may require different screening methods.

3) There is little understanding of the risk factors behind ovarian cancer, apart from age, family history and BRCA mutations. As ovarian cancer is mostly diagnosed in women of postmenopausal age, it has been suggested that any screening method should be applied only to that age group [58].

Biochemical markers such as human epididymis protein-4 (HE4), decoy receptor-3 (DcR3), osteopontin,spondin-2, SMRP, CA72-4, ERBB2, inhibin, activin, EGFR, mesothelin, kallikreins and proteomic markers have been considered, but nothing has been validated for screening purposes [72], [73], [74], [75], [76], [77], [78], [79], [80], [81], [82], [83], [84]. The use of a panel of four conventional protein biomarkers in serum has detected as high as 87-90% of stage I ovarian cancers [85]. The combination of CA125 and HE4 markers in one study showed a sensitivity of 77% for early stage disease [86]. A recent study shows that a steady increase in the levels of CA125, HE4 and mesothelin can be monitored to detect ovarian cancer 1-3 years before the clinical manifestation of the disease [87]. However, the lead time required to diagnose ovarian cancer with this method until the clinical diagnosis is confirmed is no more than one year and the impact of this on mortality remains to be determined.

The most promising development has been a combination of the serum marker cancer antigen 125 (CA125) with transvaginal ultrasonography as part of a two-stage screening, where abnormal results prompt further imaging studies. A large multicentre trial (PLCO) which evaluated the potential for screening for ovarian cancer by combining Ca125 test

and transvaginal ultrasonography concluded that there is no benefit from screening for asymptomatic patients and did not result in a reduction in deaths from this disease [88]. The completion of the subsequent UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) is expected to provide definite data, as the first results from this on-going trial show an increased detection rate for early-stage disease (48%), with adequate sensitivity (89. 4%) and specificity (99. 8%).

1.1.6 Staging of ovarian cancer

The spread of ovarian cancer at the point of diagnosis is described by the staging of the disease. It is essential to provide correct staging details for a patient as this can affect treatment decisions and later stage disease has poor survival compared to earlier stages. Currently an international system of staging is used called the FIGO system, after its authors - the International Federation of Gynaecology and Obstetrics (Figure 2). The staging of ovarian cancer is made following surgery, where a laparotomy is performed and all findings of surgical exploration are biopsied to confirm the diagnosis. The stages of ovarian cancer are summarized in Table 4.

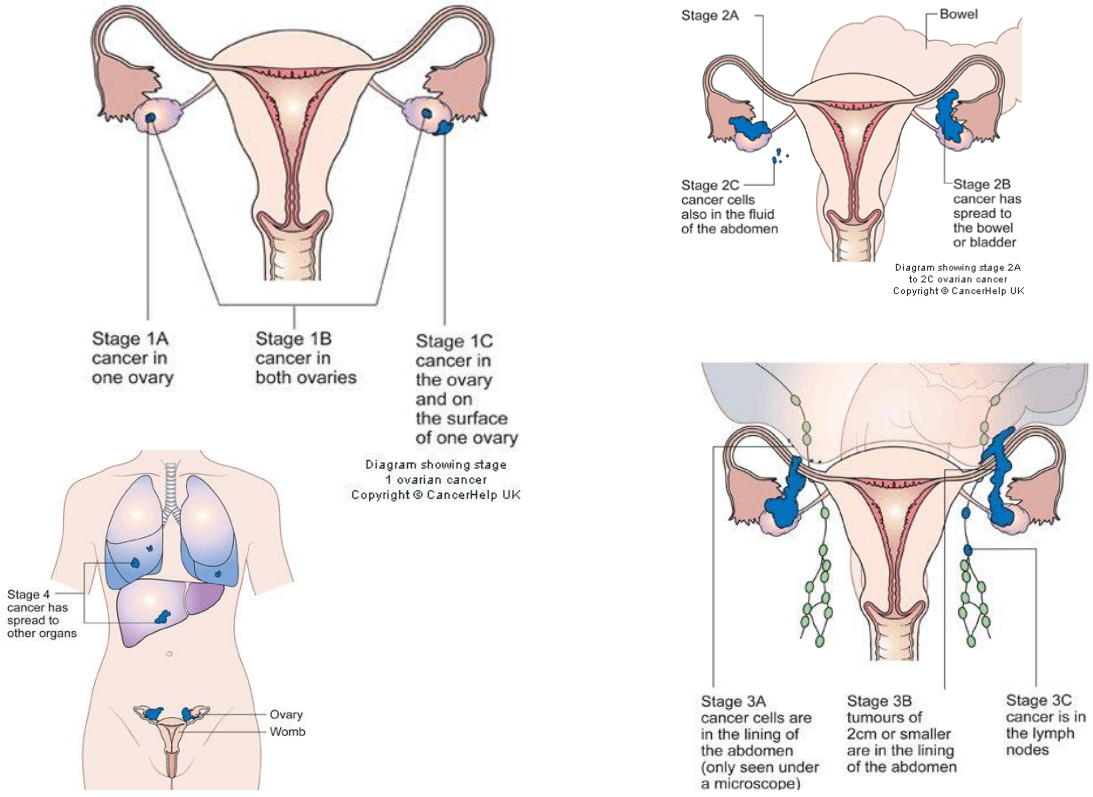

Figure 2: The stages of ovarian cancer.
(Reproduced from CancerHelp UK)

The FIGO staging system of ovarian cancer, 2014	
Stage	**Description**
IA	Tumour limited to 1 ovary, capsule intact, no tumour on surface, negative washings
IB	Tumour involves both ovaries otherwise like IA
IC	Tumour limited to 1 or both ovaries
IC1	Surgical spill
IC2	Capsule rupture before surgery or tumour on ovarian surface
IC3	Malignant cells in the ascites or peritoneal washings
IIA	Extension and/or implant on uterus and/or Fallopian tubes
IIB	Extension to other pelvic intraperitoneal tissues
IIIA	(Positive retroperitoneal lymph nodes and /or microscopic metastasis beyond the pelvis)
IIIA1	Positive retroperitoneal lymph nodes only : IIIA1(i) Metastasis ≤ 10 mm IIIA1(ii) Metastasis > 10 mm
IIIA2	Microscopic, extra pelvic (above the brim) peritoneal involvement ± positive retroperitoneal lymph nodes
IIIB	Macroscopic, extra pelvic, peritoneal metastasis ≤ 2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen
IIIC	Macroscopic, extra pelvic, peritoneal metastasis > 2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen
IVA	Pleural effusion with positive cytology
IVB	Hepatic and/or splenic parenchymal metastasis, metastasis to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity

Table 4: The FIGO staging of ovarian cancer [89].

1.1.7 Grading of ovarian cancer

The grading of ovarian cancer describes how active or aggressive the cancer is. It is done by histopathological examination of the tissue and comparison of the cancer cell's differentiation with the well differentiated tumours looking more normal than the poorly differentiated. There are three different grades of tumours: well differentiated (low grade), moderately differentiated (moderate grade) and poorly differentiated lesions (high grade), as well as types where grade cannot be assessed [5]. The tumour grade is the most important prognostic factor in stage 1 disease [90].

1.1.8 Therapy of ovarian cancer

When disease is apparently confined to the ovaries and there is no evidence of macroscopic spread, primary surgery is the standard treatment. A study which evaluated the outcome of neoadjuvant chemotherapy followed by interval debulking surgery in advanced stage disease did not exhibit any inferior survival outcomes compared to the traditional primary surgery [91].On occasions, adjuvant chemotherapy is indicated when there is microscopic evidence of metastatic spread or other poor prognostic factors such histological un-differentiation [92].

The standard treatment for advanced cancer is surgery and chemotherapy [93]. The majority of patients present with advanced disease that cannot be removed completely at surgery and in that case chemotherapy is given with intention of cure. On occasions, palliative chemotherapy is given to ameliorate symptoms [92].

The therapeutic options for the treatment for ovarian cancer are described in Table 5.

Therapeutic options in ovarian cancer at presentation	
Treatment	**Definition**
Surgery alone	Surgery as first therapy, patients can be given any further treatment
Surgery + adjuvant chemotherapy	Surgery as first therapy and then chemotherapy within 90 days from the date of surgery. Subsequently, patients can be given any further treatment.
Neoadjuvant chemotherapy + surgery	Two to four cycles of chemotherapy as first therapy and then surgery within 42 days from the end of chemotherapy. Subsequently, patients can be given any further treatment.
Radiotherapy	Radiotherapy is now rarely used and only for solitary lesions in cases of recurrent disease.

Table 5: The treatment options in ovarian cancer, reproduced with changes from [5].

1.1.8.1 Surgical management of ovarian cancer

Cytoreductive surgery in ovarian cancer can be performed before or after chemotherapy is administered to the patient. The majority of patients receive surgery first, but the option of interval debulking surgery can be considered in cases of advanced disease following two cycles of chemotherapy [92].

The surgical management in ovarian cancer is always performed with the intention to remove all macroscopic disease, regardless of the timing of surgery in relation to chemotherapy. This approach is based on evidence, which shows that in the case of ovarian cancer the reduction of the tumour burden offers a survival advantage [94]. However, it is debated whether any benefit seen is due to the surgery and the skills of the surgeon or the nature of the disease [92]. The significance of the surgical skills of the surgeon has also been investigated and the current knowledge is that optimal cytoreduction is better achieved by gynaecological oncologists compared to other surgeons [95], [92].

The current move in ovarian cancer surgical management is towards more extensive surgery and in many cases ultra-radical surgery is advocated. This often entails increased morbidity risks which should be weighed against any expected benefit for the patient [92].

1.1.8.2 Chemotherapy in ovarian cancer

The first line of chemotherapy for advanced EOC is a platinum-based drug or a combination of this with paclitaxel [96]. At present all cancer subtypes are being treated with the same regimes, however subtype-specific trials have been recommended especially for clear cell and mucinous carcinoma [97].

Platinum agents such as carboplatin induce DNA damage by the formation of intra- and inter-strand cross-links between the DNA molecules which interferes with DNA replication and transcription [98]. The resulting DNA damage response stimulates cycle checkpoints and subsequently triggers cell death [99]. The survival of subpopulations of cancer cells after platinum chemotherapy can be explained by a number of different protective mechanisms that cause drug resistance and which are discussed in more detail below [100].

Taxanes such as paclitaxel induce their chemotherapeutic effect by blocking the functional reorganization of the cellular microtubules [101]. The assembly of the mitotic spindle is arrested and this results in failure of the chromosomes to segregate [102]. This leads to prolonged M-phase arrest and subsequent cell death. Resistance to the effect of taxanes has been associated with mutations in tubulin and transcriptional changes within the cancer cell [103].

A recent advance in the field of chemotherapy has been the introduction of the intraperitoneal administration of cisplatin instead of the intravenous route [104], [105], [106]. It has resulted in an increase in the median survival [106], but the resulting toxicity remains a limiting factor [107]. The concept of “dose-dense “chemotherapy represents another advance in cancer treatment whereby the cumulative drug dose remains the same, but the time intervals between doses are shorter. The primary analysis of a recently completed trial shows that dose-dense paclitaxel and carboplatin compared to conventional regimes results in an improvement in both progression-free and overall survival of ovarian cancer patients [108].

1.1.8.3 Radiotherapy in ovarian cancer

The use of radiotherapy in the treatment of EOC is now rare and limited to cases of solitary lesions in recurrent disease.

1.1.9 Chemoresistance in ovarian cancer

Following the first line treatment 80% of the patients show a positive response and experience periods of remission. Unfortunately almost 70-80% of patients subsequently relapse [109], [110]. The chemotherapeutic regimes used in recurrent disease vary depending on whether the disease remains sensitive to the initial treatment or resistance has been acquired. Additionally to the clinical manifestations of ovarian cancer, the "gold standard" for assessing regression and recurrence of the disease is the serum CA125 (MUC16) levels. However, rising levels of CA125 in otherwise asymptomatic patients as an indication for retreatment has not been shown to confer any survival benefit [111].

The definition of chemoresistance in ovarian cancer is based upon the time when recurrent disease reappears[112] after primary chemotherapy:

De novo or intrinsic resistance.

At least 20% of patients receiving first-line chemotherapy do not have any clinical remission after treatment is completed. Based on the lack of response to chemotherapy, these tumours are considered to exhibit intrinsic drug resistance and are platinum refractory [113].

Platinum sensitivity.

Any patient with progression of disease reported after 6 months from the completion of platinum-based treatment is considered to have a chemosensitive tumour. A greater interval between primary treatment and recurrent disease is associated with an increased

likelihood that the patient will respond to retreatment for a longer period of time. Patients whose tumour is considered chemosensitive may again be treated with carboplatin and paclitaxel.

For those patients considered to have chemosensitive disease, at present, three different combination regimes are considered to be the standard of care. The use of carboplatin and paclitaxel shows a survival advantage compared to platinum monotherapy without any increased toxicity risks [114]. Alternative combination regimes which favour less toxicity include carboplatin combined with gemcitabine and carboplatin combined with pegylated liposomal doxorubicin (PLD). Comparison studies showed that there was no difference in the overall survival of patients receiving platinum agent monotherapy or the combination of carboplatin with gemcitabine. On the contrary, the carboplatin-PLD regime showed better progression-free-survival times compared to the carboplatin-paclitaxel combination but data regarding the overall survival are not conclusive [115].

Platinum-resistance.

5% of the patients progress during their first cycle of platinum-based chemotherapy and another 17% of patients progress within 6 months of completion of treatment. They are considered to have tumours that are platinum-resistant and may be considered for second line chemotherapy.

Overall, approximately 80% of patients diagnosed with EOC will relapse after first-line platinum-based and taxane-based chemotherapy and may benefit from alternative therapies. For chemoresistant patients the second-line therapy should include a

nonplatinum-based agent. The results of several trials show that although there is an impact of second-line treatment on the overall response and progression-free survival, the overall survival is not improved [114]. Existing data support the use of single-agent therapy with conventional Topotecan, pegylated liposomal doxorubicin (PLD) or gemcitabine [114].

1.1.10 Ovarian cancer prognosis

The prognostic factors that have been recognised in ovarian cancer are the volume of residual disease after cytoreductive surgery and the sensitivity to platinum-based agents [116]. The use of biomarkers (serum or tissue) as prognostic tools to predict the response of a patient to chemotherapy or the expected survival has been investigated [117], but larger cohorts of women are required before any suggested marker is fully established.

After treatment, the 5-year survival rates are [54]:

i. Stage I is >90%
ii. Stage II 70-80%
iii. Stage III 20-30%
iv. Stage IV <5%

It is accepted by many that metastases are more difficult to treat in comparison to any primary tumour [118]. For women diagnosed with advanced disease the surgical intervention is limited and the prognosis poor [119]. Serous and endometrioid ovarian cancers respond better than clear cell and mucinous cancers to platinum – taxane chemotherapeutic regimes [120]. Serous tumours are initially responsive to chemotherapy, while clear cell tumours are resistant [121], [122].

1.2 The molecular biology of ovarian cancer

1.2.1 The genetic abnormalities of ovarian cancer

It has been estimated that the process of oncogenesis in solid tumours entails a single cell undergoing five or more critical genetic aberrations. In the case of ovarian carcinogenesis approximately 30 oncogenes and tumour suppressor genes have been identified that are frequently involved. These genetic changes affect proliferation, apoptosis, adhesion and invasion, block anoikis and increase motility. The known genetic and epigenetic alterations involved in ovarian carcinogenesis are summarized in Table 6.

The cancer genome atlas project has shed light onto the biology underlying serous ovarian cancer.*TP53* is mutated in the vast majority (96%) of serous ovarian cancers. However, the disease is otherwise characterized by somatic copy number alterations, rather than mutations, which appear to be the key genetic drivers in other cancers, such as renal cancer. Changes in the Rb, PI 3-kinase and homologous recombination pathways are commonly observed (67%, 45% and 51% of tumours, respectively [123]) in serous ovarian cancer. These pathways are discussed in more detail below. Furthermore, serous cancer could be subdivided into 4 further subtypes, based on gene expression patterns.

Table 6. Genetic abnormalities in ovarian cancer			
Event	**Effect**	**Chromosome**	**Gene**
Gene amplification	Activation	1q22 3q26 5q31 8q24 19q 20p 20q13,2	*RAB25* *PRKCI,EVII* & *PIK3CA* *FGF1* *MYC* *PIK3RI* & *AKT2* *ND* *AURKA*
Gene deletion	Inactivation	4q,5q,16q,17p,17q, Xp and Xq	*ND*
Mutation	Activation	NA	*KRAS* (15%), *BRAF* (12%), *CTNNB1* (12%), *CDKN2A* (10%), *APC* (9%), *PIK3CA* (8%), *KIT* (7%) and *SMAD4* (7%)
Hypomethylation	Activation	NA	*IGF2* and *SAT2*
Loss of heterozygosity	Inactivation	17p13 and 17q21 (in 50% of cases or more) 1p, 3p, 5q, 5q, 6q, 7q and 8q (in fewer than 30% of	*ARHI*, *PEG3*, *PLAGL1*, *RPS6KA2*, *TP53*, *BRCA1, BRCA2*, *PTEN, OPCML* and *WWOX*

		cases)	
Mutation	Inactivation	NA	*TP53* (62%), *BRCA1* (5%), *BRCA2* (<5%) and *PTEN* (3–8%)
Promoter methylation	Inactivation	NA	*ARHI, DAPK1, CDH13, MLH1, ICAM1, PLAGL1, DNAJC15, MUC2, OPCML, PCSK6, PEF3, CDKN2A, CDKN1A, RASSF1, SOCS1, SOCS2*, *PYCARD* and *SFN*

Table 6: The known genetic alterations in ovarian cancer, reproduced with changes [120].

1.2.2 Oncogenes in ovarian cancer

There are at least 30 oncogenes commonly known to be involved in ovarian carcinogenesis (Table 7). The genes encoding 11 of them are amplified [124] while the remained are activated through mutation, truncation or rearrangement.

PIK3CA

PIK3CA encodes the catalytic subunit of PI3-kinase. It is involved in invasion, metastasis and the development of chemoresistance [125], [126], [127]. Through activation of the Akt pathway, PI3-kinase promotes cell survival and results in oncogenic transformation [128],[129].

CCNE1

Amplification of CCNE1, which encodes the cyclin-dependent kinase regulator cyclin E1, results in increased oncogenesis, in part through increased proliferation, and genetic instability [130], [131], [132], [133] and is associated with poor clinical outcome [134],[135].

RAB25

RAB25 encodes a small GTPase and its amplification induces increased motility and aggressiveness and can promote cancer cell survival in response to stress induced by treatment [136].

NOTCH3

Amplification is necessary for the survival of some ovarian cancers, as its inactivation lead to decreased proliferation and increased apoptosis[137].

EVI1

EVI1 is a transcription factor which increases proliferation and migration potential [138].

MYC

MYC also encodes a transcription factor and an oncogene whose amplification causes increased proliferation and migration and promotes angiogenesis and cell survival under hypoxic conditions [139],[140] ,[141] ,[142],[143],[144], [145].

KRAS

KRAS encodes a small GTPase which is activated by several growth factor receptors and stimulates the Erk pathway, thereby promoting cell proliferation. Aberrations are common in lower grade cancers and have no prognostic value [146].

ErbBB2 (or *HER2*)

ErbBB2 belongs to the epidermal growth factor (EGF) family of tyrosine kinases receptors. Its activation promotes cell proliferation, angiogenesis and metastasis [147], [148], [149]. It is frequently found in mucinous ovarian cancer (18.2%) and has not been shown to carry any prognostic value [150].

AKT2

AKT2 encodes a protein serine/threonine kinase It is implicated in pathways regulating cell survival and tumour progression. Its expression is clinically associated with advanced FIGO stage [151].

Some of the genetic alterations in ovarian cancer are of particular interest, and deserve further comments, such as the *HOX* genes and the genes which are responsible for the cell DNA repair mechanisms. The *HOX* family of homeobox genes normally regulate Mullerian duct differentiation and are expressed during gynaecological organogenesis. They are not expressed by OSE cells. It has been shown that EOC subtypes re-express homeobox genes according to their pattern of Mullerian-duct-like differentiation [152] .

BRCA1 and *BRCA2* genes are involved in DNA repair. 15% of ovarian cancer cases are associated with *BRCA1* or *BRCA2* mutations and the heritable non-polyposis colorectal cancer gene [153], [154].The tumours bearing mutations in these genes are more frequently multifocal (contrary to the fact that 90% of epithelial ovarian cancers are clonal [155]) and progress faster, but show a greater response to platinum-based therapy. *BRCA1* and *BRCA2* mutations account for 65-85% of hereditary ovarian cancers and mutations in another 4 mismatch repair genes (*MLH1, MSH2, MSH6* and *PMS2*) for another 10-15% [156], [153], [157].

Oncogenes associated with epithelial ovarian cancer					
Gene	**Chromosome**	**Percentage of cancers in which amplified**	**Percentage of cancers in which overexpressed**	**Percentage of cancers in which mutated**	**Function**
RAB25	1q22	54%	80–89%	ND	Cytoplasmic GTPase and apical vessel trafficking
EVI1	3q26	ND	ND	ND	Transcription factor
EIF5A2	3q26	ND	ND	ND	Elongation factor
PRKCI	3q26	44%	78%	ND	Cytoplasmic serine-threonine protein kinase
PIK3CA	3q26	9–11%	32%	8–12%	Cytoplasmic lipid kinase
FGF1	5q31	ND	51%	ND	Growth factor for cancer and angiogenesis
MYC	8q24	20%	41–66%	ND	Transcription factor
EGFR	7q12	11–20%	9–28%	<1%	Protein tyrosine kinase growth factor receptor

NOTCH3	9p13	20–21%	62%	ND	Cell surface growth factor receptor
KRAS	12P11–12	5%	30–52%	2–24%	Cytoplasmic GTPase
ERBB2	17q12–21	6–11%	4–12%	ND	Protein tyrosine kinase growth factor receptor
PIK3R1	19q	ND	ND	ND	Cytoplasmic lipid kinase
CCNE1	19q12	12–36%	42–63%	ND	Cyclin
AKT2	19q13.2	12–27%	12%	ND	Cytoplasmic serine–threonine protein kinase
AURKA	20q13	10–15%	48%	ND	Nuclear serine–threonine kinase

Table 7: Oncogenes implicated in ovarian cancer carcinogenesis, reproduced with changes [158],[159], [160].

1.2.3 Tumour suppressor genes in ovarian cancer

There are 16 candidate tumour suppressor genes identified in ovarian cancer to date. Three of those genes are known to be imprinted; *ARHI* (*DIRAS3*), *PLAGL1* (*LOT1*) and *PEG3*.

ARHI

ARHI encodes a small GTPase in the Ras superfamily. Its expression is associated with prolonged progression-free survival. It is down regulated in 60% of ovarian cancers and re-expression of ARHI results in reduced proliferation, motility and angiogenesis. It also induces autophagy.

PLAGL1

PLAGL1 encodes a transcription factor with antiproliferative activity. It is down regulated in 39% of ovarian cancers and re-expression inhibits cancer cell proliferation.

PEG3

PEG3 is down regulated in 75% of ovarian cancers and its re-expression inhibits growth and induces apoptosis. Recent evidence supports the view that it is involved in the p53/c-myc apoptosis pathway [161].

TP53

Undoubtedly, however, the key tumour suppressor gene associated with ovarian cancer is *TP53*. This gene has been named “guardian of the genome” and is the most commonly

mutated gene in human cancers [162]. The p53 protein is a transcription factor which is activated in response to cellular stress, particularly DNA damage. Activation of p53 results in the transcription of the gene encoding the cell cycle regulator p21, which inhibits cyclin-dependant kinases resulting in cell cycle arrest. In addition, p53 can induce the expression of pro-apoptotic genes (discussed below). Thus, p53 can be considered to promote genomic integrity by causing the elimination of cells with damaged DNA. Mutation of p53 inactivates this function, and in some cases allows p53 to act as an oncogene. 60-80% of sporadic and familial ovarian cancers carry a mutated *TP53* gene. Mutation of *TP53* is associated with a higher metastatic potential [163], [164]. There does not appear to be a statistically significant correlation between mutation of *TP53* and the chemoresistance of ovarian cancer [165].The restoration of the p53 function has been tested and although in a cellular level the effects were promising, there were no significant clinical results [166], [167].

MDM2 is an E3 ubiquitin ligase that binds to p53, catalyses the ubiquitination of p53 and targets p53 for degradation. Hdm2 (the human homolog of Mdm2) is overexpressed in one third of ovarian cancers. The inhibition of MDM2 with small molecular mass compounds in leukaemia patients with wild-type p53 resulted in increased apoptosis and p53 stability [168]. However, it is doubtful whether this strategy will be broadly effective in ovarian cancer, because only a small proportion of tumours retain wild-type *TP53*.

MLH1

MLH1 encodes a protein involved in DNA mismatch repair gene. Platinum resistance has been linked to CpG methylation of this gene and in preclinical models this was reversed with demethylating agents [169].

PTEN

PTEN is the 2nd most commonly mutated tumour suppressor gene [170], [171].

PTEN encodes a phosphatase which converts the lipid phosphatidylinositol trisphosphate (PIP_3) to phosphatidylinositol bisphosphate PIP_2. PIP_3 is necessary for the activation of the Akt signalling pathway which promotes cell survival. Consequently, mutation of PTEN leads to the activation of the Akt signalling pathway. Mutations in PTEN are found in 3-8% of sporadic ovarian cancers, of the endometrioid histotype.

1.2.4 Genetic alterations in ovarian cancer subtypes

Investigations of the mechanisms leading to ovarian cancer have not yet revealed genetic abnormalities uniquely characteristic for a particular subtype of disease. However, some molecular abnormalities have been recognised as being more frequent in certain subtypes.

Serous carcinoma

Mutations of the p53 gene are very common in high grade serous carcinoma with more than 95% of tumours carrying an inactive p53 [172]. The TCGA project also found that 21% of patients carry germline or somatic mutations in their BRCA genes[173], [70]. The PI3K pathway is often activated in these cancers conferring a survival benefit [174].On the other hand the less “aggressive” low grade serous carcinoma is characterised by mutations in the *KRAS* and *BRAF* pathway [175] or ERBB2 [176].

Endometrioid carcinoma

Endometrioid carcinoma is characterised by mutations in the gene encoding β catenin [177],[178] which is involved in two biological processes, signal transduction [179] and cell to cell adhesion [180]. These are present in 38-50% of cases [181]. Mutations of *PTEN* are also very common (43%) in this subtype [182].

Mucinous carcinoma

Mucinous carcinomas are often characterised by mutations in *KRAS* [183], [184].

Clear cell carcinoma

Clear cell carcinoma is interestingly characterised by the lack of *TP53* mutations [185] , [186]. The expression of *HER2* is twofold higher that other subtypes [187].

Hereditary ovarian cancer

The frequently involved oncogenes in the development of hereditary ovarian cancer include *c-ERBB-1* (EGFR), *c-ERBB2* (*HER2/NEU*), *MYC*, K-RAS, β-catenin (CTNNB1) [188]. Tumour suppressor genes which are involved include *TP53*, *PTE*N, *BRCA1* and *BRCA2* [189].

1.2.5 Signalling pathways in ovarian cancer

The consequence of gene copy number changes and gene mutations is the activation of several signalling pathways. Although a diverse range of genetic changes have been identified, many of these map to a discrete set of signalling pathways that are activated in ovarian cancer. Estimates of the number of pathways activated vary, and are likely to change as function is assigned to genes whose role is currently poorly understood. Thus far, seven signalling pathways have been identified which are activated in more than half of ovarian cancers.

The PI3K-Akt-mTOR pathway.

This pathway is a key regulator of proliferation, growth, survival and metabolism of cells [190]. It is activated in up to 70% of ovarian cancers (although this varies between histological type and different reports) and contributes to chemoresistance [120] to conventional cytotoxic agents and EGFR inhibitors [191]. The mechanisms which lead to the activation of this pathway in ovarian cancer include amplification or mutation of *PIK3CA*, loss of *PTEN* function or *AKT* amplification. Inhibitors of PI3K have been shown to increase the sensitivity of ovarian cancer cells to paclitaxel [192].

The Ras-MAPK pathway.

This pathway is a signal transduction pathway, which regulates cell growth, differentiation, survival and death. In ovarian cancer this pathway is activated by mutations in *HER2*, *KRAS* and *BRAF* [193], although the overall frequency of activation is thought to be less than 50% of ovarian cancers [120].

The IL-6 pathway.

IL-6 activates the IL-6 receptor which then triggers a number of signalling cascades. The downstream pathways involved include Jak2/STAT3, Ras/MAPK and PI3K–Akt pathways [194]. JAK2 is a kinase activated by the IL-6 receptor which subsequently phosphorylates and activates the transcription factor STAT3. In ovarian cancer this results in higher proliferation, enhanced angiogenesis and inhibition of apoptosis [120]. This pathway has been estimated to be activated in approximately 70% of ovarian cancers [120].

Signalling through Src.

Src is a tyrosine kinase which is highly expressed in several cancers and can be activated by several receptor types including growth factor receptors and integrins. It can regulate proliferation, invasion and migration [195], [196]. It has been estimated to be activated in more than 50% of ovarian cancers [120].

The lysophosphatidic acid (LPA) pathway.

It is thought that the activation of this pathway contributes to both cell cycle progression, cell survival and metastatic spread of ovarian cancer. LPA is a bioactive lipid that activates a family of G-protein coupled receptors. LPA increases the levels of the cell-cycle-regulator cyclin D1 [197] and the growth factors VEGF [198] and IL-8 [199] and contributes to platinum chemoresistance [200] . It has also been demonstrated that LPA promotes the production of IL-6, thus activating that pathway [201]. Autotaxin, an enzyme which catalyses the production of LPA, is overexpressed in ovarian cancer and it has been shown that it delays apoptosis caused by carboplatin [202]. It has thus been implicated in chemoresistance. Levels of LPA are frequently elevated in patients with

ascites, in which it can reach concentrations up to 80μM. It is perhaps not surprising, then, that this pathway has been estimated to be activated in 90% of ovarian tumours [120].

The MEKK3- IKK - NF-kB pathway.

More than 50% of ovarian cancers are characterised by an active MEKK3 pathway [120]. The kinase MEKK3 activates the IKK complex, which in turn phosphorylates and inactivates IκB, an inhibitor of the transcription factor NF-κB [203],[204]. This transcription factor promotes the expression of antiapoptotic genes and cell growth [120].

The Mullerian inhibitory substance (MIS) receptor.

More than 50% of ovarian cancers and stable cell lines are characterised by the presence of MIS receptors [120] . The MIS is responsible for the regression of foetal Mullerian ducts in males and constitutes a possible therapeutic target for ovarian cancer as it has been shown to inhibit ovarian cancer cell growth in vitro [205].

The PKCι pathway.

This pathway has been estimated to be activated in 78% of ovarian cancers [120]. Protein Kinase C (PKC) represents a family of kinases which regulate multiple cancer signalling pathways. Although its function is not fully understood, PKCι suppresses apoptosis, in part by providing an activating link between Src and the NFκpathway. This may explain recent data suggesting that it also plays a role in the development of ovarian cancer chemoresistance [206].

1.2.6 The role of malignant ascites in ovarian cancer

Approximately two thirds of ovarian cancer patients present with ascites [7],[207]. The collection of fluid in the peritoneal cavity of ovarian cancer patients is the result of increased vascular permeability. Factors that increase vascular permeability such as the vascular endothelial growth factor A ($VEGF_A$) [208] play a role in this process. There have been clinical studies which show that treatment with antibodies against $VEGF_A$ resulted in a reduction in the developing ascites [209], [210].

The formation of ascites in ovarian cancer patients contributes to pathogenesis through at least two mechanisms: Firstly, the collection of peritoneal fluid contributes to the flow of malignant cells within the peritoneal cavity and facilitates metastatic spread. Secondly, the secretions of tumour cells in ascites contain factors that inhibit the immune system and favour carcinogenesis [211], [212], [213], [214], [215], [216], [217], [218]. MUC16 has been shown to protect ovarian cancer cells by blocking the formation of synapses between NK cells and cancer cells [219]. Additionally, MUC16 changes the phenotype of NK cells in the peritoneal fluid to cells with less cytotoxic features [211]. As noted above, ascites contains significant quantities of the pro-survival factor LPA.

1.2.7 The molecular mechanisms of ovarian chemoresistance

Escaping cell death is considered one of the hallmarks of cancer [220]. Our knowledge of the molecular mechanisms underlying the development of platinum resistance in ovarian cancer is incomplete. However, it is clear that it is multi-factorial (Table 8). The cellular/molecular events contributing to drug-resistance are described below.

Limited availability of drug to the intracellular environment due to increased efflux

The uptake and efflux of cisplatin in ovarian cancer cells is associated with the emergence of chemoresistance. ATP7A and ATP7B are copper transporting enzymes which are highly expressed in chemoresistant cancer cells. These enzymes reduce the intracellular accumulation of platinum through increased efflux [221],[222],[223]. Real time polymerase-chain reaction (RT-PCR) studies identified a higher expression of ATP7B in resistant compared to sensitive ovarian cancer cells [224]. The combination of siRNA ATP7B silencing with cisplatin therapy in platinum resistant mouse models demonstrated a 70-80% reduction in tumour growth [225]. ABCC2, another exporter protein has been linked to clinical chemoresistance and is also considered to promote increased efflux of the drug[226]. However, trials of inhibitors of P-glycoprotein P, a drug pump associated with drug resistance, have so far not been effective.

Limited availability of drug to the intracellular environment due to drug inactivation

There are sulphur-containing molecules (such as glutathione) in the intracellular environment which show a higher reactivity than the DNA towards platinum-based compounds. The exposure of cancer cells to cisplatin has been shown to cause an increase in the levels of glutathione and a subsequent inactivation of the drug, leading to the development of progressively higher degrees of drug resistance [227], [228],[229],[230].

