Mechanism of Cell Death in Drug Resistant Human Breast Cancer Cells

By

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ABSTRACT

Anticancer drug resistance occurs as a result of altered response to cytotoxic insult, via inhibition or inactivation of apoptosis (programmed cell death type I, PCDI), which plays a major role in tumour development and progression. An alternative form of cell death - non-apoptotic, or autophagic cell death (PCD II) has recently emerged as a factor contributing to the cytotoxic response of cancer cells. We studied in vitro cell death in a drug resistant model MCF-7 human breast cancer cells with acquired resistance (c. 10-20 fold) to paclitaxel, termed MCF-7TaxR. It has been reported that the absence of caspase-3 in parental MCF-7 cells (due to chromosome deletion) may explain why they recruit apoptotic and autophagic cell death following cytotoxic insult. We investigated the induction of apoptosis response to staurosporine and Z-VAD (pan-caspase inhibitor) using the Annexin V-FITC/PI assay and studied the effect of anti-Fas on MCF-7TaxR. Results demonstrated the lack of apoptosis induction in paclitaxel resistant breast cancer cells. The oligo GEAiTay® human apoptosis microarray and qPCR analysis confirmed the absence of caspase-7 and caspase-9 genes and many other apoptosis genes in MCF-7TaxR cells and their presence in MCF-7 cells. Western blot analysis also confirmed these results. Therefore, we investigated the presence of autophagic cell death in our MCF-7TaxR model. Flow cytometry using Acridine Orange assay, Beclin 1 and LC-3 protein detection, confocal microscopy and detection of Akt/mTOR expression. Data showed evidence of autophagic cell death in MCF-7TaxR cells in the absence of an apoptotic response. Collectively, these findings indicate the lack of involvement of caspase mediated cell death in a paclitaxel drug-resistant cancer cell line MCF-7TaxR, and presence of autophagic cell death as an alternative cell death mechanism.
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I hereby declare that all tables and figures included describing pathways in this thesis were done by me by using Microsoft® Power point and Excel software.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency virus</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis protease activating factor-1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 family member, pro-apoptotic</td>
</tr>
<tr>
<td>Baf.</td>
<td>Bafilomycin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma-2</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine aspartate proteases</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP binding protein with low pi</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>E.M.</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>50 % inhibitory concentration</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukine</td>
</tr>
<tr>
<td>LC-3</td>
<td>Light chain</td>
</tr>
<tr>
<td>M.wt</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>3-MA</td>
<td>3-methyladenin</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>3-phosphatidylinositol kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl-methyl-sulfonyl-fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription/ transcriptase</td>
</tr>
<tr>
<td>±SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>St</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>Tumour necrosis factor receptor 1</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor related apoptosis inducing ligand</td>
</tr>
<tr>
<td>Ver.</td>
<td>Verapamil</td>
</tr>
</tbody>
</table>
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CHAPTER 1
GENERAL INTRODUCTION
1 General Introduction

1.1 Overview of breast cancer

Breast cancer is the most commonly diagnosed invasive malignancy in women and is the second leading cause of cancer-related deaths among females in most industrialized countries (Nkondjock and Ghadirian 2004). Globally, the number of women diagnosed with breast cancer is more than one million per year (Cancer-ResearchUK 2008). The incidence of breast cancer is highest in Northern and Western Europe and North America, with decreased rates in Northern, Eastern and Central African countries and Asia (Cancer-ResearchUK 2008). In the UK, breast cancer is the most common cancer in women (Cancer-ResearchUK 2008), and 45,500 women were diagnosed with the disease in 2005. This number means that approximately 125 women per day were diagnosed with breast cancer (Cancer-ResearchUK 2008). The incidence of breast cancer has increased by 50% over the last 25 years (Cancer-ResearchUK 2008). Moreover in the past 10 years alone in the UK, the incidence of breast cancer has increased by 13% (Cancer-ResearchUK 2008).

The occurrence of breast cancer is associated with different risk factors, including age, family history or genetic background, age at first pregnancy, age at menarche and at menopause, use of oral contraceptives, and lifestyle (McPherson et al. 2000). The incidence and mortality rate for breast cancer increases with age, doubling approximately every 10 years (McPherson et al. 2000; Lester 2005). Nearly 70% of breast cancer cases occur in women over the age of 50 (McPherson et al. 2000; Lester 2005). Women who start menstruating early (under 11 years of age), or who have a late menopause, have an increased risk for developing breast cancer (McPherson et al. 2000; Lester 2005). Women
who have stopped taking oral contraceptive agents after using them for a period of 10 years have a small increase in risk of developing breast cancer (McPherson et al. 2000; Lester 2005). Moreover, women who began taking birth control pills before the age of 20 appear to have a higher relative risk than women who began taking oral contraceptives when they were older (McPherson et al. 2000). Lifestyle is another factor considered in the occurrence of breast cancer (McPherson et al. 2000). Obesity may increase the risk of breast cancer by two-fold in postmenopausal women (McPherson et al. 2000). Alcohol intake is also correlated with the incidence of breast cancer (McPherson et al. 2000).

Family history or genetic background is a vital factor that affects the incidence of breast cancer, but less than 10% of breast cancer cases in Western countries are due to genetic background (McPherson et al. 2000; Schulz 2007). A certain number of women have a higher risk of breast cancer due to the presence of mutations in two breast cancer genes, BRCAl and BRCA2 (McPherson et al. 2000). These two genes are located on chromosome 17 and 13, respectively (McPherson et al. 2000). Both genes are very large and mutations can occur at any position, therefore molecular screening should be used to detect the mutation in individuals if a family history of breast cancer exists (McPherson et al. 2000). BRCAl and BRCA2 encode proteins involved in DNA repair and the control of genomic integrity (Schulz 2007). These genes act as classical tumour suppressors in many familial cases of breast cancer, with one defective copy inherited and the second copy inactivated by mutation, recombination, deletion, or epigenetic inactivation in the tumour (Schulz 2007). Thus, blocking the inactivation of these genes reduces susceptibility to breast and ovarian cancers (Nkondjock and Ghadirian 2004). Mutation of BRCAl and/or BRCA2 is commonly found in women who have a family history of breast and ovarian cancer occurrence (Ford et
1.2 Classification of breast cancer

Different systems have been developed to classify breast cancers (Schulz 2007). Breast cancer tumours are adenocarcinomas and are classified as *in situ carcinomas* or *invasive carcinomas* (Robbins 1999). *Carcinoma in situ* is a cancer that has not spread beyond the area where it began. *In situ* breast cancers are confined within the ducts (ductal carcinoma *in situ* or DCIS) or the lobules (lobular carcinoma *in situ*, or LCIS) of the mammary tissues and have not invaded the basement membrane (Lester 2005). The majority of these tumours will not spread to lymph nodes and blood vessels and will not progress to become invasive tumours (Lester 2005). *Carcinoma in situ* is an early stage of cancer and nearly all of these cancers can be cured (Lester 2005). However, *LCIS* can be a marker for an increased risk of developing an invasive cancer in the future (Robbins 1999; Lester 2005). *Invasive breast carcinoma* is a condition whereby a cancer that started in the ducts or lobules of the breast infiltrates the basement membranes, duct or gland walls and, subsequently, invades the lymph nodes and surrounding fatty tissue of the breast to become metastatic (Lester 2005).

Molecular markers have been used in the classification of breast cancers (Schulz 2007). Several proteins, the estrogen receptor (ER), progesterone receptor (PR), and HER2, have been used as breast cancer markers (Lester 2005). Testing for expression of these proteins is carried out by immuno-histochemistry techniques (Lester 2005). The profile of the tumour influences the prognosis and helps the oncologist to select the appropriate treatment for the
patient (Lester 2005). The ER is expressed in about 80% of all breast cancers and ER positive cancers generally have a better prognosis and often respond to hormonal therapy (Lester 2005). However, ER negative breast cancers are more aggressive and unresponsive to anti-estrogens (Lester 2005).

Grading and staging of the breast cancer tumour uses a histopathology classification scheme and is very important in determining the prognosis of the disease (Schulz 2007). When the tumour is well differentiated and similar to normal tissue, it is low grade (Lester 2005; Schulz 2007). If the tumour is poorly differentiated and consists of disorganized cells with abnormal neuropathy, it is described as high grade (Lester 2005; Schulz 2007). Moderately differentiated tumours (intermediate grade) exhibit a morphology intermediate to that of the low and high grade tumours (Lester 2005). The staging method of classifying tumours is an important determinant in prognosis of the disease (Schulz 2007). The accepted clinical method for breast cancer staging is the tumour lymph node metastases (TNM) classification system (Mammen 2005). Staging involves the size of the tumour itself, whether it has reached lymph nodes, and whether there are any distant metastases to organs other than the breast and lymph nodes (Mammen 2005). Table 1-1 shows a summary of breast cancer staging.
CHAPTER 1

Breast cancer staging

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description of staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Ductal carcinoma in situ (DCIS)</td>
</tr>
<tr>
<td>I</td>
<td>(T) not involving (N)</td>
</tr>
<tr>
<td>IIA</td>
<td>(T) size 2-5 cm, but (N) negative</td>
</tr>
<tr>
<td>IIB</td>
<td>(T) size &gt;5 cm and (N) negative, or (T) size 2-5 cm and (N) positive (size &lt;4cm in axillary nodes)</td>
</tr>
<tr>
<td>IIIA</td>
<td>(T) size 5 cm and (N) positive, or (T) 2-5 cm with involvement of 4 or more axillary nodes.</td>
</tr>
<tr>
<td>IIIB</td>
<td>Tumour has spread to chest wall or skin and may have spread to more than 10 axillary lymph nodes</td>
</tr>
<tr>
<td>IIIIC</td>
<td>Tumour has spread to more than 10 lymph nodes, one or more supraclavicular or infraclavicular nodes, or spread to internal mammary nodes.</td>
</tr>
<tr>
<td>IV</td>
<td>Distant metastasis.</td>
</tr>
</tbody>
</table>

Table 1-1: A summary of breast cancer staging.
(T) tumour, (N) axillary lymph node. Adapted from (Mammen 2005).

1.3 Breast cancer treatments

When a breast tumour is localized treatment with surgery is the primary treatment protocol.

Other treatments, depending on tumour staging, include potential adjuvant chemotherapy and hormonal therapy (chemotherapy after operation) or neoadjuvant chemotherapy (chemotherapy prior to operation) and/or radiotherapy and, less frequently, immunotherapy.

1.3.1 Surgery and radiotherapy

Surgery and radiotherapy are the local regional treatments for breast cancer. Surgery in breast cancer is the main and essential standard treatment (Kaufmann et al. 2007). The surgical management of the breast is performed according to the breast surgery guidelines prior to systemic chemotherapy (Kaufmann et al. 2007). Different factors are considered and can affect successful breast cancer surgery (Chen et al. 2005). These factors include tumour size, lymphatic invasion and nodal status, or spread of microcalcifications (Chen et
AL. 2005). Lumpectomy, wide excision and quadrantectomy are breast surgery conservation techniques (Spear et al. 1998). Another option in surgical treatment is subcutaneous mastectomy, which is the removal of breast tissues only, sparing the nipple-areola complex, skin and nodes (Mammen 2005). Simple mastectomy, which is the total removal of the breast (removing the breast tissue, nipple-areola complex and skin) without axillary node dissection, is one type of surgical management for metastatic breast cancer (Mammen 2005). Another type of mastectomy is the modified radical mastectomy (MRM) which involves the removal of breast tissue, pectoralis fascia, nipple-areola complex, skin and axillary lymph nodes (Mammen 2005).

In the majority of breast cancer patients who are recommended for mastectomy, radiotherapy after mastectomy is recommended in most cases (Kaufmann et al. 2007). A recommendation for radiotherapy in patients who have had a mastectomy with a stage I cancer, or a stage II cancer with one to three positive lymph nodes, is not always made due to the reduced 5 year risk of local/regional recurrence in these patients (Garg et al. 2004; Kaufmann et al. 2007). However, radiotherapy for patients with stage III and stage IV breast cancer is strongly recommended (Huang et al. 2004).