Increased DNA repair activity in cancer cells

Cisplatin interacts with DNA, forming adducts which modify its structure and deregulate its ability for transcription. Increased repair of the DNA through the nucleotide excision repair (NER) pathway by removal of cisplatin adducts is considered to contribute to the chemoresistance of ovarian cancer to cisplatin [231], [232],[233]. The excision repair cross-complementation group 1 (ERCC1) protein mediates the NER pathway and is found to cause *in vitro* resistance to cisplatin [234]. 75% of patients with chemoresistant disease have positive ERCC1 expression and siRNA silencing of ERCC1 expression increased the sensitivity of platinum-resistant stable cell lines [235].

Bypass of DNA damage

Poly ADP-ribose polymerase (PARP) is an enzyme which is essential for the repair of single strand DNA breaks [236] Loss of function of this mechanism results eventually in double strand breaks. These may be repaired by homologous –recombination repair mechanisms in which BRCA1 and BRCA2 are major regulators. Studies in animal models showed that the combination of a PARP inhibitor with chemotherapy resulted in improved survival [237] while in phase I clinical trials the inhibition of PARP had an improved clinical outcome and stabilized disease [238], [239].PARP inhibitors are also being evaluated as monotherapy. In patients whose tumours have a defect in homologous recombination, for example those with BRCA defects, DNA repair depends on PARP, and consequently these tumours are highly sensitive to PARP inhibition. This is referred to as "synthetic lethality" – because inhibition of either BRCA or PARP on its own is ineffective, but is lethal when both the BRCA and PARP are non-functional. In these cells, double strand break repair is dependent on non-homologous end joining which is highly error prone and subsequently leads to cell death.

There is evidence to suggest that platinum-resistant ovarian cancer cells exhibit a replicative bypass activity against the DNA adducts formed by cisplatin. Such an activity was not observed when these cells were exposed to UV light stress or a different chemotherapeutic agent, suggesting that it is specific to the lesions induced by cisplatin. Thus, although DNA damage still occurs in response to cisplatin, it does not result immediately in cell death and so manifests itself as drug resistance.

Deregulation of apoptosis in cancer cells

A mechanism which confers to the development of chemoresistance is the inhibition of apoptosis that is otherwise induced following DNA damage. The AKT signalling pathway conveys protection to cancer cells from cisplatin through several mechanisms, including by inhibiting the x-linked inhibitor of apoptosis (XIAP), blocking caspase 3 activity and subsequent apoptosis [240] . It also acts by blocking other proapoptotic proteins [241], [242].

Aurora kinases are involved in cell cycle regulation and they are considered to contribute to drug resistance [243]. Aurora-A was found to be overexpressed in 83% of EOC patients [244] and there is evidence to suggest that it promotes cell survival by activating the AKT pathway and deregulating the cell cycle checkpoint mechanisms [245], [246]. Inhibition of the Aurora-A activity in chemoresistant ovarian cancer cells lead to reduced tumour growth and the combination of Aurora inhibitors with cisplatin demonstrated synergistic effects [247].

The *BCL2L1* gene encodes the anti-apoptotic protein, Bcl-X_L. In ovarian cancer Bcl-X_L expression is associated with shorter disease-free interval after therapy and chemoresistance, [248], [249], [250],[251],[252]. Inhibition of Bcl-X_L by "BH3-mimetics"has been shown to increase sensitivity to cytotoxic agents [253], [254], [255] and these are discussed in more detail below (Figure 3) [256].

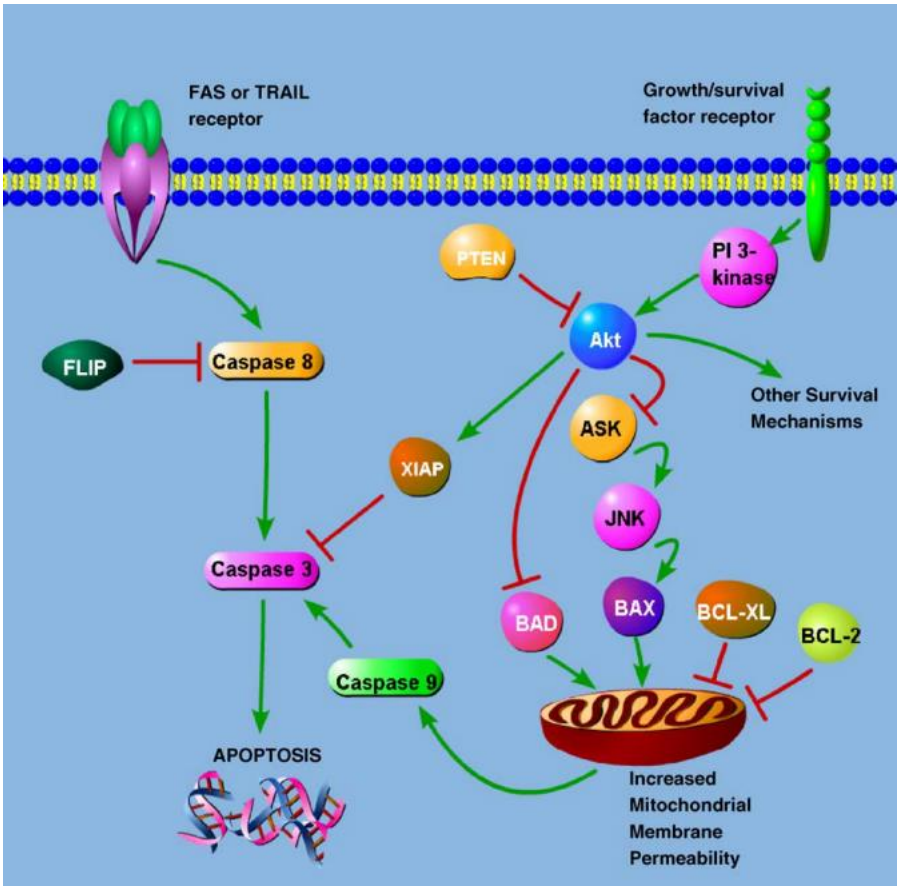

Figure 3: Apoptosis signalling pathways implicated in drug resistance in ovarian cancer ,reproduced from [257], [258].

Changes to the tumour microenvironment

Many of the genes which are involved in the organization of the extracellular matrix (ECM) are activated in chemoresistant ovarian cancer cells [259]. It has long been suggested that the ECM interacts with tumour cells and confers protection against chemotherapy [260] .The production of collagen VI from cancer cells has been reported to be contributing to their chemoresistance [261] and studies where cisplatin sensitive cells were cultured in collagen VI containing conditions resulted in the emergence of cisplatin resistance [259]. This may in part reflect the ability of integrins, receptors for extracellular matrix, to activate cell survival pathways.

Genes and molecular pathways implicated in ovarian cancer drug resistance	
Function	**Gene or pathway**
DNA damage and repair	the nucleotide-excision repair system-*ERCC1* gene (NER), DNA mismatch repair (MMR)- *MLH1* gene, *BRCA1/2* genes
Apoptosis regulation	caspase inhibitor XIAP, BCL-2 family anti-apoptotic members, FLIP apoptosis inhibitor
Growth factor receptors	IGF-R, TrkB, EGF receptor family, *HER-2*
Cell proliferation-migration	*Src*
Cell survival	*Akt-PI3*-kinase pathway, *PTEN*,

Table 8: Genes and molecular pathways implicated in ovarian cancer chemoresistance[238].

1.3 The role of apoptosis in ovarian cancer

1.3.1 What is apoptosis?

The phenomenon of cell death in multicellular organisms is an essential part of the natural cell turnover process. It helps promote homeostasis by controlling normal cell numbers during development and disease. If damaged cells were to accumulate as a result of injury, infection or stress then the functional capacity of tissues would be impaired. One form of controlled and programmed cell death is known as apoptosis [262]. The first medical use of the word "apoptosis" is found in Hippocrates's book "Mochlicon" to describe the "falling off of the bones" and Kerr & Wylie reintroduced it in 1972 with its current meaning.

Apoptosis is characterized by specific morphological changes (Figure 4) which include cellular shrinkage and fragmentation into apoptotic bodies, which are subsequently opsonized for cleavage by macrophages [263], [264].

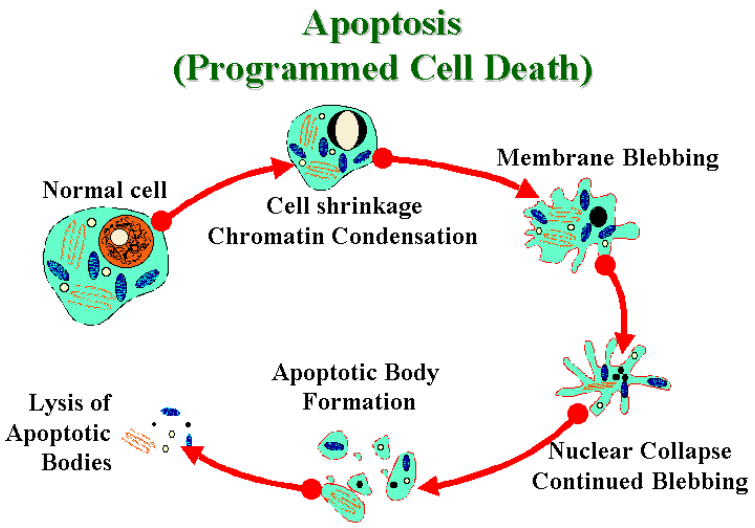

Figure 4: Overview of the process of apoptosis, reproduced from [265].

The cell death machinery which initiates apoptosis operates by having sensor molecules which detect cellular stress or damage through transcriptional mechanisms [266]. The biochemical changes that underpin apoptosis are mediated by a family of cysteine proteases (caspases) initially expressed as inactive zymogens which are proteolytically processed to their active state by an apoptotic signal [267] (Figure 5). Caspase proteins exist as inactive silent pro-enzymes and are activated by proteolytic cleavage in hierarchical order. The initiator caspases (caspases -6, -8, -9 and -10) are activated first which then activate downstream effector caspases (-2, -3 and -7). The release of cytochrome c from mitochondria in the intrinsic apoptosis pathway results in the activation of caspase-9 and the formation of the apoptosome which confirms the commitment of the cell to programmed death. The evasion of apoptosis is considered a hallmark of cancer which can explain the survival of cancer cells after chemotherapy and the development of chemoresistance [220], [268].

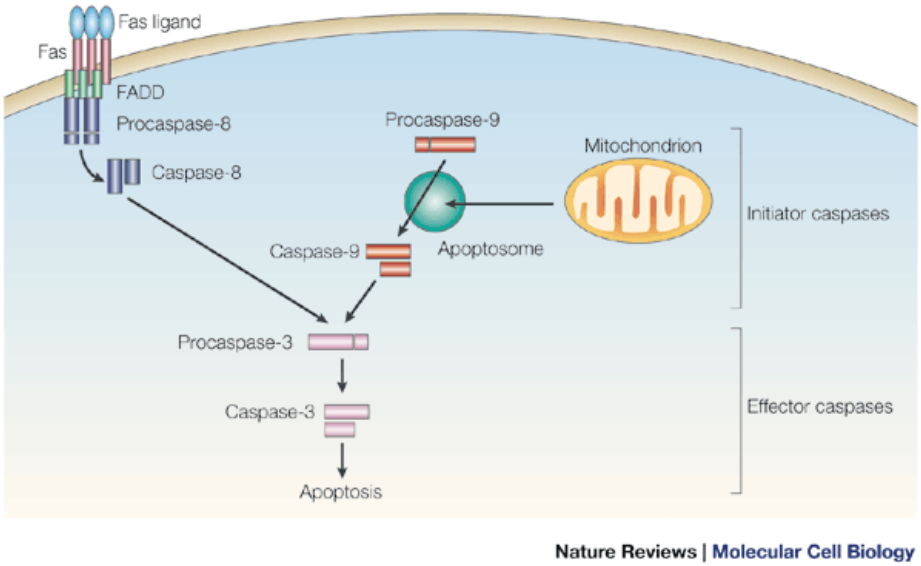

Figure 5: The caspase cascade at apoptosis, reproduced from **[269]**.

1.3.2 The cellular pathways that activate apoptosis

Apoptosis signalling can be initiated from outside the cells via plasma membrane receptors (referred to as the extrinsic pathway) or through stress that originates from within the cell (referred to as the intrinsic pathway) [270], [271]. The extrinsic pathway is triggered when either receptors for Fas or TRAIL are activated by their ligands [272]. This leads to the formation of a death-inducing signalling complex which in turn activates caspase-8 and 10. Mutations and deletions of the Fas gene have been found in haematological malignancies [273], [274]. The intrinsic pathway, also referred to as the mitochondrial pathway is characterized by the mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome-c, an enzyme normally found in the space between the inner and outer mitochondrial membrane (OMM) [275]. This pivotal event represents a “point-of-no-return” step because once it is passed it constitutes an irreversible decision to commit cellular suicide (Figure 6).

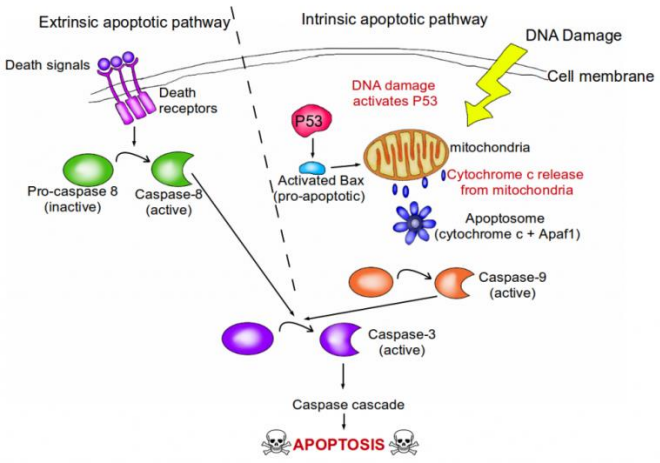

Figure 6: Overview of the apoptotic pathways, reproduced from [276].

1.3.3 The role of the Bcl-2 family of proteins in apoptosis

The intrinsic apoptosis pathway is controlled by the Bcl-2 family of proteins [277].This family comprises evolutionary related proteins with possess either pro-apoptotic or anti-apoptotic action. There are four different classes of proteins in this family; the proapoptotic “effectors” (Bax, Bak), the anti-apoptotic “inhibitors” (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, Bfl/A1, Bcl-B), the proapoptotic “activators” (Bim, Bid, Puma [2-4], possibly Bmf and Noxa [5]) and the pro-apoptotic “sensitizers” (e.g. Bad, Bmf, Noxa) (Figure 7).

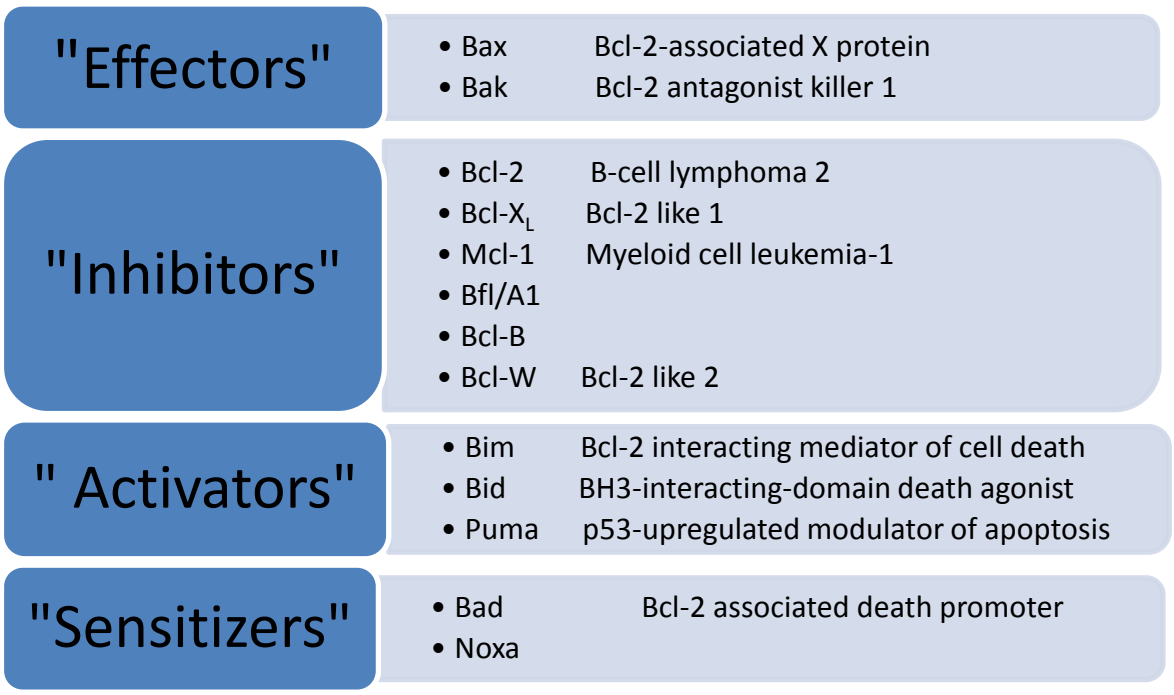

Figure 7: The division of the Bcl-2 family of proteins to anti- and pro-apoptotic members.

The members of the family are defined by their unique α-helical composition of up to 4 Bcl-2 homology domains termed BH1,BH2,BH3 and BH4 (Figure 8). The genetic background of the Bcl-2 control of apoptosis was first discovered when mutants of the organism *Caenorhabditis elegans* were observed to cause failure in apoptosis to cells which normally undergo this procedure during development [278]. The proteins responsible for blocking apoptosis were found to share homology with mammalian proteins including B-cell CLL/lymphoma 2(Bcl-2) [279]. The *BCL-2* gene was originally identified at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma [280] and is considered the founding member of the family. The study of the function of *BCL-2* revealed its contribution as preventing cell death rather than promoting cell proliferation [281].

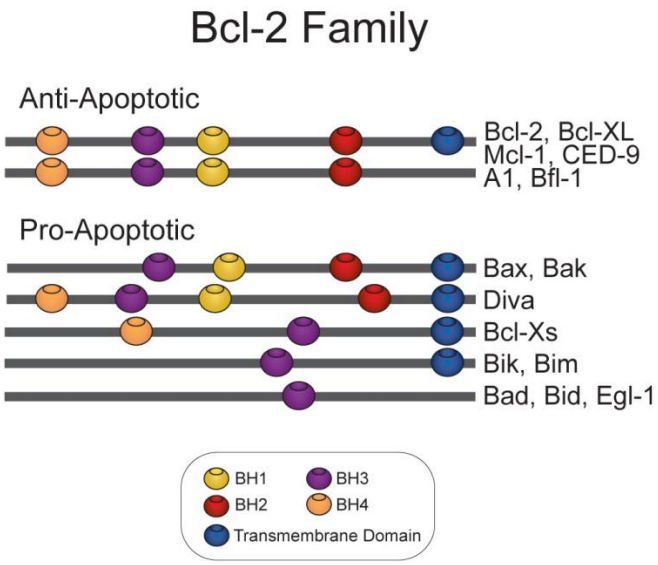

Figure 8: The Bcl-2 family of proteins, reproduced from [282].

The pro-apoptotic "effector" molecules of the Bcl-2 family

The pro-apoptotic "effector" molecules of the family include the Bcl-2 antagonist killer 1 (Bak) and the Bcl-2-associated X protein (Bax). These proteins contain BH domains 1 to 4 [283]. Bak and Bax can independently permeabilize the OMM [284], [285], [286, 287]. Bax and Bak are essential since mitochondria deficient for Bax and Bak fail to release cytochrome c [288]. The loss of Bax or Bak renders cells resistant to permeabilization caused by BH3-only proteins [289].

The pro-apoptotic BH3-only proteins of the Bcl-2 family; "activators" and "sensitizers"

The BH3-only proteins are defined by the presence of a single BH domain in their molecule, the BH3 domain. They include the "activators" such as the Bcl-2-interacting mediator of cell death (Bim), the BH3-interacting-domain death agonist (Bid) and the p53-upregulated modulator of apoptosis (Puma) and the "sensitizers" such as the Bcl-2-associated death promoter (Bad) and Noxa (the latin word for damage; also known as PMAIP1). All these BH3-only proteins can be activated by various different stimuli via several mechanisms including transcriptional up regulation, subcellular localization and/or post-translational modifications [290].

The anti-apoptotic “inhibitors” of the Bcl-2 family

The anti-apoptotic “inhibitors” currently known are Bcl-2, Bcl-X_L (Bcl-2 like 1), Mcl-1 (myeloid cell leukemia-1), Bfl/A1, Bcl-b and Bcl-W (Bcl-2 like 2) and they contain BH domains 1 to 4. They all share a conserved hydrophobic groove formed by 6 alpha (α) helices and which binds to the BH3 domain. The structure of the groove varies slightly from one member to the other and these molecular variations can explain how different pharmacologic inhibitors that target these anti-apoptotic BCL-2 “inhibitors” have different binding affinities for various family members.

1.3.4 The regulation of apoptosis in ovarian cancer

The exposure of a cell to stress or any other pro-apoptotic treatment triggers transcriptional and post-translational regulation of individual members of the Bcl-2 family. It is the dynamic equilibrium between the opposing functions of the pro- and anti-apoptotic members that affects the integrity of the outer mitochondrial membrane.

The activation of Bak and Bax involves a conformational change which exposes the N-terminus of the proteins that is otherwise hidden [291]. Bak is localized primarily in the OMM with only a small fraction sited at the endoplasmic reticulum (ER). Bax is cytosolic and once a death stimulus has been received it migrates to the OMM and becomes an integral membrane protein that can induce permeabilization [292]. The homo-oligomerization of Bax and Bak leads to MOMP through the formation of proteolipid pores on the mitochondrial surface. This causes the release of mitochondrial proteins such as the cytochrome c and second mitochondria-derived activator of caspase (SMAC also known as DIABLO) from the mitochondrial intermembrane space into the cytosol, where they interact with caspases and activate them.

Two competing models have been proposed for how Bax and Bak are activated, but recent data supports a “direct activation” model in which the BH3-only “activators” bind directly but transiently to the “effectors” Bax or Bak to activate them [293]. The alternative “indirect” model envisages that the effectors” do not require any activation and are continuously sequestered by the “inhibitors”. Apoptosis is induced indirectly by the BH3-only proteins as they bind to the “inhibitors”, allowing for the “effectors” to be

released fulfilling their pro-apoptotic potential. Other variations of both of these models have been suggested and it is possible that aspects of both of them are correct.

The anti-apoptotic “inhibitors” preserve the integrity of the outer mitochondrial membrane by binding to both the “effectors” and the BH3-only “activators”. When the BH3 domain of Bax or Bak is sequestered by antiapoptotic Bcl-2 family proteins their apoptotic function is lost. However, the binding of the “sensitizers” through their BH3 domain to the hydrophobic BH3-binding groove of the “inhibitors” can reduce their capacity to sequester the “activators” or the “effector” molecules. The overall reduction in the cellular reservoir of the “inhibitors” results in the effective transmission of pro-apoptotic signalling from the “activators” to the “effectors” Bak and Bax (Figure 9).

Many types of cancer including ovarian cancer, haematological malignancies and gastrointestinal cancers are characterised by an elevated expression of various prosurvival proteins [250], [294]. In more than 70% of ovarian cancers the expression of anti-apoptotic proteins Bcl-2, Bcl-X_L and survivin is increased compared to normal ovarian tissue [295]. High levels of Bcl-X_L have been linked to resistance to platinum and taxane therapy and disease recurrence [248], [296]. Altered expression of survival proteins is often due to translocation, gene amplification or mechanisms non-related to genomic alterations. In leukemia cell lines chemoresistance was induced *in vitro* by the overexpression of Bcl-X_L [251]. Overexpression of Bcl-X_L also contributes to the resistance of other types of cancer such as pancreatic [249] .

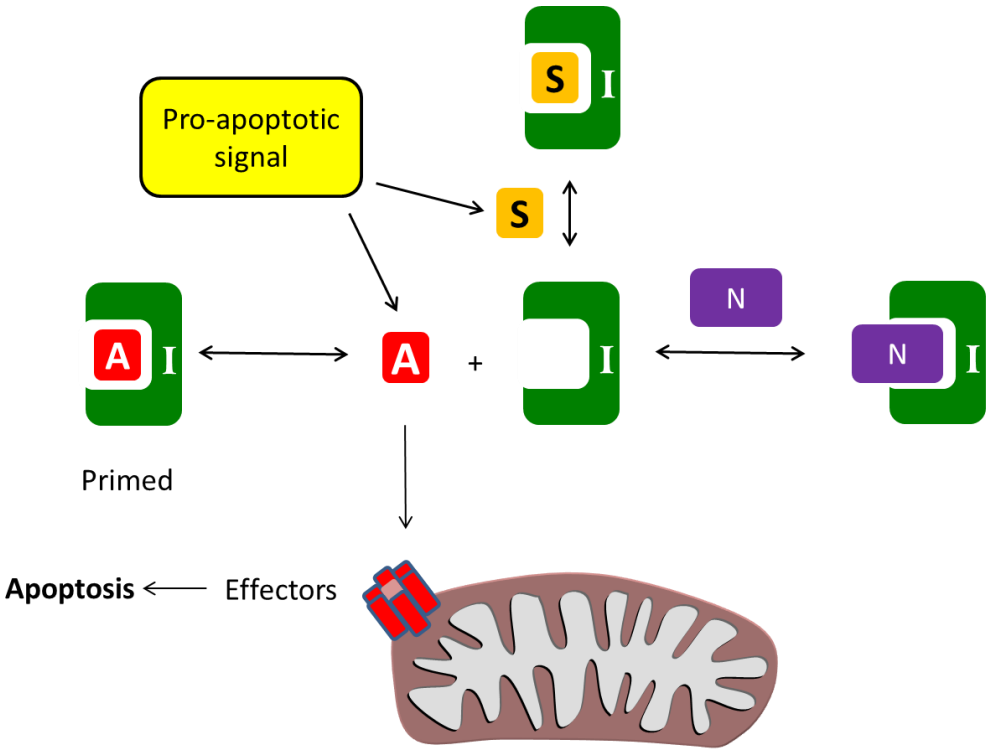

Figure 9: The regulation of the intrinsic apoptosis pathway by the Bcl-2 proteins.

Apoptosis is triggered by the formation of a pore in the mitochondrial outer membrane formed from the effectors. This is controlled by the activator molecules (Bid, Bim, Puma, labelled "A") which directly stimulate the effectors (Bak and Bax). A pro-apoptotic signal (such as oncogene stress or chemotherapy) induces the expression of apoptosis activators and sensitizers. The activators may be sequestered by the inhibitors (labelled "I", e.g. Bcl-2, Bcl-X_L, Mcl-1) and this prevents apoptosis. However, sensitizer molecules (labelled "S", eg Noxa, Bad) or BH3 mimetics (e.g. ABT-737, labelled "ABT") can occupy the inhibitors, preventing them from binding the activators. In cells in which the inhibitors are already primed with an activator, the effective displacement of the activator from the inhibitor by ABT-737 induces apoptosis.

The interaction between pro- and anti-apoptotic proteins of the Bcl-2 family is also regulated by the different binding capacity between the "inhibitors" and their ligands. Some BH3-only proteins can bind to all the apoptosis inhibitors, whereas other BH3-only proteins exhibit specificity for a subset of the apoptosis inhibitors. For example, Bad bind preferentially to Bcl-2, Bcl-X_L and Bcl-W [7, 8], whereas Noxa binds preferentially to Mcl-1 [7, 8] and possibly Bfl/A1 [7]. The BH3-only apoptosis activators Bim and Puma are each able to bind to all the apoptosis inhibitors whereas Bid has a more restricted binding profile [7, 9]. Whether the induction of a BH3-only protein leads to apoptosis depends on whether the repertoire of apoptosis inhibitors expressed is adequate to dampen the pro-apoptotic signal arising from the activation of BH3-only proteins (Figure 10).

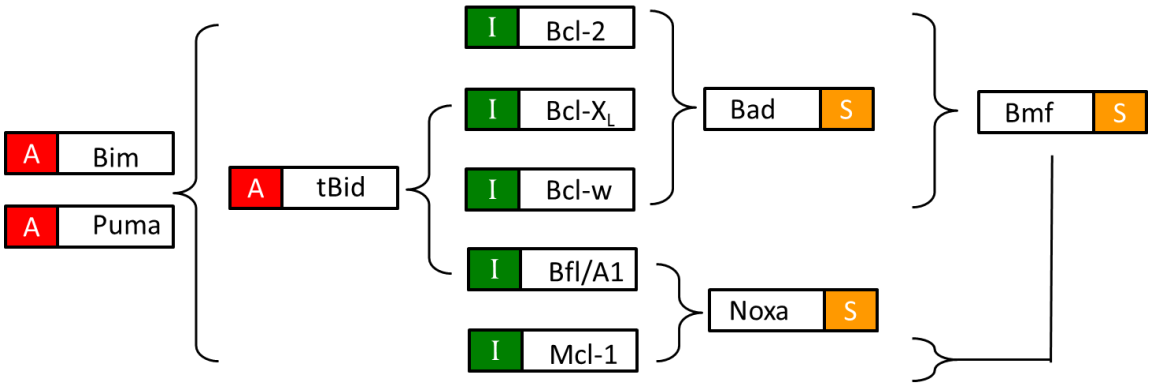

Figure 10: The binding specificity of BH3 only proteins. The activators (denoted with an "A"), Bim and Puma bind to all the apoptosis inhibitors (denoted with an "I"). However, tBid and the sensitizers (denoted with an "S") bind to a subset of the apoptosis inhibitors. There are some discrepancies in the literature (e.g., whether Bmf binds Mcl-1) and it is important to note that this diagram summarizes interactions (adapted from [7-9] in a binary fashion (interaction or no interaction) whereas in reality the interactions are better described by a range of binding affinities.

The Bcl-2 family is also responsible for interacting with non-apoptotic cell-death machinery such as that which regulates autophagy [297]. Autophagy is a type of cell death characterised by the formation of double-membrane vacuoles called autophagosomes [298],[299] which merge with lysosomes and degrade the cellular components [300]. Bcl-2 family proteins also bind to the endoplasmic reticulum, where they modulate autophagy, another important pathway regulating cell survival and death [294]. The proteins Bcl-2 and Bcl-X_L can sequester beclin 1 (BECN1), a tumour suppressor protein which activates autophagy. Bcl-2 and Bcl-X_L bind the BH3 domain of the molecule of beclin and inhibit autophagy [301]. The addition of a BH3-only protein or a drug which binds to the Bcl-2 family inhibitors can reverse this and induce autophagy [302]. Autophagy plays a dual role in cell survival as it can provide the cell with energy at times of starvation or, when there are excessive stress conditions can become a suicide pathway [303].

1.3.5 The use of BH3 mimetics as treatment for ovarian cancer

The introduction of platinum-based compounds and taxanes over the last forty years has resulted in an improvement in survival for patients diagnosed with ovarian cancer but it appears likely that conventional chemotherapy has achieved its maximum potential (although incremental improvements are being explored with differing schedules such as dose-dense chemotherapy and different routes such as intraperitoneal administration). Standard chemotherapy could however be used in tandem with a type of targeted therapy that acknowledges the different molecular abnormalities which characterize every subtype of EOC [181].

It has been shown that where the anti-apoptotic Bcl-2 “inhibitors” are overexpressed the apoptotic cell death is prevented. In some cancer cells, constitutive expression of pro-apoptotic BH3-only proteins renders the cells dependent on BH3-only proteins for survival. Such cells have been described as being “primed for death”. Alternatively, treating cells with pro-apoptotic stimuli such as chemotherapy can increase the expression of BH3-only proteins. In both cases, the cells become dependent on the expression of Bcl-2 family inhibitors for survival. Evidence in support of this hypothesis was provided by the observation that a modified peptide derived from the BH3-only protein Bid displayed anticancer activity in haematological malignancies and xenografts [304].

In the last two decades our progress in understanding the function of the Bcl-2 family of proteins has contributed to the development of drugs, which prevent Bcl-2 family

inhibitors from binding the BH3-only pro-apoptotic proteins. There are several agents of this category that have been developed so far. The first drug (oblimersen sodium) targeting Bcl-2 was a Bcl-2 antisense oligodeoxynucleotide which showed chemosensitising effects when combined with chemotherapy drugs in chronic lymphocytic leukaemia (CLL) patients[305]. However, this molecule has failed to progress in clinical trials and any efficacy observed may reflect off-target effects. Other small molecule inhibitors have recently been developed. These include molecules that affect expression of anti-apoptotic Bcl-2 members (e.g. sodium butyrate, depsipeptide, fenretinide, flavopiridol).

BH3-mimetics are a recently described class of molecules. These are drugs which bind to the same binding site as the BH3 domain of pro-apoptotic Bcl-2 family members, thereby diminishing the capacity of the “inhibitors” to sequester the pro-apoptotic proteins. These drugs are termed BH3-mimetics because they bind to the hydrophobic groove of the anti-apoptotic Bcl-2 proteins. These BH3-mimetics antagonise the Bcl-2 family apoptosis “inhibitors” and are expected to induce apoptosis in cancer cells that are primed for death by liberating pre-bound BH3-only “activators”. This can explain why BH3-mimetics have exhibited remarkable single agent activity in primed cancer cells [306]. Alternatively, in cells treated with chemotherapy, the expression of BH3-only proteins is usually increased. BH3 mimetics effectively lower the threshold at which cells undergo apoptosis because they prevent the Bcl-2 family “inhibitors” from sequestering the BH3-only proteins. Consequently, BH3-mimetics have shown synergy with several chemotherapeutic agents that induce BH3-only proteins in cells that are not primed already with a BH3-only activator [307]. In both cases (primed cells or cells in which BH3-

only proteins have been induced), the BH3-mimetics elicit their effect because the cancer cells become dependent on Bcl-2 family proteins for survival. Thus, BH3-mimetics are not thought to directly induce apoptosis on their own, but require the induction of the pro-apoptotic BH3-only proteins. This suggests that targeting the Bcl-2 family could be an effective way to facilitate cancer cell death either as single agent or combination therapy [308].

Gossypol is the first compound that demonstrated inhibition of Bcl-2,Bcl-X_L and Mcl-1 and was originally discovered during the 1950s [309]. Its analogue, apogossypol is still being developed and seems to possess less systemic toxicity and target Bcl2- and Mcl-1 better [310].