1.3.2 Adjuvant and neo-adjuvant chemotherapies

In women with metastatic breast cancer, systemic treatments are still the most effective and reduce the risk of breast cancer death (Mamounas 2005). In the last 20 years, adjuvant therapy has been shown to have a major impact on treatment of breast cancer patients with either node-negative or node-positive disease (Mamounas 2005). Adjuvant chemotherapy was first established in the 1950s in response to concerns that the cancer might be spread
CHAPTER 1

during surgery, resulting in a recurrence of the disease, as well as the possibility of death from most metastatic cancers (Robin Stuart-Harris 2005). Chemotherapy used in the pre-operative period, referred to as neo-adjuvant chemotherapy, in the first clinical trials in 1958, but no differences were found in survival or recurrence of the disease (Fisher et al. 1968). A subsequent study of post-operative administration of Thiotepa or placebo for 2 days in a randomized trial with 826 premenopausal women, with at least four positive nodes, showed a considerably better incidence of survival at 5 years (Fisher et al. 1968). Therefore, post-operative adjuvant chemotherapy was highly recommended and most chemotherapeutic administration has been post-operative since the 1960s (Fisher et al. 1975). Also, a number of clinical trials were carried out using a post-operative combination of chemotherapy agents instead of single agent (Fisher et al. 1975; Fisher et al. 1986; Group 1992). The results showed that a combination of chemotherapies was more effective than long-term administration of a single chemotherapeutic agent (Fisher et al. 1975; Fisher et al. 1986; Group 1992).

The adjuvant chemotherapy currently used to treat metastatic breast cancer consists of the alkylating agent cyclophosphamide and two anti-metabolites, methotrexate and 5-fluorouracil, (the CMF regimen). The CMF regimen is correlated with a reduction in breast cancer deaths (Mamounas 2005; Ring and Ellis 2005). The use of CMF was established in the 1970s and used for 20 years (Bonadonna et al. 1976). More recently, anthracycline containing regimens (doxorubicin or epirubicin) have reduced the levels of death and recurrence, more effectively than the CMF regimen (Robin Stuart-Harris 2005). The Early Breast Cancer Trialists Collaborative Group (EBCTCG) evaluated the effect of the anthracycline regimens in 1998 in over 11 randomized studies comparing anthracycline
containing regimens to CMF (Robin Stuart-Harris 2005). The results showed that anthracycline containing regimens produced a reduction in recurrence (12%) and death (11%) equivalent to CMF (Robin Stuart-Harris 2005). Anthracycline treatment has not always shown a benefit similar to CMF in clinical trials (Robin Stuart-Harris 2005). The advantages of anthracycline containing regimens compared to the CMF chemotherapy is that they are suitable for newly diagnosed breast cancer patients, premenopausal patients aged <50, and postmenopausal patients aged 50-69, both ER negative and positive, and both node negative and node positive patients (Gluck 2005). There have been some adverse side effects resulting from treatment with high doses of anthracycline (Bernard-Marty et al. 2003). In one of the trials, an analysis was carried out that noted the subsequent development of secondary malignancies in 16 patients administered a high dose of epirubicin compared with epirubicin at an intermediate dose (Bernard-Marty et al. 2003).

Taxanes are one of the most effective groups of anticancer drugs that have been incorporated into the treatment of metastatic breast cancer (Robin Stuart-Harris 2005). Most clinical trials of taxane containing regimens, in early breast cancer cases, showed greater efficacy than CMF (Robin Stuart-Harris 2005). The first strategy involved sequential therapy with anthracycline treatment followed by taxane treatment (Mamounas 2005). In four clinical trials (CALGB 9344, NSAB B-28, [PACS] 01, and NSBP B-27), paclitaxel or docetaxel were used in patients who had previously undergone anthracycline therapy (Mamounas 2005). These trials demonstrated a significant increase in disease-free survival of approximately 14-17% (Mamounas 2005). In two of the clinical trials (CALGB 9344 and PACS 01), results indicated a significant reduction in breast cancer mortality of approximately 18-23%, in patients treated with regimens including taxanes (Mamounas...
An alternative approach is to integrate taxanes into adjuvant regimens and to administer them simultaneously with the anthracycline (Ring and Ellis 2005). This approach enables a shorter treatment duration, but potentially increases toxicity (Ring and Ellis 2005). The BCIRG 001 clinical study randomized 1491 women who had lymph node positive breast cancer and completed regional treatment (Ring and Ellis 2005). These patients were treated with docetaxel, doxorubicin and cyclophosphamide (TAC), or with 5-fluorouracil, doxorubicin and cyclophosphamide (FAC) (Ring and Ellis 2005). There were significant improvements with TAC compared with FAC in disease-free survival (5 years DFS 75% vs. 68%) (Ring and Ellis 2005). However, the benefits of TAC over FAC were only significant in those with 1-2 positive lymph nodes (Ring and Ellis 2005). In several cases of breast cancer, the use of taxanes rather than anthracyclines is preferable in order to prevent anthracycline-induced cardiotoxicity (Mamounas 2005). Taxanes have also been used with trastuzumab (Herceptin®) containing regimens, which is one of the most recent advances in adjuvant treatments for breast cancer (Mamounas 2005).

However, the benefit of specific treatments to individual breast cancer patients and the adverse effects they experience varies considerably (Stearns et al. 2004). The efficacy and safety of anticancer therapies may depend on the tumour, the treatment and the individual patient’s characteristics (Stearns et al. 2004). The antitumor activity or safety of specific agents may depend not only on drug dose and timing but also on functional targets, drug metabolizing enzymes and transporters (Stearns et al. 2004).
1.3.3 Molecular mechanism of chemotherapies and hormonal therapy

There are different types of anticancer drugs, some of which target cell cycle components, cause cell cycle inhibition, and induce apoptosis (Steams et al. 2004; McGrogan et al. 2008). In general, chemotherapy can be described as DNA damaging and includes a number of different classes of agent (Steams et al. 2004). Chemotherapies include alkylating agents and antimetabolites, as well as microtubule and topoisomerase inhibitors (Figure 1-1). Hormonal targeted therapy includes agents such as tamoxifen and aromatase inhibitors and a new, targeted therapy for breast cancer, which includes human epidermal growth factor HER2 targeted therapy such as lapatinib and trastuzumab.

Microtubule Inhibitors (paclitaxel, docetaxel), Epothilones, Vinca alkaloids (vinblastine, vincristine, vinorelbine).

Alkylating agents (Cyclophosphamide).

Anthracyclines (Doxorubicin, epirubicin).

Platinum agents (cisplatin, carboplatin).

HER2 (trastuzumab).

Antimetabolites (5-fluorouracil, methotrexate, capecitabine, gemcitabine).

Topoisomerase Inhibitors (etoposide).

Figure 1-1: The action of anticancer drugs on different targets in the cell cycle (McGrogan et al. 2008).
1.3.3.1 Cyclophosphamide

Cyclophosphamide is one of the main alkylating anticancer drugs and has been used in the treatment of breast cancer for more than 30 years (Stearns et al. 2004). Cyclophosphamide (Cytoxan™) is used in combination with other anticancer drugs, such as adjuvant chemotherapy CMF regimens, or can be given as single high dose chemotherapy (Stearns et al. 2004). Cyclophosphamide is a mechlorethamine-analog, which possesses potent, broad-spectrum anticancer activity (Stearns et al. 2004). Cyclophosphamide bio-activation involves enzymatic conversion by cytochrome P450 enzymes in the liver to yield phosphoramidemustard (Figure 1-2) following the initial reaction producing 4-hydroxycyclophosphamide (Mahoney et al. 2003). Phosphoramidemustard is the active compound generated from cyclophosphamide; it is the active DNA alkylating metabolite which causes both single-stranded DNA breaks and DNA cross-links in dividing cells (Mahoney et al. 2003).

The toxicities commonly associated with cyclophosphamide treatment of breast cancer are myelosuppression such as leukopenia, nausea and vomiting, in addition to skin and nail hyper-pigmentation and gonadal dysfunction (Stearns et al. 2004). Administration of a high dose of cyclophosphamide may cause haemorrhagic cystitis or secondary leukaemia (Stearns et al. 2004).
1.3.3.2 Methotrexate

Methotrexate is one of the oldest antimetabolite drugs used as part of the CMF regimen for breast cancer treatment (Mamounas 2005; Ring and Ellis 2005). Many women tolerate methotrexate comparatively well (Steams et al. 2004). However, a small number of women experience severe myelosuppression (Steams et al. 2004). Methotrexate is a structural analogue of folic acid and antagonizes the functions of this vitamin by suppressing dihydrofolate reductase (DHFR) activity (Myciek et al. 2000). Folic acid is important in nucleic acid and protein synthesis (Myciek et al. 2000). Methotrexate regimens act through regulating the level of intracellular folate by inhibiting DHFR, resulting in a depletion in nucleic acid synthesis and, ultimately, inducing cell death (Figure 1-3) (Myciek et al. 2000; Stearns et al. 2004).
1.3.3.3 Fluoropyrimidines

Fluoropyrimidines such as 5-fluorouracil and capecitabine are antimetabolite drugs which have been used in the treatment of a number of solid tumours, such as breast cancers, for many years (Mycek et al. 2000; Steams et al. 2004). The 5-fluorouracil analogue is a uracil ring that contains a stable fluorine atom (Mycek et al. 2000). In normal cells, deoxyuridylic acid is converted to thymidylic acid, which is an important precursor for DNA synthesis (Mycek et al. 2000). This process is inhibited by the presence of the fluorine containing ring of 5-fluorouracil, thus depriving the cell of one of the essential precursors for DNA synthesis (Figure 1-4) (Mycek et al. 2000). The drug targets thymidylate synthase (TS), which affects cell proliferation and cell death (Liu et al. 2002). However, 5-fluorouracil is
severely toxic to the gastrointestinal tract, and also causes myelosuppression, nausea and vomiting, diarrhoea, hand foot syndrome, and, less frequently, ocular and neurological toxicity (Steams et al. 2004).

Capecitabine is a 5-fluorouracil pro-drug. This drug requires three enzymatic reactions to be activated (Hamilton and Hortobagyi 2005). Capecitabine as an oral formulation (Xeloda) is widely used in breast cancer treatment (Steams et al. 2004). The action of capecitabine is the same as 5-fluorouracil, but involves bio-activation via thymidine phosphorylase (Steams et al. 2004; Hamilton and Hortobagyi 2005). Since thymidine phosphorylase is over-expressed in cancer cells, a degree of target selectivity is achieved (Hamilton and Hortobagyi 2005). The pro-drug prevents exposure of normal cells and tissue to the active drug and, thus, offers protection from its toxic effects (Hamilton and Hortobagyi 2005).
Unlike other chemotherapies, which are taken intravenously, capecitabine is taken orally for 14 days in a 3-week cycle (Blum et al. 1999). Capecitabine was registered in the US after it showed a 20% improvement in response rate in treating breast cancer patients (Blum et al. 1999).

1.3.3.4 Taxanes

Paclitaxel is an effective chemotherapy that has recently been introduced for breast cancer treatments (Braakhuis et al. 1994). Paclitaxel is a microtubule binding agent and a natural product extracted from the Pacific Yew Taxus brevifolia (Schiff and Horwitz 1980). The US Food and Drug administration (FDA) were the first to approve paclitaxel for treating ovarian cancer in 1992 and subsequently for the treatment of metastatic breast cancer in 1994 (Gueritte 2001). The hydrophobic nature of paclitaxel requires that it be administered in a solution containing alcohol and Cremophor® EL (polyoxyethylated castor oil) to enhance delivery (Roy and Perez 2006). Therefore, treatment with dexamethasone prior to paclitaxel treatment is recommended due to hypersensitivity reactions resulting from Cremophor® EL (Roy and Perez 2006). Docetaxel is a second generation taxane, taken intravenously as standard three week management therapy (Martin et al. 2005). However, docetaxel is poorly tolerated clinically and less effective than paclitaxel and is, therefore, used less frequently (Mackey et al. 2004; Martin et al. 2005).