ABT-737 is a selective inhibitor of Bcl-2, Bcl-X_L and Bcl-W [311]. Nuclear magnetic resonance–based screening and structure-based design targeting the Bcl-2 family of proteins was employed for the development of this compound, with linking of two different molecules of modest affinity into a single molecule of high affinity [310]. Preclinical studies have shown in ovarian cancer cells that it increases the sensitivity to carboplatin [312]. As a single agent ABT-737 showed limited potency in ovarian cancer [312], in contrast to its activity against lung cancer and leukaemia cells. This may in part be attributed to the fact that it binds to Mcl-1, an anti-apoptotic Bcl-2 protein expressed in several ovarian cancer cell lines, with a weak affinity [312]. Mcl-1 has become recognized as one of the key mediators of resistance to ABT-737. Alternatively, ovarian cancer cells may not be “primed” for death. However, ABT-737 lacks adequate oral bioavailability, and this prompted the development of ABT-263 (navitoclax), an orally bio-

available analogue of ABT-737. ABT-263 has similar pharmacological properties to ABT-737, including relatively poor affinity for Mcl-1.

In contrast to this, GX15-070 (Obatoclax) is a pan-Bcl-2 inhibitor that inhibits all of the anti-apoptotic Bcl-2 proteins. This overcomes the potential for resistance due to expression of Mcl-1. It has been shown to induce features of apoptosis even without the activation of the intrinsic apoptosis pathway because cell death is reduced (but not prevented) in cells lacking Bax or Bak. It is possible that its activity involves additional targets relating to mitochondrial damage [313]. It is being assessed in phase I and II clinical trials for haematological malignancies. It is discussed in more detail in chapter 4.

HA14-1 is another small-molecule inhibitor of the anti-apoptotic Bcl-2 proteins which was developed by structure-based screening [314]. It seems to be potent both as single agent and in combination with other chemotherapeutics and has a very high binding affinity for Bcl-2 [314]. Other “BH3 mimetics” such as BH3 inhibitor-1 and -2 and Antimycin A [315] are still in preclinical development and appear to possess other, additional mechanisms of restoring the “pending” apoptosis of primed cancer cells. However, several of these drugs induce cell death in cells lacking Bax and Bak. Apoptosis is critically dependant on the latter two proteins, suggesting that these compounds induce cell death through some additional undefined mechanism and are not “pure” BH3-mimetics.

Table 9 summarizes the various BH3-mimetics currently being evaluated in preclinical and clinical development [310].

Agents targeting anti-apoptotic BCL-2 family proteins			
Agents	**Target Proteins**	**Sponsor**	**Stage**
Apogossypol	Bcl-2, Bcl-XL, Mcl-1	Burnham (NCI)	Preclinical
HA-14	Bcl-2	Maybridge Chem	Preclinical
Antimycin A	Bcl-2, Bcl-XL	University of Washington	Preclinical
BH3Is	Bcl-XL	Harvard University	Preclinical
Oblimersen sodium	Bcl-2	Genta	Phase III
Gossypol (AT-101)	Bcl-2, Bcl-XL, Bcl-2, Mcl-1	Ascenta (NCI)	Phase I/II
ABT-737 (ABT-263)	Bcl-2, Bcl-XL, Bcl-w	Abbott	Phase I
GX15-070	Bcl-2, Bcl-XL, Bcl-w, mcl-1	GeminX	Phase I
Abbreviations: BH3Is, BH3 inhibitors; NCI, National Cancer Institute; Maybridge Chem, Maybridge Chemical Co. Ltd; U, University			

Table 9: Various BH3-mimetics currently being evaluated reproduced with changes from [310].

The foregoing discussion has highlighted the need for new therapies to treat ovarian cancer. When this study was commenced, ABT-737 had been shown to increase sensitivity of ovarian cancer cells to carboplatin both as a single agent and also in combination with carboplatin [312]. These studies demonstrated activity in *vitro* (cell lines and primary cultures) as well as *in vivo* (xenografts) and also suggested that apoptosis was induced faster by this drug. However, it had not been investigated in combination with carboplatin and paclitaxel, the therapy most commonly used to treat ovarian cancer. Furthermore, although it was anticipated that navitoclax would also show synergy with carboplatin, this had not formerly been demonstrated. Thus, one goal of this work was to further investigate the potential for navitoclax in ovarian cancer.

The realization that Mcl-1 and A1 conferred resistance to ABT-737 suggested that ABT-263 might only be effective in tumours where these Bcl2 family proteins were not expressed. Obatoclax represented a newly described panBcl2- family inhibitor. Therefore, a further goal of this work was to investigate the potential for obatoclax in the treatment of ovarian cancer.

1.4 Models of ovarian cancer disease

1.4.1 Permanent cancer cell lines

A cell line is defined as a permanently established cell culture that will proliferate indefinitely given appropriate medium and conditions. It differs from a cell strain in that it has escaped the Hayflick limit and has become immortal [316]. The Hayflick limit is the number of times a normal human cell population will divide until cell division stops. The current body of research on ovarian cancer has been predominantly based on the use of ovarian cancer derived cell lines. Their use is invaluable considering their advantages, such as the long lifespan and high proliferation rate.

However, it is argued that these models are not without weaknesses and consequently do not represent the drug resistance in ovarian cancer adequately. This argument becomes particularly important when one considers the diversity of ovarian cancer as a disease and that the future of cancer treatment potentially lies in individualised care with novel "targeted" therapies rather than traditional chemotherapy [67]. Permanent cell lines are known to be different genomically from primary cultures of the same origin as they have acquired genetic alterations over the length of time [317],[318],[319], [320], [321], [322] . This is related to the fact that they have been cultured for years in non-physiological conditions and have been exposed to oxidative stress. Additionally, their drug resistance does not necessarily correlate with the clinical response of the tumours they were derived from [323] but is much more dynamic and can be influenced even by the method

of drug administration [324]. It is also impossible to compare their chemosensitivity to that of the tissue of origin and establish a link with the clinical chemoresistance. Thus, the existing repertoire of ovarian cancer cell lines do not, on their own, provide an adequate experimental model of ovarian cancer.

1.4.2 Primary cultures from ascites of ovarian cancer patients

The heterogeneity of ovarian cancer emphasizes the significance of an unmet need to develop a plethora of models which can adequately represent the diverse nature of this malignancy and allow for the optimal evaluation of new anticancer drugs. One model that has been used by many researchers is to test drugs on primary cultures of patient tumours, with the expectation that the growth of the cells in culture provides a reasonable model of clinical disease. Our group has previous experience of establishing primary cultures of ovarian cancer cells (PCOCC) from ascitic fluid and ethical approval was obtained for this study to use samples of ascites drained from ovarian cancer patients at the University Hospital of North Staffordshire [312]. The sensitivity of these cultures to obatoclax was evaluated under standard laboratory conditions (21% O_2). Although this is often referred to as normoxia, this is significantly higher than the oxygen tension in cancer tissue, which often experiences an O_2 tension of 2% or lower [325]. This is often (inaccurately) referred to as hypoxia. To approximate physiological conditions and compare this to routine laboratory conditions, it seemed appropriate to test drug activity in both 21% and 2% O_2.This is particularly relevant to drug evaluation, because O_2 tension can have profound effects on cancer cell biology [326].

The growing body of evidence regarding the validity of permanent cancer cell lines has resulted in a search for improved models of ovarian cancer disease which more closely resemble the tumour subtypes [1], [327]. Our group has previous experience of primary cultures of ovarian cancer cells (PCOCC) as a model to study drug resistance in EOC [312]. The ascites in ovarian cancer patients is an exudative fluid with a cellular fraction. It

contains mainly ovarian cancer cells, mesothelial cells and lymphocytes [328]. The cancer cell fraction is a mixture of single cells and aggregates or spheroids. These spheroids have been shown to contribute to the dissemination of the disease by adhering to the extracellular matrix [329] and the ascitic fluid contains both angiogenic factors and growth factors [328] and is thought to be playing a role in the tumour microenvironment. There are several protocols for establishing primary cultures of ovarian cancer cells (PCOCCs) from malignant ascites with the simplest one being seeding culture vessels directly with ascitic sample, which leads to cultures of EOC cells [318]. The current experience is that once established these cells usually proliferate for 6-8 passages before reaching senescence [330] and so any planned experiments should optimally be done with low passage number cells. The process of distinguishing EOC cells from other contaminating cells in the cultures is based on the use of a combination of appropriate markers as there is no unique marker for EOC cells. Potential contaminating cells include fibroblasts, endothelial and mesothelial cells and other ovarian type cells. Leucocytes, red blood cells and platelets are of minor concern as they do not survive or are removed from the culture at first passage. These distinguishing markers are summarised in Table 10.

Marker	Distinguishing cells
keratins 7, 8, 18, and 19	other ovarian cell types
17b-OH steroid dehydrogenase and mucin	extraovarian mesothelial cells
Laminin/ keratin	stromal fibroblasts
absence of factor VIII and Ulex lectin receptors	morphologically similar endothelial cells

Table 10: Distinguishing markers of epithelial ovarian cancer cells.

1.4.3 Three dimensional (3D) cell cultures

Drug discovery in oncology relies heavily on animal testing, an expensive practice neither completely representative of the human body behaviour towards the drug nor without ethical concerns. Traditionally two-dimensional cell culture models have been used *in vitro*, but the configuration of cells on a flat surface remains unnatural and cannot adequately predict how the tumours in the human body will react to a drug [331]. The two-dimensional environment lacks the ability to create areas of hypoxia with extracellular matrix influences, heterogeneous cell populations with different replication phases and the effects of soluble signalling molecules and nutrients/waste transport [332]. Additionally, it has previously been observed that chemoresistant tumour cancer cells show greater sensitivity to chemotherapy when cultured in monolayers [333]. All the above can at least explain why drug discovery in cancer very often fails in the clinical phase despite the promising pre-clinical assessments.

Three-dimensional cell cultures can bridge the gap between the *in vitro* and *in vivo* methods and create a microenvironment that more closely resembles the human physiological conditions [1]. At present a number of techniques have been described to allow for such models to be created. Previous studies on mesothelioma cancer have shown that 3D models set up from either cell lines or tumour cells exhibit a higher expression of Bcl-2 molecules and develop a degree of apoptotic resistance [334], which can be overcome by the addition of the BH3-mimetic ABT-737. This observation confirms that the involvement of the Bcl-2 family in the development of multicellular resistance to

chemotherapy [334]. Therefore, in this study ovarian cancer cell spheroids were developed as three-dimensional models in order to evaluate the effect of anticancer agents and BH3 mimetics. These were grown from the existing stock of permanent ovarian cancer cell lines.

1.4.4 Xenograft studies

Xenograft studies can be performed by implanting human cancer cells into animal models. The use of murine models as preclinical cancer models in ovarian cancer to study the activity of any compound is a well-established practice and can be considered complementary to the *in vitro* human tumour cell lines, the three-dimensional cell cultures and the primary ascitic cultures[335],[336],[337],[338]. Human tumour xenografts are the most widely used models where human cancer cells are transplanted into immunocompromised mice so that they are not rejected. The tumours are transplanted subcutaneously, intraperitoneally or orthotopically regarding the organ specific for the type of cancer. Once these tumours are developed the response to a certain therapeutic regime can evaluated by measuring tumour size.

These models have been shown to resemble both the histological characteristics and the degree of differentiation observed in the patient's tumours [339],[340]. Any limitations in their clinical predictive value are thought to be associated with the immunodeficiency of the hosts and the differences in the tumour microenvironment and drug metabolism [339],[340].

1.5 Aims & objectives

Overcoming the chemoresistance of ovarian cancer poses a major challenge for researchers today and is the main focus of my project. The major goal of the research presented in this thesis was to provide the preclinical data necessary to support the clinical development of navitoclax and obatoclax in ovarian cancer. The following hypotheses were tested.

I. Navitoclax will have weak single agent activity, but will enhance the cytotoxicity of carboplatin and paclitaxel, both in double and triple combinations.

II. Obatoclax may have significant single agent activity but will sensitize ovarian cancer cells to carboplatin and paclitaxel.

III. Furthermore, these studies would be conducted where possible using additional experimental models other than monolayer cultures maintained in 21% O_2.

Chapter 2: Materials & Methods

2.1 Ovarian cancer permanent cell lines

The ovarian cancer cell lines used in these studies were A2780, cisA2780, Ovcar-3, Ovcar-4, Ovcar-5, Ovcar-8, Igrov-1 and Sk-Ov-3 cells and were purchased at the beginning of the study from the American tissue culture collection (ATCC).

A2780, cisA2780, Ovcar-4, Ovcar-5, Ovcar-8, Igrov-1 and Sk-Ov-3 cells were grown in Roswell Park Memorial Institute medium (RPMI 1640; Lonza) supplemented with 10% fetal bovine serum (FBS; Lonza) ,2mM L-Glutamine (Lonza) and penicillin/streptomycin (Lonza, 50 U/ml).

Ovcar-3 cells were grown in RPMI supplemented additionally with 0,01mg/ml insulin (Lonza) and 1mM sodium pyruvate (Lonza).

Cells were incubated in a water jacketed incubator, at 37 °C in a humidified 5% CO_2 atmosphere. cisA2780 cells were periodically (once per month) treated with Cisplatin (1μM for 1 week, from a 1 mM DMSO stock) to maintain their chemoresistance.

2.1.1 Trypsinization

Cells were grown in T25 or T75 cell culture flasks (Triple red). Cells were routinely sub-cultured in sterile conditions twice weekly. Confluency was confirmed microscopically and flasks more than 80% confluent were deemed to require sub-culturing.

To passage the cells, the cultures were washed with 5 ml phosphate buffered saline (PBS, Lonza) and then treated with 1ml 0.01% trypsin/1 mM EDTA (Lonza) in PBS at 37°C. Cells were agitated to encourage them to detach. The trypsin was neutralized by the addition of equal volume of growth medium containing 10% FCS. The re-suspended cells were transferred to a sterile 15ml polypropylene tube (Triple red) and collected by centrifugation at 150 g for 2 minutes at room temperature. The supernatant was aspirated and the cell-pellet was resuspended in fresh medium.

2.1.2 Cell counting

Cell counting was performed with the use of the improved Neubauer hemocytometer Cells were resuspended at an estimated concentration of between 10^5 and 10^6 cells per ml. At least 100 cells were counted to determine cell number.

2.1.3 Cryopreservation

Cells were grown in T75 culture flasks to 50% confluence. Only cells of low passage number which appeared microscopically to be healthy and were growing in logarithmic phase were considered appropriate for cryopreservation.

The cells were collected by trypsinization (see section 3.1.1) and quenched with 5 ml growth medium. The freezing medium comprised complete growth medium containing 8% (v/v) sterile DMSO (Sigma Aldrich) The pellet was resuspended in 1 ml of freezing medium (estimated 2-4 million cells per ml). 200µl aliquots of this cell suspension were transferred into 2ml cryovials (Triple Red). The cells were frozen slowly (approximately -1°C per minute) by being placed inside an insulated box in a -70°C freezer overnight. The next day the cryovials were transferred to the liquid nitrogen tank (-196°C) for long-term storage.

2.1.4 Reviving cryopreserved cells

To revive the frozen cells the frozen cells are thawed rapidly by immersing in a water bath at 37°C. The content of each vial was diluted in 10 ml of pre-warmed culture medium. In the case of Ovcar-3 cells, the cells are washed at this stage once by centrifugation (150g, 3 minutes) in 10 ml growth medium to immediately remove DMSO. Other cells are directly seeded into T25 culture flasks. The medium was changed the following day to remove any residual DMSO and dead cells that have not attached to the bottom of the culture dish.

2.2 siRNA interference studies

The siRNAs used in these experiments were obtained as siGENOME SMARTpool reagents (Dharmacon) and were complementary to mRNA encoding Atg-5 or Beclin. A non-targeting SMARTpool was used as a negative control as well as an individual non-targeting control siRNA. The siGENOME SMARTpool siRNAs consist of four distinct RNA oligoduplexes per target gene.

The optimal conditions for transfection have previously been determined by work in the laboratory [312]and afforded more than 95% transfection efficiency. Cells were collected by trypsinization, seeded in 96-well plates at 5,000 cells/well for all cell lines, except Ovcar-8, A2780 and CisA2780 where 2,500 cells/were used. The cells were seeded in 80µl of antibiotic-free RPMI medium containing 10% FCS and the culture vessels were incubated overnight as described (see section 3.1).

The next morning cells were transfected with siRNA with the use of the transfection reagent Dharmafect-1. The siRNA were diluted in optimum (Invitrogen) to 10 times the desired final concentration. In parallel, a 1% Dharmafect-1 solution in Optimem was prepared. The two solutions were mixed and incubated at room temperature for 20 minutes to allow the formation of siRNA liposome complexes. During this time, the medium on the cells was replaced with 80 µl of fresh growth medium containing 10% FCS. Subsequently 20 µl of the liposome siRNA complex was added to the cells. The cells were returned to the incubator and medium was changed after 24 hours.

The reduction in the expression of the target genes was confirmed by Q-PCR and western blotting.

2.3 Cell cytotoxicity studies

In drug development the study of pharmacodynamics to evaluate the physiological and biochemical effects on human cells can be performed with a number of techniques. The toxicity testing methodology applied in our studies is described here.

2.3.1 Drug preparation

Navitoclax (Chemitek) and paclitaxel (Sigma) were dissolved in DMSO at 20 mM and 10 mM respectively and carboplatin (Sigma) was dissolved in PBS (13.5 mM). Obatoclax (GeminX) was dissolved in DMSO at 5mM.

2.3.2 SRB colorimetric assay

The sulforhodamine B (SRB) assay was used to measure the growth of cultures of ovarian cancer cells [312]. Cells were collected by trypsinization and were seeded in 96-well plates (Triple red). 5,000 cells were seeded per well for all cell lines except 2,000 cells per well for Ovcar8, cisA2780 and A2780 cells) in 80 µl of growth medium. The cells were incubated for 24 hours at 37 °C in 5% CO_2 in a humidified atmosphere before drugs were added. For dose response curves, 18 different drug concentrations were prepared (and a control containing only the drug solvent) at 5 times the desired final concentration. 20 µl of these drug dilutions was added to the cells. After further 72-hr incubation the medium

was removed and the cells were fixed in 100 µl of cold 10% trichloroacetic acid on ice for 30 minutes. The plates were then washed in water, air dried and then stained in 0.4% sulforhodamine B for 30 minutes. Excess SRB was removed by washing with 1% acetic acid (3x100µl) and the dye was solubilized in 100µl of 10mM Tris (pH 10). The optical density was measured at 570nm with a microplate reader. In samples in which the measure absorbance exceeded 2.0, a fraction of the sample was removed, and the sample supplemented with Tris before remeasuring at A_{570}.

2.3.3 Non-linear regression for IC_{50} value determination

The IC_{50} concentration of drugs in cell growth assays using ovarian cancer cell lines was determined from the dose-response curves. In every dose-response curve a certain drug concentration (independent variable) led to a certain degree of growth inhibition (dependent variable). Only data from complete dose–response curves were used. The data were analyzed by non-linear regression using the Graphpad Prism software to fit a 4 parameter (also called Hill-equation) sigmoidal dose–response curve. The averages IC_{50} values from all experiments are reported together with the standard deviation.

2.3.4 Drug Combination Studies

For drug combinations, carboplatin and paclitaxel were combined at the ratio of their IC_{50}s. Where navitoclax was included, a fixed concentration estimated from preliminary IC_{50} determinations on its own to inhibit proliferation by 5 % was used while the concentration of the chemotherapeutic agent was varied. The fixed concentrations of navitoclax were: A2780, 3.3 µM; cisA2780, 1.5 µM; Ovcar-3, 1.2 µM; Ovcar-4, 2.1µM; Ovcar-5, 0.7 µM; Ovcar-8, 1.5 µM; Igrov-1, 0.4 µM; Sk-Ov-3, 0.6 µM. Using a fixed concentration overcomes problems associated with drug insolubility that can occur at high concentrations if a drug that has a high IC_{50} is included in combination at a fixed ratio. Drugs were prepared at 6x the final drug concentration, and 20 µl of each drug added to the cells in 80 µl of growth medium. After incubation for 72 hours, the cells were stained with SRB as described (2.3.2) and IC_{50} values determined (2.3.3).

2.3.5 Combination Index (CI) value determination

The concept of the Combination Index (C.I.) was introduced by Chou and Talalay [341] for the quantification of synergism or antagonism between two drugs. It can be defined as a quantitative measure based on the mass-action law, of the degree of drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement [341]. The equation for calculating the Combination Index can be seen at Figure 11.

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} = \frac{(D)1}{(Dm)1[\frac{fa}{(1-fa)}]1/_{m1}} + \frac{(D)2}{(Dm)2[\frac{fa}{(1-fa)}]^{1/_{m2}}}$$

Figure 11: The Combination Index (CI) equation.

In the denominator, $(D_x)1$ is for Drug 1 "alone" that inhibits a system x%, and (D_x) 2 is for Drug 2 "alone" that inhibits a system x%. The (D_x) 1 and (D_x) 2 values can be calculated from the results of non-linear regression obtained from fitting a Hill equation to the dose-response data. In the numerators, $(D)_1$ $(D)_2$ represent the concentrations of drug 1 and drug 2 respectively in the combination that also inhibit by x%. If the sum of these two fractional terms is equal to 1, an additive interaction is indicated. If the C.I. value is smaller than 1, synergism is indicated, and if the C.I. value is greater than 1, antagonism is indicated [341].

In our drug combination studies with Obatoclax, Carboplatin and Paclitaxel the Combination Indices or "C.I. values" were calculated to measure the combined effect of the drugs in doublet combinations. The drugs were combined in a fixed ratio of their respective individual IC_{50} values measured in preliminary experiments with the single agent using every cell line separately. The C.I. values described represent the averages from the experiments with the associated standard deviation.

In the studies with navitoclax, for each single agent, double or triple drug combination, complete dose-response curves were determined in every experiment, using 18 different drug concentrations to establish IC_{50} values and Hill coefficients. For drug combinations of carboplatin and paclitaxel, the drugs were combined at the ratio of their IC_{50}s. Where navitoclax was included, a fixed concentration estimated from preliminary IC_{50} determinations on its own to inhibit cell growth by 5 % was used while the concentration of the chemotherapeutic agent was varied. The fixed concentrations of navitoclax were: A2780, 3.3 µM; cisA2780, 1.5 µM; Ovcar-3, 1.2 µM; Ovcar-4, 2.1µM; Ovcar-5, 0.7 µM; Ovcar-8, 1.5 µM; Igrov-1, 0.4 µM; SkOv-3, 0.6 µM. Using a fixed concentration overcomes problems associated with drug insolubility that can occur at high concentrations if a drug that has a numerically high IC_{50} is included in combination at a fixed ratio (ref 4). The C.I. values are quoted at fraction affected = 0.5 (i.e. the concentration at which the combination affected 50% of the cells).

2.3.6 Trypan Blue viability studies

Trypan blue is a vital dye which is used to determine cell viability. The chromophore is negatively charged and only penetrates the damaged membrane of dead cells. It is used to stain dead cells or tissue and exclude them from live cells. Under microscopy, the cytoplasm of dead cells is stained blue while the viable cells have a clear cytoplasm.

12-well plates were seeded with 50,000 cells/well and after 24hr obatoclax was added at multiples of the IC_{50} (0x,1x,3x,6x,10x,20x) previously measured in 72 hour cell growth assays. After an incubation period of 72 hours the adherent cells were collected by trypsinization (see section 3.1.1) with 1.2ml trypsin/EDTA and combined with the non-adherent floating cells. The cells were then collected by centrifugation at 150 g for 3 minutes and the pellet was resuspended in an appropriate volume of 0.4% Trypan Blue. The viable and non-viable cells were then counted with a hemocytometer as described (see section 3.1.2).

2.3.7 Measurement of drug sensitivity after siRNA transfection

To measure the effect of knockdown of Atg-5 or Beclin on the sensitivity of cells to obatoclax, cells were transfected with siRNA as described (section [2.2]). 24 hours after the transfection the medium was replaced with fresh antibiotic-free 10% FCS/RPMI medium. After a further 24 h, the cells were treated for 48 hours with the indicated concentration of carboplatin, paclitaxel or obatoclax. The drug sensitivity assay was performed as described (section 3.3.2).

2.3.8 Colony formation assay

100,000 cells from the Ovcar-5 and Ovcar-8 cell lines were seeded in 6-well plates in 1 ml of growth medium. The next day the cells were treated with obatoclax at multiples (0, 1,3,6,10,20 x IC_{50}) of the IC_{50} measured in cell growth assays. The cells were incubated in the presence of the drug for 48 hours. The cells were collected by trypsinization and counted using a hemocytometer. Cells were then reseeded at multiple densities (10,100,1000,10,000,100,000 cells per well) in a 6-well plate The cells were kept in a humidified 5% CO_2 atmosphere at 37°C for 15 days. The colonies were stained with 1 ml of 0.5% methylene Blue in 50% ethanol, before washing with water. Colonies larger than 1 mm in diameter were counted macroscopically.

2.3.9 Spheroid assays

To measure the activity of drugs using spheroids, cells were resuspended in complete medium (2 x 10^6 cells/ml) and 20 µl pipetted on the up-turned lid of a 48 well plate. Only the inner 'wells' of the lid were used and 20 µl of medium was placed in the remaining lid 'wells'. 300 µl of PBS were placed in the well beneath the lid to ensure a humid atmosphere. After 1 week, the spheroids were exposed to drug by addition of 5 µl of RPMI containing the drug or solvent. After a further 72 hours, the spheroids were collected with a wide bore pipette into a 96 well plate and 25 µl of Cell-titer Glo (Promega) reagent were added to measure ATP. Microscopic inspection confirmed that this method fully lysed the spheroids, avoiding issues of the reagent penetration into the spheroid affecting the measurement. For IC_{50} measurements, the data were analyzed using Graphpad Prism to fit a four parameter Hill equation using non-linear regression. In drug combinations, single concentrations of each drug were tested. To compare the observed effect of the drug combinations in these experiments with the expected effect, the Bliss independence criterion was used to calculate the expected effect of the combination from the effect of the individual single agents [342].

$$E_{Expected} = E_A + E_B - E_A E_B$$

Where $E_{Expected}$ is the expected effect of the combination assuming Bliss additivity, E_A and E_B represent the effect of drugs A and B when used on their own. The actual effect of the combination (the "observed effect") was then compared to the expected effect using a paired t-test.

2.3.10 Caspase 3/7 assays

To measure caspase 3/7 activity, cells were collected by trypsinization, counted and 5,000 cells were seeded per well of a 96 well plate in 80 µl of growth medium. The following day cells were exposed to the indicated concentration of drug by addition of 20 µl of drug at 5_X times the desired final concentration. After 30 hours the cells were collected by centrifugation (150 g, 1min) to collect any floating cells at the bottom of the well and 75µl of the supernatant was removed. Caspase activity was measured by addition of 25µl using Caspase 3/7 Glo (Promega), incubating for 30 min and measuring luminescence using a luminometer.

2.4 Molecular biology methods

2.4.1 Gene expression analysis

To confirm effective knockdown of gene expression by siRNAs, Q-RTPCR studies were performed on parallel samples prepared at the same time as the siRNA experiment that was used to measure changes in drug sensitivity. Alternatively, protein was isolated and expression measured by western blotting.

2.4.2 Total RNA extraction from cell lines

Cells were plated for siRNA studies as described (section [2.2]). To isolate RNA for QPCR studies, RNA was isolated from 4 wells in a 96 well plate (a minimum of 10^5 cells) 96 hours after transfection with siRNA. Cells were lysed in 350 µl RLT buffer before isolation of RNA), according to the manufacturer's protocol (Qiagen RNAeasy kit).

The RNA concentration was determined spectrophotometrically by absorbance at 260 nm and the purity of RNA samples was assessed by the 260/280 nm ratio.

2.4.3 cDNA synthesis

For the production of cDNA, 11.5µl of RNA were mixed with 1.5µl (2.5 µg/µl) anchored oligodT primer (Invitrogen) and heated at 65 °C for 5 minutes to denature the secondary structure of the RNA before being placed on ice. A “master mix” was prepared comprising (per sample) 4 µl Transcriptor reverse transcriptase buffer (Roche) 2µl of deoxyribonucelotides (10 mM) and 0.5 µl reverse transcriptase (Roche). 7 µl of the master mix were added to each sample. The reaction was incubated at 55 °C for 30 min to allow cDNA synthesis and then at 85 °C for 5 minutes to inactivate the reverse transcriptase. Each sample was diluted 5-fold by adding RNA free water and stored at -20 °C.

2.4.4 Semi quantitative RT-PCR

qRT-PCR was performed using SybrGreen (Abgene) according to the manufacturer's instructions using a 7900HT Fast Real-Time PCR machine. 7.5µl per well of a master mix consisting of primers (GAPDH, Atg5 and Beclin respectively) & SYBR Green were prepared by mixing 6.25µl SYBR Green with 0.125µl 10 µM primers (final concentration 100nM) and 1.13µl H_20. 5µl of 5-fold diluted RT reaction cDNA was added to the mix which was then centrifuged to remove any bubbles from the bottom of the wells. The qRT-PCR reaction was performed with dissociation stage setting to check for non-specific PCR product.

For each primer set, the efficiency of each primer was measured using 4 serial 2-fold dilutions of the template. Data were analyzed by plotting log [DNA] versus Ct and the slope of the resulting graph determined by linear regression. Efficiency for each round of amplification was calculated as

$$Efficiency = 10^{-1/gradient}$$

100% efficiency amplification corresponds to two copies of each template being synthesized per amplification cycle. To calculate knockdown following siRNA transfection, QPCR reactions were performed for the target gene and for GAPDH on each sample (cells transfected with non-targeting control siRNA, and cells transfected with siRNA designed to the gene under investigation).This efficiency was then used during the calculation of the “fold-knockdown” with siRNA.

$$\%Knockdown = 100\ x\ \frac{\left(\frac{{E_{gene}}^{Ct_{gene}}}{{E_{GAPDH}}^{Ct_{GAPDH}}}\right)}{\left(\frac{{E_{gene}}^{Ct_{gene}}}{{E_{GAPDH}}^{Ct_{GAPDH}}}\right)}$$

Primers for Atg5 and Beclin1 autophagy genes were designed using the NCBI Entrez Gene database and the Primer3 software. The sequences for primers used were as follows: forward1 Beclin, 5'-AGATGCGTTATGCCAGAC- 3'; reverse1 Beclin 5'-GATTGTGCCAAACTGTCCAC- 3'; forward2 Beclin, 5'- CAGGCTGAGGCTGAGAGAC- 3'; reverse2 Beclin 5'-TTCAGCTCATCATCCAGCTC - 3'; forward1 Atg5, 5'-GCATCAAGTTCAGCTCTTCC - 3'; reverse1 Atg5 5'- GATGGACAGTGCAGAAGGTCT- 3'; forward2 Atg5, 5'-CAGATGGACAGTTGCACACA - 3'; reverse2 Atg5 5'-TGTTGGCTGTGGGATGATAC- 3' (Sigma).

2.4.5 Transfection of GFP-LC3

Cells (5000 cells per well) were seeded in 96 well plates in 100µl antibiotic-free RPMI containing 10% FCS. After 16 hours the cells were transfected with 0.1 µg DNA (pCI-neo-GFP-LC3) encoding GFP-LC3 and 0.2µl Lipofectamine 2000 in 50 µl Optimem as described [343]. Cells transfected with the vector were included as a control on every 96-well plate. After 6 hours the cells were washed once with PBS and placed in fresh medium.

2.4.6 Western Blotting

Cell extracts were either prepared from T25 flasks or from 6 well plates. For the extraction of cellular proteins following siRNA transfection a sample was obtained by combining 6 wells of a 96 well plate that had been transfected using 5,000 cells/well and which were cultured in a 96-well plate for 96 hours after transfection. Cells were washed with PBS, and then lysed with modified RIPA ("radioimmune precipitation assay") buffer (use 1 ml for every 100 cm^2 of culture vessel). The modified RIPA comprised 20 mM Hepes, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 1% NP40 adjusted to pH7.0 with NaOH and freshly supplemented with 100 µM leupeptin and 15 µM pepstatin. The samples were cleared by centrifugation (15,000g, 10 min, 4°C) before the supernatant was collected. Protein concentration was estimated with a bicinchoninic acid (BCA) assay and samples were stored at -70°C.

The BCA assay (Sigma Aldrich) was used to determine protein concentration. Bovine serum albumin (BSA, Sigma Aldrich) was used to construct a calibration curve (0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 mg ml^{-1} BSA). 10 µl of the sample or standard were treated with 100 µl of the BCA assay reagent comprising bicinchoninic acid solution (containing bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH with final pH 11.25; Sigma-Aldrich) mixed with 4% (w/v) $CuSO_4$ • 5H2O solution in a ratio of 50:1. The samples were incubated for 30 min at 37°C and A_{570} determined spectrophotometrically. The sample protein concentration was calculated following linear regression analysis of the standard curve of the sample.

For electrophoresis protein samples (10-20µg) were denatured at 70°C in NuPAGE sample buffer (Invitrogen) containing 5% β-mercaptoethanol for 15 minutes and subjected to gel electrophoresis using 3–12% BisTris gels (Invitrogen) using Hepes running buffer (100 mM Tris, 100mM Hepes, 1% SDS) in an XCell surelook minicell (Invitrogen) . Protein markers (Invitrogen) were included on every gel. The separated proteins were electrotransferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare, Buckinghamshire, UK) in using 25mM Tris, 200 mM Glycine 0.075% SDS in 10% methanol at 30 V for 1 hour. The blotted membranes were incubated in blocking solution (5% non-fat dry milk in TBST (0.02 M Tris hydrochloride, pH7.5, 150 mM sodium chloride, 0.1% Tween) for 1 hour at room temperature and incubated overnight with the antibody at 1:1000-1:2000 dilutions. The membranes were then exposed to secondary antibody at 1:2500 dilution for 1 hr.

Chemiluminescence was detected using an ECL Chemiluminescent Substrate kit as per the manufacturer's instructions.

2.5 Flow Cytometry

Cells in the G_1 phase of the cell cycle have diploid DNA while cells in G_2/M phase have doubled this DNA content. Propidium iodide (P.I.) is a fluorescent stain which binds to DNA and RNA and so can be used to measure progression through the cell cycle. The fluorescence emission is proportional to the DNA content of the cell, therefore G_1 cells fluorescence with half the intensity of cells in G_2 or M phases. Cells in the S phase will exhibit intermediate fluorescence intensities. Apoptotic cells have reduced DNA content and exhibit a hypodiploid peak (sub-G_1 peak) when stained with P.I. and analyzed by flow cytometry. That is due to the partial loss of DNA resulting from the effect of endogenous nucleases and the diffusion of low-molecular weight DNA out of the cells.

Ovarian cancer cell line were seeded in 6-well plates at a density of 10^5 cells per well in 2ml of growth medium. The following day an appropriate volume of 25 µM obatoclax solution was added to each well to a final drug concentration equal to a 1x, 3x, 6x, 10x or 20x multiple of the IC_{50} measured in 72 cell growth assays. Control cells were treated with solvent alone. Cells were incubated for 48 hours at 37°C in a 5% CO_2 humidified atmosphere. At high drug concentrations some cells detached, so both detached and adherent cells were collected. The culture supernatant was removed and the adherent cells collected by with 0.5 ml trypsin EDTA (section [2.1.2]) before being recombined with the culture supernatant. The cells were then collected by centrifugation (150g, 3 min). The supernatant was removed and the cells in the pellet fixed by dropwise addition of ice-

cold 70% ethanol while vortexing the sample. The cells were stored for up to 2 weeks at 4°C.

For flow cytometry, the ethanol was removed by centrifugation (200xg, 10 min, 4°C) and the cells washed twice with 5 ml ice-cold PBS by centrifugation. The cells were resuspended in 0.5 ml PBS and stained by addition of 50 µl propidium iodide/RNAse (0.5 mg/ml heat treated RNASE, 500 µg/ml Propidium Iodide) for 15 minutes in the dark. Flow cytometry data were acquired using a BD FACsort flow cytometer with CellQuest software and data collected on the FL2 channel. Cells were gated to exclude small fragments using the forward scatter/side scatter plot and to exclude doubles using the FL2 doublet discrimination module plot of FL2 area versus FL2 width plot. FACS data were analyzed with the use of Cyflogic software.

2.6 Primary cultures from malignant ascites

In this study we collected ascitic fluid from untreated patients diagnosed with ovarian cancer at the University Hospital of North Staffordshire, who attended the Radiology Department to have ultrasound-guided drainage. This was done strictly anonymously and was approved by the South Staffordshire Research Ethics committee (reference 09/H1203/61). All the patients were consulted and provided with an information leaflet and about the purposes of the study and the processing of the ascitic fluid. The process of the aspiration itself was not modified in any way from the usual protocols. A signed consent form was required in order for the samples to be collected and processed. The patient's respective GPs were later informed by letter regarding the participation of the patients in this study and their right to access more information or withdraw.

Primary cultures were established by the method described by Nachtigal et al [318]. Equal volumes of medium and ascitic fluid were seeded in T75 flasks and after 24 hours the supernatant was removed and replaced with fresh medium. For the primary cultures the media used was a 1:1 mix of MDCB105 and M199 (both from Sigma Aldrich), supplemented as described above [Section2.1.1]. Cells were used between passages 2 and 8.

2.6.1 Fluorescence microscopy of primary cultures

Cells were grown on glass coverslips and fixed with 3% paraformaldehyde for 20 min. After washing 3 times with PBS the cells were incubated for 1 hour with Hoechst-33342 or anti cytokeratin antibody clone MNF116 (Dako) diluted 1:500 in PBS. The cells incubated with the antibody were then washed three times with PBS before incubating with FITC-anti-mouse antibody (1:1000). After washing a further three times, the cells were mounted in Vectorshield and viewed with a fluorescent microscope.

2.7 Tumour xenograft experiments

Due to lack of appropriate facilities and licensing at Keele University, the xenograft experiments were performed by Suzanne A. Eccles at the Institute of Cancer Research, Sutton, United Kingdom in accordance with the U.K. Home Office regulations under the Animals (Scientific Procedures) Act 1986 and the U.K. Coordinating Committee on Cancer Research guidelines for animal experimentation [344].

In brief, tumors were established from cultured Ovcar-8 cells harvested from culture vessels after trypsinization, wash, and resuspension in PBS. Cells were injected s.c. bilaterally in mice. Dosing commenced when tumors were well established (f5-6 mm diameter). Obatoclax was prepared in 9.6% polyethylene glycol 300, 0.4% polysorbate 20, and 5% dextrose. Animals received either vehicle alone or obatoclax (5mg/kg) intravenously every day for the first 5 days of the study. Animals were observed daily, weighed, and tumors measured thrice weekly. Tumors were measured across two perpendicular diameters and volumes calculated from the following formula:

$$V = 4/3\pi \left[\frac{d1 + d2}{4}\right]^3$$

as previously described [345].

Chapter 3: Evaluation of navitoclax in ovarian cancer

3.1 Aims & Objectives

The BH3-mimetic ABT-737 has previously been shown to enhance the cytotoxic effects of chemotherapy in ovarian cancer cells. However, ABT-737 has poor oral availability and this has led to the development of its successor, navitoclax, which has sufficient bioavailability to justify its entry into clinical trials. However, the effect of navitoclax in ovarian cancer cells had not yet been assessed. The goal of the research presented in this chapter was to investigate whether navitoclax was also synergistic with the chemotherapy used to treat ovarian cancer.

3.2 Introduction

A common assumption in cancer studies is that the over-expression of a gene in a large fraction of tumours indicates that the encoded gene product supports tumorigenesis. The intrinsic apoptotic pathway provides an example of this, and is considered to be central to the survival of cancer cells in which anti-apoptotic proteins which regulate this pathway are overexpressed. This is profoundly important in ovarian cancer where the emergence of resistance to drug treatment is a major hurdle that must be overcome. The discovery of the Bcl-2 family of proteins (Chapter1) in the last 20 years and the clarification of the functions of its different family members has contributed significantly to identifying new targets for cancer therapy. The development of molecules which can effectively antagonize the Bcl-2 family apoptosis "inhibitors "has made considerable progress and has delivered agents such as the "BH3-mimetics" to the clinic. These drugs are able to bind the hydrophobic groove of anti-apoptotic Bcl-2 proteins mimicking BH3-only proteins. A number of agents has been developed and entered clinical trials and these innovative drugs have shown synergy with several chemotherapeutic agents [307].

ABT-737 was discovered by Abbott Laboratories in the mid-2000s using nuclear magnetic reasonance-based screening and structure-guided design to exploit the hydrophobic binding groove of the prosurvival proteins Bcl-2 and Bcl-X_L which binds to the BH3 domain of pro-apoptotic proteins. By occupying the hydrophobic grove, the apoptosis inhibitors are no longer able to sequester the pro-apoptotic BH3-only protein, thereby increasing their propensity to induce apoptosis [346],[347],[348]. ABT-737 binds with high affinity to

Bcl-2, Bcl-X_L and Bcl-W (IC_{50}<10nM), but is far less potent against Mcl-1 and A1 [349]. It is a selective inhibitor that requires functional Bax and Bak to induce apoptotic cell death by disrupting the interactions between antiapoptotic and proapoptotic proteins [349] . Thus it does not directly induce apoptosis, rather it sensitizes cell to other pro-apoptotic stimuli which induce the expression of pro-apoptotic BH3-only proteins.

ABT-737 has been shown to be a potent single agent in lymphoma and small cell lung cancer [311], AML [350],[351], multiple myeloma [352], CLL [353] and acute lymphoblastic leukemia [354]. Although this may seem in contradiction with the foregoing discussion of ABT-737 as an apoptosis sensitizer, in fact it reflects pre-existing pro-apoptotic stimuli in these cells. In many of these cells the constitutive expression of BH3-only proteins does not lead to cell death because they are sequestered by Bcl-2 family inhibitors. ABT-737 displays potent single agent activity in these cells because they are “addicted” to Bcl-2 family inhibitors to survive. ABT-737 liberates the proapoptotic proteins and thereby induces cell death. In other cells where BH3-only proteins are not constitutively expressed, the cells are not dependant on Bcl-2 family apoptosis inhibitors for continual survival and ABT-737 shows weak single agent activity.

Our group has shown that in ovarian cancer ABT-737 has modest potency as a cytotoxic single agent in *in vitro* assays using ovarian cancer cell lines [312]. In ovarian cancer, combination with carboplatin resulted in a shortening of the time to apoptosis and an increase in cell growth inhibition, especially when cells were treated with ABT-737 after

exposure to chemotherapy [312]. In xenograft studies in mice the combination of both drugs showed enhanced efficacy. Other workers have found that combinations of ABT-737 with paclitaxel in ovarian and breast cancer cell lines successfully enhanced the observed cell death [355], [356]. Thus, ABT-737, and other BH3-mimetics, has the potential to increase the sensitivity of ovarian cancer cells to chemotherapy. This is particularly important in ovarian cancer where the development of drug resistance prevents effective treatment. Bcl-2 family apoptosis inhibitors have been shown to be over-expressed in drug resistant disease [357].This suggests that BH3-mimetics might be considered for use in patients whose disease has become resistant to chemotherapy.

The potential for ABT-737 to be used in patient therapy is limited by its poor oral bioavailability. The prospect of administering it intravenously is also hampered by its low aqueous solubility [358]. The structural characteristics of ABT-737 which were considered responsible for these liabilities were identified and then analogues were designed with modified structures in an attempt to allow oral administration without adversely affecting the drug's pharmacodynamics activity [358]. Navitoclax is a second-generation BH3-mimetic with considerably higher oral bioavailability which was developed from ABT-737 and has the same pharmacological profile and inhibits the same members of the Bcl-2 family. Navitoclax has low plasma clearance values and low volumes of distribution in animal models with near 50% bioavailability in lipid-based formulations [358]. The selectivity of navitoclax for Bcl-2 family members resembles that of ABT-737 as well as that of the BH3-only protein BAD [349]. Navitoclax displays a high affinity for Bcl-2, Bcl-X_L and Bcl-W and binds less to Mcl-1 an A1. Extensive studies have confirmed that its

cytotoxicity is, as with ABT-737, mechanism based and dependant on the mitochondrial apoptotic pathway [358]. Unsurprisingly it has been described already that cells overexpressing Mcl-1 show resistance to navitoclax.

The evaluation of navitoclax in pre-clinical studies has shown that it causes cell death in a panel of leukemia and lymphoma cell lines and tumour regression in xenograft models [359]. Additionally, it enhances the therapeutic effect of chemotherapy for B-cell lymphoma and multiple myeloma xenograft models [358]. Combinations of navitoclax with rapamycin in lymphoma cells showed enhanced cell killing when compared to the effect of each single agent [359]. In ovarian cancer, combinations of navitoclax with paclitaxel or gemcitabine exhibited synergistic effects across a number of cell lines [360].So far, navitoclax has been evaluated in phase I and II studies in small-cell lung cancer, haematological malignancies and other solid tumours [361], [362]. Although there was no evidence of efficacy in lung cancer, 35% of patients with chronic lymphoid leukemia achieved a partial response and 27% showed stable disease for 6 months [363]. These observations encouraged us to extend the observations previously made with ABT-737 in ovarian cancer cells to navitoclax. This led to the hypothesis that navitoclax would synergize with carboplatin in ovarian cancer cells.

The current gold-standard therapy for ovarian cancer patients consists of the combination of carboplatin and paclitaxel. This has been extensively reviewed for its haematological toxicity and found to be overall well tolerated by patients with high response rates. Thrombocytopenia remains a clinical challenge which often affects the quality of life and

causes treatment dose reductions or treatment delays. Carboplatin has been shown to cause thrombocytopenia and leukopenia and is associated with the need for transfusional support. It causes dose-dependent myelosuppression and this may occur in patients with renal impairment or patients concurrently receiving other myelotoxic drugs. Paclitaxel also possesses dose-dependent myelotoxicity, but is less associated with thrombocytopenia. Rather, paclitaxel is known to have “platelet-sparing” activity and reduce the thrombocytopenia induced by carboplatin [364],[365],[366].

The use of ABT-737 on mice has also shown the induction of rapid and reversible thrombocytopenia due to accelerated cell death of platelets (especially of the older cells), but no bone marrow toxicity [367]. This is a result of the inhibition in platelets of the prosurvival Bcl-2 proteins by the drug, directly causing an apoptotic-type of cell death. Despite the reduction in platelet counts, in other animal models plasma concentrations of ABT-737 several folds higher than the efficacious level were well tolerated [358].

Navitoclax similarly causes dose -dependent thrombocytopenia and T-lymphopenia. The levels of endogenous Bcl-X_L have been found to play a critical role *in vivo* for the survival of platelets and the high affinity of navitoclax for this molecule can explain the observed platelet apoptosis. It is interesting that unlike conventional chemotherapy where myelosuppression is observed, the selective action navitoclax only affects the circulating platelets. The reduction in the number of circulating cells is rapid, but multiple daily dosing in animal models did not significantly reduce the cells to levels lower from the initial first-dose effect [358]. This could be explained by the ability of the animal to

produce more platelets or by the possibility of separate platelet populations with distinct sensitivity to navitoclax. It has also been shown that navitoclax interferes with the mechanism of calcium signalling in platelets and contributes to the depletion of the cellular calcium stores and this may also affect the observed thrombocytopenia.

Despite these concerns, all the available evidence from phase 1/2 clinical studies of navitoclax at the time this study was initiated showed that navitoclax was safe and well tolerated with minimal systemic toxicities [368],[369], [361]. Thus, it still appeared reasonable to evaluate the hypothesis that navitoclax would synergize with carboplatin. However, the thrombocytopenia observed with both carboplatin and navitoclax raised the concern that these drugs might display additive toxicity. Bearing in mind that paclitaxel has “platelet sparing” activity, and that combinations of carboplatin and paclitaxel are the current standard of care in ovarian cancer, the hypothesis was modified to evaluate whether navitoclax would synergize with combinations of carboplatin and paclitaxel. Paclitaxel might also have platelet sparing activity when added to the carboplatin-ABT-263 combination and ameliorate the anticipated additive thrombocytopenia. However, previous work from our group failed to find significant synergy between paclitaxel and ABT-737 and several groups have published preclinical work which has demonstrated an antagonistic interaction between carboplatin and paclitaxel [370], [371]. This made the outcome of the navitoclax carboplatin-paclitaxel combination unpredictable. Therefore all the pairwise combinations of carboplatin, paclitaxel and navitoclax were investigated alongside the triple combination of all three agents.

3.3 Results

3.3.1 Single agent studies of carboplatin, paclitaxel and navitoclax

In order to evaluate the combined effect *in vitro* of carboplatin, paclitaxel and navitoclax on ovarian cancer cell lines, studies were first performed to assess the activity of the drugs as single agents. This information was subsequently used to select the concentrations of drugs to use in combination studies. The IC_{50} values for carboplatin and paclitaxel measured in the panel of cell lines in this study (Tables 11 & 12) were comparable to those previously reported by our group [312]. The navitoclax single agent studies demonstrated that the growth of cell cultures was inhibited in all 8 cell lines with IC_{50} values ranging from 3-8μM (Table 13). This was comparable to the potency of ABT-737 against the same cell lines measured previously by our group, although navitoclax was approximately, 2-fold more potent than ABT-737 [312].

Cell Line	IC_{50} (μM)
A2780	10.9 ±1
cisA2780	108.5 ±15.9
Ovcar-3	4.6 ±0.9
Ovcar-4	25.2 ±11.9
Ovcar-5	63.6 ±10.1
Ovcar-8	69.6 ±10.7
Igrov-1	20.8 ±3.8
Sk-Ov-3	30 ±4.1

Table 11: The IC_{50} values (mean ± S.D., n=3) of carboplatin as evaluated in single agent studies against 8 different ovarian cancer cell lines. Cells were treated with indicated drug for 72 hours after which relative cell number was estimated by staining with SRB.

Cell Line	IC_{50} (nM)	Hill Slope	Number of experiments
A2780	2.7 ±0.78	-4.1 ±0.9	n=13
cisA2780	2.8 ±1.9	-3.7 ±1.1	n= 10
Ovcar-3	5.1 ±6	-3.3 ±1.2	n=11
Ovcar-4	8.6 ±6.7	-3.8 ±1.1	n=8
Ovcar-5	6.7 ±1.7	-3.9 ±1.1	n=9
Ovcar-8	4.8 ±1.3	-3 ±0.9	n=10
Igrov-1	6.2 ±7.8	-2.7 ±1.7	n=9
Sk-Ov-3	7.9 ±4.1	-1.8 ±0.3	n=8

Table 12: The IC_{50} values (mean ± S.D., n=3) of paclitaxel as evaluated in single agent studies against 8 different ovarian cancer cell lines. Cells were treated with indicated drug for 72 hours after which relative cell number was estimated with SRB.

Cell line	IC_{50} (μM)	n
A2780	6.8 ± 2.5	9
cisA2780	4.7 ± 2.0	5
Ovcar-3	5.9 ± 0.4	3
Ovcar-4	5.9 ± 3.5	6
Ovcar-5	3.2 ± 1.3	10
Ovcar-8	5.3 ± 1.5	13
Igrov-1	8.5 ± 5.7	16
Skov-3	5.2 ± 1.5	6

Table 13: Single agent potency of navitoclax.

Cells were treated with a range of concentrations of navitoclax for 72 hours, stained with SRB and the IC_{50} (mean ± S.D.) calculated from the indicated number (n) of experiments.

3.3.2 Combination Studies

Pairwise drug combination studies of carboplatin and paclitaxel, carboplatin and navitoclax, paclitaxel and navitoclax as well as the triple combination of all three agents were performed. In these studies navitoclax was used at a fixed concentration that is comparable to the levels observed in phase 1 clinical trials with navitoclax [361],[372].

In agreement with previous reports describing antagonism between carboplatin and paclitaxel, data consistent with modest antagonism (C.I. > 1) between these two drugs was obtained in all of the cell lines. [370],[371]. However, this only reached statistically significant in two cell lines (Figure 12).

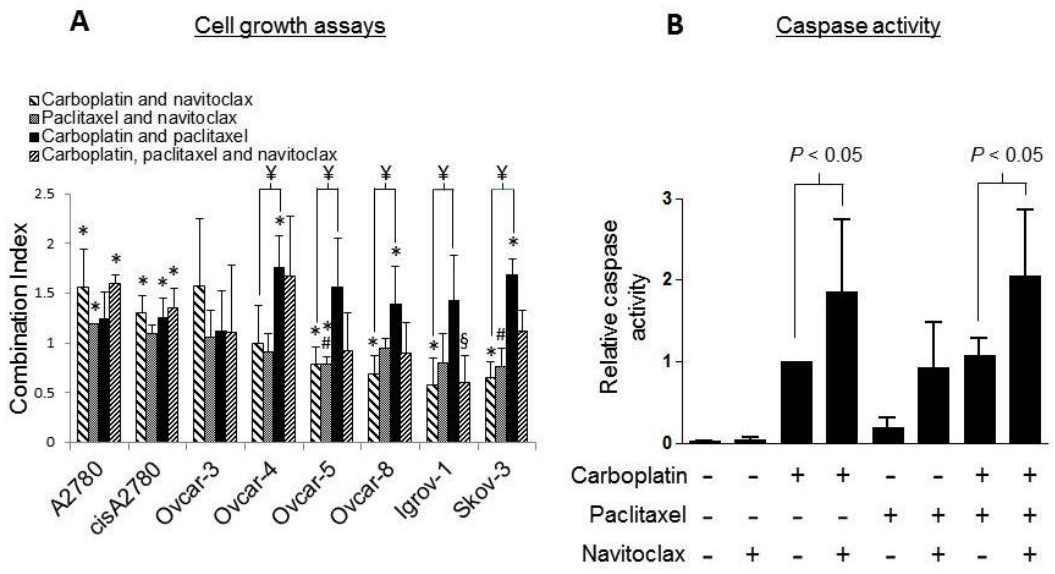

Figure 12: The effect of drug combinations containing navitoclax on ovarian cancer cells . *A*. The indicated cells were treated with carboplatin, paclitaxel or navitoclax drug combinations. Carboplatin and paclitaxel were evaluated at 18 different concentrations, combined at a ratio of the respective single agent IC_{50}s. Where navitoclax was included, a fixed concentration was used (see methods). The C.I values (mean ± S.D., n= 3-8) are quoted at fraction affected f_a=0.5 and are significantly different from C.I.= 1.0 where indicated (*, $P < 0.05$, t-test). The CI values obtained with carboplatin/navitoclax (¥) or paclitaxel/navitoclax (#) differed significantly from the values observed with carboplatin/paclitaxel combination (Kruskal-Wallis test, $P < 0.05$) and the CI obtained with carboplatin/paclitaxel (§) differed significantly from carboplatin/paclitaxel/navitoclax where shown. *B.* Igrov-1 cells were treated with the indicated combinations of carboplatin (50 µM), paclitaxel (20 nM) or navitoclax (0.4 µM) for 30 hours and caspase activity measured. The results (mean ± S.D, n=6) were normalized for cell number by staining duplicate samples with SRB and are expressed as a fraction of the caspase activity measured in cells treated with carboplatin alone. The results were statistically different where indicated ($P < 0.05$, 1-way ANOVA). Error bars represent S.D.

Our group has previously observed synergy between ABT-737 and carboplatin. In this study synergy was observed between navitoclax and carboplatin in 4 of 8 cell lines evaluated (C.I. < 1; (Figure 12). This was most prominent in Igrov-1 cells, the cell line in which synergy between carboplatin and ABT-737 was most evident in previous studies [312]. Antagonism was observed between carboplatin and navitoclax in the A2780 cell line and its platinum-resistant daughter cell-line cisA2780. These observations are consistent with the previously observed antagonism between carboplatin and ABT-737 [312]. Why antagonism is observed in these two related cell lines is unclear, although, of the cell lines evaluated, they are the least genetically related to authentic serous ovarian cancer [373]. The carboplatin-navitoclax combination performed significantly better (smaller C.I. value) than the standard-of-care doublet combination of carboplatin-paclitaxel in 5 of the 8 cell lines evaluated.

Previous studies from our group demonstrated an additive rather than a synergistic interaction between ABT-737 and paclitaxel in cell proliferation studies. Those experiments were performed using the same methods used here - by measuring a combination index after 72 hour cell proliferation assays [312]. In contrast, Wong *et al* observed a more than additive effect between navitoclax and paclitaxel in ovarian cancer cell lines [312]. In those studies a 72 hour cell growth assay was also used, but a combination index was not reported, rather the Bliss independence criterion was used to identify super-additive effects. In the studies presented here (which used the combination index method), consistent with the work previously published by our group, additivity was observed between navitoclax and paclitaxel in most of the cell lines

examined, although modest synergy between navitoclax and paclitaxel was observed in one cell line (Ovcar-5, Figure 12).

When the triple combination of carboplatin, paclitaxel and navitoclax was evaluated the pattern of synergy and antagonism across different cell lines matched that observed with the carboplatin and navitoclax doublet combination. In Igrov-1 cells, in which synergy between carboplatin and navitoclax was observed, the triple combination was also synergistic. In A2780 and cisA2780 cells antagonism was again evident (Figure 12). In addition, in all of the cells in which synergy between carboplatin and navitoclax had been observed, the C.I. value for the triple combination was less than that obtained with the standard-of-care doublet combination of carboplatin and paclitaxel. In Igrov-1 cells, where navitoclax and carboplatin showed the most synergy, the triple combination was also the most synergistic. Importantly, in those cells where antagonism was observed between carboplatin and paclitaxel, antagonism was no longer evident when navitoclax was included. This suggests that in cells in which navitoclax is synergistic with carboplatin, navitoclax is able to improve upon the activity obtained with the standard-of-care carboplatin/paclitaxel combination.

To confirm these observations using an endpoint other than cellular biomass, caspase 3/7 activity was measured in cells exposed to single, double or triple combinations of carboplatin, paclitaxel and/or navitoclax. For these experiments Igrov-1 cells were used because this is the cell line in which the most significant synergy between carboplatin,

paclitaxel and navitoclax was observed. Relatively short term exposure (30 hours, compared to 72 hours used in the cell proliferation studies) was used because the signal from this assay eventually decays after cell death has occurred. Carboplatin increased caspase 3/7 activity, and this was enhanced by inclusion of navitoclax, consistent with the synergy seen between these two agents in the cell growth studies using this double combination. In agreement with the work of Wong and co-workers [360], navitoclax also enhanced the caspase 3/7 activity measured after 30 hours exposure to paclitaxel. However, this was not consistent with the lack of synergy observed between navitoclax and paclitaxel in the cell proliferation studies presented here. One potential explanation is that navitoclax accelerates apoptosis induced by paclitaxel when it is measured after 30 hours drug exposure but has a relatively modest effect on the potency of paclitaxel measured after 72hr drug exposure. Alternatively, measuring caspase 3/7 activity may be a more sensitive means to detect modest synergy. The results with the triple combination were, however, consistent with the data obtained in cell proliferation studies. The caspase-3/7 activity induced by the combination of carboplatin, paclitaxel and navitoclax, was significantly greater than that in cells exposed to the carboplatin and paclitaxel doublet combination.

To confirm further these observations, the activity of the drug combinations was measured using Igrov-1 cells grown as spheroids. Firstly, the activity of the single agents was evaluated. Carboplatin inhibited the survival of spheroids (assessed by measuring cellular ATP) with only a modest (3-fold) decrease in potency compared to that measured using monolayer cultures (Figure 13). In contrast, the sensitivity of cells in spheroids to

navitoclax was increased approximately 5-fold. Strikingly, the spheroids were approximately 2000-fold more resistant to paclitaxel than the cells grown in monolayer cultures (Figure 13).

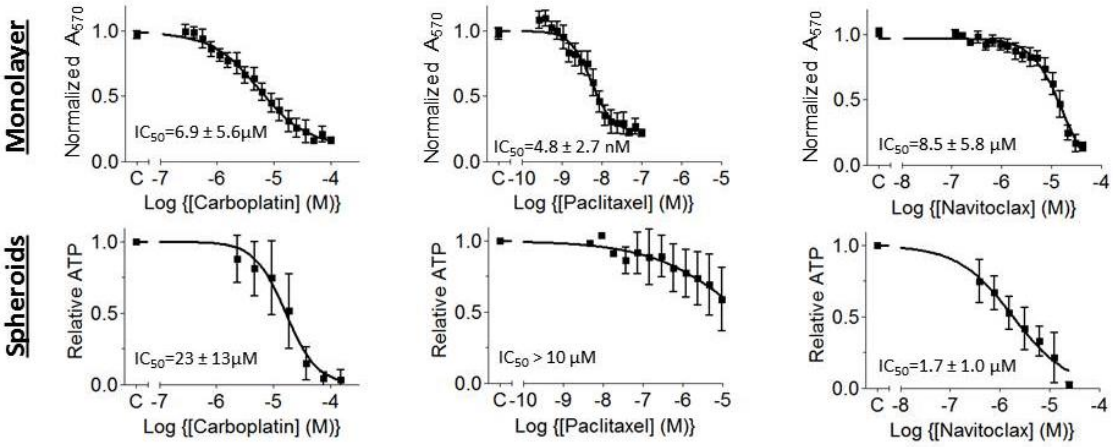

Figure 13: Evaluation of navitoclax, carboplatin and paclitaxel in Igrov-1 spheroids. The potency of navitoclax, carboplatin and paclitaxel against Igrov-1 cells was compared against monolayer and spheroid cultures. The results (mean ± S.D., n=7-11) are expressed as a fraction of the ATP measured in cells treated with drug vehicle alone. Error bars represent S.D.

The effect of the drug combinations was then evaluated. For these experiments, the Bliss independence criterion was used to assess drug interactions. Although evaluating synergy by measuring a combination index is a robust approach, it is best supported by performing a full dose-response analysis which is technically challenging using spheroids due to the difficulties in obtaining sufficient data points. Instead single drug concentrations were used and chosen to inhibit survival on their own by approximately 50% (25% for paclitaxel due to its poor potency in spheroids). These concentrations were comparable to drug concentrations achieved in patients [361],[372],[374],[375].The Bliss independence criterion has been used by other workers to analyse drug interactions in the absence of complete dose-response curves e.g. [360]. In these studies, antagonism between carboplatin and paclitaxel was not observed with Igrov-1 spheroid cultures. However, when navitoclax was included with either carboplatin, or with paclitaxel, or with the combination of carboplatin and paclitaxel, a more than additive interaction was observed. The number of cells surviving in each case was significantly fewer than that anticipated for the corresponding drug combination estimated from the Bliss independence criterion and the effects of each drug as a single agent (Figure 14).

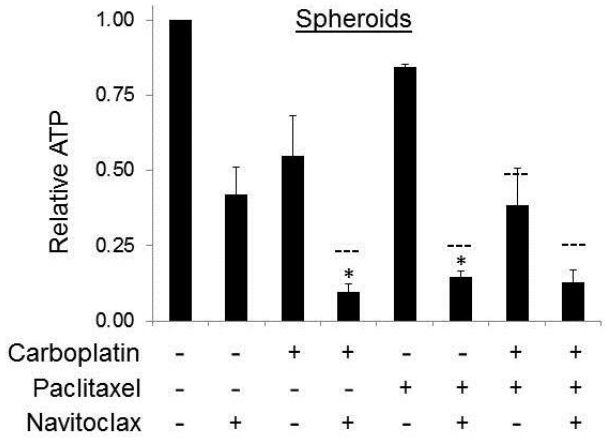

Figure 14: Evaluation of navitoclax drug combinations in Igrov-1 spheroids. Igrov-1 spheroids were treated with the indicated combinations of 30 µM carboplatin, 2 µM navitoclax and 1.2 µM paclitaxel. The results (mean ± S.D., n=4-5) are expressed as a fraction of the ATP measured in cells treated with drug vehicle alone. Drug combinations were compared to the expected effect (shown with a dashed line for each drug combination) calculated from the measured effect of the individual drugs in each individual experiment and assuming Bliss independence. *, significantly different from the Bliss expected effect (t-test, $P < 0.01$). Error bars represent S.D.

3.4 Discussion

Platinum agents and taxanes are at the core of our current gold standard for treatment of ovarian cancer. Carboplatin induces its effect by forming DNA adducts at any phase of the cell cycle, although the response to this is cell cycle-dependent and platination causes arrest of cells in S-phase [16, 26]. In contrast, paclitaxel causes M-phase arrest and exerts its activity by preventing normal microtubule dynamics. The strategy of targeting the intrinsic apoptosis pathway in cancer cells with the use of BH3-mimetics which bind to the Bcl-2 family of proteins has yielded new drugs which have shown enhanced toxicity when combined with traditional chemotherapeutics[376]. Various BH3-mimetics are currently undergoing further pre-clinical and clinical assessment as single therapy and in drug combinations for different types of cancer. The work in this chapter explored combinations of these drugs.

The addition of navitoclax to combinations of carboplatin and paclitaxel in monolayer and spheroid cultures appears to increase their potential therapeutic effect. Several studies, including the results presented here, have reported antagonism between platinum based compounds and taxanes in monolayer cultures. The antagonism between carboplatin and paclitaxel has been proposed to be due to carboplatin causing arrest, preventing cells from reaching M-phase when they are sensitive to paclitaxel [16, 17]. However, carboplatin can still attach to DNA in cells that are first exposed to paclitaxel and which have arrested in M-phase. Thus, pre-exposing cells to paclitaxel has generally been found to be less antagonistic than other schedules of administration. However, in clinical

practice, patients may receive carboplatin and paclitaxel on the same day of treatment. Thus, the simultaneous exposure to both carboplatin and paclitaxel in our experiments approximates common clinical practice. In several cell lines the inclusion of navitoclax with either carboplatin or paclitaxel in monolayer led to combination indices significantly lower those obtained with the carboplatin and paclitaxel combination. Whether antagonism occurs between carboplatin and paclitaxel in the clinic is unclear. Two chemotherapeutic drugs, even if mutually antagonistic, may elicit more cell kill than would occur if either agent were used on its own so antagonism *per se* does not necessarily prohibit the combination of two drugs. However, the results presented here suggest that the inclusion of navitoclax with the carboplatin-paclitaxel combination may increase the therapeutic effect.

After these studies were completed, clinical trials of navitoclax were suspended due to the thrombocytopenia induced by navitoclax. This is a particular concern in the present study, because carboplatin also causes thrombocytopenia, raising the potential for additive toxicity when these drugs are combined. Abbott, the company that discovered navitoclax, has subsequently developed a new analogue of navitoclax "ABT-199", which lacks appreciable affinity for Bcl-X_L [377]. The development of ABT-199 from Abbott is expected to resolve the problem of thrombocytopenia [378]. This agent has been developed from the re-engineering of navitoclax and on-going studies confirm that it possesses weak *ex vivo* activity on platelets [377]. Clinical studies have demonstrated efficacy against haematological malignancies and show an improved toxicity profile [377], [379]. Unfortunately, previous work from our group suggests that Bcl-X_L is a key mediator

of chemoresistance in ovarian cancer cells, suggesting that ABT-199 is unlikely to be useful in ovarian cancer.