Intracellularly, paclitaxel binds to β-tubulin (the taxane target site) in the mitotic spindle (Figure 1-5) (McGrogan et al. 2008). Paclitaxel stabilizes microtubules, inhibiting their depolymerisation and interfering with kinetochore attachment (Kelling et al. 2003; Jordan and Wilson 2004; Kienitz et al. 2005). The net result is to change the tension through the
kinetochores in mitosis, thus disrupting mitosis and inducing apoptosis (Kelling et al. 2003; Jordan and Wilson 2004; Kienitz et al. 2005). Docetaxel shares the same target as paclitaxel, the microtubule (Gligorov and Lotz 2004). Docetaxel interrupts centromere organization and, thus, affects the late stage of S phase, as well as the G2 and M phases, resulting in defects in mitosis and increased numbers of cells in the G2 and M phase, ultimately resulting in cell death (Gligorov and Lotz 2004). The major toxic effects associated with paclitaxel treatment are myelosuppression (leukopenia, myalgias), arthralgias, sensory peripheral neuropathy, and sporadic anaphylactoid reactions (reversed by corticosteroid treatment) (Steams et al. 2004). Accumulation of docetaxel results in toxic effects including edema and nail changes (Steams et al. 2004).

Figure 1-5: Paclitaxel mediated disruption of the mitotic phase of the cell cycle resulting cancer cell death (McGrogan et al. 2008).
Microtubule targeting drugs can be divided into two groups. The first group are microtubule-stabilizing agents such as the taxanes, epothilones A and B, discodermolide and eleutherobin (Jordan and Wilson 2004). These agents stabilize microtubules by binding to tubulin polymers and preventing microtubule disassembly (Zhou and Giannakakou 2005). The second group are the microtubule destabilizing agents including the vinca alkaloids (Vincristine, Vinblastine) (Jordan and Wilson 2004). These agents destabilize microtubules, thus preventing microtubule attachment to kinetochores and suppressing microtubule assembly (Orr et al. 2003).

1.3.3.5 Anthracyclines

Doxorubicin and epirubicin are antibiotic anthracyclines, and are the most common chemotherapeutic agents used in breast cancer treatment (Steams et al. 2004). Anthracyclines are generally used in combination with other anticancer regimens such as cyclophosphamide and 5-fluorouracil, for treatment of early breast cancers (Hamilton and Hortobagyi 2005). Doxorubicin is isolated from Streptomyces peucetius var. caesius (Arcamone et al. 1969). Doxorubicin intercalates between DNA base pairs, producing a local uncoiling in the DNA through non-specific binding to the sugar phosphate backbone (Painter 1978; Mycek et al. 2000). The intercalation results in blocking of DNA and RNA synthesis (Painter 1978; Mycek et al. 2000). In addition, this intercalation can interfere with topoisomerase II, which mediates breakage repair of DNA strands (Figure 1-6) (Mycek et al. 2000). Doxorubicin is also a topoisomerase type II inhibitor (Mycek et al. 2000). Anthracycline cytotoxicity is largely dependent on the inhibition of topoisomerase II activity, resulting in DNA strand breaks (Mycek et al. 2000).
Epirubicin is the 4' isomer of doxorubicin but its action in the liver is slightly different from that of doxorubicin (Mycek et al. 2000). The common toxic effects of both anthracyclines are cardiotoxicity which is caused by the production of free radicals in the cell (Stearns et al. 2004), although epirubicin is much less cardiotoxic (Stearns et al. 2004). Other side effects include myelosuppression, commonly leukopenia, acute and delayed nausea and vomiting, mucositis, and skin and nail hyperpigmentation (Stearns et al. 2004).

1.3.3.6 Tamoxifen

Breast cancers are routinely screened for the presence of estrogen or progesterone receptors (Stearns et al. 2004). Approximately 50% of women presenting primary breast cancers express the estrogen receptor (ER+) and or progesterone receptor (PR+), and, thus, should be offered some form of hormonal adjuvant therapy, for example, tamoxifen (Stearns et al. 2004). Tamoxifen is one of the most commonly used hormonal therapies (Mocanu and Harrison 2004). It is a non-steroidal synthetic anti-estrogen and belongs to the selective
estrogen receptor modulator (SERM) family (Mocanu and Harrison 2004). In 1973, tamoxifen was approved in the UK; in 1977, it was approved by the FDA in the US for use in adjuvant chemotherapy for postmenopausal, node positive breast cancer patients (Cole et al. 1971). Most hormones mediate their effects by activating cell surface receptors (Mocanu and Harrison 2004). However, steroid hormones have a different mechanism of action, first entering the cell and then binding to a nuclear receptor (Mocanu and Harrison 2004). Therefore, tamoxifen enters the cell, and binds to the nuclear receptor (ER) in place of estrogen (Nilsson et al. 2001). The hormone receptor complex normally binds to a specific site on the DNA, the estrogen response element (ERE) (Figure 1-7) (Mocanu and Harrison 2004). These sites regulate the genomic responses in all cells expressing ER (Mocanu and Harrison 2004).

![Figure 1-7: The action of tamoxifen on the estrogen receptor in the nucleus (Mocanu and Harrison 2004).](image)
Tamoxifen reduces the risk of breast cancer recurrence in hormone receptor positive women by 50% and reduces the incidence of newly diagnosed breast cancer in women at high risk for the disease (Group. 1998; Stearns et al. 2004). Tamoxifen is well tolerated and has short term side effects that are similar to menopause (Group. 1998; Stearns et al. 2004). The adverse effects of tamoxifen are hot flushes, skin rash, vaginal bleeding and discharge (Clemons et al. 2002). Another side effect of long term tamoxifen treatment is an increased risk of endometrial cancer (Clemons et al. 2002).

1.3.3.7 Aromatase Inhibitors

Aromatase inhibitor therapy is recommended for postmenopausal women (Stearns et al. 2004). The most commonly used aromatase inhibitor is anastrozole (Carpenter 2008), which inhibits the synthesis of estrogen from androgens by inhibiting aromatase (Stearns et al. 2004; Carpenter 2008). There are a number of reports that describe aromatase inhibitors used as first line treatment in metastatic breast cancer over tamoxifen, with data showing a lower recurrence of breast cancer (Smith and Dowsett 2003). However, the advantage of aromatase inhibitors is not apparent for all hormone receptor positive breast cancers (Stearns et al. 2004). Aromatase inhibitor side effects include a decrease in circulating estrogens, resulting in bone demineralisation, arthralgias/myalgias and hot flushes (Stearns et al. 2004).

1.3.3.8 Trastuzumab

Human epidermal growth factor receptor 2 (HER2/neu) targeted therapy has recently become one of the most vital components of breast cancer treatment (Gluck 2005). Trastuzumab (Herceptin®) was approved in 1998 as a first line adjuvant therapy treatment...
to be administered with paclitaxel for metastatic breast cancer patients positive for HER2 expression (Slamon et al. 2001). Trastuzumab is a human monoclonal antibody that binds specifically to the human epidermal growth factor receptor on the cell surface (HER2) (Gluck 2005). In normal cells, the function of HER2 is to promote growth and division (Gluck 2005; Hamilton and Hortobagyi 2005). In cancer cells, HER2 is over-expressed and promotes abnormal cell proliferation and tumour growth (Gluck 2005). Trastuzumab binds to the HER2 receptor and suppresses its function (Gluck 2005; Hamilton and Hortobagyi 2005). Trastuzumab reduces HER2 signalling through the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades (Nahta and Esteva 2006). The decrease in downstream signalling through these pathways induces expression of the cyclin-dependent kinase inhibitor p27kip1, which promotes cell cycle arrest and apoptosis (Sliwkowski et al. 1999; Baselga et al. 2001).

A number of randomized clinical trials have been carried out investigating the effect of trastuzumab as an adjuvant therapy, in high risk, early stage breast cancer patients with HER2-positive disease (Slamon et al. 2001; Gluck 2005). In these trials, trastuzumab exhibited significant therapeutic advantages over anthracyclines and taxanes (Gluck 2005). The NSABP B31 and the North Central Cancer Treatment Group (NCCTG) N9831 clinical trials for breast cancer treatment used adjuvant chemotherapy consisting of an anthracycline as a first line treatment, followed by paclitaxel plus trastuzumab (Gluck 2005). The results of these clinical trials were significant (Gluck 2005). The addition of trastuzumab to anthracycline-based chemotherapy either with or without taxanes produced a reduction in the recurrence risk by approximately 50% in patients over-expressing the HER2 receptor compared with patients who were HER2-negative (Gluck 2005; Romond et al. 2005).
1.4 Development of chemoresistance in breast cancer

There are several problems that may develop in breast cancer patients undergoing chemotherapy leading to a failure of treatment associated with chemoresistance (Gottesman 2002). These problems involve poor patient tolerance to anticancer drugs, as well as genetic or epigenetic changes within the cancer cell (Gottesman 2002). Factors that can give rise to clinical problems are the rapid metabolism or excretion of the anticancer drug and limited absorption of the drug (common in elderly patients), which reduces the drug dose below optimal levels (Pluen et al. 2001). There may also be a failure to distribute the drug to the tumour location, particularly in bulky tumours (Pluen et al. 2001). Genetic alterations in the cancer cells involve changes in the molecular components of the cell, such as a lack of cell surface receptors, or efflux transport of the drug, or mutation of the drug target (Gottesman 2002; Gottesman et al. 2002; Leonard et al. 2003; Leonessa and Clarke 2003). There may also be defects in apoptotic mechanisms, alterations in protein expression related to tumour resistance, and changes in DNA repair mechanisms after drug-induced damage (Figure 1-8) (Gottesman 2002; Gottesman et al. 2002; Leonard et al. 2003; Leonessa and Clarke 2003).
CHAPTER I

Lack surface transporter "Anti cancer drug Pump efflux
Change in drug target
Change DNA repair
Change in apoptosis
Apoptosis inhibition

Figure 1-8: Different molecular mechanisms of drug resistance in cancer cells
(Gottesman 2002; Coley 2008).

Metastatic breast cancers treated with first line chemotherapies containing anthracyclines or
taxanes have a response rate ranging from 30-70% (Bonneterre et al. 2004; Ishikawa et al. 2004; Vassilomanolakis et al. 2005). When metastatic breast cancers continue to progress following first line therapies, response rates are reduced to 20-30% with use of either a single agent or a combination of anticancer drugs (Porkka et al. 1994). More than 90% of the failures in breast cancer treatment are due to the development of chemoresistance (Longley and Johnston 2005). The development of drug resistance following the exposure of cancer cells to chemotherapeutic agents is referred to as "acquired resistance" (Coley 2008). When tumour cells become resistant to one chemotherapeutic agent, they may also show resistance to other structurally different anticancer drugs, a process referred to as cross-resistance or multi-drug resistance (MDR) (Coley 2008). However, in some cancers,
drug resistance occurs without prior exposure to chemotherapy; this type of multi-drug resistance is referred to as intrinsic resistance (Gottesman 2002; Coley 2008).

The standard chemotherapy treatment for breast cancer, a regimen containing anthracyclines plus taxanes, showed improved activity in patients who had developed resistance to treatment with a single anthracycline (Fisher et al. 1990; Lister-Sharp et al. 2000). In their meta-analysis, Jones and Smith suggested that the inclusion of taxanes and anthracycline in treatment is more effective than anthracyclines alone or in combination with cyclophosphamide, 5-fluorouracil and or vincristine [Gensia Sicor Pharmaceuticals Inc., Irvine, CA] (Jones and Smith 2006; Coley 2008). In cases in which a metastatic breast tumour is resistant to both anthracycline and taxanes, another option is the administration of capecitabine (Coley 2008). If chemoresistance to taxanes, anthracyclines and capecitabine develops in tumour cells, metastatic breast cancer patients currently have no other treatment options (Coley 2008).