The reduced sensitivity of spheroids to chemotherapeutic agents has been previously well characterized. In our experiments, the spheroid cultures were much less sensitive to paclitaxel than cells in monolayer culture. In part, this may be due to poor penetration of paclitaxel into tumour spheroids [27]. This is unlikely be the sole explanation for the reduced sensitivity because paclitaxel can penetrate cultured tumour fragments within 24 hours [27] and in our experiments spheroids (and monolayers) were exposed to drug for 72 hours. Furthermore, the spheroids retained their sensitivity to both carboplatin and navitoclax, suggesting that drug penetration is not completely prevented. Alternatively, a more likely explanation may be that the relatively low mitotic index of cells in spheroid culture may reduce the potency of cell cycle specific drugs such as paclitaxel [28]. Cells which proliferate slowly are likely to be relatively resistant to agents such as paclitaxel whose mechanism of action depends on cells progressing through the cell cycle. This reduced potency of paclitaxel may also contribute to the lack of antagonism observed between carboplatin and paclitaxel in spheroid culture. The spheroids were modestly less sensitive to carboplatin than were cells in monolayer. In contrast, the spheroids were somewhat more sensitive to navitoclax. This latter observation may reflect the change in expression of Bcl-2 family members. The expression of Bcl-2 and Bcl-X_L, both of which are sensitive to inhibition by navitoclax, is increased in spheroids [29-32]. In contrast, expression of the navitoclax insensitive family member Mcl-1 is decreased [29, 30]. The expression of the BH3-only protein Bim is also increased [33] which would effectively

neutralized Bcl-2 apoptosis inhibitors. This may indicate a greater dependence of spheroid cultures for survival on Bcl-2 family members that are sensitive to navitoclax. These observations underline the value of using different experimental models to measure drug sensitivity and interactions. It also suggests that studies which seek to identify drugs which restore the sensitivity of cells to paclitaxel should consider using spheroid culture rather than monolayers, as these may provide a better model.

Our group has previously reported synergy between carboplatin and ABT-737 in a number of ovarian cancer cell lines [8]. ABT-737 was also found to augment the inhibition of the growth of Igrov-1 xenografts. In the studies presented here, synergy was also observed between navitoclax and carboplatin. Our group initially reported additivity between paclitaxel and ABT-737 in several ovarian cancer cell lines [5]. In contrast, Wong and co-workers [15] observed a more than additive interaction in an even broader panel of ovarian cancer cells. Combining navitoclax with paclitaxel is an attractive approach, because paclitaxel does not cause significant thrombocytopenia. One possible explanation for the discrepancy between the work presented here and that of *Wong et al* is the different methods of analysis of cell proliferation assays used by us (calculation of Combination Index) and by Wong and co-workers (comparison of the observed effect with the effect expected estimated from the Bliss independence criterion). However, in the work presented here and that of Wong and co-workers, an increase in caspase activity was identified when navitoclax was included with paclitaxel for relatively short periods (24-30 hours). These observations may suggest that navitoclax can potentiate the activity of paclitaxel against ovarian cancers, but it is less pronounced when measured in

72 hour cell proliferation assays using monolayer cultures. It also raises an issue of the clinical relevance of these observations If ABT-737 or navitoclax, when combined with paclitaxel, simply accelerate cell death without affecting the number of cells that die, is there likely to be any clinical benefit? Navitoclax appears to accelerate apoptosis induced by paclitaxel [15] and ABT-737 has previously been observed to accelerate apoptosis induced by carboplatin. Nonetheless, navitoclax was able to increase the effectiveness of the carboplatin-paclitaxel combinations. Thus it wold seem sensible to prioritize the clinical evaluation of carboplatin-paclitaxel-navitoclax over paclitaxel-navitoclax combinations.

The binding of navitoclax to Bcl-X_L affects platelet survival and causes mechanism-based predictable transient thrombocytopenia, with a dose-limiting relationship [363], [380]. At present the majority of clinical trials assessing navitoclax have been completed and the focus is on the adverse effects on the patients. In the present study, navitoclax improved the activity of carboplatin and paclitaxel combinations, making it desirable to evaluate this combination in clinical trials. It will be essential to determine if paclitaxel is able to reduce the impact on platelets of the carboplatin and navitoclax. These studies still appear worthwhile, if the thrombocytopenia can be carefully monitored, because of the lack of suitable treatment options in patients with drug-resistant disease. Thus, it remains important to be determined if navitoclax can be tolerated in ovarian cancer patients.

In summary, navitoclax improved the activity of the carboplatin/paclitaxel combination, the doublet combination that most closely matches the current standard of care, both in monolayer and spheroid cultures. Combined with our previous studies demonstrating synergy between ABT-737 and carboplatin [8], and those of Wong [15] showing more than additive interaction between paclitaxel and navitoclax, this leads to the conclusion that navitoclax warrants clinical evaluation in ovarian cancer, but the platelet effects of the combination must be carefully monitored. One set of patients likely to benefit from this combination are those whose tumours express high level Bcl-X_L. High expression of Bcl-X_L correlates with a poor response to paclitaxel [15] and Bcl-X_L is also a key determinant of synergy between carboplatin and ABT-737 [15]. However, multiple factors determine sensitivity to navitoclax [5] and other patient groups, such as those with elevated expression of BH3-only proteins, may also benefit from inclusion of navitoclax.

Chapter 4: Evaluation of obatoclax in ovarian cancer

4.1 Aims & objectives

In this study the efficacy of obatoclax was evaluated in models of ovarian cancer to explore the potential for this agent as a treatment for patients with ovarian cancer. This study was performed using established ovarian cancer cell lines and primary cultures established from patients with varying degrees of chemoresistance. The mechanism(s) of action of this drug were also investigated as part of this analysis.

4.2 Introduction

The induction of apoptosis has been identified as a therapeutic strategy for drug discovery in oncology. Molecules such as the BH3-mimetics were designed to target cancer cells considered “primed” for apoptotic cell death because of their “addiction” to overexpressed pro-survival Bcl-2 “inhibitors” or cells in which BH3-only proteins were induced by chemotherapy [306]. These drugs are thought to elicit their effect by liberating BH3-only proteins which are apoptosis activators thereby causing the activation of Bax and Bak and subsequently MOMP [381], [353]. A number of these drugs has been developed and these differ in their binding capacities for the anti-apoptotic members of the Bcl-2 family of proteins [376], [382]. The overexpression of the apoptosis inhibitor Mcl-1, which does bind neither ABT-737 nor navitoclax with high affinity, has emerged as a mechanism of resistance to the aforementioned drugs [376], [351], [349].

Obatoclax mesylate (GX15-070) is a prodiginine derivative with an indole ring developed through structure-activity relationship studies of the pyrrolic ring A of GX15 [383]. It is a pan-Bcl-2 protein inhibitor originally developed by GeminX (Montreal, Canada) and having the ability to bind to all pro-survival Bcl-2 protein, obatoclax is not anticipated to suffer from the same resistance mechanisms that limits the activity of ABT-737[384]. In the foregoing chapters, the efficacy of ABT-737 and navitoclax in models of ovarian cancer has been discussed. These initial studies provided proof-of-principle for the therapeutic targeting of the prosurvival Bcl-2 family of proteins in ovarian cancer.

However, the anticipated broader activity of obatoclax warranted its evaluation in ovarian cancer.

A number of preclinical *in vitro* studies have examined the activity of obatoclax in several types of cancer and demonstrated that this drug possesses significant single agent efficacy. The most prominent activity of obatoclax has been demonstrated against a number of haematological malignancies including acute myeloid leukemia (AML), mast cell leukemia, acute lymphoid leukemia (ALL), chronic lymphocytic leukemia (CLL), lymphoma and multiple myeloma [385]. The spectrum of the published IC_{50} values in these studies lies primarily in the nanoMolar range, spanning 150 nM for sensitive lines to 5 μM relatively resistant cell lines [386], [387], [388], [389], [390], [391], [392]. Breast cancer cell lines with increased Bcl-2 expression showed increased apoptosis when treated with obatoclax on its own or combined with radiotherapy, suggesting that these cells were dependant on Bcl-2 for survival [393],[394]. In neoplastic mast cells obatoclax exhibited potent activity against primary cultures established from patients as well as permanent cell lines [395]. In head and neck squamous cell carcinoma (HNSCC), a cancer with very poor prognosis, obatoclax showed potent monotherapeutic activity, which was enhanced by the addition of chloroquine, an autophagy inhibitor [396]. In cholangiocarcinoma, a type of malignancy where the cancer cells are not considered "primed" for apoptotic death, obatoclax was also potent as single agent treatment and its activity has been associated with the activation of Bax [397]. In hepatoblastoma cells obatoclax was able to augment apoptosis and additionally inhibit tumour cell migration [398]. Thus, there is a significant body of evidence demonstrating the single agent activity of obatoclax in a range of cancer cell types.

The use of obatoclax in drug combinations has also been examined *in vitro* and these combinations have shown an overall increased effectiveness in a number of malignancies. In haematological malignancies such as acute myeloid leukemia, sensitivity to bortezomib was enhanced by the inhibition of Mcl-1 by obatoclax [399] and in Hodgkin lymphoma cells synergy was seen when obatoclax was combined with entinostat [400]. In colon cancer cells obatoclax showed synergy with chemotherapy to induce apoptosis [401]. In hepatoblastoma cells obatoclax demonstrated an additive effect when it was combined with cisplatin, etoposide, irinotecan, paclitaxel, or doxorubicin [402]. The use of the obatoclax derivative SC-2001 together with bortezomib in acute leukemia to inhibit the action of Mcl-1 protein or as single agent against hepatocellular carcinoma cells, restored the sensitivity of cancer cells to treatment [403], [404]. In pancreatic tumours the combination of obatoclax with the multikinase inhibitor sorafenib and histone deacetylase inhibitors sensitized the cells to treatment [405]. Melanoma cells underwent apoptosis with greater sensitivity when treated with the endoplasmic reticulum stress inducer tunicamycin when it was combined with obatoclax [406]. Pretreatment of central nervous system cancer cells with obatoclax increased their sensitivity to lapatinib and resulted in prolonged host survival in xenograft studies [407]. Similarly, orthotopic xenograft studies of obatoclax in mouse models have assessed this drug in hepatoblastoma and cholangiocarcinoma cells and tumour size reduction and increased survival were also observed [398], [397]. Thus, there is also a significant body of evidence demonstrating the activity of obatoclax in combination with several other drug classes in a range of cancer cell types.

The efficacy of obatoclax demonstrated through *in vitro* preclinical studies, either as monotherapy or in combinations with chemotherapy, has justified and led to the clinical evaluation of obatoclax. Phase I clinical trials of obatoclax aiming to detect a safe dose range and assess possible side effects were conducted with patients with haematological malignancies [408],[387],[409]. Other phase I trials have evaluated the combination of an obatoclax infusion over 3 hours or 24 hours with carboplatin and etoposide in patients with small cell lung cancer (SCLC) or the combination of obatoclax with topotecan for (other) solid tumours [410]. In all of the trials obatoclax was administered intravenously and it was seen that steady-state levels of the drug were easily achieved [387]. The half-life of obatoclax appears to range from 39 to 60 hours [408]. The trials demonstrated that obatoclax is safe for use in humans and the most commonly occurring adverse events were grade 1/2 central nervous system symptoms such as somnolence, euphoria and ataxia, which rapidly resolved at the end of the infusion. Neuropsychiatric side effects have been found to be related to the dose and frequency of administration and these can be reduced by increasing the duration of drug infusion from 1 hour to 3 hours [409]. This is a strong indication that the drug can cross the blood-brain barrier, and the adverse effects may reflect the high levels of Bcl-X_L expressed in neurons [409]. 24 hour infusions have also allowed dose escalation without dose limiting toxicity being observed [387]. A further study found that the incidence of CNS events was reduced with 24 hour compared to 3 hour infusions. However, the data were consistent with greater efficacy when the drug was infused for 3 hours (81% response rate with 3 hour infusion, 44% response rate with 24 hour infusion) although relatively small numbers of patients were treated [411].

The successful completion of a number of phase I trials of obatoclax was followed by respective phase II studies. In patients diagnosed with relapsed or refractory classical Hodgkin lymphoma obatoclax was very well tolerated, but showed limited clinical activity. No objective responses were observed and consequently this study did not complete the plans for enrolment beyond the initial small number of patients (ten patients). Adverse events were all limited to grade I severity and half of the patients showed signs of stable disease while the remaining experienced disease progression within 8 weeks [412]. In relapsed SCLC patients the addition of obatoclax to 2nd line topotecan treatment after 1st line platinum therapy did not result in an improved response rate and for this reason the sample population was not expanded [413]. This study recruited 9 patients of which 5 had stable disease for the duration of treatment while the remaining developed progressive disease. No partial or complete responses were noted and the median progression-free survival was 2 months (range 2-5 months). The main adverse events noted were similar to those described previously with neurologic symptoms of somnolence and ataxia being the most frequent ones. These were transient and occurring during the period of the actual drug infusion and resolved within 2 hours after completion of treatment. In relapsed non-small-cell lung cancer the combination of obatoclax with docetaxel showed minimal response [414]. 11% of the patients showed a partial response and the median duration of response was 4.8 months with a median progression-free survival of 1.4 months. Due to two episodes of dose-limiting toxicities, the intended maximum dose was not reached. Neutropenia was the most common adverse event described. Monotherapy with obatoclax infusion in patients diagnosed with myelofibrosis did not exhibit significant clinical activity [415]. None of the 22 patients enrolled for this trial showed complete or partial response, though one patient exhibited clinical improvement which was

maintained for 4 cycles of therapy. Low-grade ataxia and fatigue were common adverse events in half of the patients. A phase II trial of obatoclax in combination with carboplatin and etoposide versus conventional chemotherapy for SCLC patients has been reported at the world conference on lung cancer 2011 (and abstract published, [416]). The final publication of this data is eagerly anticipated because a trend for improved ORR, PFS and overall improved survival was claimed. Obatoclax appeared to reduce the rate of refractoriness during the first six chemotherapy cycles by 37%. In this trial, obatoclax was administered as a 3-hour infusion, rather than a 24-hour infusion [411]. It is interesting to note that all the other obatoclax phase II trials that were apparently less successful used either 1-hour or 24-hour infusion rates for obatoclax and the participants had been heavily pre-treated with chemotherapy, making them intrinsically less likely to respond. It is therefore possible that the 1-hour infusion may have prevented sufficiently high doses being used and the 24-hour infusion might have prevented sufficiently high plasma concentrations being achieved. Controlling the rate of drug administration may be critical to obtain a satisfactory therapeutic window.

The adverse effects observed in phase II trials reflected those in previous studies .The most common adverse events described during the phase II trials of obatoclax were haematological such as neutropenia, thrombocytopenia and anaemia or neurological like ataxia, fatigue and somnolence [413],[414].

Thus, there is a sound rationale based on previous studies of BH3-mimetics by our group and a significant body of evidence, both preclinical and clinical, supporting the evaluation of obatoclax in ovarian cancer. Many of the clinical trials cited above were published after the current research commenced, but they suggest that adverse effects might be managed using appropriate infusion schedules and doses which achieve a balance between efficacy and toxicity. It still seems reasonable, therefore, to investigate the use of obatoclax in ovarian cancer.

4.3 Results

4.3.1 Single agent studies of obatoclax

To investigate the sensitivity of ovarian cancer cells to obatoclax the drug was initially evaluated in single-agent cell growth studies. For the purposes of these studies 8 ovarian cancer cell lines were used: A2780, cisA2780, Ovcar-3, Ovcar-4, Ovcar-5, Ovcar-8, Igrov-1 and Sk-Ov-3. Obatoclax inhibited the growth of cultures of all of the ovarian cancer cell lines with nanoMolar potency (Table 14 and Figure 15).

Cell Line	IC_{50} (nM)	Hill Slope	Number of experiments
A2780	59 ± 30	-3.6 ± 1	n=17
cisA2780	72 ± 39	-3.9 ± 0.8	n= 15
Ovcar3	35 ± 28	-2.1 ± 1.1	n=11
Ovcar4	140 ± 130	-1.9 ± 0.7	n=10
Ovcar5	78 ± 29	-3.1 ± 0.9	n=15
Ovcar8	97 ± 35	-4.3 ± 1.1	n=15
Igrov-1	150 ± 78	-4.7 ± 2.6	n=15
Skov-3	220 ± 92	-1.6 ± 0.4	n=9

Table 14: Single agent activity of obatoclax against eight ovarian cancer cell lines. The activity of obatoclax against ovarian cancer cell lines was evaluated in cell growth assays. IC_{50} and Hill coefficients (mean ± S.D.) were determined by the SRB assay following 72 hours exposure to obatoclax. Error bars represent S.D.

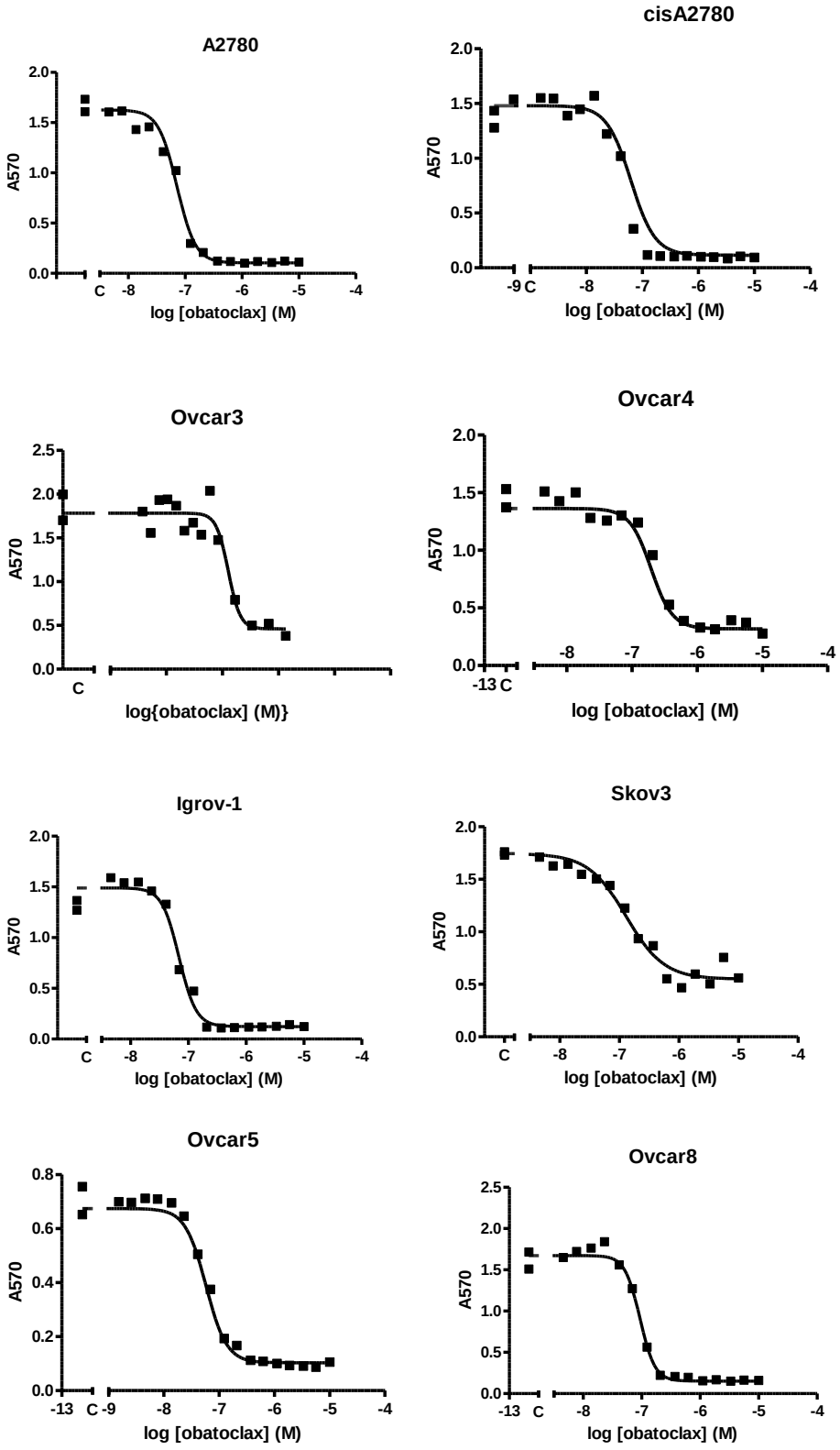

Figure 15: Activity of obatoclax in cell growth assays. The activity of obatoclax against ovarian cancer cell lines was evaluated in cell growth assays by the SRB assay following 72 hours exposure

to obatoclax. The figures show representative experiments, the results of which are summarized in Table 14. “C” denotes cells treated with vehicle alone.

The evaluation of the single agent activity of obatoclax in the same eight ovarian cancer cell lines was repeated in preliminary experiments in 2% O_2 to mimic those found physiologically (Table 15). Although these experiments were only performed once, in 2% O_2 obatoclax mostly retained activity in all of the cell lines although the IC_{50} increased modestly, on average 2.4-fold across all 8 cell lines (range 1.4 - fold to 4.3 – fold).

Cell Line	**Average IC_{50} (nM)**
A2780	90
CisA2780	100
Ovcar3	90
Ovcar4	340
Ovcar5	140
Ovcar8	330
Igrov-1	230
Skov-3	950

Table 15: The single agent activity of obatoclax in reduced oxygen tension conditions. The activity of obatoclax against ovarian cancer cell lines was evaluated in cell growth assays in 2% O_2. IC_{50} and Hill coefficients were determined by the SRB assay following 72 hours exposure to obatoclax, n=1.

4.3.2 Trypan blue viability studies

Measuring the growth of cell cultures does not distinguish between a drug inhibiting cell proliferation, inducing cell death, or a combination of the two. To discriminate between these two, the viability of cells following exposure to obatoclax for 72 hours was assessed by staining with trypan blue. The study was performed on all eight ovarian cancer cell lines (Figure 16). In all of the cell lines, exposure to obatoclax (at 10 times the IC_{50} measured in cell growth assays) led to a significant increase in the number of dead cells compared to the number measured in a sample in which the cells were treated with vehicle. However, this increase was less marked in Ovcar-3, Ovcar-4 and Sk-Ov-3 cells.

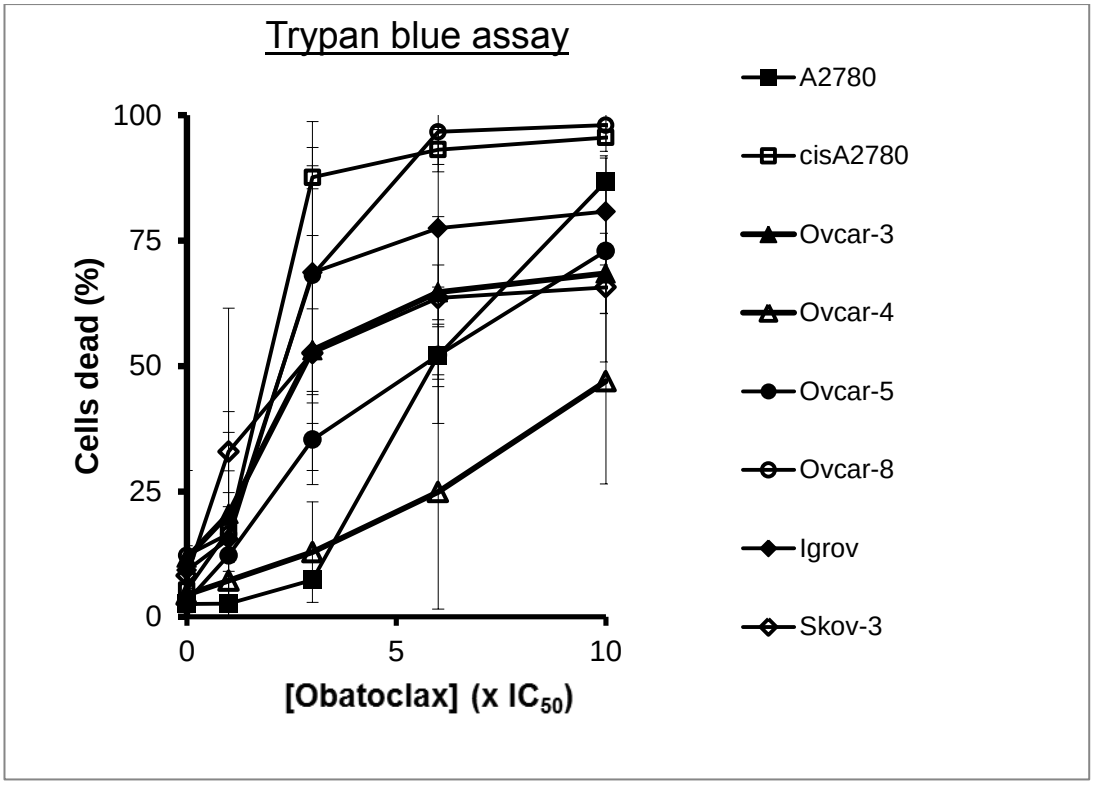

Figure 16: The trypan blue viability assay of obatoclax. Eight ovarian cancer cell lines were treated with obatoclax for 72 hours at multiples of the IC_{50} values for each cell line measured in cell growth assays. The results are expressed as a fraction of the total cells present at each time point (mean ± S.D., n=2-4). In all of the cell lines, there was a significant increase in dead cells ($P < 0.05$, paired t-test) when cells were treated with the highest concentration of obatoclax compared to cells treated with vehicle. Error bars represent S.D.

4.3.3 Colony formation assays

Colony forming assays were performed to confirm that obatoclax is cytotoxic. Once a cell population has been exposed to a drug, if the drug is cytotoxic then it is anticipated than only a fraction of the initial population will retain its capacity to produce colonies even the drug is subsequently removed [417]. If however, the drug is purely cytostatic, when the drug is removed the cells are anticipated to grow again and form colonies. Obatoclax dose-dependently reduced the ability of Ovcar-8 cells to form colonies, consistent with it causing cell death (Figure 17). Thus, this confirmed the results obtained by trypan blue staining.

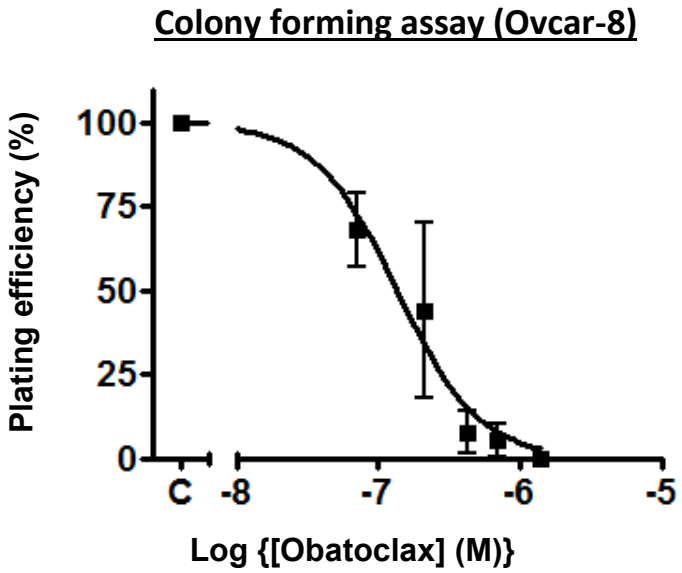

Figure 17: Colony formation assay of obatoclax.

Cells were treated with the indicated concentration of obatoclax for 48 hours, trypsinized and replated. The number of colonies was measured after 2 weeks. The results (mean ± S.D., n=3) are expressed as a percentage of the plating efficiency measured in cells treated with vehicle alone. The IC_{50} of obatoclax calculated from these combined experiments (140 nM) is comparable to that measured in the cell growth studies (100 nM), although in the later studies the duration of exposure was 72 hours. Error bars represent S.D.

4.3.4 Ovcar-8 spheroids treated with obatoclax

The experiments so far made use of cells growing as a monolayer. This is not an accurate reflection of a tumour which possesses a 3-dimensional architecture. Growing cells in 3-dimensional culture has been found to alter their sensitivity to several drugs (for example, see Chapter 3). One way in which this can be mimicked in the laboratory is to grow the cells as spheroids. Therefore, the activity of obatoclax was evaluated using Ovcar-8 cells grown as spheroids. Spheroids were prepared as previously described [418] and were 0.47 ± 0.05mm in diameter (E. Robinson & A. Richardson, unpublished observations). The cytotoxic activity of obatoclax was confirmed in these studies (Figure 18) although the potency was diminished somewhat (IC_{50} = 610 nM) compared to monolayer cultures.

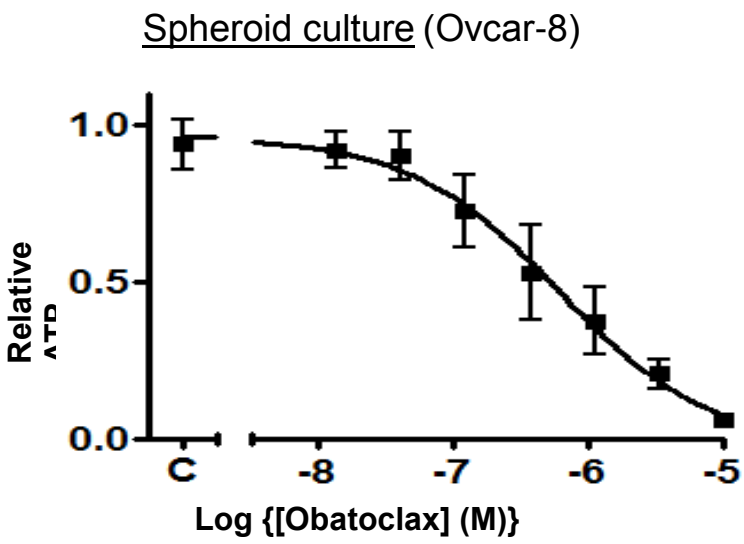

Figure 18: Evaluation of the activity of obatoclax in spheroid cultures.

Cells were allowed to form spheroids over 1 week, before the indicated concentration of drug was added. After 72 hours relative cell number was estimated by measuring intracellular ATP. The results (mean ± S.D., n= 3) are expressed as a fraction of the ATP measured in cells treated with vehicle alone (marked "C". Error bars represent S.D.

4.3.5 Single agent studies of obatoclax on primary cultures

To further validate the usefulness of Obatoclax as a potential treatment for ovarian cancer, its activity on primary cultures of ovarian cancer cells (PCOCC) was investigated. The process of establishing primary cultures of ovarian cancer cells (PCOCCs) and harvesting an adequate number of cells for our experiments remained quite problematic throughout the course of this study. This was mostly due to failure of many of the samples to generate a viable culture. The success rate was approximately 33%. Although a total of 25 ascitic samples were collected, only 8 samples led to viable cultures. A frequent problem was the appearance of cell culture infections, which were resistant to antibiotic supplementation. Thus, only a limited number of successful experiments were completed.

All the investigations were performed using low passage number of primary cancer cells due to the well described problem of senescence after later passages [330]. The initial experiments were performed by seeding 5,000 cells per well (PCOCC2 to PCOCC6) and did not provide cultures which grew reliably. This was improved by increasing the number of cells seeded to 8,000 cells per well. To determine how long the cells could be maintained in culture in either 21% O_2 or 2% O_2, the time between successive passages (which were performed when the cultures became confluent) was recorded (Figure 19).

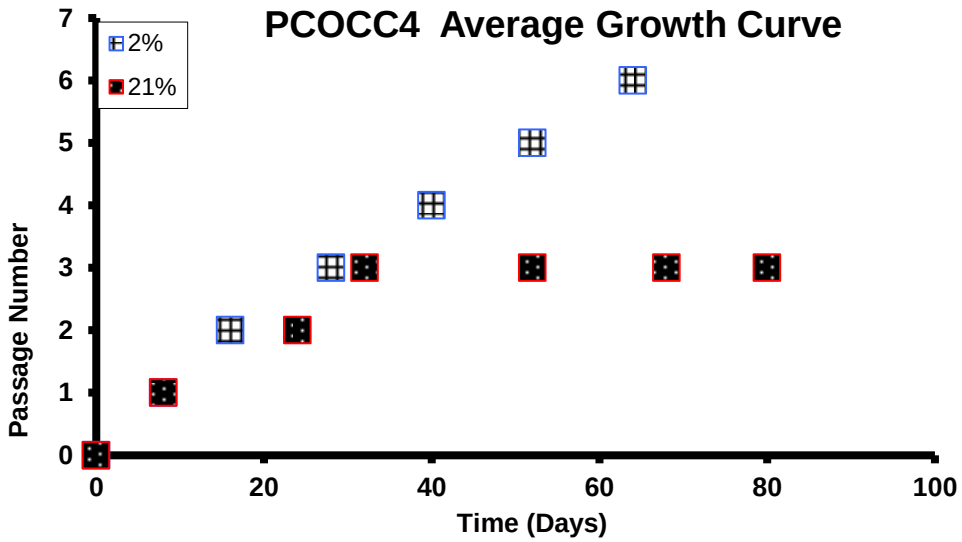

Figure 19: Growth curve of PCOCC4 cells established in different O_2 conditions.

Both primary cultures were established from the same ascitic sample and were kept in separate atmospheric O_2 conditions.

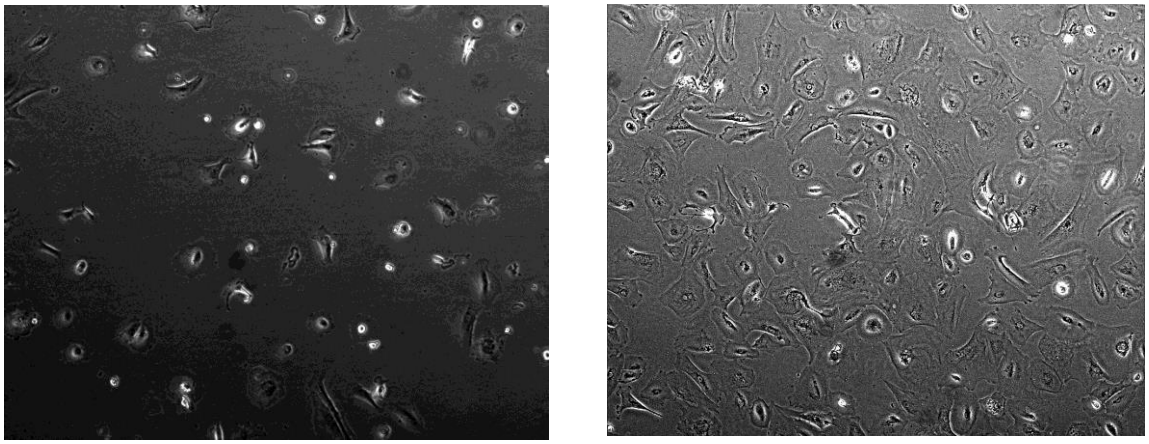

Figure 20: Primary cultures of ovarian cancer grown in 21% O_2 and 2% O_2.