The most common cellular mechanism connected to tumour resistance is the drug efflux mechanism mediated by members of the ATP binding cassette (ABC) transporter family, particularly P-glycoprotein (P-gp), multidrug resistant protein 1 (MRP1), and the breast cancer resistance protein (BCRP) (Table 1-2) (Coley 2008). The function of these transporters in normal cells is clearance and protection against unnecessary extracellular and intracellular xenobiotics and toxins (Borst et al. 2000; Leonard et al. 2003). Hence, in the case of cancer cells, ABC transporters effectively block the accumulation of adequate amounts of cytotoxic agents (e.g. doxorubicin) intracellularly (Coley 2008). Low levels of the drugs are insufficient to provoke cell death (Coley 2008). These ABC transporters are
found primarily in the plasma membrane of the cell (Coley 2008).

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<tr>
<th>P-gp (MDR1)</th>
<th>MRP1</th>
<th>BCRP</th>
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<td>Anthracyclines</td>
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Table 1-2: Drug efflux transporters and associated anticancer drug substrates. (P-gp) P-glycoprotein, (MRP) multidrug resistance protein, (BCRP) breast cancer resistance protein. Adapted from (Coley 2008).

The classical drug efflux transporter is P-glycoprotein (molecular weight 170 kDa) (Chen et al. 1986; Sauna et al. 2001; Gottesman et al. 2002), which is encoded by the MDRI gene, and consists of 12 transmembrane domains attached to an ATPase domain (Chen et al. 1986). ATP is hydrolysed when a substrate binds to the transporter, resulting in a conformational change that releases the substrate into the extracellular compartment (Sauna et al. 2001; Gottesman et al. 2002). P-gp is expressed in approximately 40-50% of primary breast cancers (Leonessa and Clarke 2003). Small tumours, < 2 cm in size, showed lower P-gp expression than tumours > 2 cm in size (Leonessa and Clarke 2003). Over-expression of P-gp in breast cancer patients increases following chemotherapy, from 40-50% to 60-70% (Chevillard et al. 1997; Vargas-Roig et al. 1999). The other type of drug efflux proteins involved in anticancer drug resistance are the multidrug resistance protein (MRP) family members, MRP1-9 (Coley 2008). MRP1 is a 190 kDa protein, which is composed of 17 transmembrane domains and has a central region similar to P-gp (Cole et al. 1992; Borst et al. 2000; Gottesman et al. 2002). Substrates for the MRP1 transporter include
anthracyclines, antifolates, and vinca alkaloids (Cole et al. 1994; Hooijberg et al. 1999; Borst et al. 2000; Leonessa and Clarke 2003). However, taxanes and cisplatin are not substrates for MRP1 (Cole et al. 1994; Hooijberg et al. 1999; Borst et al. 2000; Leonessa and Clarke 2003). The size of the tumour, lymph node grade and histological stage are not correlated to MRP1 expression (Leonessa and Clarke 2003). The incidence of MRP1 expression in breast cancers, 62% of cases before chemotherapy, increases following chemotherapy to about 88% (Sauna et al. 2001). The breast cancer resistance protein transporter BCRP (ABCG2) is a drug efflux protein (molecular weight 72 kDa) consisting of six transmembrane domains plus an N-terminal domain with an ATP binding site (Coley 2008). Cultured MCF-7 human breast cancer cells express BCRP constitutively (Coley 2008).

The alterations in proteins and enzymes in cancer is associated with anticancer drug resistance (Coley 2008). The topoisomerase enzymes change as a consequence of anthracycline resistance (Coley 2008). However, studies on the MD-MB-231 breast cancer cell line with acquired resistance to etoposide showed a lack of P-gp and MRP overexpression and a reduction in topoisomerase II alpha and beta (Lewis et al. 2007). The p53 gene codes for a vital tumour suppressor which has a role in the apoptotic response to DNA damage (O'Connor et al. 1997). p53 is mutated at a high frequency in cancers (O'Conor et al. 1997). In one study, approximately 50% of all tumours and 25% of breast cancers exhibited deletions and point mutations in the p53 gene (Gasco et al. 2003). Resistance to doxorubicin is associated with p53 mutation and early recurrence in breast cancer patients (Aas et al. 1996). In addition, dysfunction of the cell cycle in breast cancer cells is strongly linked to the loss of p53 activity (Coley 2008). DNA repair processes are correlated to
tumour resistance in breast cancer (Coley 2008). Mutations in the breast cancer susceptibility gene 1 (BRCA1) result in a deficiency of homologous recombination repair (HHR) of DNA double strand break repair and cross-links, thus producing unstable DNA (Bernstein et al. 2002). Furthermore, defects in the mismatch repair mechanism (MMR) in breast cancer are associated with the development of anti-cancer drug resistance (Coley 2008). Genes involved in MMR (hMLH1 and hMSH2) can be subject to epigenetic silencing and are associated with low sensitivity to cyclophosphamide, methotrexate and fluorouracil in breast cancer (Son et al. 2004). The cell death response is also correlated with anticancer drug resistance (Coley 2008). The cell death mechanism known as apoptosis may act via two main routes, mitochondrial (intrinsic) and cell surface receptor (Fas) mediated (extrinsic) pathways (Coley 2008). A number of studies found that drug resistance was associated with the intrinsic pathway rather than the extrinsic pathway (Coley 2008). However, many breast cancer cell lines are Fas-resistant, and the Fas receptor is downregulated in many breast cancers (Keane et al. 1996).

1.5 Types of cell death

There are several classes of cell death: apoptosis, autophagy, necrosis, and mitotic catastrophe (Table 1-3). The classification of cell death is often based on biochemical and morphological features (Kerr et al. 1972; Kerr 2002). Apoptosis and autophagy are programmed cell death mechanisms (Kerr et al. 1972; Kerr 2002).
Apoptosis, or programmed cell death I (PCD I), is cell suicide by physiological means (Kerr et al. 1972). Apoptosis is important for embryonic development, immune system function, and maintenance of tissue homeostasis (Kerr et al. 1972; Ellis et al. 1991; Jacobson et al. 1997). Defects in apoptosis may be associated with pathological states such as neurodegenerative disease, autoimmunity, and cancer (Okada and Mak 2004). Apoptosis involves the breakdown of cellular components and their engulfment by nearby cells (Okada and Mak 2004). Morphological features of apoptosis include condensation and fragmentation of the nuclei, membrane blebbing and apoptotic body formation (Kerr 2002; Okada and Mak 2004). Under normal conditions, apoptotic cells will be engulfed by macrophages or nearby cells (Ricci and Zong 2006). Many chemotherapeutic agents kill tumour cells by apoptosis and this is an active area of research allied to the discovery and
design of new, more effective anticancer drugs (Ricci and Zong 2006).

Autophagic cell death, or programmed cell death II (PCD II), is a non-apoptotic form of cell death (Okada and Mak 2004). Unwanted proteins in normal cells are degraded by two independent mechanisms: ubiquitin-mediated proteolysis and autophagy (Okada and Mak 2004). Activation of autophagic cell death is triggered as a response to nutrient depletion, developmental triggers, and metabolic stresses, and involves degradation of intracellular proteins and organelles (Shintani and Klionsky 2004; Lum et al. 2005). Excessive autophagy in cells tends to induce PCD II, and dysregulation of autophagy is associated with pathological conditions such as neurodegenerative diseases, cardiomyopathy, and cancer (Kalimo et al. 1988; Schwartz et al. 1993; Anglade et al. 1997; Bursch et al. 2000; Tanaka et al. 2000). The main morphological features of autophagic cell death are the formation of vacuoles or autophagic vesicles that engulf organelles in the cytoplasmic compartment (Klionsky and Emr 2000). The cytoplasm is condensed with tightly packed organelles and, along with evidence of mitochondrial condensation, autophagy is associated with partial chromatin condensation (Ricci and Zong 2006). Cells dying by autophagy tend to be in clusters and phagocytes are not primarily responsible for clearance of the dead cells (Schweichel and Merker 1973; Clarke 1990).

Apoptosis is a genetically controlled and defined mechanism of cell death, whereas necrosis is the opposite—uncontrolled cell death—and is associated with pathological states (Ricci and Zong 2006). Necrosis is a result of a pathophysiological state such as infection, inflammation, or ischemia leading to trauma that causes a failure in normal cellular mechanisms that balance cellular homeostasis, including energy production and ion
transport (Nicotera and Melino 2004; Okada and Mak 2004). When cellular energy is reduced in necrotic cells, swelling starts to develop followed by membrane lipid damage, lack of homeostatic ion pump function, mitochondrial dilatation, and ending in rupture of organelles and the plasma membrane, releasing cytoplasmic material (Ricci and Zong 2006). An imbalance in intracellular calcium flux and ROS production can lead to the stimulation of non-apoptotic proteases (Ricci and Zong 2006). Increased production of ROS causes oxidative stress that damages intracellular molecules and organelles resulting in necrosis (Figure 1-9) (Ricci and Zong 2006). ROS also cause DNA strand cleavage and oxidation of purines, as well as lipid oxidation, resulting in loss of membrane integrity (Marnett 2000). These changes often elicit an inflammatory response, which is designed to deliver leukocytes to sites of necrosis (Mateo et al. 1999). Thus, leukocytes clear any invading microbes and begin the process of breaking down necrotic tissues (Mateo et al. 1999). Cellular events include emigration of the leukocytes from the microcirculation and accumulation at the focus of the injury (Robin Stuart-Harris 2005). The main inflammatory responses that are mediated by necrosis are haemodynamic changes, neutrophil accumulation, and release of chemical mediators (Robin Stuart-Harris 2005). The haemodynamic changes involve initial transient vasoconstriction, followed by massive vasodilatation mediated by histamine, and increased vascular permeability (Robin Stuart-Harris 2005). Neutrophils and macrophages accumulate in response to necrosis, and chemical mediators of inflammation, such as histamine and cytokines are produced (Robin Stuart-Harris 2005). The major outcomes of inflammation are abscess formation and transition to chronic inflammation (Robin Stuart-Harris 2005).
Mitotic catastrophe is a mechanism involving the disruption of mitosis caused by inappropriate separation of the chromosomes to produce sister chromatids (Weaver and Cleveland 2005). In itself, mitotic catastrophe is not considered a form of cell death but is a trigger of cell death (Weaver and Cleveland 2005). In normal cells there is a complex mechanism that stimulates cell survival via activation of multiple signalling pathways after detection of DNA damage (Ricci and Zong 2006). These responses result in suppression of the cell cycle and stimulation of DNA repair mechanisms (Ricci and Zong 2006). When damage is severe the cell cycle will be permanently arrested and cell death may be induced (Ricci and Zong 2006). The lack of cell cycle checkpoints (DNA structure checkpoints) during mitosis will result in mitotic catastrophe-associated cell death (Castedo et al. 2004).
The ‘DNA structure checkpoints’ arrest cells at the G2/M transition in response to unreplicated DNA or DNA damage, and the spindle assembly checkpoint prevents anaphase until all chromosomes have obtained a bipolar attachment to the spindle (Castedo et al. 2004). Therefore, the presence of checkpoint deficiencies and DNA damage would lead to mitotic catastrophe (Castedo et al. 2004). The morphological characteristics of mitotic catastrophe induced cell death include large cells containing many micronuclei or two nuclei (Ricci and Zong 2006). The nuclear envelope, which collects chromosomal fragments during a mitotic catastrophe, develops into the micronuclei (Ricci and Zong 2006).

1.6 Cancer and cell death defects

Cancer cell progression and prolonged proliferation occur due to a number of defects, including defects in apoptosis that result in a high survival rate in cancer cells (Borst and Rottenberg 2004). To date, a number of studies have suggested that the induction of apoptosis in breast cancer cells is the main endpoint of different chemotherapies (Mashima and Tsumo 2005). However, several factors affect the ability of cancer cells to induce apoptosis (Okada and Mak 2004; Mashima and Tsuruo 2005). Hence, inactivation of apoptotic cell death may be associated with the development of chemoresistance to a number of unrelated anticancer drugs that are linked with tumorigenesis (Okada and Mak 2004; Mashima and Tsuruo 2005).

Apoptosis can be mediated by two pathways: the intrinsic pathway, which is mitochondrially mediated, and the extrinsic pathway, which is cell surface receptor (Fas) mediated (Okada and Mak 2004). In many breast cancers and breast cancer cell lines, the
CHAPTER 1

Fas receptor is down regulated, leading to Fas resistance (Keane et al. 1996). Other described defects in breast cancer include the absence of caspase-3 in the MCF-7 breast cancer cell line (Janicke et al. 1998; Kurokawa et al. 1999; Simstein et al. 2003). Caspase-3 is one of the important executioner caspases in apoptosis (Janicke et al. 1998; Kurokawa et al. 1999; Simstein et al. 2003). In addition, the inhibitor of apoptosis proteins (IAPs) that are involved in the apoptotic pathway are over-expressed in many types of cancers (Nachmias et al. 2004).

One of the most common defects in cancer, as mentioned earlier, is mutation in the p53 tumour suppressor gene (Mashima and Tsuruo 2005). This defect can cause inactivation of the apoptotic machinery and lead to tumour development (Mashima and Tsuruo 2005). Inactivation of apoptosis may results from defects in Apaf-1 and caspase-9, the apoptosome complex core machinery in the intrinsic pathway of apoptosis (Soengas et al. 1999; Mashima and Tsuruo 2005). The function of the apoptosome has been by investigated by Mashima and Tsuruo (2005) who found that induction of apoptosis was increased in many tumours whilst being reduced in others. The Bcl-2 family members are important factors in regulating the apoptotic pathway (Johnstone et al. 2002). Bcl-2 is over-expressed in a variety of cancers (Reed 1999). In addition, over-expression of Bcl-2 is associated with tumorigenesis in transgenic mice (Adams et al. 1999). In contrast, the pro-apoptotic Bcl-2 family members are inactivated in a number of cancers and defects in these genes also stimulate tumorigenesis in mice (Adams et al. 1999). Moreover, changes in expression or mutations in upstream regulators of Bcl-2 proteins accompany cancer progression (Johnstone et al. 2002). For example, the Bad-kinase Akt is regulated by different oncoproteins and by the PTEN tumour suppressor (Datta et al. 1999). Thus, Akt over-
expression and PTEN mutations have frequently been observed in a variety of solid cancers (Datta et al. 1999). The extrinsic pathway or death receptor pathway is also often inactivated in tumour cells, and mutations in CD95, TRAIL receptors, and downstream signalling pathways may occur (Johnstone et al. 2002). For example, in a metastatic breast cancer, TRAIL-R1/R2 was mutated, leading to the suppression of death receptor mediated apoptosis (Shin et al. 2001).

Other defects in non-apoptotic pathways have also been reported, such as an alteration in autophagic cell death (Okada and Mak 2004). Defects in protein degradation are often associated with cancer and there are oncogenes and tumour-suppressor genes that collectively have been shown to influence the autophagic pathway (Okada and Mak 2004). The autophagic cell death pathway is important in restricting cellular transformation and regulating protein degradation (Okada and Mak 2004). Hence, mechanisms that inhibit this pathway may lead to tumour progression (Okada and Mak 2004). Inhibition of autophagy disrupts the natural turnover of proteins which function as active regulators of cell growth and, thus, promotes cell survival (Okada and Mak 2004). The phosphatidylinositol 3-kinase class I (PI3K/Akt) signalling pathway stimulates cell growth in response to mitogenic signals (Ng and Huang 2005). This pathway is up-regulated in a number of cancer cells, leading to inhibition of autophagy (Mochizuki et al. 2002). A study using cultured breast cancer cells treated with tamoxifen, showed that induction of autophagy involved Akt down-regulation (Scarlatti et al. 2004). Moreover, the downstream effector of Akt (mTOR) was associated with the suppression of the autophagic pathway, since hyperactivation of mTOR and Akt signalling could inhibit both apoptosis and autophagic cell death (Arico et al. 2001; Mochizuki et al. 2002).
1.7 MCF-7 breast cancer cells: a model for the study of cell death induced by chemotherapy

The human breast cancer MCF-7 cell line is derived from a pleural effusion of a patient who had metastatic breast cancer in 1970 (Soule et al. 1973; Levenson and Jordan 1997). Findings by Levenson and Jordan indicated that the MCF-7 cell line was the first hormone-responsive breast cancer cell line (Soule et al. 1973; Levenson and Jordan 1997). Follow-up studies in the 1970s and 1980s on MCF-7 cells led to the development of monoclonal antibodies to ER (Levenson and Jordan 1997). This advance was shortly followed by the establishment of new techniques for measuring estrogen and progesterone receptors in tumours to determine the applicability of hormone treatment in individual patients with breast cancer (Simstein et al. 2003). A study by Yang et al. (2001) showed that the MCF-7 cell line is insensitive to a number of chemotherapeutic agents, but is highly responsive to doxorubicin and etoposide and undergoes apoptosis when caspase-3 is reconstituted (Yang et al. 2001). Further, MCF-7 cells were studied for their inconsistent apoptotic responses to tumour necrosis factor-α (TNF-α) inducing agents and anti-Fas antibodies (Simstein et al. 2003). When MCF-7 cells are subjected to long-term exposure to TNF or chemotherapeutic drugs such as doxorubicin they develop resistance (Simstein et al. 2003). In addition, there is some recent evidence demonstrating that MCF-7 cells can undergo autophagic cell death. A study by Lamparska-Przybysz et al. (2006) on BID-deficient MCF-7 cells revealed a connection between apoptosis and autophagy. When placed under metabolic stress MCF-7 cells lose their ability to induce apoptosis and, therefore, switch to autophagic cell death (Lamparska-Przybysz et al. 2006). All these findings suggest that the MCF-7 cell line is an excellent tool for studying breast cancer chemoresistance and susceptibility to cell death (Simstein et al. 2003).
1.8 Aim and objectives of the project

The work in this thesis focuses on the use of *in vitro* drug resistant tumour cell line models to study cell death pathways and associated mechanisms underlying the altered responses of these cells to cytotoxic agents. My work has concentrated on cancer cell line models developed in the laboratory of my supervisor, Dr. Helen Coley. I particularly focused on a model cell line derived from MCF-7 cells, MCF-7TaxR. The MCF-7TaxR cell line has *in vitro* acquired resistance to the chemotherapeutic agent paclitaxel (Taxol®). Previous work carried out by Dr. Coley demonstrated an absence of caspase-9 in the MCF-7TaxR cell line, in addition to the absence of caspase-3 already apparent in the parental MCF-7 cells. Furthermore, due to overexpression of the MDR transporter P-glycoprotein (P-gp) in the MCF-7TaxR cell line, these cells are expected to exhibit a classical MDR (multidrug resistant) phenotype. However, unexpectedly, we saw a lack of cross-resistance to a range of agents such as VP-16 and doxorubicin, which we would normally expect to see in a P-gp expressing MDR cell line. Moreover, there was a collateral sensitivity of MCF-7TaxR cells to platinum anticancer agents.

Thus, the current study set out to answer an important question: if the MCF-7TaxR cells lack significant components of the apoptotic machinery, what is the molecular basis underlying the relatively drug sensitive phenotype of these cells?

The first part of the project focused on confirming the lack of involvement of caspase-mediated cell death in MCF-7TaxR cells in response to anticancer drugs. Further, I monitored temporal changes in apoptotic genes/proteins during the development of paclitaxel resistance in MCF-7TaxR cells, compared with parental cells MCF-7.
In the next part of the study, I considered whether alternative mechanisms of cell death might be operating in the cells and studied PCD II autophagic cell death induction in the resistant MCF-7TaxR cell line. This part of my work included an assessment of the role of the Akt/mTOR pathway in the MCF-7 cell line models.

In the final part of the project, I looked for evidence of any epigenetic changes, chiefly epigenetic silencing due to methylation of caspase genes in MCF-7 and MCF-7TaxR cells in an effort to characterise the drug resistance profile further.
CHAPTER 2

MATERIALS AND METHODS
2 Materials and Methods

2.1 Purchase of chemicals and reagents, by source

Alexis Biochemicals, Nottingham, UK.
Rapamycin (purity ≥ 98.0%), staurosporine (purity ≥ 98.0%), LY-294002 [2(4-morpholiny1)-8-phenyl-4H-1-benzopyran-4-one] (purity ≥ 98%).

Bayer, Pharmaceutical Division, Newbury, Berkshire, UK.
Ciproxin®, infusion antibiotic (ciprofloxacin).

Bachem®, Switzerland.
Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD) (purity > 91.9%).

Beckman Coulter, UK.
Mouse Monoclonal Anti-Fas/CD95 (purified).

Bio-Rad Laboratories Ltd, Herts, UK.
Bio-Rad DC protein assay kit.

Biosource International, Inc, USA.
Akt rabbit monoclonal antibody and phospho-specific Akt/PKB[pS473] rabbit monoclonal antibody.
Bristol Myers Squibb, UK.
Paclitaxel (Taxol®) as a pharmacy preparation for IV infusion purposes.

Calbiochem® Oncogene™, UK.
Annexin V-FITC (fluorescein-isothiocyanate) apoptosis detection kit, β-actin mouse antibody.

Cell Signalling Technology® Inc, (distributed by Hitchin, Hertfordshire, U.K)
Caspase-7, Caspase-8 and Caspase-9 mouse monoclonal antibodies, mTOR, phospho-mTOR (Ser2448) rabbit monoclonal antibodies, p70 S6 Kinase and phospho-p70 S6 Kinase (Ser371) rabbit polyclonal antibodies, Bim rabbit antibody, Bad and phospho-Bad (Ser112) rabbit polyclonal antibodies, and LC3B rabbit monoclonal antibody.

Eastman Kodak Company, (distributed by Sigma Aldrich, Poole, U.K)
Kodak BioMax XR Light Film.

Invitrogen™ Life Technologies, Paisley, UK.
NuPAGE® Novex Bis-Tris gels, MOPS, MES and Tris-Acetate electrophoresis running buffers, NuPAGE® PVDF 0.2 µm membranes, NuPAGE® Nitrocellulose membrane, Western blotting transfer buffer, HiMark™ Prestained high molecular weight protein standards, Novex® sharp protein standards, secondary antibody solutions (alkaline phosphatase conjugated anti-mouse and anti-rabbit), Western Breeze® chemiluminescent Western blot immunodetection kit, foetal bovine serum (FBS®), Glutamax®, TRIzol® for RNA extraction.
Santa Cruz Biotechnology Inc (distributed by Autogen Bioclear, Wiltshire, UK)

Bcl-2, BECN1 and Bax mouse monoclonal antibodies. MDR1 H-241 rabbit polyclonal antibody.

Sigma, Poole, Dorset, UK.

Porcine Trypsin-EDTA, 0.4% Trypan blue, RPMI-1640 and DMEM HEPES modified cell culture medium, cisplatin, carboplatin, doxorubicin, verapamil, zebularine, 5-azacytidine, trichostatin A, 3-methyladenine, Nonidet® P-40 detergent, sodium dodecyl sulphate (SDS), phenylmethylsulphonylfluoride (PMSF), aprotinin, leupeptin, sodium orthovanadate (Na3VO4), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33358, acridine orange.

SuperArray, Bioscience Corporation, USA

RT² qPCR assay-SYBR® Green and primers for Human CDKN2A (p16), CASP7, and CASP9.

Vector Laboratories Inc. Burlingame, CA.

Vectashield Mounting Medium for Fluorescence H-1000.

2.2 Buffers and Solutions

Hypotonic Cell lysis buffer

50 mM Tris-HCl (C4H11NO3 HCl), 150 mM NaCl, adjusted to pH 7.0 with concentrated HCl, in milliQ water. The buffer was supplemented with 1% Nonidet® P-40 detergent, 0.2% SDS, 1 mM PMSF.
**NuPAGE® MOPS SDS**

20% MOPS SDS running buffer prepared in 1 L of milliQ water.