The image on the left shows primary culture PCOCC3 cells in 21% O_2 and the image on the right the same culture seeded and grown in 2% O_2. The normoxic conditions appear to contribute to the cells proliferating less and going into a state of senescence.

The cobblestone morphology of the cultures (particularly evident in Figure 20, 2% O_2) provided some reassurance that the cells grown in the primary cultures were of epithelial origin and thus likely to be EOC cells. To support this, cells were stained with an antibody to detect the epithelial cell marker cytokeratin. More than 90% of the cultured cells were stained with the cytokeratin antibody (Figure 21). These images show cells grown on a microscope coverslip. Under these conditions the cells did not adopt the typical epithelial cell morphology seen on cell culture plasticware. The cytokeratin staining is not satisfactory because no cellular morphology is evident, raising concerns about the specificity of staining (although no staining was observed with the cells when the primary antibody was omitted). Due to the issues with culturing the ascites cells which led to further studies with these cells being abandoned, no further experiments to overcome this technical problem were attempted.

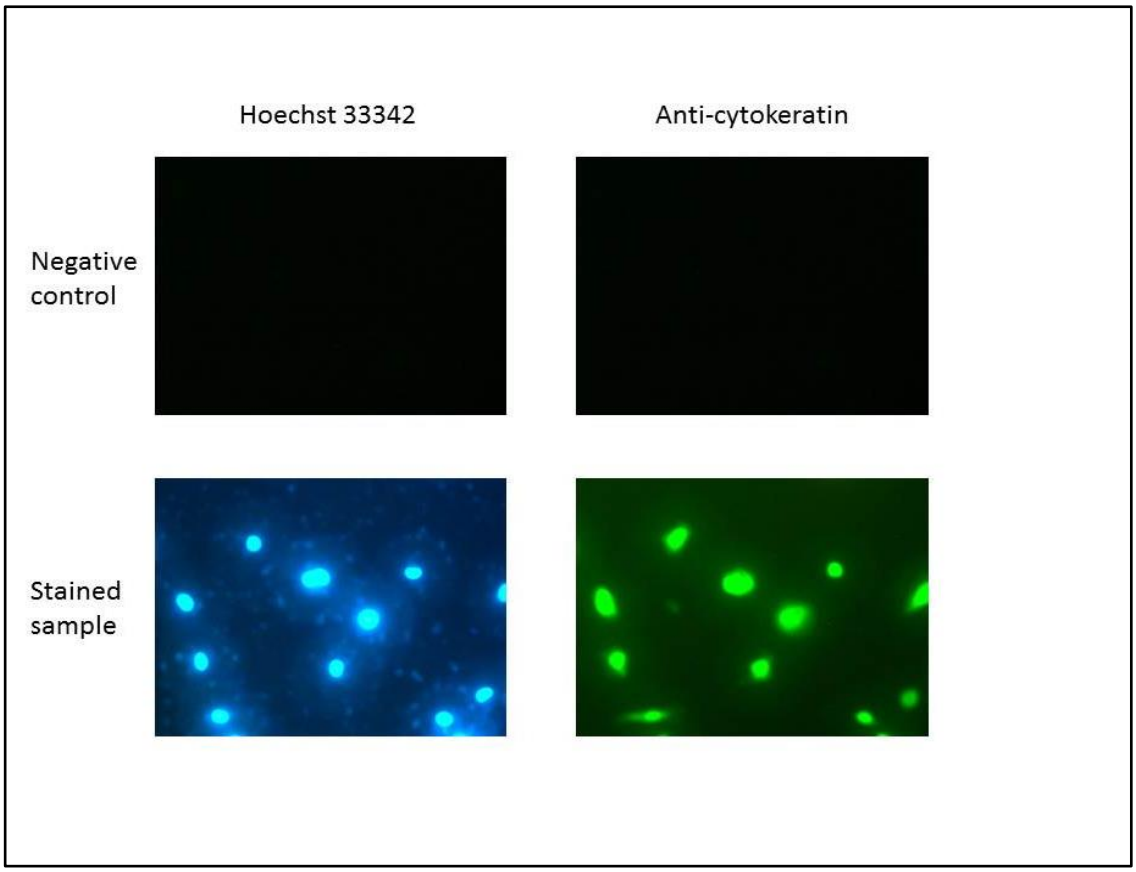

Figure 21: Staining of ascites cells. PCOCC20 cells were grown on glass coverslips, fixed and stained with either Hoechst 33342 (nuclear dye) or anti-cytokeratin (epithelial cell marker). In the "negative control" samples Hoechst or the primary antibody were omitted respectively. The image is representative of 3 experiments.

The sensitivity of PCOCC to carboplatin, paclitaxel and obatoclax was evaluated in cell growth assays. The measured IC_{50} of carboplatin using 2 primary cultures (PCOCC2 & PCOCC3) cells was 8μM. Although in some experiments satisfactory dose response curves were obtained using paclitaxel (e.g. Figure 22), in many experiments the drug showed poor activity. This may reflect the fact that paclitaxel requires cells to be actively progressing through the cell cycle to cause cell death. In Chapter 3, the decreased potency of paclitaxel in spheroids was also noted and attributed to the low mitotic index likely in spheroids. All of the cell cultures were sensitive to obatoclax (Table 16 and Figure 23), although an inadequate number of experiments were performed to assess the effect of oxygen tension on obatoclax sensitivity. In 21% O_2 using the cultures which grew sufficiently for the experiments to be replicated, the sensitivity of the cultures to obatoclax was approximately 10-fold less than the sensitivity of the ovarian cancer cell lines to obatoclax.

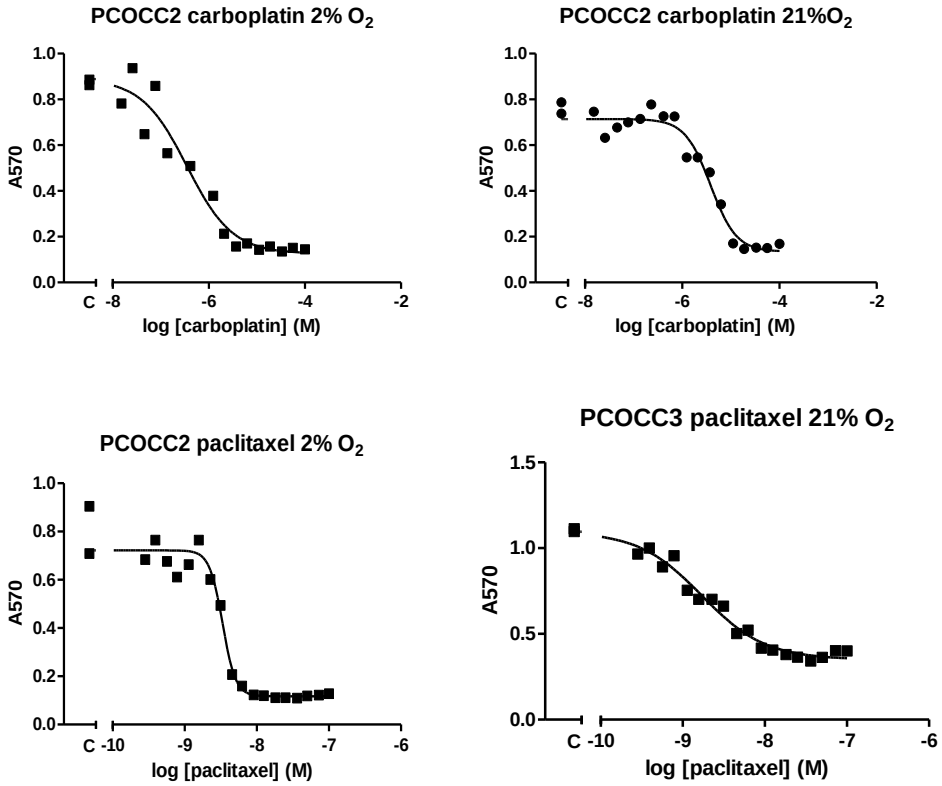

Figure 22: Activity of carboplatin and paclitaxel in cell growth assays of primary cultures. Single agent studies were conducted in primary cultures of ovarian cancer cells (PCOCC2 & PCOCC3) cultured in normoxia and reduced oxygen tension. Each drug response was measured in singlicate, with the exception of the cell number measured in the absence of drug (indicated "C" on the x-axis) which was measured in duplicate.

Cell Line	[O_2]	IC_{50} (nM)	Hill Slope	Number of experiments
PCOCC2	21%	950	-3.3	n=1
PCOCC2	2%	46	-1.4	n=1
PCOCC3	2%	39	-0.8	n=1
PCOCC4	21%	1500 ± 360	-1.2 ± 0.02	n=1
PCOCC4	2%	520 ± 510	-1 ± 0.9	n= 4
PCOCC5	21%	880 ± 680	-1.3 ± 0.4	n=2
PCOCC8	21%	730 ± 420	-1.1 ± 0.9	n=4

Table 16**:** Cell growth assays evaluating the activity of obatoclax against primary cultures of ovarian cancer cells in normoxic (21% O_2) and reduced oxygen tension (2% O_2) conditions. The activity of obatoclax was evaluated in cell growth assays using primary cultures of ovarian cancer cells obtained from ascites. IC_{50} values (mean ± S.D.) were determined in all of the cell growth assays with primary cultures. In some cultures, the IC_{50} was only measured once due to the problem described in the text.

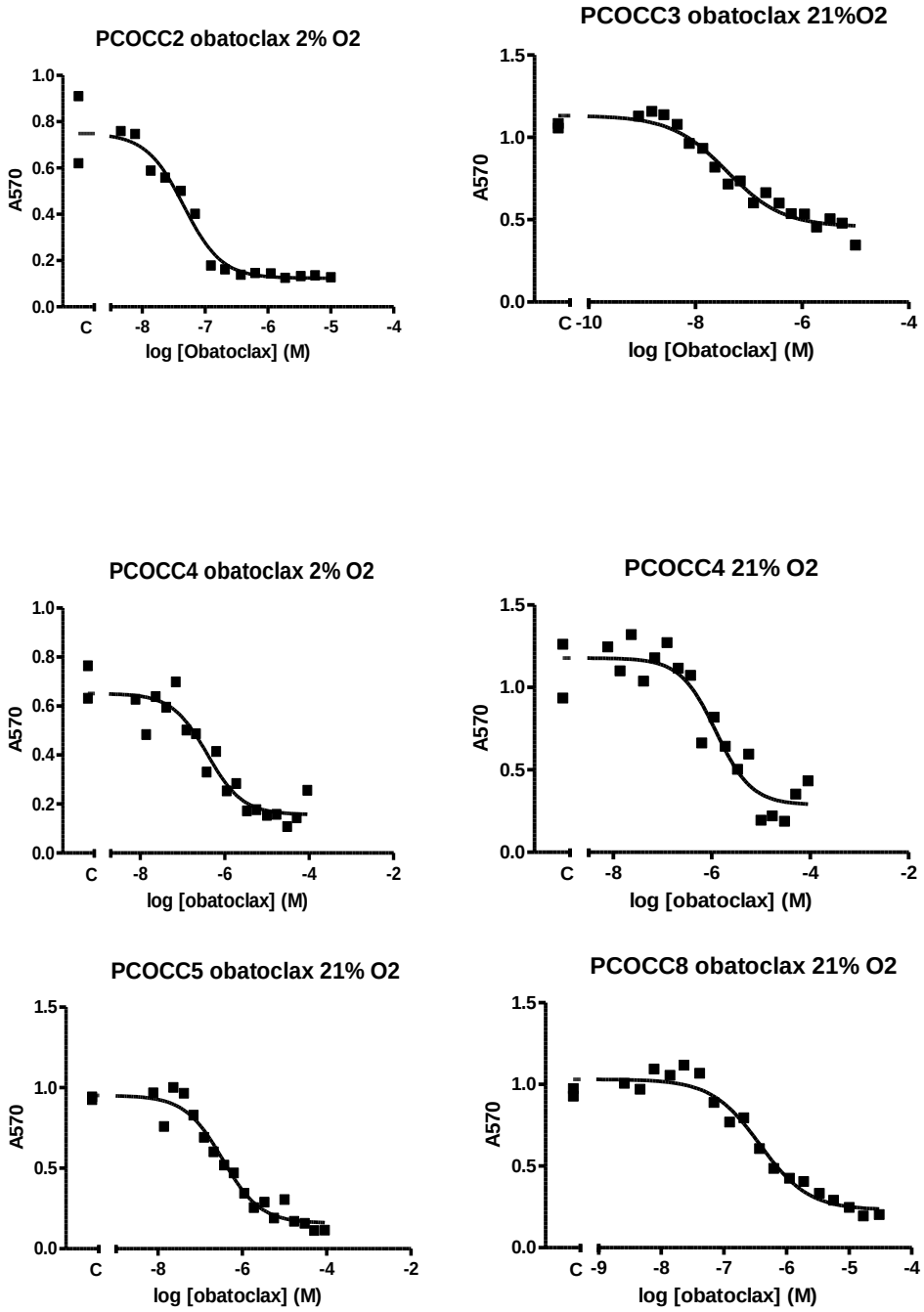

Figure 23: Activity of obatoclax in primary cultures of ovarian cancer cells. The figures show representative experiments which are summarized in Table 17. Cells were treated with the indicated concentration of obatoclax and the number of cells surviving after 72 hours estimated with an SRB assay. "C" denotes cells treated with vehicle alone.

4.3.6 Caspase 3/7 studies of ovarian cancer cells treated with obatoclax

The foregoing data demonstrated that obatoclax caused cell death in established ovarian cancer cell lines and inhibits the growth of cultures of primary EOC derived from ascites fluid, presumably also by causing cell death. BH3-mimetics can cause cell death by activating the intrinsic apoptosis pathway (Chapter 1). Subsequent experiments were performed to confirm that obatoclax induces apoptosis. Caspases 3 and 7 are considered effector caspases which are activated by the release of cytochrome c from the mitochondria [419]. Their activation provides an indication of the irreversible commitment to apoptosis. Caspase 3/7 activity was measured using a commercially available kit (Caspase 3/7 Glo) following exposure to two different concentrations of obatoclax and compared to apoptosis induced by carboplatin (a drug known to induce apoptosis). Obatoclax dose-dependently increased caspase 3/7 activity in most of the cell lines (Figure 24). However, no increase was evident in Ovcar-3, Ovcar-4 and Sk-Ov3 cells. It is notable that these were the cells in which obatoclax caused the least cell death in the trypan blue staining experiments (Figure 24). Note that the relatively high apparent activation of caspases in cisA2780 reflects the fact that the results were normalized to the activation induced by carboplatin in each cell line. The cisA2780 cells are relatively resistant to carboplatin when the caspase activity induced by obatoclax is expressed as a fraction of the carboplatin-induced caspase activity, it consequently appears more pronounced than in other cell lines.

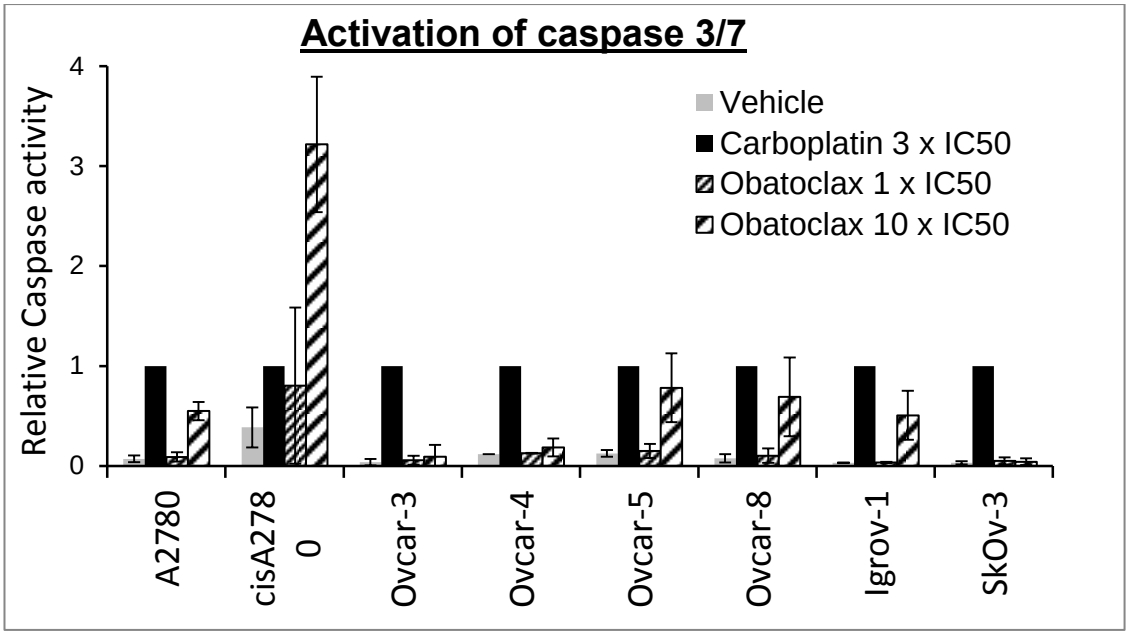

Figure 24: The activation of caspase 3/7 substrate in after treatment with obatoclax. The study was performed using eight ovarian cancer cell lines. The results (mean ±S.D., n=2-3) are expressed as a fraction of the caspase 3/7 activity measured in cells exposed to carboplatin at its IC_{50} concentration (measured in 72 hour cell growth assays) in each cell line. Obatoclax was evaluated at 1x and 10x its IC_{50} concentration (measured in 72 hour cell growth assays) in each cell line. Obatoclax induced significantly increased (P<0.05, paired t-test) caspase 3/7 activity compared to carboplatin in cells treated with vehicle in A2780, cisA2780, Ovcar-8 and Igrov-1 cells. Error bars represent S.D.

4.3.7 Western blotting studies - The induction of apoptosis and autophagy after treatment with obatoclax

To confirm the results of the caspase 3/7 assays, cleavage of PARP was also measured. PARP is a substrate of caspases, and it cleavage therefore provides an additional measure of apoptosis. Each of the cell lines was treated with obatoclax at multiples of its respective IC_{50s} and PARP cleavage measured by western blotting (Figure 25). PARP cleavage was evident in Ovcar-5, Ovcar-8 and Igrov-1 cells. These cells were the ones in which the most significant activation of caspase 3/7 was observed. Relatively modest cleavage of PARP was observed in A2780 cells, consistent with the modest activation of caspase 3/7 in these cells. Cleavage of PARP was not evident in Ovcar-3, Ovcar-4 and Sk-Ov-3 cells, consistent both with the trypan blue studies and the lack of activity in the caspase 3/7 assay. This suggests that in these cell lines, obatoclax did not induce apoptosis.

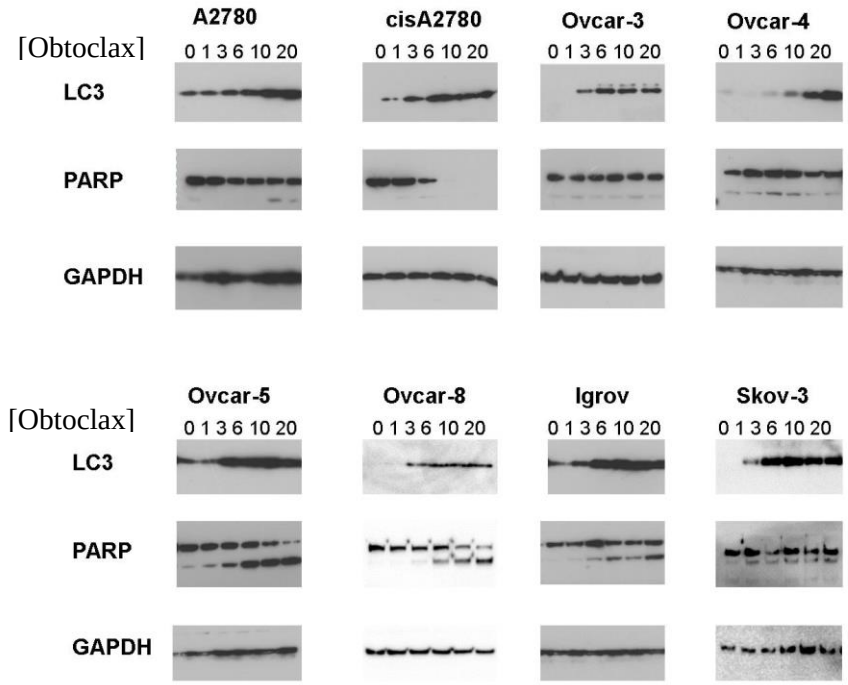

Figure 25: The induction of PARP cleavage and LC3 after treatment with obatoclax. W Cells were treated with obatoclax at the indicated multiple of the IC50 measured in cell growth assays. After 48 hours, cells were lysed and samples processed for western blotting with antibodies directed to LC3, PARP or GAPDH. The results shown are representative of 3 experiments. Note that the band labelled LC3 represents LC3-II. Unprocessed LC3, which appears as a band 2kd larger, was difficult to detect and only visible on prolonged exposure.

4.3.8 Nutlin and obatoclax

One possible explanation for the lack of induction of apoptosis in some cell lines was that these cells had defects in the p53 signalling pathway. Under normal conditions, any type of cellular stress can result in the activation of the tumour suppressor p53.Ovarian cancer is characterised by deleted or mutated copies of *TP53* (the gene encoding p53). The transcriptional activity of p53 is regulated by the mouse double minute 2 homolog (MDM2) protein, which is encoded by the *MDM2* gene [420]. MDM2 acts by binding to the N-terminal domain of p53 and catalysing its ubiquitination. *MDM2* is very often amplified in cancer and its increased expression promotes turnover of p53 and inhibition of apoptosis allowing the survival of cancer cells. Nutlin-3 can inhibit the binding of MDM2 to p53, preventing ubiquitination and turnover of p53 and effectively cause its re-activation. Cells which have a functional p53 pathway are relatively sensitive to Nutlin-3, whereas cells with defects in the p53 pathway are relatively resistant. Nutlin-3 sensitivity can thus be used to estimate the status of the p53 pathway without having to evaluate genetic (and potentially epigenetic) abnormalities in all pathway members.

These studies were performed prior to the publication of the cancer genome atlas research network which highlighted the high frequency of p53 abnormalities in ovarian cancer [123]. With the exception of two cell lines, (A2780 and its daughter line cis-A2780), most of the cell lines were relatively insensitive to Nutlin-3 (Figure 26). This is consistent with TGCA data indicating that these cell lines have aberration in the p53 pathway, but

A2780 does not [421]. It does not, however, provide an explanation for the differential induction of apoptosis in these cells.

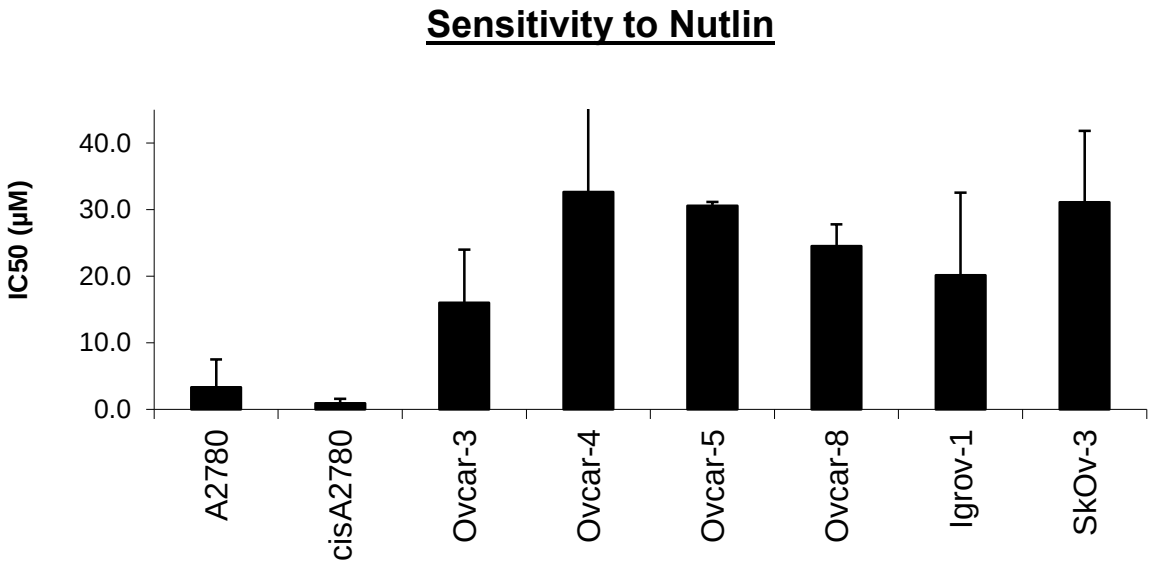

Figure 26: Sensitivity of eight ovarian cancer cell lines to nutlin.

The cells were treated with Nutlin-3 for 72 hours and their sensitivity estimated using an SRB assay. Nutlin IC_{50} values (mean ±S.D. n= 2-3) were determined by non-linear regression to fit a Hill equation. Error bars represent S.D.

4.3.9 The formation of autophagosomes after treatment with obatoclax

Prior to this work, ABT-737 had been shown to induce autophagy by displacing Beclin-1 from Bcl-2 [422]. Obatoclax has relatively high affinity for Bcl-2, and this raised the possibility that obatoclax might also induce autophagy. Autophagy can be considered to be either a protective mechanism which allows the cell to recycle existing structures and be energy efficient or a type of death if it becomes excessive. The process of autophagy involves the formation of autophagosomes, organelles which captivate cellular components and digest them. The recruitment of proteins into autophagosomes is regulated by proteolytic processing of LC3 and its subsequent lipidation to generate LC3-II. Measurement of LC3-II has been used as a marker of autophagy. The samples analysed for PARP cleavage were therefore also analysed to measure the effect of obatoclax on LC3-II. A robust induction of LC3-II was observed in all cell lines raising the possibility that this also contributes to cell death.

To confirm the induction of autophagy in these cells we transfected cell lines with an LC protein tagged with green fluorescent protein. Live cell imaging was used to visualise those cellular organelles that displayed the fluorescent LC3 protein chain.

Autophagosomes

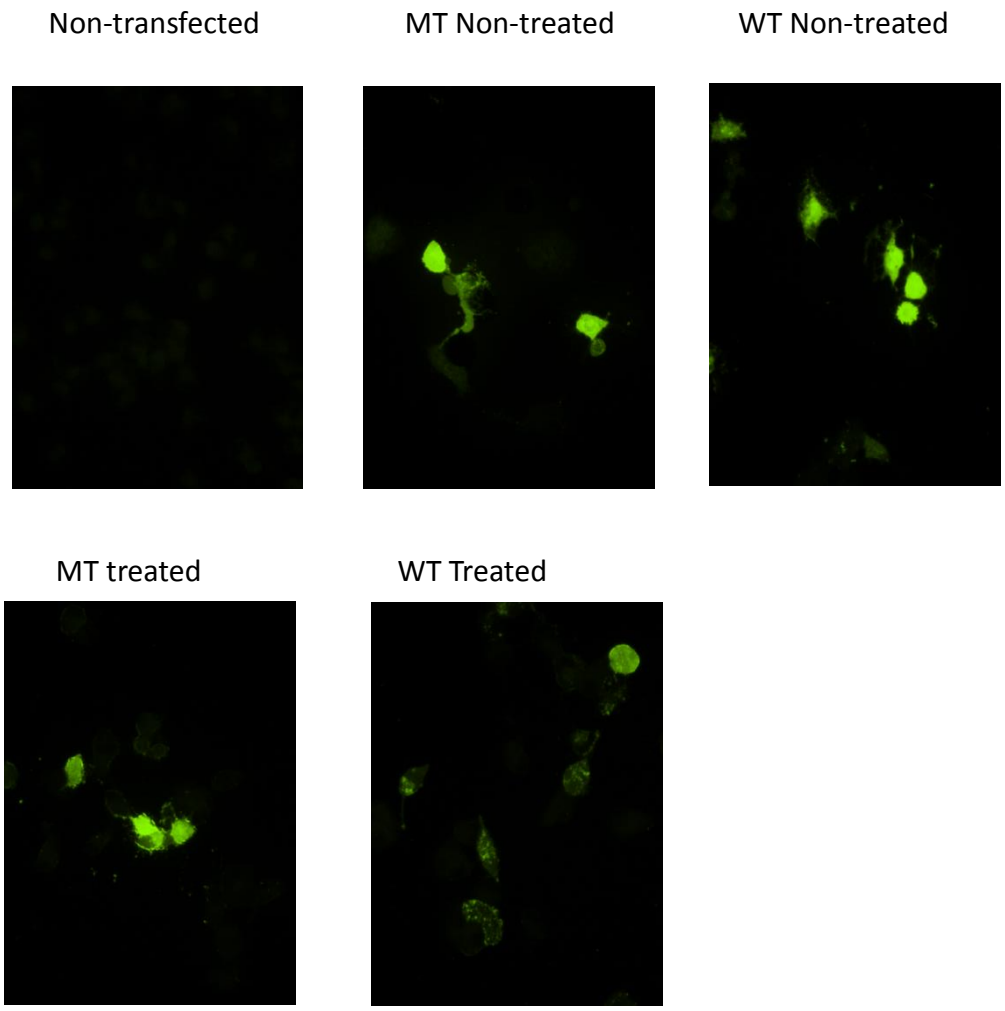

Figure 27: The formation of autophagosomes in LC3-GFP transfected Ovcar-8 cells treated with obatoclax.

Ovcar-8 cells were transfected with plasmids encoding wild ("WT") and mutant "MT" LC3-GFP. The mutant LC3 does not undergo lipidation and so is not recruited to autophagosomes in an autophagy dependent manner. It therefore provides a negative control for autophagy independent staining of autophagosomes. The cells were treated with obatoclax before fluorescence microscopy was used to evaluate the formation of autophagosomes. The results are representative of 3 experiments.

4.3.10 siRNA inhibition studies

The foregoing work suggested the possibility that autophagy may contribute to cell death induced by obatoclax. To investigate this, an RNAi approach was taken. The expression of two genes known to be essential for the regulation of autophagy, Beclin1 and Atg5 was inhibited in Ovcar-4, Ovcar-5 and Ovcar-8 cells by transfection with Dharmacon SMARTpools (a pool of 4 siRNAs) directed to the corresponding mRNAs. QPCR studies were performed first to identify the appropriate concentration of SMARTpool. The 20nM concentration was selected so that mRNAs was significantly reduced 48 hours after transfection without significantly reducing cell number (Figure 28). Western blotting studies confirmed that the levels of each protein were also reduced 48 hours after transfection (Figure 29).

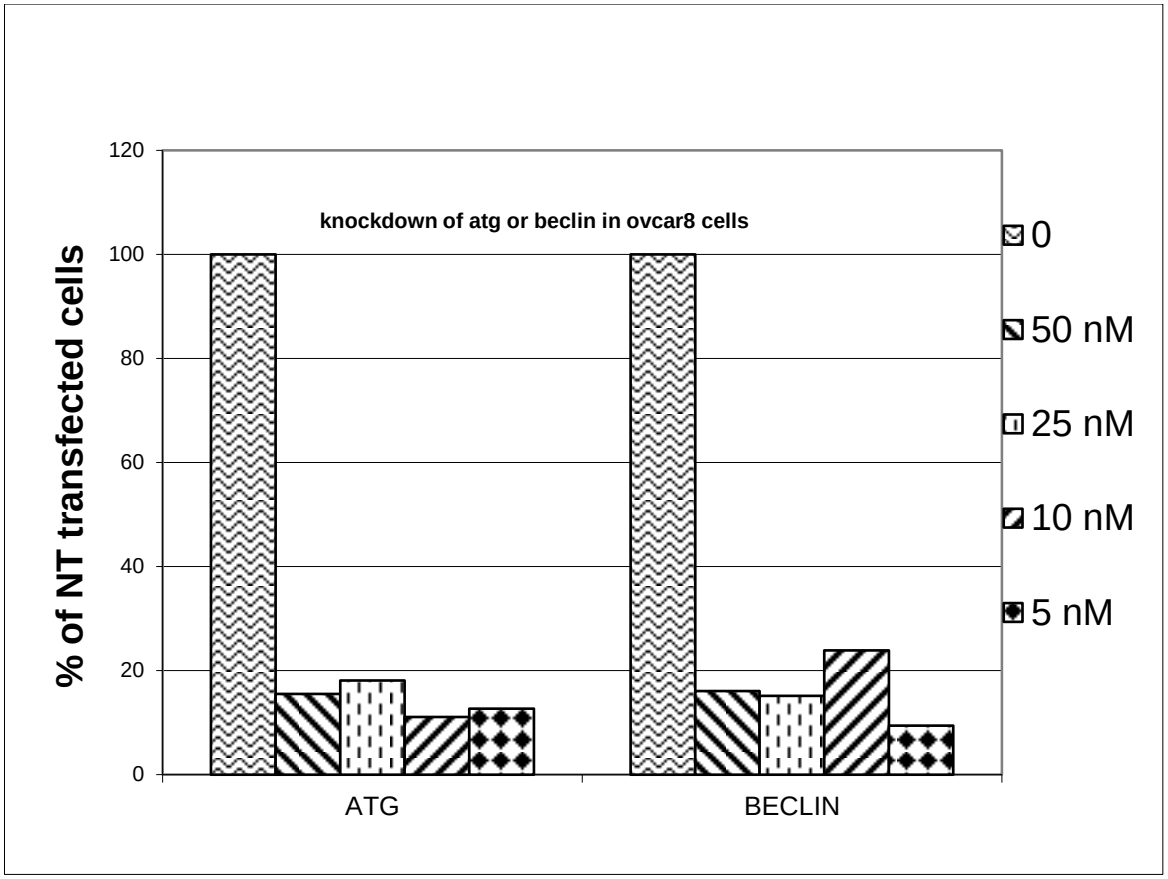

Figure 28: Inhibition of Beclin1 and Atg5 using siRNA. Ovcar8 cells were transfected with siRNA to Beclin1 or Atg5. After 48 hours mRNA was isolated and the expression measured by Q-RTPCR. The results are expressed as a fraction of the mRNA measured in cells transfected with the non-targeting control siRNA. The experiment is representative of two experiments.