**NuPAGE® Transfer Buffer**

20% transfer buffer with 10% methanol in 1 L of milliQ water.

**Phosphate buffered saline (PBS)**

For 1× strength: 80.0 g NaCl, 11.5 g Na_2HPO_4_, 2.0 g KCl, 2.0 g KH_2PO_4_ in 10 L deionised water.

**Acridine Orange buffer I**

20 mM citrate-phosphate, pH 3, 0.1 mM EDTA, 0.2 mM sucrose, 0.1% Triton X-100, in milliQ water.

**Acridine Orange buffer II**

10 mM citrate-phosphate, pH 3.8, 0.1 NaCl, in MilliQ water.

**Cytotoxic drugs**

Paclitaxel, cisplatin and carboplatin were diluted in sterile 0.9% saline as stock solutions and stored at -20 °C until use. Doxorubicin was dissolved in sterile distilled water, stored frozen as stock solution. Stock solutions were thawed prior to use. All other compounds were prepared in DMSO and stored as frozen aliquots prior to use.
PBS/Triton solution

0.3% Triton-X-100 in PBS (i.e., 100 ml of sterile PBS + 300 µl Triton-X 100).

Hi Salt/PBS solution

23.4 g NaCl per 1 L PBS.

2.3 Cell culture

The human breast carcinoma cell lines MCF-7, T47D and MDA-MB231, and the human cervical cancer HeLa cell line, were obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, UK (ECACC)). All cell lines were cultured in DMEM Hepes modified medium with 2 mM Glutamax® and supplemented with 10% heat-inactivated foetal bovine serum (FBS) at 37 °C. Drug-resistant cell lines with acquired resistance to paclitaxel were generated by growth in increasing doses of the appropriate selecting agent on successive passages (by Dr Helen Coley, University of Surrey). The MCF-7 and T47D paclitaxel resistant cell lines, designated MCF-7TaxR and T47DTaxR, were maintained at 6 nM and 4 nM paclitaxel, respectively. VP16 resistant MDA-MB-231 cells, designated MDA-MB-231VP16R were maintained in 1 µM VP16. Cultures were grown in drug free medium for at least one passage before experimental use. In addition, MCF-7TaxRREV cells were established MCF-7TaxR-7 cells (i.e., in excess of 35 passages) that had been grown in the absence of paclitaxel for several weeks.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type and Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Human Caucasian breast adenocarcinoma derived from pleural effusion. Cells show epithelial morphology and adherent growth mode.</td>
</tr>
<tr>
<td>MCF-7TaxR</td>
<td>Human breast cancer cells derived from the MCF-7 cell line with acquired resistance to the anticancer drug paclitaxel.</td>
</tr>
<tr>
<td>MCF-7TaxRREV</td>
<td>Breast cancer cells derived from the MCF-7 cell line with acquired resistance to paclitaxel and left to grow in the absence of paclitaxel for several weeks.</td>
</tr>
<tr>
<td>T47D</td>
<td>Human breast tumour cell derived from pleural effusion of a ductal carcinoma. Cells show epithelial morphology and adherent growth mode.</td>
</tr>
<tr>
<td>T47DTaxR</td>
<td>Breast cancer cells derived from the T47D cell line with acquired resistance to the anticancer drug paclitaxel.</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Human Caucasian breast adenocarcinoma derived from pleural effusion. Cells show epithelial morphology and adherent growth mode.</td>
</tr>
<tr>
<td>MDA-MB-231VP16R</td>
<td>Breast cancer cells derived from the MDA-MB-231 cell line with acquired resistance to the anticancer drug etoposide (VP-16).</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human Negroid cervical epitheloid carcinoma derived from cervical carcinoma. Cells show epithelial morphology and adherent growth mode.</td>
</tr>
</tbody>
</table>

Table 2-1: Types and characteristics of cell lines used.

2.4 Chemosensitivity Testing

Cytotoxicity was determined by the MTT assay, as described previously (Mosmann 1983). The method relies on the measurement of metabolic activity due to the presence of actively respiring mitochondria. Cell lines in cell suspension were seeded at a density $2 \times 10^4$ or $3 \times 10^4$ cells/ml (depending on cell doubling times) in 200 µl of medium in a 96-well plate.
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Cells were allowed to grow and attach in a humidified incubator for 24 h at 37 °C and 5% CO₂. Increasing amounts of the appropriate drug (50 µl volume), or control medium were added. Cells were then incubated with continuous drug exposure for 72 h or 96 h (for cells with longer doubling times, e.g. T47D). The assay was terminated by incubation with 0.05 mg MTT in PBS solution for approximately 4 h at 37 °C. The medium was removed by careful aspiration and the resulting tetrazolium crystal product was then dissolved in 200 µl DMSO. The absorbance of each well was measured at 540 nm using an automated plate reading spectrophotometer (Labsystems Multiskan RC Plate Reader) with Genesis 3.05 software. The IC₅₀ values were determined as the drug concentration necessary to cause a 50% reduction in cell viability compared to untreated controls. Thus, a graph showing cell viability versus drug concentration was produced from the mean absorbance values, by calculating the percent growth of drug treated cells relative to control cells. For each drug concentration, treatments were set up in triplicate or quadruplicate which were then averaged and the percent of control growth calculated.

2.5 Trypan Blue Assay for cell viability

As a guide for the major experiments, preliminary testing was carried out in order to examine the inhibitory effect of Z-VAD (caspase inhibitor) by treating with paclitaxel as an anticancer agent in MCF-7 and MCF-7TaxR cell lines to examine the induction of apoptotic cell death in these cell lines. The test was set as a 96 well plate with cells being grown at a density of 2x10⁴ or 3x10⁴ cells/ml in a 200 µl volume overnight. The next day cells were treated with 100 µM Z-VAD for 1 h, and paclitaxel was then prepared in a range of concentrations by serial dilutions, added to the wells, and incubated over 72 h. Cells were then trypsinised, stained with Trypan blue, and counted using a haemocytometer.
2.6 Annexin V-FITC/propidium iodide apoptosis assay

The Annexin V-FITC conjugated apoptosis detection kit was used as described by the manufacturer (Calbiochem® Oncogene™, UK). The Annexin V-FITC/PI method is based on the use of two fluorescent probes, Annexin V-FITC and PI, in order to distinguish between viable, early apoptotic, late apoptotic, and necrotic cells. When Annexin V-FITC binds to the phospholipids of the plasma membrane, phosphatidyl serine (PS) has been externalised in an apoptotic cell. However, if cells are dead via necrotic death, their degraded cell membrane will allow nuclear staining of PI which will differentiate them from apoptotic cells. Hence, PI staining allows for the differentiation between viable and early apoptotic cells. The phospholipids of viable cells are located on the inner surface of the plasma membrane. The data are presented in a four quadrant format: F1, F2, F3 and F4. F1 contains cells that are dead or in end-stage apoptosis/necrosis (Annexin negative, PI positive), F2 contains cells in mid-phase and late-phase apoptosis (positive for both Annexin and PI), F3 contains viable cells (negative for both Annexin and PI), F4 contains cells in early apoptotic phase (Annexin positive, PI negative).

The MCF-7, MCF-7TaxR and MDA-MB-231 cells were plated at a density of $2 \times 10^4$ or $3 \times 10^4$ in T25 flasks and allowed to adhere for 24 h. Cells were treated with either 100 µM Z-VAD (apoptosis inhibitor) or 0.5-1 µM staurosporine, or both (100 µM Z-VAD for 1 h then 0.5 µM staurosporine), or medium free of inhibitors for 24 h or 48 h. For the experiments looking at the effects of Fas, cells were seeded in T25 flasks as control untreated and treated with 200 ng/ml Fas or 1 µg/ml cycloheximide, or with both 200 ng/ml Fas and 1 µg/ml cycloheximide, and incubated for 48 h or 72 h. Cells were harvested and assayed using the Annexin V-FITC/PI method. In addition, different experimental settings
were also used with the MCF-7 and MCF-7TaxR cell lines. Cells were incubated in normal medium, 1% serum medium, or serum free medium for 72 h.

Images of the MCF-7, MCF-7TaxR, and HeLa cells were captured post-treatment by light microscopy (Nikon ECLIPSE TS-100) before processing the cells. Both floating and attached cells were harvested and washed twice in cold PBS. Cells were resuspended in ice cold binding buffer to develop Annexin V-FITC binding to the phosphatidyl serine on the cell surface. Annexin V-FITC (0.002%) was added and incubated at room temperature for 30 minutes in the dark. Cells were then centrifuged for 5 minutes at 1000 rpm. The medium was removed and the cells resuspended in ice cold binding buffer and 0.002% propidium iodide (PI) was added on ice and away from light and analyzed using a Beckman Coulter Epics XL flow cytometer (argon laser, excitation wavelength 488 nm). Samples were analyzed by flow cytometry, using the FL1 and FL3 settings, each reading using a collection of 10,000 events.

2.7 Fluorescence microscopy of Hoechst 33258 stained cells

In order to detect the cell morphology characteristic of cell death, MCF-7, MCF-7TaxR and T47D cells were stained with Hoechst 33258 for the analysis of changes in nuclear morphology as a direct marker for apoptosis (Jones et al. 1998). Cells were seeded in T25 flasks at $2 \times 10^4$ or $3 \times 10^4$ cells and grown and treated with 1 μM staurosporine for 48h or left untreated. Cells were harvested with their medium and 200 μl of each cell suspension was added to a slide for cytopsin (Cytospin 4 Thermo Electron Corporation, Basingstoke,
Hants), forming a pellet on the slides. The pellet was fixed with a few drops of ice cold methanol for 5-8 minutes, and then washed with PBS for 1 minute, then one drop of a stock solution (2 µg/ml Hoechst 33258) was added to the pellet for 5 minutes. Afterwards, the pellet was washed with PBS, left to dry, and Vectashield® mounting solution was added to the cell pellet to preserve the fluorescence. A coverslip was placed over the pellet on the slide and sealed with nail polish. Slides were left to dry for 10-20 minutes and covered with foil to avoid light. Stained nuclei were detected by fluorescence microscopy (Nikon Eclipse TE2000-S by JENCONS-PLS) Magnification, ×40. Images of nuclear staining were captured by a Nikon digital camera.

2.8 Whole cell lysis preparation

Different breast cancer cell lines were grown in tissue culture T25 or T75 flasks to approximately 40% confluence for 24h. Flasks of cells were treated with different drugs or left untreated for 24, 48 or 72 h. Cells were harvested by scraping the monolayer of adherent cells, combining them with the floating cell population and washing with PBS. The supernatant was removed and the cell pellet was incubated for 20 minutes on ice with freshly prepared cell lysis buffer. Cells were lysed using a 1 ml syringe and resuspended several times for approximately 20 seconds on ice. Cells were centrifuged for 10 minutes, and the supernatant was then collected and stored at -20 °C.

The cell protein concentration was estimated using the Bio-Rad DC protein assay. Following the manufacturer's instructions, cell lysate samples were diluted 1:10 in milliQ water (100 µl total volume) and added to 96 well plates. SDS was added to the NaOH solution at a ratio of 1:50 and 25 µl was added to the cell lysate samples or the BSA protein
standards in the range of 1.5-2.0 mg/ml. The folin reagent (200 µl) was added to the cell lysate samples or BSA protein standards. After 10 minutes, the absorbance was measured at 690 nm using a Labsystem Multiscan RC plate reader. The protein concentration of the cell lysates was estimated by interpolation of the BSA protein standard curve and equal protein amounts were then used for Western blotting analysis.

2.9 Western Blotting

The Western blotting analysis was performed on cell lysates using the Invitrogen™life technologies system. Following the manufacturer’s instructions, cell lysate samples ranging from 30-50 µg were prepared in NuPAGE® LDS sample buffer (1:4, diluted in milliQ water) and NuPAGE® reducing agent (1:10, diluted in milliQ water), heated at 70 °C for 10 minutes, and then subjected to electrophoresis. The proteins were separated on NuPAGE® Novex Bis-Tris Gels (7%, 8%, 10%, 12% or 4-12% gradient gels) depending on the protein molecular weight subject to detection. Using NuPAGE® MOPS SDS as the running buffer, samples were transferred to PVDF or nitrocellulose membranes in an apparatus provided by the manufacturer. Blocking solution was provided in the Chemiluminescent Western Blot Immunodetection Kit from Invitrogen™ prepared with 2-5% milk or BSA to block the nonspecific binding sites on the membrane, according to the manufacturer’s instructions. Primary antibody was prepared in antibody diluent provided with the kit in order to probe the membrane. The membrane was incubated with the primary antibody solution at 4 °C overnight with gentle shaking. The membrane was washed and a suitable secondary antibody solution, provided with the kit, was used to probe the membrane for 30-45 minutes with gentle shaking. The membranes were washed with prepared antibody solution, provided with the kit, for 5 minutes for a total of three washes. Membranes were rinsed
with milliQ water. The signal was detected using a chemiluminescent substrate provided with the kit and Kodak Biomax Light film. To detect equivalent protein loading, the membrane was stripped by incubating at room temperature for 2 h in 1 mM sodium azide in PBS and gentle shaking. The membrane was then exposed to an anti-mouse β-actin antibody at a dilution 1:20,000.

2.10 Total RNA extraction

Total RNA was extracted with the TRizol® reagent (Invitrogen™) according to the manufacturer’s instructions, from T25 or T75 monolayers of the breast cancer cell lines MCF-7, MCF-7TaxR, T47D, T47DTaxR and MDA-MB-231, 48 h after the cells reached 80% confluence. The Trizol reagent was added to cells to disassociate nucleoprotein complexes. Chloroform was added and thoroughly mixed to separate DNA and RNA from proteins, incubated for 3 minutes, and then centrifuged for 10 minutes at 10,000 g at room temperature. The RNA was transferred to isopropanol and incubated for 10 minutes at room temperature. After 10 minutes centrifugation at 8000 g at 4 °C, the RNA pellet was washed with 75% ethanol and dissolved in RNase free water. The quantity and quality of total RNA was checked, RNA was diluted 1:10 with RNase free water and measured using a Nano-drop spectrophotometer (Agilent). The RNA samples were stored at -80 °C until use for gene microarray or qPCR analysis.

2.11 Gene microarray analysis

RNA was extracted from the MCF-7 and MCF-7TaxR cell lines as described in section 2.10. After the quantity and quality of RNA samples were checked, as described in 2.10 using the Nano-drop spectrophotometer (Agilent), RNA samples were sent on dry ice to
perform oligo GEArray® human apoptosis microarray profiling of the expression of 112 apoptosis related genes by the SuperArray Bioscience Corporation, Frederick, USA.

2.12 Quantitative PCR analysis

Two methods for the qPCR analysis were carried out, a one step method and a two step method. However, both methods gave the same results. As described previously, in section 2.10, RNA was extracted with the Trizol reagent. The qPCR analysis was carried out using the SYBR Green reagent; the synthesis of cDNA is all in one step with RT-PCR. The kit used was a Qiagen® One-step QuantiTect® SYBR® Green RT-PCR kit. Following the manufacturer's instructions, a mixture was prepared with an RNA concentration of 50-100 ng/μl, 6.25 μl of QuantiTect SYBR Green RT-PCR master mix, 0.125 μl QuantiTect RT mix, 1 μl each of forward and reverse primers, or 1.25 μl forward and reverse combined primer mix, and 1-2 μl RNase-free water. The total volume for the reaction is 12.5 μl/reaction. Primers were provided from SuperArray® and reconstituted to give a 10 μM working solution. The PCR reactions were carried out as follows: 30 minutes at 50 °C for reverse transcription; 15 minutes at 95 °C for the PCR initial activation step; 40 cycles of denaturation for 20 sec at 95°C and annealing for 60 sec at 60 °C.

The second method is a two step analysis or indirect method, which involved first strand cDNA synthesis in a separate step and was carried out using a reaction mixture of the ImProm-II™ Reverse Transcription System from Promega®. Following the manufacturer's instructions, the reaction mixture was made using 0.5 μg RNA, 0.5 μg random primer, 2 μl nuclease-free water, and the sample was then heated for 5 minutes at 70 °C. To add the reverse transcription reaction mix, the mixture was prepared as follows: 5× ImProm-II™
reaction buffer, 0.5 mM dNTPs, 1.5 mM MgCl2, 20 U recombinant ribonuclease inhibitor, 1 U ImProm-II™ Reverse Transcriptase, for each sample mixture. The reaction mixture was left at 25 °C for 10 minutes followed by 60 minutes at 42 °C. The reaction was terminated by incubation at 70 °C for 15 minutes. The cDNA was then subjected to qPCR using the SYBR Green reagent obtained from Stratagene®. The reaction mixture was prepared as follows: 1 µl of each primer, 1 µl cDNA template, 12.5 µl SYBR Green reagent, RNase free water to 25 µl total volume. Samples were subjected to qPCR analysis. Primers were provided from SuperArray® and reconstituted to give a 10 µM working solution. The PCR reactions were carried out as follows: 10 minutes at 95 °C for initial activation; 40 cycles of denaturation for 20 sec at 95 °C, and annealing for 60 sec at 60 °C (Stratagene®-Mx 3000P QPCR system).

Primers were human CDKN2A (p16), caspase-7 and caspase-9, obtained from SuperArray Bioscience Corporation.

*(p16)* human gene sequence primer 5'-3': Forward, CAGAGGATTTGAGGGACAGG; Reverse, CTCCTCTTTCTTCCTCCGGT.

*Caspase-7 human gene sequence primer 5'-3':* Forward, CCGTGGAACGTAAGAAGAA; Reverse, GTCTTGATGGATCGCATGG.

*Caspase-9 human gene sequence primer 5'-3':* Forward, CCTGCTTAGAGGACACAGG; Reverse, TTCGACAACTTTTGTGCTGCTTGG.
2.13 Quantitation of acidic vesicles by acridine orange staining

Autophagic cell death was assessed in breast cancer cell lines by the quantitation of acidic vesicles stained with acridine orange, as described previously (Datzynkiewicz 1990). The breast cancer cell lines MCF-7, MCF-7TaxR, and MDA-MB-231 were plated at a density of 2×10^4 or 3×10^4 cells in T25 flasks and allowed to adhere for 24 h. Cells were then treated with 0.5 μM staurosporine, or untreated for 24 h or 48 h. Cells were harvested by trypsinisation and washed with PBS, and, then 500 μl of buffer was added to all samples. To inhibit autophagy, cells previously treated with staurosporine were treated with 5 μM bafilomycin for 1 h followed by staining with acridine orange (2 μg/ml stock solution diluted 1:1000 in buffer II) for 1 h at room temperature in the dark. Samples were then analyzed by flow cytometry using FL1 (525 nm) and FL4 (630 nm). The cytoplasm and nucleoli of stained cells fluoresced bright green and dim red, respectively, whereas autophagic cells containing acidic vesicles fluoresce bright red.

2.14 LC-3 Confocal microscopy imaging

MDA-MB-231, MCF-7, and MCF-7TaxR cells were analysed for LC-3. MDA-MB-231 cells were used as a negative control for LC-3. All cells were grown in T25 flasks at densities of 2×10^5 or 3×10^5 cells per flask and left untreated, as a control, or treated with 0.5 μM or 0.25 μM staurosporine. Cells were incubated for 24 h and then harvested and processed for cytospin (Cytospin 4 Thermo Electron Corporation, Basingstoke, Hants) forming a monolayer pellet on the slides. The cytospin slides were fixed with 2-4% formaldehyde in PBS for 15 minutes and rinsed with PBS. Slides were covered with ice-
cold methanol (100%) and were kept in the freezer for 10 minutes (methanol permeabilization stage). Samples were then rinsed twice with PBS for a duration of 5 minutes. Samples were blocked with 5% anti-goat secondary antibody for 1 h and washed with PBS three times. This was followed by incubation with the primary antibody LC-3 using a dilution of 1:200 to 1:100 overnight using PBS/Triton. After draining off the blocking serum solution from each slide, 50-100 µl of diluted antibody per cytospin or coverslip was added. Slides were incubated overnight in a humidifying chamber in the cold room. The next day, slides were washed with PBS and incubated with Alexa-Fluor diluted with PBS/Triton to approximately 1:200 (to conjugate to LC-3) for 2 h. Slides were rinsed with PBS and nuclear dye (1:200 dilutions) was added to the slides for 10 minutes and finally washed with high salt PBS. Slides were left to dry and, then, anti-fade and sealed cover slips with nail polish were added. Imaging of the slides was carried out using confocal microscopy at 40x magnification (Carl Zeiss Laser Scanning System LSM510).

### 2.15 Electron microscopy for detection of autophagosomes.

For detection of autophagosomes in MCF-7TaxR cells and in MCF-7 cells, we performed an electron microscopy analysis. MCF-7 and MCF-7TaxR cells were grown in T75 flasks at a density of 3×10^4, the cells were untreated, or were treated with 0.5 µM or 1 µM staurosporine for 48 h. Cells were harvested and collected in 200 µl solution forming a pellet. The processing and embedding of cells was performed with collaboration of researchers at Brunel University. Pellets were fixed with 2% glutaraldehyde for 10 minutes at room temperature, washed with phosphate buffer for 10 minutes three times, and fixed
with 1% osmium tetroxide in phosphate buffer for 1 h at room temperature. Cells were embedded in 2% agar, heated on a hot block to 50 °C and stirred using a glass rod. The consistency was correct when droplets set firmly around the edges on a cold surface. Approximately 0.5 ml agar was added to the pellets and very gently mixed so that cells were in contact with the agar, but still highly concentrated at the tip of the tube. The pellet must have no excess phosphate buffer present as this would prevent the agar setting to the correct consistency. The agar with cells was set and cut into small chunks approximately 2-3 mm² using a blade on some dental wax, before being added to a small tube. The samples were washed with distilled water 2 times for 5 minutes and dehydrated with ethanol for 15 minutes in each of the following ethanol solutions: 30%, 50%, 70%, 90% 2 times, and 100%. Samples were incubated overnight in propylene oxide at room temperature. The next day cells were incubated with fresh resin for 6 h. Cells were embedded by transferring the agar to a plastic mould and adding fresh resin. This was gently mixed with a cocktail stick so that no air bubbles were present at the bottom of the tube and the agar was pushed into the tip of the tube. Resin was incubated at 65 °C with lids open. Ultrafine sections of 100 nm thickness or less were made. Sections were collected on grids and allow to dry for 10-15 minutes. These sections were stained with 2% uranyl acetate made in distilled water for 1-2 h by placing the grids on droplets on dental wax in a glass dish followed by washing with distilled water. Sections were stained on lead citrate droplets for 4 minutes and left on dental wax in a covered glass dish. Potassium hydroxide pellets were added to the dish to absorb CO₂. The grids were taken for electron microscopy imaging. Samples (grids) were analysed by scanning transmission electron microscopy STEM (assisted by Dr. Vladimir Stolojan, UniS) (Philips CM200 Transmission Electron Microscopy- using 2 um magnification.) at the MSSU- University of Surrey.
2.16 Statistical analysis

Data were expressed as means ± standard deviation. Significance was determined using a two-tailed, paired, Student's T-test, using the SPSS software or Microsoft Excel. A P-value <0.05 indicated a significant difference.
CHAPTER 3

INDUCTION OF APOPTOTIC CELL DEATH IN DRUG RESISTANT HUMAN BREAST CANCER CELLS
3 Induction of Apoptotic Cell Death in Drug Resistant Human Breast Cancer Cells