Following silencing of Beclin1 and Atg5 changes in sensitivity to obatoclax were measured. Knockdown of Beclin1 and Atg5 had a very modest, although statistically significant, effect on the sensitivity of Ovcar-5 cells to obatoclax (Figure 30). No significant effect was on obatoclax sensitivity was observed in Ovar-4 or Ovcar-8 cells. It should be recalled that in Ovcar-4 cells, obatoclax did not induce apoptosis. These data are not inconsistent with autophagy playing a role in obatoclax induced cell death, however they suggest that autophagy is not necessary for this process.

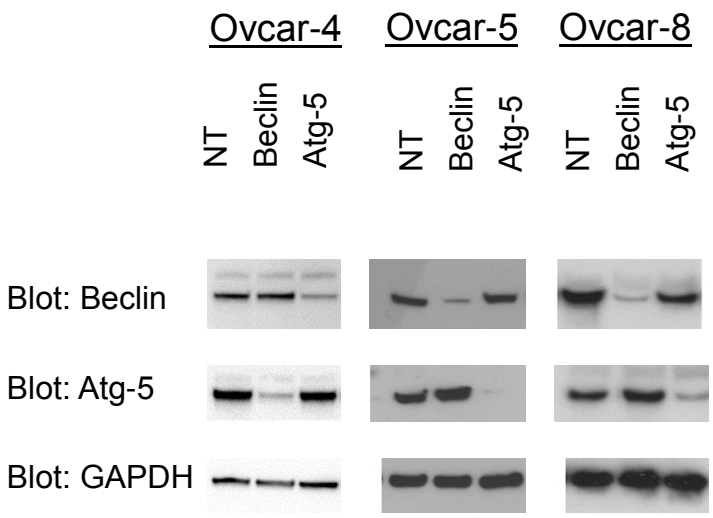

Figure 29: Knockdown of Beclin-1 and Atg-5.

Cells were transfected with the indicated siRNA and lysed after 48 hours before the samples were processed. Western blotting was performed to confirm the knockdown (representative of 2-3 experiments).

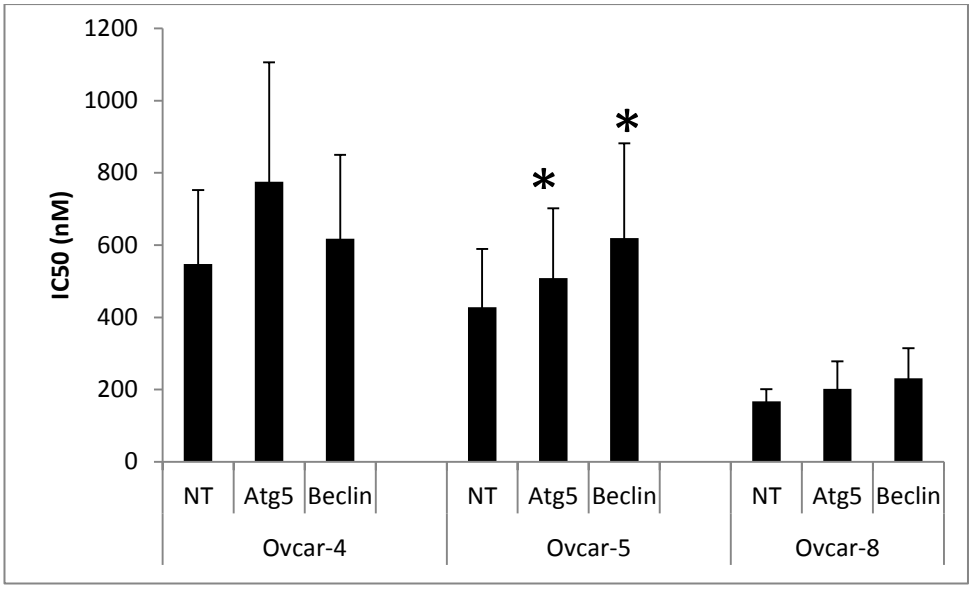

Figure 30: siRNA inhibition studies. In order to evaluate the contribution of autophagy to the activity of obatoclax RNAi studies were performed. Following knockdown of Atg-5 and Beclin, the IC_{50} of obatoclax (mean ± S.D., n= 3-4 separate transfections) was compared to that measured in cells treated with a non-targeting (NT) siRNA. *, IC_{50} significantly different (P<0.05, paired t-test) from that measured in cells transfected in the non-targeting control. Error bars represent S.D.

4.3.11 N-acetylcysteine assay rescue experiments of obatoclax

Recent work has proposed that the combination of obatoclax and lapatinib kills breast cancer cell lines through the generation of reactive oxygen species (ROS) [423]. To test the possibility that obatoclax killed ovarian cancer cells by generating ROS, the effect of N-acetylcysteine on the potency of obatoclax was evaluated in a selection of the ovarian cancer cell lines. N-acetyl cysteine protects cells against oxidative stress by scavenging ROS [424]. Thus, if obatoclax induces apoptosis through generation of ROS, the measured IC_{50} of obatoclax would be anticipated to increase in the presence of N-acetylcysteine. However, when cells were treated with N-acetylcysteine, the potency of obatoclax was not significantly altered in 4 of 5 cell lines evaluated (Figure 31). There was a modest, but significant, increase (not decrease) in obatoclax potency in cisA2780 cells. Notably, N-acetylcysteine had no consistent effect on obatoclax potency even in Ovcar-3 and Ovcar-4 cells in which apoptosis was not evident following exposure to obatoclax. These data suggest that the generation of ROS is not essential for obatoclax-induced cell death.

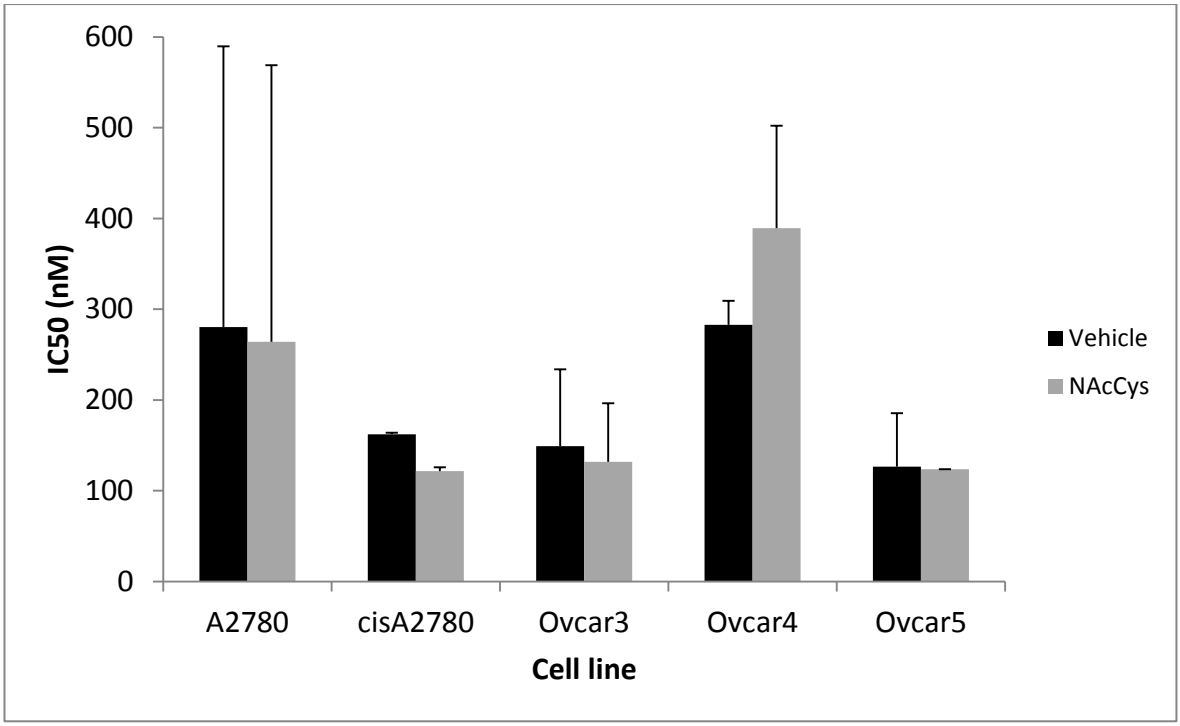

Figure 31: Effect of N-acetylcysteine on the activity of obatoclax. The indicated cells lines were treated with a range of concentrations of obatoclax in the presence or absence of 10 mM N-acetylcysteine (labelled "NAcCys"). After 72 hours, the number of cells surviving was estimated with the SRB assay and the IC_{50} (mean ± S.D., n =2 -3) determined. No consistent change in the potency of obatoclax was noted although in cisA2780 cells the change in IC_{50} was significantly reduced (P<0.05, paired t-test). Error bars represent S.D.

4.3.12 Flow cytometry studies

The results thus far failed to fully clarify the mechanism by which obatoclax inhibits the growth of cultures of ovarian cancer cells. The possibility was considered that obatoclax might also cell cycle arrest in addition to inducing apoptosis, and that this might contribute to the inhibition of growth of cell cultures treated with obatoclax (Figure 15). Precedent for this was provided by the report that at non-cytotoxic concentrations, obatoclax can cause S-phase arrest in AML [391].

Flow cytometry can be used to measure the proportion of cells in different phases of the cell cycle. Following staining with propidium iodide, changes in DNA content can be assessed and used to identify cells in G_1, S or G_2/M phases of the cell cycle [425], [426]. Furthermore, the appearance of cells that have a DNA content less than that measured in diploid cells (i.e. sub G1) has been taken as a measure of apoptosis [427].

Each of the ovarian cancer cell lines was treated with obatoclax at a range of drug concentrations (Figure 32). There was no consistent pattern of accumulation of cells in S or G2/M phases of the cell cycle. In particular, an increase in cells in S-phase, as reported for AML cells exposed to a non-cytotoxic concentration of obatoclax, was only observed in Ovcar-5 cells [391]. These data suggest that the major mechanism by which obatoclax inhibits the growth of cultures of ovarian cancer cell lines is not mediated by cell cycle arrest. However, there was a significant increase in the sub-G_1 fraction in several of the cell lines, particularly in the Ovcar-5, Ovcar-8 and Igrov-1 cells in which apoptosis was observed previously, consistent with apoptotic cell death[428].

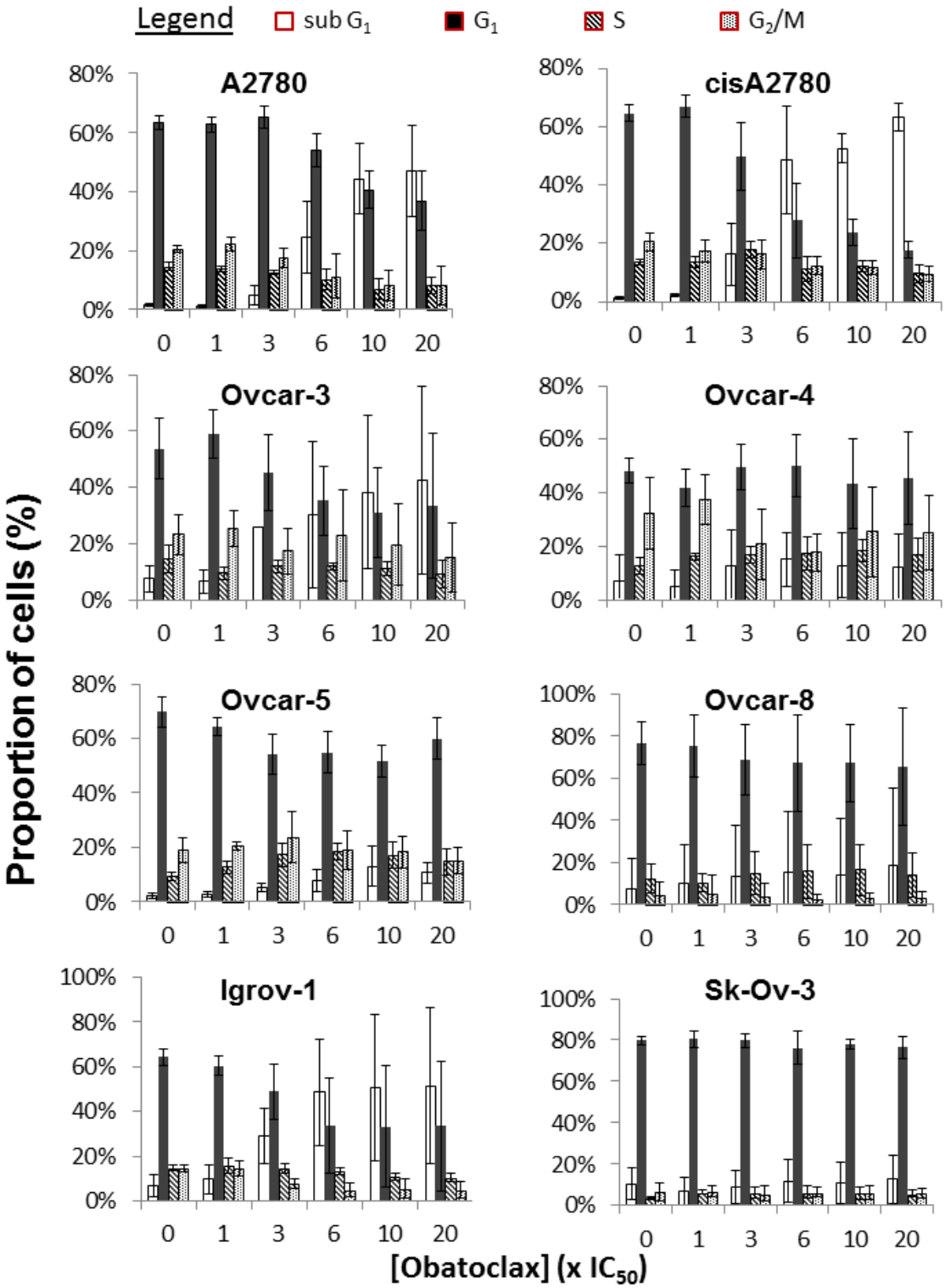

Figure 32: Effects of obatoclax on the cell cycle.

The indicated cell lines were treated with vehicle or obatoclax for 48 hours at the indicated multiple of the IC_{50} measured in cell growth assays. Cells were stained with propidium iodide and analysed by flow cytometry. The results (mean ± S.D., n = 2-4) show the % of cells in different

phases of the cell cycle. Note that the increase in cells in S phase (1 -10 x IC_{50}) in Ovcar-5 cells was significantly different (*P < 0.05*, paired t-test) from that measured in cells treated with vehicle alone. A comparable increase was not observed in the other cell lines. Error bars represent S.D.

4.3.13 Synchronous combination studies of obatoclax with carboplatin and paclitaxel

The use of drug combination therapy in cancer is a long-established practice which has evolved greatly over the years. It is based on the concept that inhibiting multiple drug targets can lead to synergistic activity of the drugs and potentially overcome chemoresistance. Previous work from our group has demonstrated that the BH3-mimetic ABT-737 can resensitize ovarian cancer cells to chemotherapy. The work presented in chapter 3 demonstrated that navitoclax is also synergistic with carboplatin and paclitaxel. This suggested that obatoclax would also show synergy with the standard-of-care drugs carboplatin and paclitaxel. Studies were designed to combine obatoclax with these drugs in a ratio of their IC_{50} values. To do this, the obatoclax IC_{50} values from the single agent studies performed previously were used and the IC_{50} values for carboplatin and paclitaxel (Tables 11 & 12, Chapter 4) were obtained from single agent cell growth assays with each of the cell lines. The combination studies were conducted using the same ovarian cancer cell lines. In order to evaluate the effect of the drug combination, a combination index (C.I.) was calculated [429]. A C.I. value <1 is considered indicative of synergy, while values over 1 are indicative of antagonism between the two drugs.

Unexpectedly, the results from the combination of obatoclax with carboplatin (Table 17) indicated that these drugs have a synergistic activity in only two of the seven cell lines, while additivity is observed for the majority of cases. For example, in Igrov-1 cells, the cell line in which synergy was most evident between carboplatin and ABT-737 [312] or with

navitoclax (Chapter 3), there was no evidence of synergy between carboplatin and obatoclax. The results from the obatoclax and paclitaxel combinations (Table 18) surprisingly indicated that obatoclax and paclitaxel displayed antagonist activity. These results are also shown graphically (Figure 34).

Combination study of obatoclax and carboplatin					
Cell Line	Exclusive CI values	t-test	Non-exclusive CI values	t-test	Number of experiments
A2780	1.4 ±0.5	$P < 0.05$	1.9 ±1.0	$P < 0.05$	n=10
cisA2780	1.5 ±1.2	$P = 0.2$	2.2 ±2.4	$P < 0.1$	n=8
Ovcar4	1.0 ±0.3	$P = 0.7$	1.0 ±0.3	$P = 0.2$	n=7
Ovcar5	1.0 ±0.1	$P = 0.9$	1.0 ±0.1	$P = 0.2$	n=4
Ovcar8	0.9±0.3	$P = 0.5$	1.0 ±0.3	$P = 0.6$	n=10
Igrov-1	1.0 ±0.1	$P = 0.6$	1.0 ±0.1	$P = 0.1$	n=3
Skov-3	1.2 ±0.2	$P = 0.1$	1.5 ±0.4	$P < 0.05$	n=4

Table 17: Figure 33: The activity of obatoclax and carboplatin in combination.

The activity of combinations of paclitaxel and obatoclax was evaluated in cell growth assays in which the drugs were combined at the ratio of their respective IC_{50}s. C.I. values (mean ± S.D.) were calculated as described in section 2.4. C.I. values were calculated assessing exclusivity and non-exclusivity. Exclusivity assumes the drugs have similar mechanisms of action while non-exclusivity assumes that they have different mechanisms. The C.I. values were statistically different from unity where indicated ($P < 0.05$, t-test, with Welch's correction).

Combination study of obatoclax and paclitaxel					
Cell Line	Exclusive C.I. values	t-test	Non-exclusive C.I. values	t-test	Number of experiments
A2780	1.5 ±0.1	$P < 0.01$	2 ±0.2	$P < 0.01$	n=6
cisA2780	1.3 ±0.1	$P < 0.05$	1.7 ±0.2	$P < 0.01$	n= 4
Ovcar3	1.4 ±0.4	$P < 0.01$	1.9 ±0.7	$P < 0.01$	n=10
Ovcar4	1.3 ±0.2	$P < 0.05$	1.7 ±0.3	$P < 0.01$	n=5
Ovcar5	1.5 ±0.1	$P < 0.01$	1.9 ±0.2	$P < 0.01$	n=7
Ovcar8	1.4 ±0.1	$P < 0.01$	1.8 ±0.2	$P < 0.01$	n=6
Igrov-1	1.3 ±0.1	$P < 0.01$	1.6 ±0.2	$P < 0.01$	n=5
Skov-3	1.4 ±0.3	$P < 0.05$	1.7 ±0.4	$P < 0.01$	n=8

Table 18: The activity of obatoclax and paclitaxel in combination against eight ovarian cancer cell lines. The activity of combinations of paclitaxel and obatoclax was evaluated in cell growth assays in which the drugs were combined at the ratio of their respective IC_{50}s. C.I. values (mean ± S.D.) were calculated as described in section 2.4. C.I. values were calculated assessing exclusivity and non-exclusivity. Exclusivity assumes the drugs have similar mechanisms of action while non-exclusivity assumes that they have different mechanisms. The C.I. values were statistically different from unity where indicated ($P < 0.05$, t-test, with Welch's correction).

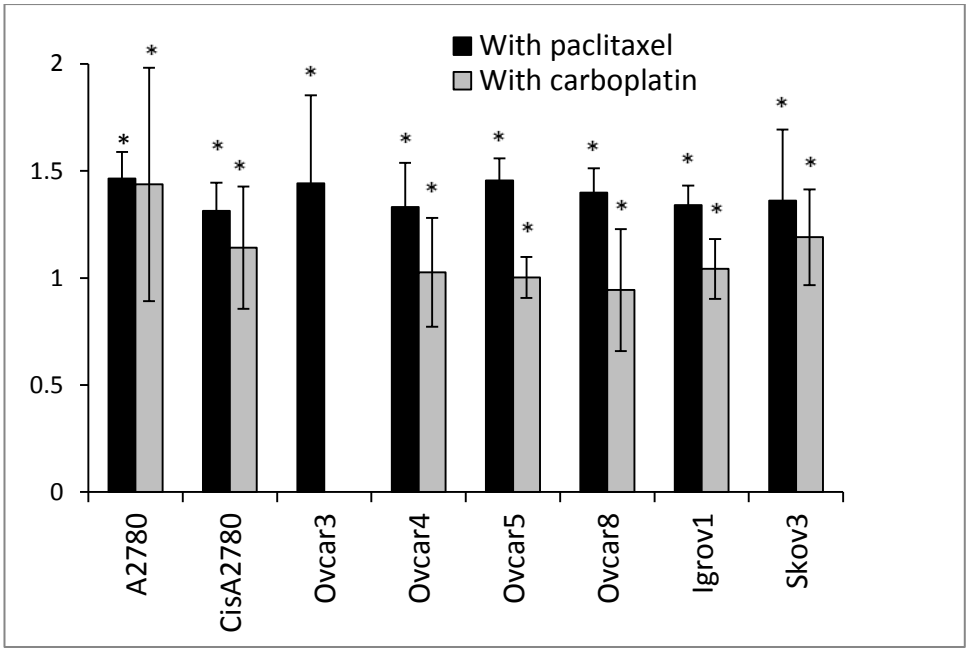

Figure 34: Activity of obatoclax in combination with carboplatin and paclitaxel in 7 ovarian cancer cell lines. A C.I. value <1 is considered indicative of synergy, while values over 1 are indicative of antagonism between the two drugs. The C.I. values were statistically different from unity where indicated ($P < 0.05$, t-test, with Welch's correction). Error bars represent S.D.

4.3.14 Sequenced combination studies of obatoclax & carboplatin

The lack of synergy between obatoclax and both carboplatin and paclitaxel was unexpected. Work in our group previously found that the order of addition of ABT-737 and carboplatin substantially affected the extent of synergy. Addition of carboplatin to cells prior to ABT-737 was the most synergistic, presumably because the BH3-only proteins induced by carboplatin are subsequently liberated by ABT-737. Furthermore, drugs which have effects at different stages of the cell cycle may be antagonistic. For example, carboplatin and paclitaxel are also antagonistic (Chapter 3), most likely because they exert effects at different stages of the cell cycle, and the antagonism can be altered by changing the schedule of addition. The possibility was therefore considered that altering the schedule of addition of obatoclax and carboplatin or paclitaxel would increase the effectiveness of the drug combinations.

A range of different schedules of administration were evaluated. Obatoclax was administered before, during or after exposure to carboplatin. The results (Figure 35) suggested that pre-treatment of ovarian cancer cells with carboplatin before obatoclax was marginally preferable to other sequence of drug administration. When obatoclax was used after carboplatin then a more additive/less antagonistic effect was seen in five of the eight cell lines (but none of these reached statistical significance). However, pre-treatment with carboplatin appeared comparable to synchronous treatment with obatoclax and carboplatin for the majority of cell lines evaluated (six out of eight) and significant antagonism was still observed in several of the cell lines. Thus, in most of the

cell lines altering the schedule of administration did not substantially improve the interaction between these drugs. This is a further surprising result with obatoclax, bearing in mind the schedule dependant effects observed with ABT-737 and carboplatin [312].

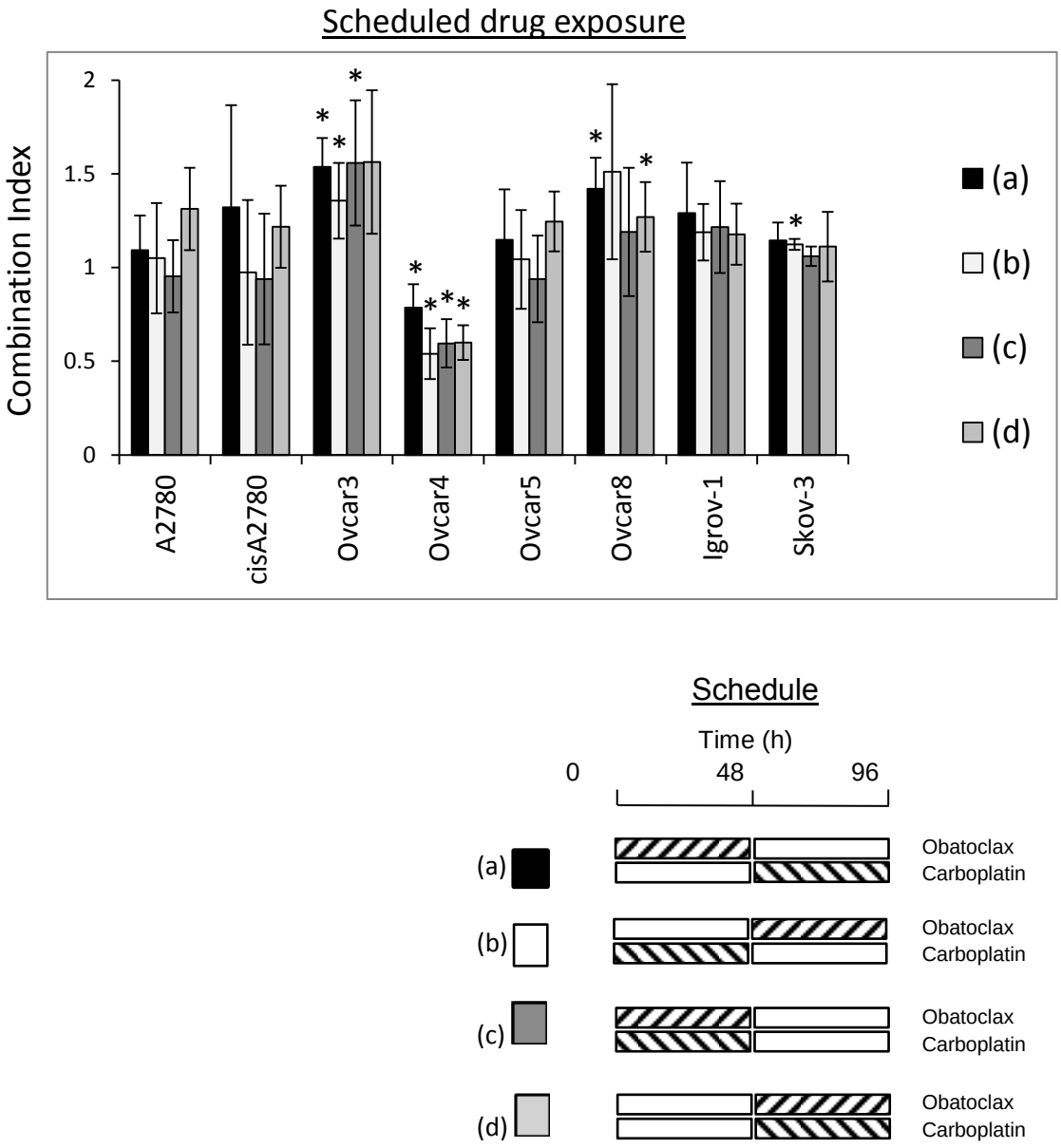

Figure 35: Scheduled combinations of obatoclax and carboplatin.

Cells were exposed to: (a) obatoclax for 48 hours then carboplatin for 48 hours; b) carboplatin for 48 hours, then obatoclax for 48 hours; c) carboplatin and obatoclax for 48 hours then culture medium for a further 48 hours; d) culture medium for 48 hours, then carboplatin and obatoclax for 48 hours. In each case, the cells were treated with carboplatin and obatoclax at the ratio of the IC50s determined in experiments with the individual drugs. Results are expressed as the mean combination index (mean ± S.D. n=3-4) and were significantly different from 1.0 where indicated (*, $P < 0.05$, t-test using Welch's correction). Error bars represent S.D.

4.3.15 Obatoclax and lovastatin combinations

Statins are drugs that have been widely established for the treatment of hyperlipidaemia [430]. In many instances they have been shown to contribute to the prevention and treatment of cancer [431] and clinical trials have investigated their activity [432]. It is believed that this effect of statins is due to their ability to interact with the cellular pathways often activated in oncogenesis [433]. In ovarian cancer lovastatin has been shown to induce apoptosis as a single agent and to synergize with chemotherapeutic drugs such as doxorubicin ,[434] [435]. Results in our laboratory have demonstrated that statins have the potential to treat ovarian cancer [436].

Combination studies of obatoclax and lovastatin were performed to investigate their potential in ovarian cancer. The two drugs were combined in a fixed ratio of their respective IC_{50}s. The respective C.I. values are described in Table 19. The combination of the two drugs was synergistic in one of the cell lines additive in two of the cell lines and antagonistic in A2780 cells.

Cell Line	Exclusive CI values	Non-exclusive CI values
A2780	1.3	1.6
Ovcar5	0.6	0.6
Ovcar8	1.1	1.1
Igrov-1	4.4	5.2

Table 19: Combination studies of obatoclax and lovastatin in four ovarian cancer cell lines. The Combination Index (C.I.) values, n=1.

4.3.16 Xenograft studies of obatoclax

The lack of synergy, and in some cases antagonism, between obatoclax and standard-of-care chemotherapy, together with the potent single agent activity of obatoclax, suggested that obatoclax may preferably be used as a single agent in ovarian cancer. Clinical trials generally require demonstration of a drug's activity in animal models. Xenograft studies of obatoclax were not performed by the author but carried out in collaboration with Dr Sue Eccles, Institute for Cancer Research. Obatoclax was administered to mice bearing Ovcar-8 tumours using a schedule (o.d. days 1-5, i.v.) which had previously been shown to be successful in several cancer xenograft models [384]. No statistically significant reduction in tumour volume was observed at the end of the treatment. The most pronounced differences between vehicle and drug-treated tumours were seen during the period of drug administration (days 1-5), and a small (though neither statistically nor biologically significant) difference remained throughout most of the period of study (Figure 36).

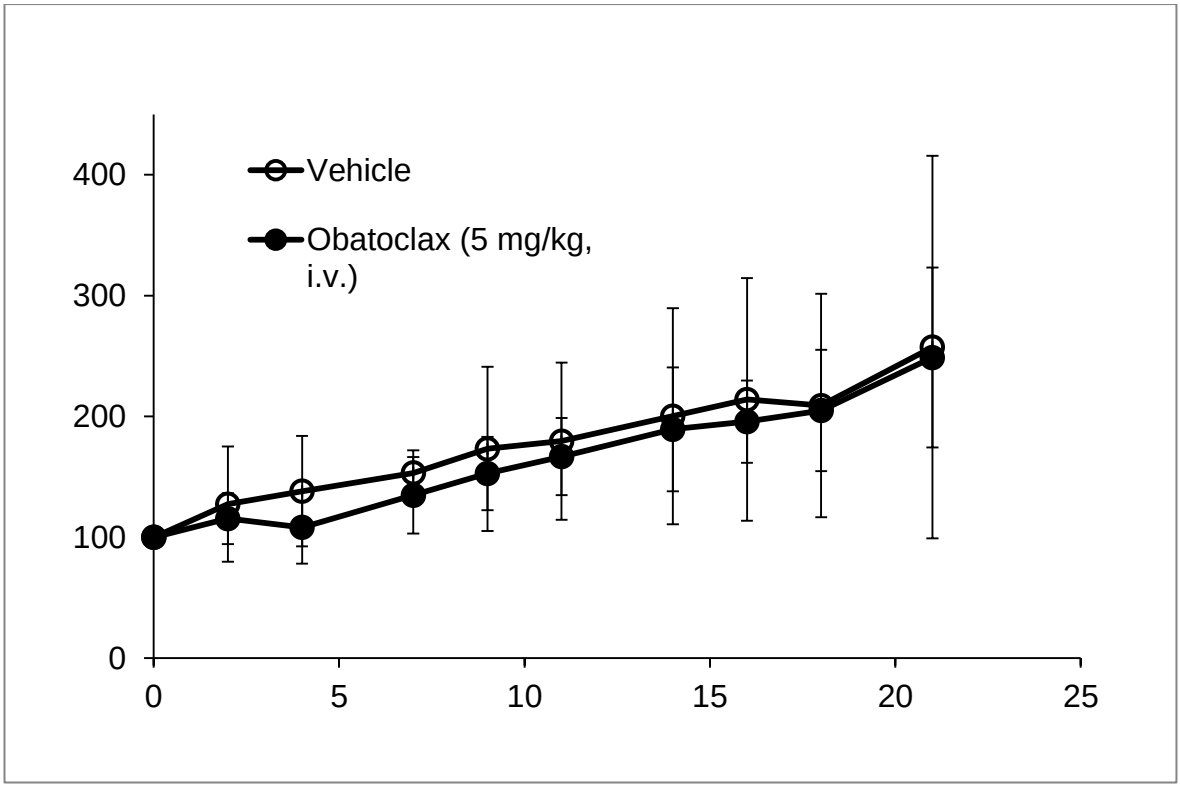

Figure 36: The xenograft study of Ovcar8 cell origin tumour treated with obatoclax. Mice were injected with Ovcar-8 cells and once the tumours were established, received either vehicle or obatoclax (5 mg/kg, i.v.) for days 1-5 of the study. Error bars represent S.D.

4.4 Discussion

Since the introduction of platinum agents and taxanes into the treatment of ovarian cancer almost forty years ago there has not been any other breakthrough and the development of new therapies has not significantly altered the survival rates in ovarian cancer. One exception to this may be bevacuzimab, a monoclonal antibody that sequesters VEGF and inhibits angiogenesis. Recently completed trials have shown that bevacuzimab extends the progression free survival of patients [437]and can result in an increase in overall survival for a subgroup patients at high risk of progression [438]. Nonetheless, new therapies are still needed. It is reasonable to suggest that overcoming the chemoresistance of ovarian cancer is likely to be achieved through the inclusion of targeted agents, especially as our understanding of the molecular pathways underlying this disease increases [439].

This work described in this chapter aimed to evaluate obatoclax, a new agent which targets the Bcl-2 family of apoptosis regulators and test the hypothesis that obatoclax could increase the sensitivity of ovarian cancer cells to existing chemotherapy. Previous work from our group in the field of ovarian cancer and by others evaluating similar BH3 mimetics in a number of malignancies has provided us with evidence that BH3 mimetics warrant further investigation as anticancer drugs [376].

The evaluation of obatoclax as a single agent has demonstrated striking nanoMolar potency in all the models studied. Obatoclax was significantly more potent than ABT-737 [312] or Navitoclax (Chapter 3). Although this may be due to several factors such as differences in cell permeability, metabolism, protein binding or affinity for its target, it may in part also reflect the ability of obatoclax to inhibit all Bcl-2 family members. Alternatively obatoclax may have additional, as yet undefined, pharmacological activity (discussed below) that contributes to the measured potency. Whatever the cause of the notable potency, obatoclax retained its activity in cells which displayed varying degrees of resistance to carboplatin. This is perhaps best exemplified by A2780 cells and its cisplatin/carboplatin resistant derivative cisA2780. A2780 cells are approximately 10 times more sensitive to carboplatin than are cisA2780 cells [312] yet they have indistinguishable sensitivities to obatoclax. Of the other cell lines, Ovcar-3 is the most sensitive to carboplatin and Ovcar-8 the least sensitive. Ovcar-3 cells are approximately 16-fold more sensitive to carboplatin than are Ovcar-8 cells, yet Ovcar-3 cells are only 2.8 fold more sensitive than Ovcar-8 cells to obatoclax. The activity of obatoclax in cells which are otherwise drug-resistant has implications for the design of clinical trials of obatoclax and are discussed below.

The establishment of primary cultures of ovarian cancer cells from the ascitic fluid of ovarian cancer patients has in itself at times been delayed or partially hindered because of a variety of reasons including new technique and skills being developed, failure of samples to establish as a culture and periodic lack of ascitic samples. However, 8 different PCOCC were successfully cultured. Excitingly, significant success was obtained culturing

the cells in conditions of reduced oxygen tension The primary cultures maintained in reduced oxygen tension do not undergo senescence as quickly as those in atmospheric oxygen. This supports the use of reduced oxygen tension conditions for culturing both permanent ovarian cancer cell lines and primary cultures. Ideally, it was expected that some of these primary cultures would overcome their expected senescence and become permanent cell lines. Unfortunately, no immortalized cell lines were obtained within the period of this project.

It was also anticipated that during the course of this project the majority of patient – donors of ascitic samples would relapse and die from this disease. This would have provided us samples from patients who were initially chemosensitive and who then progressed to chemoresistant disease. The availability of paired cell lines under would have provided an important model of drug resistance and this is a potential further avenue of investigation. A future aim is to examine the value of primary cultures as representative models of this dynamic disease and establish a link between the response of primary cultures to treatment in *vitro* and the clinical response of patients *in vivo.*

Obatoclax clearly induced cell death – this was supported by both trypan blue staining and colony forming assays. However, obatoclax was anticipated to function as a BH3 mimetic and induce apoptosis through the intrinsic apoptosis pathway. It was striking that using two markers of apoptosis (caspase 3/7 activation and PARP cleavage), only 3 of the cell lines showed clear apoptosis. In 3 further lines, Ovcar-3, Ovcar-4 and Sk-Ov-3 cells,

apoptosis was minimal or undetectable. It was notable that these later cell lines were the one in which trypan blue staining was least evident following exposure to obatoclax. Thus there appears to be a mechanism in addition to apoptosis that leads to cell death induced by obatoclax and the contribution of this mechanism varies between cell lines. In the cell lines where apoptosis was observed, it is possible this additional mechanism also contributes to cell death alongside apoptosis. The existence of additional mechanism of cell death following exposure to obatoclax is supported by the observation that obatoclax-induced cell death is only partly blunted in $Bak^{-/-}/Bax^{-/-}$ cells which are unable to undergo apoptosis [391]. Our group has also found that the caspase inhibitor ZVAD-FMK also failed to blunt the activity of obatoclax (V. Stamelos, N. Fisher & A. Richardson, unpublished observations). Additionally, synergy between obatoclax and carboplatin was anticipated, following the synergy between navitoclax and carboplatin. The lack of synergy (see below) may also reflect an additional activity of obatoclax.

A number of experiments were conducted to identify the potential additional mechanism of obatoclax induced cell death. The first possibility evaluated was that obatoclax caused cell death by activating autophagy. This seems reasonable because the autophagy activator beclin-1 is sequestered by Bcl-2 family proteins, and it was anticipated that obatoclax would liberate beclin. Although currently debate rages whether autophagy is a pathway contributing to cell death or cell survival, at the time these experiments were performed it was considered a potential contributor to cell death. Obatoclax did indeed induce autophagy, as evidenced by the accumulation of LC3-II and its recruitment to autophagosomes. However, inhibition of autophagy using siRNA to Atg5 or beclin did not

substantially alter the sensitivity of the cells to obatoclax. In one cell line, a modest decrease in sensitivity was observed, suggesting that autophagy may contribute to the response to obatoclax, but it does not appear to be essential for obatoclax-induced cell death.

Obatoclax has also been reported to cause the generation of reactive oxygen species (ROS) [423]. N-Acetylcysteine is a free radical scavenger that can reduce ROS-induced cell death. However, there was no clear pattern of altered sensitivity to obatoclax in cells simultaneously with N-acetylcysteine. Related to this, obatoclax belongs to a class of compounds termed prodiginines. These compounds have been claimed to be proton ionophores which could potentially uncouple mitochondrial energy production. In preliminary studies, no change in intracellular ATP was observed following short-term exposure (24 hours) to obatoclax (data not shown, n=1). Obatoclax has been shown to be involved in the induction of another type of cell death named necroptosis [440]. However, inclusion of the necroptosis inhibitor necrostatin-1 had no effect on the activity of obatoclax (N. Fisher & A. Richardson, unpublished observations).

Obatoclax has also been reported to induce S-phase arrest in AML cells [391]. However, in the studies presented here, obatoclax only caused accumulation of cells in S phase in one of the cell lines. Cells also did not appear to arrest in other phases of the cell cycle after exposure to a range of concentrations of obatoclax that inhibited the growth of cell cultures.

These observations lead to the rather unsatisfactory conclusion that although obatoclax is a *bona fide* BH3-mimetic, it possesses an additional, as yet unidentified, pharmacological activity that contributes to cell death. Nonetheless, the induction of apoptosis in some cell lines provides a rationale for the development of obatoclax in ovarian cancer, if appropriate patient populations can be identified.

Clinical trials of a new oncology drug are usually evaluated as monotherapy, or in combination with the existing standard-of-care treatment. This raised the possibility that obatoclax could be evaluated in clinical trials in combination with carboplatin and paclitaxel. Previous studies in our laboratory [312] and in the present work (chapter 3) have highlighted synergy between BH3-mimetics and carboplatin and created an expectation that carboplatin would also be synergistic with obatoclax. However, in the studies presented here, the combination of obatoclax with carboplatin was generally additive and the combination with paclitaxel was even antagonistic. Altering the schedule of administration did not ameliorate these results. The reasons for the lack of synergy between obatoclax and carboplatin are unclear. However, it is possible that the additional activity of obatoclax proposed here masks any synergy between carboplatin and obatoclax that results from the activity of obatoclax as a BH3-mimetic.

The key significance of these observations of the activity of obatoclax in combination with chemotherapy is that they inform the clinical development of obatoclax. From the data presented here, it is questionable whether there will be any significant clinical benefit

from adding obatoclax to current standard of care regimens. Rather the data suggest any clinical trial of obatoclax in ovarian cancer should evaluate it as a single agent treatment. Of course such a development carries all the ethical limitations of administering experimental treatment to humans as it is known that any investigator is ethically obliged to provide only that treatment that is most effective [441]. There are also the toxicity related risks, but recent studies show that combinations of obatoclax with topotecan for solid tumours have been well tolerated [442]. The results presented here showed that obatoclax retained its activity even in cells (e.g. cisA2780, Ovcar-5 and Ovcar-8) which are relatively resistant to chemotherapy. It is therefore reasonable to consider obatoclax as second line therapy for patients who have relapsed with acquired drug resistant disease or as an option for patients with evident intrinsic chemoresistant disease. However, in xenograft studies, only minimal activity of obatoclax was noted, and appeared to be restricted to the period of drug administration (if indeed activity was present at all). It should be noted that this xenograft study was performed with cells which underwent apoptosis *in vitro* following exposure to obatoclax. These data suggest that different dosing schedules deserve evaluation (eg daily for several weeks) in xenograft models before the clinical evaluation of obatoclax is warranted.

Emerging evidence from on-going preclinical and clinical investigations suggests that the full potential of obatoclax mesylate as a novel anticancer agent may be realized as monotherapy, in rational combination treatments other than those investigated here and guided by molecular predictors of therapeutic response. By understanding the molecular underpinnings of obatoclax response, along with optimal therapeutic regimens and

indications, the potential of obatoclax mesylate for the treatment of haematological malignancies may be further clarified [385]. The issue of which patients should be considered for therapy with a BH3 mimetic is the subject of the remaining chapter of this thesis.

Chapter 5: Conclusion & Future Work

Approximately four decades after the introduction of platinum agents in the treatment of ovarian cancer, gynaecological oncology is facing the great challenge of discovering novel anticancer drugs to overcome the chemoresistance that occurs in this disease. The current practice in chemotherapy is to offer treatment based on the tissue of origin of the tumour. In advanced ovarian cancer, the standard-of-care for the majority of patients comprises surgery followed by chemotherapy, where platinum agents and paclitaxel are administered. However, this traditional approach does not fully reflect our understanding of the extensive heterogeneity of ovarian cancer which can partially explain why there has not been any significant breakthrough in overcoming the chemoresistance of ovarian cancer.

The growing body of knowledge of the biology underlying ovarian cancer has contributed to the identification of a number of potential therapeutic targets and the development of a plethora of targeted cancer therapies. These events can be regarded as the early characteristics of the entry into an era of personalized medicine, where the traditional chemotherapy approach is replaced by a more patient-tailored treatment. This new strategy of personalized medicine, whereby a patient's treatment is chosen to address the molecular defects associated with their personal disease, can allow for the individual pharmacodynamic and pharmacokinetic factors to be taken into account as they may affect drug safety and efficacy.

In principle, the process of mapping the genetic (or epigenetic) abnormalities in a patient's tumour and selecting therapy accordingly appears relatively straightforward. However, knowledge of the genes affected is often inadequate and does not necessarily

translate into a thorough understanding of the signalling pathways involved in carcinogenesis. A common feature of ovarian cancer and other malignancies is the evasion of apoptosis through the deregulation of the equilibrium between anti- and pro-apoptotic members of the Bcl-2 family of proteins. For the “primed” cancer cells, whether the induction of a BH3-only protein leads to apoptosis depends on whether the repertoire of apoptosis inhibitors expressed is adequate to dampen the pro-apoptotic signal. The recognition that Bcl-2 family proteins regulate apoptosis has led to the development of “BH3-mimetics”. Can we understand what determines whether cancer cells are sensitive to BH3-mimetics and use such drugs in the personalized medicine setting?

Identifying the factors which increase sensitivity to BH3-mimetics will enable the clinician to rationally decide whether to deploy these drugs. Understanding the cancer signalling pathways in detail also allows the rational design of drug combinations which may improve on the efficacy of a drug. In the case of BH3-mimetics, whose mechanism of action is dependent on activation of the intrinsic apoptosis pathway, (such as ABT-737 and navitoclax), activity has been shown to be limited by the selectivity of these drug for some of the anti-apoptotic Bcl-2 proteins. This limitation does not necessarily apply to obatoclax, a BH3-mimetic that binds all Bcl-2 family inhibitors and that also elicits apparently off-target effects. Expression of Bcl-2 family apoptosis inhibitors that do not bind ABT-737/navitoclax confers resistance to these drugs. Several reports indicate that Mcl-1 confers resistance to ABT-737 because of the poor affinity of ABT-737 for Mcl-1 and Bfl/A1 also binds ABT-737 weakly [443]. This suggests that the expression of BH3-only proteins which can occupy these apoptosis inhibitors should increase the sensitivity of

cells to ABT-737. Thus, understanding which patients should receive BH3-mimetics requires an understanding of the expression of both Bcl-2 family inhibitors and the BH3-only proteins that bind to them. Considering the BH3-only proteins, both activator and sensitizer proteins determine the sensitivity of cells to ABT-737/navitoclax.

Bid, Bim and Puma are BH3-only activator proteins that can trigger Bax/Bak to induce apoptosis. Early work in the field highlighted the importance of the association of Bim with Bcl-2 in determining sensitivity to ABT-737 [444]. The existence of Bcl-2 primed with Bim predicted sensitivity to ABT-737, because ABT-737 can displace Bim from Bcl-2 and induce apoptosis. Puma has also recently emerged as an activator BH3-only protein. In the presence of ABT-737 to occupy Bcl-2 inhibitors, the expression of Puma may be sufficient to induce apoptosis [445]. Puma co-operates with other activators and ABT-737 to induce apoptosis [446] while loss of Puma reduces the apoptotic activity of ABT-737 [445, 447]. Bid is an activator BH3-only protein which plays a role in the intrinsic apoptosis pathway. Caspase-mediated cleavage of BID promotes its pro-apoptotic activity. ABT-737 has been shown to trigger the cleavage of BID in leukaemia [448] and neuroblastoma cells [449]. How this occurs is unclear but it suggests cross-talk between the intrinsic and extrinsic apoptosis pathways, suggesting it may be necessary to consider the status of the extrinsic pathway as well when trying to predict sensitivity to BH3-mimetics.

BH3-only sensitizer proteins (Figure 7) can also contribute to the sensitivity of cells to ABT-737/navitoclax. Noxa is a particularly significant BH3-only protein because it binds to both Mcl-1 and Bfl/A1, both of which are insensitive to ABT-737/navitoclax. Noxa

increases the sensitivity of cells to ABT-737 by occupying the apoptosis inhibitors to which ABT-737/navitoclax does not bind. Bad is classified as a sensitizer which, in contrast to Noxa, has a similar binding specificity for Bcl-2 family apoptosis inhibitors similar to that of ABT-737. The liberation of BAD by ABT-737 indirectly induces apoptosis by reducing the reservoir of apoptosis inhibitors.

The overall conclusion from these observations is that there is no single determinant of sensitivity to ABT-737 or navitoclax and the over-arching factor that determines the induction apoptosis appears to be whether sufficient pro-apoptotic proteins can be released to exceed the buffering capacity of the anti-apoptotic proteins when cells are exposed to navitoclax. This is determined by the repertoire of inhibitors, activators and sensitizers expressed in a particular cell. Thus, navitoclax may require a broad panel of biomarkers to predict accurately its activity in patients.

Can the sensitivity of cells to navitoclax be increased? One obvious strategy that should be evaluated further is to decrease the expression of Mcl-1. This approach carries with it significant challenges because it has been shown that exposure to ABT-737 can itself increase expression of Mcl-1 [450-452] thus providing a mechanism by which ABT-737 may partly induce “resistance to itself”. Several drugs have been shown to reduce the expression of Mcl-1 (Figure 37) and future work should consider their combined use with navitoclax.

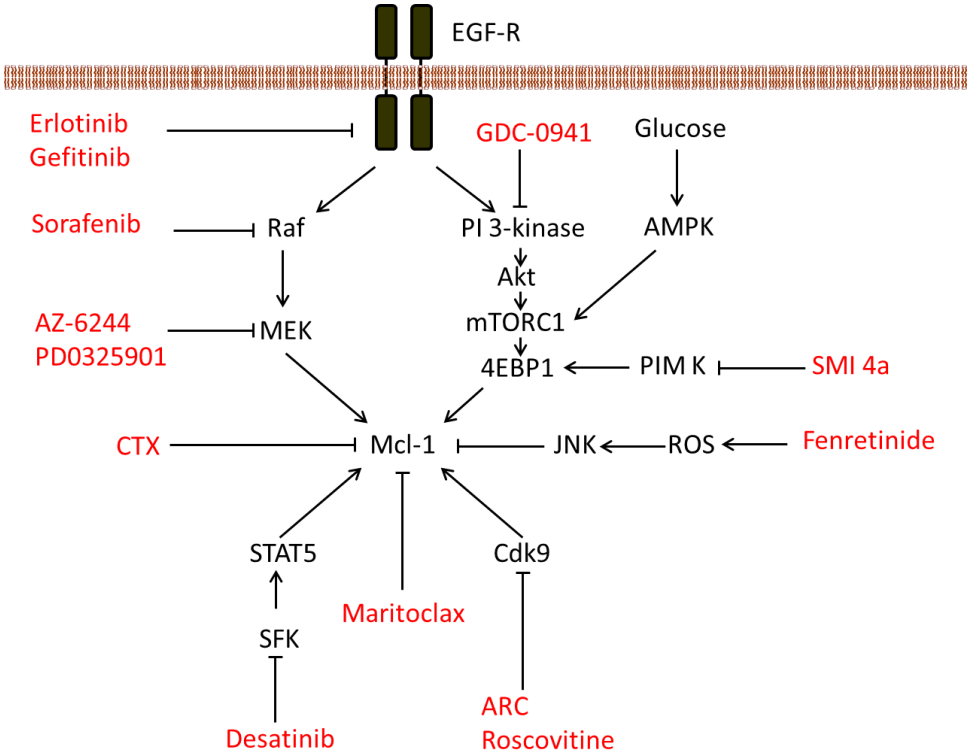

Figure 37: Strategies to increase sensitivity to navitoclax. The sensitivity of cells to navitoclax can be increased using drugs which decrease the expression of Mcl-1. The Erk (including Raf and MEK) and PI 3-kinase pathways are key regulators of Mcl-1 expression so drugs inhibiting this path show synergy with navitoclax. Drugs inhibiting other regulators of Mcl-1 are also shown, and these drugs also increase sensitivity to navitoclax. ARC, 4-amino-6-hydrazino-7-beta-D-ribofuranosyl-7H-pyrrolo (2, 3-d)-pyrimidine-5-carboxamide; CTX, chemotherapy; EGF-R, EGF receptor; ROS, reactive oxygen species; SFK, src family kinase;

Future combinations of navitoclax with drugs expected to reduce Mcl-1 expression

Raf inhibitors. The inhibition of Raf with sorafenib or the inhibition of MEK can reduce the expression of Mcl-1. ABT-737 combined with a MEK or Raf inhibitor has been shown to be more effective in xenograft studies than the single agents [453-455].

Src family kinase inhibitors. Desatinib is an inhibitor of BcrAbl and Src-family kinases which also reduces the expression of Mcl-1 by inhibiting the src family kinase lyn. Lyn is widely expressed, so it will be of interest to evaluate this strategy in ovarian cancer.

PI 3-kinase inhibitors. The PI-3 kinase pathway is frequently activated in cancer including ovarian cancer (Chapter 1). There are several points on the PI3K/Akt/mTORC1 pathway that could be inhibited and this is an active area of research, e.g. GDC-0941 is a PI 3-kinase inhibitor which reduces expression Mcl-1[450].

Glycolysis. Mcl-1 expression is maintained in the presence of extracellular glucose, but it rapidly diminished in its absence and therefore the inhibition of glycolysis with 2-deoxyglucose can also inhibit the expression of Mcl-1 [456, 457]. The combination of 2-DG with ABT-737 or navitoclax improved the survival of animals with an experimental model of lymphoma [458] or prostate cancer [459] beyond that seen in animals treated with either agent alone.

ROS. Navitoclax could also be used together with fenretinide, a synthetic retinoid whose cytotoxic activity depends in part on the generation of reactive oxygen species (ROS). Subsequent activation of Jnk by ROS leads to phosphorylation and decreased levels of Mcl-1 [448].

Transcription and translation inhibitors. Pim kinases are involved in the regulation of transcription, translation, cell survival and energy metabolism [460] and it has been seen that inhibition of Pim kinases decreases translation of Mcl-1 mRNA, as well as increasing degradation of Mcl-1 protein [461].ARC is a nucleoside analogue which inhibits Cdk9 and reduces the expression of Mcl-1 [462]. In combination with ABT-737, ARC induces apoptosis and causes a synergistic inhibition of cell survival. Transcription of Mcl-1 can also be reduced by exposure of cells to roscovitine which inhibits several cyclin dependant kinases including cdk9 and this also increases sensitivity to ABT737 [349, 443, 463]. Synergy with flavopiridol, another pan cdk inhibitor that down-regulates Mcl-1, has also been observed [464]. The transcriptional inhibitor actinomycin has been shown to reduce Mcl-1[465, 466]. Other agents which have shown synergy with ABT-737 by virtue of down-regulating Mcl-1 include Methylseleninic acid [467] and L-asparaginase [468].

Chemotherapy. Several chemotherapeutic agents like etoposide or cisplatin have shown synergy with ABT-737 (reviewed in[308]) and in some cases this reflects the down regulation of the expression of Mcl-1 by the chemotherapy [469, 470]. Their combined use with navitoclax is expected to show further synergistic effects.

Proteosome inhibitors. The induction of the pro-apoptotic protein Noxa may lead to the ubiquitination and proteasomal degradation of Mcl-1 [471] and agents that induce this effect should be evaluated in combination studies with navitoclax or obatoclax.

The alternative approach to decreasing the expression of Mcl-1 is to promote the induction of BH3-only proteins which would then occupy Mcl-1 and hence increase sensitivity to navitoclax. For the purposes of this both chemotherapeutic and molecularly-targeted agents have been used successfully and future work in ovarian cancer therapeutics should aim to examine their potential uses.

Future combinations with navitoclax with drugs expected to induce BH3-only proteins.

JAK2 inhibitors. An inhibitor of JAK2 induces Bim and also shows synergy with ABT-737 in cells harbouring a JAK2 activating mutation [472].

Bad. Although Bad does not bind to Mcl-1, it is possible that it could still improve sensitivity to ABT-737 by reducing the total capacity of free apoptosis inhibitors. Both sorafenib [473, 474] and ABT-737 [475] can increase Bad protein levels. Bad is also likely to play a role in the change in sensitivity to ABT-737 when cells are deprived of glucose or exposed to 2-DG (discussed above). In its unphosphorylated state, BAD promotes apoptosis, but when phosphorylated it regulates glucose metabolism [476]. Dephosphorylation of BAD is promoted by glucose deprivation [477], suggesting that 2-DG may do the same, and that this may contribute to the synergy observed between ABT-737 and 2-DG. However, 2-DG also induces the expression of Bim, Bmf and Noxa [478, 479] and glucose deprivation induces Puma, Bid and Bim [456, 480], again making it difficult to assess the contribution of Bad.

Noxa. The induction of Noxa is one key strategy, because Noxa binds to the Bcl-2 family proteins Mcl-1 and Bfl/A1 that are insensitive to antagonism by ABT-737 and Noxa has

also been reported to induce the degradation of Mcl-1 [471, 481, 482] but not in all cells [483]. Noxa is induced by exposure to several chemotherapeutic agents including fludarabine [443] , CPT11 [484], imiquimod [485], dacarbazine [485] and actinomycin [465] and in each of these cases, synergy with ABT-737 has been observed. Expression of Noxa is also increased by exposure to targeted agents including several proposed BH3 mimetics [452], bortezomib [443, 464], a Pim kinase inhibitor [461], and inhibition of signalling through the notch pathway using a γ-secretase inhibitor [486].

HDAC inhibitors. These agents have been shown to induce expression of Bim and the preferential priming by Bim of Bcl-2 and Bcl-X_L compared to Mcl-1. Consequently, the HDAC inhibitor suberoyl bis-hydroxamic acid was found to induce apoptosis synergistically with ABT-737 [487]. ABT-737 released Bim from complexes with Bcl-2 or Bcl-X_L, and knockdown of Bim substantially blunted the effect of the drug combinations. Vorinostat (also known as SAHA) induces histone acetylation, the expression of the genes encoding Bmf, Bim and Noxa and consequent priming of Bcl-2 [488]. As a result, vorinostat shows synergy with ABT-737 which is reduced by inhibition of Bmf expression by shRNA.

Bim. Bim binds to all known Bcl-2 family proteins, including Mcl-1. Inhibition of the epidermal growth factor receptor (EGFR) with erlotinib or gefitinib leads to increased expression of Bim [444, 489, 490], probably through transcriptional and translational regulation [489], and apoptosis. In these experiments, ABT-737 exhibited minimal activity when tested on its own, but when combined with erlotinib or gefitinib, a supra-additive increase in cell death was observed. Sorafenib (which inhibits several kinases including Raf) induces Bim and apoptosis and is synergistic with ABT-737 in AML cells [474].

Obatoclax

Unlike navitoclax, whose activity seems to be principally mediated by on-target effects, the evaluation of obatoclax in ovarian cancer in this study and previous work done by others for other malignancies has clearly demonstrated that obatoclax has off-target effects. Although these can be seen as a problem, this additional activity may be of significant value that perhaps should be utilised further. Obatoclax did not measurably induce apoptosis in Ovcar-3, Ovar-4 and Sk-Ov-3, despite killing the cells, and it is therefore imperative to identify the additional mechanism of action by which these cells were killed. The very nature of personalized medicine requires that the mechanism of action of a drug is understood, in order to determine which patients should receive it. In the case of obatoclax, the data presented in this work makes it clear that our understanding is incomplete. One approach worth considering is to perform first xenograft studies using cells that respond via apoptosis and compare the results with xenografts of cells that respond via the "additional" mechanism. In the present study, minimal activity was observed in xenograft studies with Ovcar-8 cells, which do undergo apoptosis. From that perspective, it would be worth evaluating the activity of obatoclax with xenografts of cells that do not undergo apoptosis in response to obatoclax, such as in Sk-Ov-3 xenografts. It would also be worthwhile simply repeating the studies with obatoclax in another cell line (e.g. Igrov) that did undergo apoptosis because significant problems were encountered in growing Ovcar-8 as xenografts. Obatoclax also deserves further investigation using different schedules of drug administration (as discussed in Chapter 4). It seems sensible to conduct these studies to assess the efficacy of obatoclax before further studies are performed to identify the additional mechanism of action.

Although obatoclax binds to all Bcl-2 family inhibitors, it may be useful to consider some of the same drug combinations discussed for ABT-737/navitoclax with obatoclax. Decreasing the expression of Bcl-2 family apoptosis inhibitors, or increasing the expression of BH3-only proteins, may augment the activity of obatoclax.

The importance of experimental conditions

The underlying goal of investigating the activity of BH3-mimetics in ovarian cancer at a preclinical level is to prepare the ground for these agents to be used in the clinic to improve patients' response. This presupposes that all the *in vitro* measurements made in the laboratory are predictive of clinical outcome. It is therefore reasonable to question the value of all of our preclinical models and emphasize the use of those that are as realistic as possible, so appropriate hypotheses are tested in clinical studies. It is without doubt that today worldwide the most common setting in which sensitivity to drugs is measured is based on the use of cancer cell lines grown in nutrient and growth-factor rich media, as a monolayer attached to cell culture plasticware, in 21% O_2 (i.e. atmospheric oxygen). Over the last few years an increasing amount of evidence shows that this is perhaps not the most realistic model, despite its ready availability, convenient access and cost-efficiency. In patients, cancer cells may be located in hypoxic, nutrient-poor environments, comprising three-dimensional tumours. Each of these factors has already been reported to affect cell sensitivity to BH3-mimetics and other antineoplastic drugs.

Hypoxia appears to increase the sensitivity of tumour cells to ABT-737 [491-493]. This could either be due to down-regulation of Mcl-1 [491], or up-regulation of BH3-only proteins including PUMA, Noxa and BNIP3 [493]. It is noteworthy that cells resistant to anoxia show increased expression of Bcl-2 family proteins [493]. In xenograft studies, ABT-737 induces apoptosis preferentially in hypoxic regions of the tumour [491]. It has been seen that the degree of synergy observed *in vitro* between chemotherapeutic agents and ABT-737 is dependent on oxygen concentration [491]. Thus, it may be advisable to evaluate the activity of navitoclax and obatoclax in laboratory experiments under a more physiological oxygen tension, considerably lower than the one commonly applied. An initial step towards this was reported in this thesis.

The utilisation of three-dimensional cultures such as spheroids offers an alternative to the conventional monolayer cultures currently in use. These aggregates of proliferating cancer cells are prevented from adhering to a solid surface and their formation can mimic a small tumour. It can be said that they resemble more closely the cancer cell spheroids found in the ascites of advanced ovarian cancer patients. It has already been reported that in these models the repertoire of Bcl-2 family proteins appears to change, compared to the same cell line grown as a monolayer. Increases in the expression of Bcl-2 and Bcl-X_L have been observed in spheroids [402, 494-496], and decreases in the expression of Mcl-1 have also been reported [402, 494]. This implies a switch towards a BH3-sensitive phenotype in cells grown as spheroids. The above observations highlight the usefulness of incorporating cancer cell spheroids as tumour models into the laboratory investigations, but some caution is appropriate. The physiology of an avascular cancer cell formation

affects the pharmacodynamic properties of any drug and any observed decrease in drug sensitivity in a spheroid culture can also be explained by poor diffusion of the drug into the spheroid inner mass [497], or a reduced rate of cell proliferation. The changes in the expression of both pro- and anti-apoptotic Bcl-2 family members observed in spheroids may also be linked to the oxygen gradient between the inside and outside of the spheroid [491].

Implementing personalized medicine with BH3-mimetics

By definition, the success of tailored anticancer therapy will depend grossly on the accurate selection of patients who are most likely to benefit from it. The choice of drug and sequence of administration to the patient should preferably be based on a dynamic monitoring of the biological responses of the tumour and the clinical condition of the patient. Both of these aspirations imply that for future clinical studies with navitoclax and obatoclax we would need to be able to identify which patients should receive the drug(s), either alone or in combinations. Bearing in mind the number of factors discussed previously which influence the sensitivity to BH3-mimetics, it is no small challenge to decide how to address this.

Until today there has not been any evidence to suggest that a correlation exists between drug sensitivity and the expression of an individual Bcl-2 family member [443, 483, 498, 499]. In the case of BH3-mimetics, Mcl-1 does confer resistance and from that point of view one strategy for patient selection would be to measure Mcl-1 in order to select

which patients should receive navitoclax alone, or which would benefit from a combination with a drug that would repress Mcl-1. The predictive value of Mcl-1 measurements has been investigated and can be improved by measuring multiple anti-apoptotic Bcl-2 family members [464, 499, 500]. We also have evidence to suggest that the pro-apoptotic Bcl2 family members influence the sensitivity of cancer cells to ABT-737 as it has been shown from reports which describe correlations between Noxa/Mcl-1 ratios [443, 483] or Bim/Mcl-1 ratios with the activity of ABT-737 [501]. This suggests that the process of designing a panel of biomarkers to select patients for treatment should consider the inclusion of measurements of all the Bcl-2 family members. For those tumours overexpressing more than one of the anti-apoptotic Bcl-2 proteins, determining the distribution of pro-apoptotic proteins among the various apoptosis inhibitors is likely to be a better predictor of sensitivity to ABT-737 than simply measuring expression [381, 502]. The dependence of tumours on different apoptosis inhibitors may be measured by a test termed “BH-3 profiling”, which has the potential to be used clinically [503]. Considering that short-term exposure to ABT-737 has been reported to elevate expression of Mcl-1 [450-452], Bfl/A1 [504] as well as Bad [473, 475] and Noxa, [483] it is very likely that measurement of Bcl-2 family members prior to drug treatment may provide an inaccurate estimation of drug response. One solution to this might be to briefly expose patients to navitoclax prior to surgery and measure Bcl-2 family members in the excised tumour. Similar biomarker evaluations could very well become part of the clinical care even during the course of treatment, in case chemoresistance was suspected.

Undoubtedly, the usefulness of any biomarkers will remain to be seen in practice. If the adverse events induced by BH3-mimetics are only modest, then the false-positive frequency (the frequency with which patients inappropriately receive the BH3-mimetic) is potentially less relevant. However, experience to date suggests thrombocytopenia can be a serious issue (Chapter 3), so the false-positive frequency of any biomarker panel should be appropriately low to ensure patient safety.

Conclusion

The discovery of BH3-mimetics has delivered a class of drugs with evidence of promising preclinical activity in many types of cancer. Both navitoclax and obatoclax advanced in phase I & II clinical trials, where they were found initially to be overall well tolerated by patients although thrombocytopenia has been a significant concern. In the case of navitoclax, on-going trials have been suspended due to haematological adverse events and the results of these trials assessing the efficacy of navitoclax are currently awaited. However, the experience with navitoclax has greatly influenced the development of ABT-199, an agent which avoids Bcl-X_L mediated thrombocytopenia through its low affinity for Bcl-X_L, but unfortunately at the same time this means it is not likely to be effective in ovarian cancer [312]. The development of obatoclax, initially produced by GeminX, bought by Cephalon and now owned by Teva, has stopped as the current proprietor has chosen to focus on different therapeutic areas. These developments have not stopped the characterisation of new compounds with improved profiles [505] and currently BH3-mimetics are being assessed in a wider range of cancers [506]. These updates exemplify how rapidly things change in drug discovery and how the discovery of a drug can

stimulate interest and eventually lead to a diverse number of newer agents with improved profiles and better clinical applicability.

The transition from the conventional model of cytotoxic chemotherapy to the newer concept of targeted therapeutics will hopefully result in improved efficacy and will be accompanied by a reduction in the incidence of adverse effects. At present, cancer therapies are designed based on a 'one size fits all' approach with regard to the tumour histological type and the stage of disease. The aim of this study is to utilise targeted therapeutics such as BH3-mimetics, to increase efficacy and reduce toxicity. This proposed change in cancer treatment will inevitably signal the coming of a new era and the adjustment of our standard of practice of cancer therapeutics. The concept of personalised medicine is an appealing solution to the problem of chemoresistance today, but demands a depth of understanding of disease biology that is only just beginning to be translated from the laboratory to the clinic.

Chapter 6: References

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