3.1 Programmed cell death I - Apoptosis

The term apoptosis is derived from the Greek for ‘falling off’, like leaves fall from trees (Kerr et al. 1972). This process is important for tissue development and homeostasis (Wu 1996; Taylor et al. 2008). Apoptosis was first described by Kerr and colleagues (1972), who coined the term ‘apoptosis’ for programmed cell death and reported the occurrence of apoptosis in adults in relation to health and disease. Normally, apoptosis is a physiological process that is vital to embryonic development and the immune system and also occurs in the elimination of excess neurones during development (Okada and Mak 2004; Schimmer 2004). During early development of the embryo the role of apoptosis is important in configuring the shape of organs and the removal of the interdigital webs of fingers and toes (Renehan et al. 2001; Schimmer 2004). In cases such as the immune and nervous systems, which produce a high number of cells to serve different functions in the body, when these cells eventually fail to function, as in making synaptic connections or producing specific antigens, apoptotic cell death plays a role in subsequently clearing the unwanted cells (Renehan et al. 2001; Okada and Mak 2004). Apoptosis maintains the balance of cellular turnover in adulthood, since approximately ten billion cells die every day and new cells are produced from the stem cell populations in the body (Renehan et al. 2001; Fischer and Schulze-Osthoff 2005). The control of tissue homeostasis by apoptosis is a highly regulated process (Renehan et al. 2001; Okada and Mak 2004). During ageing, however, the mechanisms of apoptosis become less controlled and less sensitive to DNA damage (Renehan et al. 2001; Okada and Mak 2004; Fischer and Schulze-Osthoff 2005).
Hence, there is a relationship between the development of degenerative diseases and delayed progression of cell death (Renehan et al. 2001; Okada and Mak 2004; Fischer and Schulze-Osthoff 2005). Moreover, there is now accumulating evidence that defects in apoptosis are associated with a number of diseases (Thompson 1995). Apoptosis is up-regulated in AIDS, as well as in ischemic injury following myocardial infarction and stroke, and increased apoptosis is a feature of autoimmune diseases such as hepatitis (Renehan et al. 2001). Conversely, a reduction in the apoptotic response is a factor in carcinogenesis, autoimmune diseases and some viral infections (Martin et al. 1998; Renehan et al. 2001). Therefore, impaired apoptotic function is involved in many pathological conditions and, thus, suggests that apoptotic pathways/components represent potential therapeutic targets (Fischer and Schulze-Osthoff 2005).

The morphological and biochemical characteristics of apoptosis show a sequence of events resulting in cell death (Kerr 2002; Okada and Mak 2004; Shiozaki and Shi 2004; Tardy et al. 2006). Mechanisms of apoptosis can be divided into three stages: (1) initiation of apoptosis via inducing agents, (2) activation of caspases, and (3) proteolytic cleavage of cellular materials (Simstein et al. 2003). Apoptosis is quite distinct from necrosis, with morphological features involving membrane blebbing, cellular shrinkage, nuclear and cytoplasmic condensation, and degradation of chromosomes into nucleosomal fragments (Okada and Mak 2004; Shiozaki and Shi 2004). A major feature of the apoptotic cascade is the activation of a family of proteases called caspases followed by formation of 'apoptotic bodies' that are cleared and removed by phagocytosis (Okada and Mak 2004; Shiozaki and Shi 2004). The biochemical events of apoptosis are complex and induced by signal transduction events that lead to activation of caspases, which, in turn, cleave
cellular proteins (Schulze-Osthoff et al. 1998; Tardy et al. 2006). Biochemical changes include translocation of phosphatidylserine to the outer leaflet of the plasma membrane and extensive inter-nucleosomal genomic DNA degradation carried out by selectively activated endonucleases (Schulze-Osthoff et al. 1998; Tardy et al. 2006). There are two pathways of apoptosis that have been described in the literature, the extrinsic and intrinsic pathways (Figure 3-1). Both pathways involve an activation of caspase cascades (Srinivasula et al. 1996; Thornberry and Lazebnik 1998).

![Extrinsic Pathway](image)

**Extrinsic Pathway**
- Ligands (FasL, TNF-α, TRAIL)
- Death receptors (Fas, TNFR)
- FADD, Pro-C-8
- Caspase-8
- Caspase-3

**Intrinsic Pathway**
- Stress, DNA damage, UV, Chemotherapy
- Mitochondria
- Bcl-2
- Cytochrome c
- dATP
- Smac/DIABLO
- Apoptosome

**APOPTOSIS**

Figure 3-1: Schematic representation of the two apoptotic pathways. (Srinivasula et al. 1996; Thornberry and Lazebnik 1998; Simstein et al. 2003; Okada and Mak 2004; Schimmer 2004).
3.1.1 Apoptotic key regulators- caspases

Caspases are a group of cysteine-dependent aspartate-specific proteases (Thornberry et al. 1992; Alnemri et al. 1996). Caspases cleave substrates at Asp-Xxx bonds (Thornberry et al. 1992; Alnemri et al. 1996). Approximately fourteen types of caspases have been identified in mammals, but not all are involved in apoptotic cell death mechanisms (Cohen 1997). Caspases can be classified into three groups based on their functional activity: (1) initiator caspases for apoptosis include caspase-2, -8, -9 and -10; (2) executor caspases of apoptotic cell death include caspases 3, 6 and 7; (3), caspases involved in cytokine activation include caspase-1, -4, -5, -11, -13 and -14 (Zhang 2004-2005). Not all caspases, however, are fully understood in terms of their functionality (Zhang 2004-2005).

Caspases are synthetised as inactive single polypeptides termed zymogens or pro-caspases (Zhang 2004-2005). The zymogen consists of an N-terminal prodomain followed by sequences containing first large and then small subunits (Zhang 2004-2005). The zymogen component of caspases possesses an intrinsic proteolytic activity in order to initiate the apoptotic pathway (Stennicke and Salvesen 1999). When a pro-caspase is activated, it requires two cleavages to transform the zymogen or pro-caspase into a catalytically active enzyme (Zhang 2004-2005). These two cleavage reactions involve separation of the prodomain from the large subunit and separating the large and small subunits, involving Asp-X bond cleavage (Zhang 2004-2005).

Activation of initiator and executioner caspases takes place by two mechanisms, the intrinsic and extrinsic pathways (Zhang 2004-2005). The initiator caspases are stimulated by recruitment of their zymogen to the corresponding regulator protein (Zhang 2004-
CHAPTER 3

2005). Executioner caspase activation requires the activation of initiator caspases leading to induction of the downstream proteolytic apoptotic pathway (Zhang 2004-2005). The initiator caspases play an important part in the apoptotic process by initiating the execution phase of the process; hence, different initiators, adaptors and regulators of caspases are needed for regulating the proteolytic mechanisms of different cell death inducers (Zhang 2004-2005). In Fas- and TNFRI-mediated signal transduction, the activation of caspase-8 is important and requires the Fas-associated death domain protein (FADD) to initiate catalytic activation (Figure 3-1) (Varfolomeev et al. 1998; Zhang et al. 1998). Activation of caspase-9 and Apaf-1 is essential for DNA damage, corticosteroid-, and staurosporine-induced cell death in thymocytes and embryonic fibroblasts (Hakem et al. 1998; Yoshida et al. 1998; Vu et al. 2001). The recruitment of caspase-10 requires FADD for TRAIL and Fas mediated signalling of apoptosis (Sprick et al. 2002). In human T-cells, both caspase-8 and caspase-10 have been shown to be associated with the death effector domain and are important in Fas-induced apoptosis (Wang et al. 2001). Caspase-12, which functions in the maturation of cytokines, is also considered an initiator caspase, since it was shown to be activated by stress-induced apoptosis in the endoplasmic reticulum (Nakagawa et al. 2000; Hitomi et al. 2004).

The executioner caspases are activated following the recruitment of initiator caspases as part of the apoptotic cascade (Fernandes-Alnemri et al. 1995; Zheng et al. 2000). The most important executioner caspase is caspase-3, which was found to be vital for embryonic development; caspase-7, another key executioner caspase, has been shown to compensate for caspase-3 function (Fernandes-Alnemri et al. 1995; Zheng et al. 2000). Studies of caspase-7 showed that a caspase-7 gene defect in cells confers resistance to
common apoptosis-inducing agents such as anti-cancer drugs and disrupts externalisation of phosphatidylserine on the plasma membrane and DNA fragmentation (Korfali et al. 2004).

Caspase function is controlled by a group of inhibitor of apoptosis proteins (IAPs), consisting of a family of regulatory proteins which include XIAP, cIAP1, cIAP2, NAIP, ML-IAP, and survivin (Deveraux et al. 1999; Salvesen and Duckett 2002; Sanna et al. 2002). The IAP family binds selectively to caspase-3, -7 and -9, hence blocking apoptosis (Deveraux et al. 1999; Salvesen and Duckett 2002; Sanna et al. 2002; Schimmer 2004). IAPs can, however, be inhibited by the second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pl (Smac/DIABLO), which is released from the mitochondria with cytochrome c (Figure 10) (Mita et al. 2006). Overexpression of IAPs can be caused by the anti-apoptotic transcription factor, nuclear factor kappa B (NF-κB), which can be indirectly stimulated by growth factors via the phosphoinositol-3 phosphate (PI3K/Akt) pathway (Conradt and Horvitz 1998).

### 3.1.2 Intrinsic pathway

The main body of evidence to date confirms that the mitochondria are the main core of the intrinsic pathway along with a number of apoptotic regulators that inhibit or activate apoptosis (Adrain and Martin 2001; Wang 2001; Zamzami and Kroemer 2001; Logue and Martin 2008). This pathway is induced by different cellular stresses such as heat shock, DNA damage or other forms of stress, and the mitochondria starts to release cytochrome c from the inter-membrane space into the cytoplasm (Adrain and Martin 2001; Wang 2001; Zamzami and Kroemer 2001; Logue and Martin 2008). Subsequently, cytochrome c binds
to and activates oligomerization of Apaf-1 in the presence of dATP, which assemble together in a wheel structure with pro-caspase-9 to build the apoptosome complex (sometimes referred to as the “wheel of death”) (Li et al. 1997; Srinivasula et al. 1998; Slee et al. 1999). The apoptosome activates caspase-9, followed by activation of the executioners caspase-3 and -7, leading to activation of a downstream caspase cascade (Li et al. 1997; Srinivasula et al. 1998; Slee et al. 1999).

Although caspase-7 shows no involvement in activation of downstream caspases, caspase-3 drives the activation of caspase cascades by proteolysis and leads to activation of caspase-2 and 6 (Rodriguez and Lazebnik 1999; Slee et al. 1999; Slee et al. 2001; Acehan et al. 2002). Different studies on knockout mice have explored the importance of caspase-9 and Apaf-1 in activating downstream caspases in this pathway (Logue and Martin 2008). Other studies have shown that using different stress-inducing agents, such as cytotoxic drugs and radiation, on cells derived from caspase-9 null animals, results in resistance to these agents (Hakem et al. 1998; Kuida et al. 1998). Apaf-1 usually works as a monitor to detect cell damage that results in release of mitochondrial proteins into the cytosol, which is not usually found in healthy cells (Logue and Martin 2008). Apaf-1 exists as a monomeric protein when cytochrome c is absent, making it unable to bind to pro-caspase-9 (Logue and Martin 2008).

According to a number of studies, the Bcl-2 family proteins function as powerful and important regulators of apoptotic pathways (Kroemer 1997; Adams and Cory 1998). Evidence that has accumulated in the last few years shows that the Bcl-2 family proteins consist of seventeen members: some proteins promotes apoptosis and others inhibit
apoptotic cell death (Logue and Martin 2008). Members of the Bcl-2 family typically consist of four conserved α-helical domains, BH1, BH2, BH3, and BH4, that are involved primarily in the interactions between these proteins (Chittenden et al. 1995; Cheng et al. 1996; Adams and Cory 1998; Kelekar and Thompson 1998; Reed 1998). The anti-apoptotic subfamily members are Bcl-2, Bcl-XL, Bcl-w, Bcl-b, Mcl-1, and A1. The pro-apoptotic proteins include Bax, Bak and Bok, which have domains homologous to the BH1-3 domains, and the pro-apoptotic BH3-only proteins, Bid, Bim, Bad, Bik and PUMA, which act as ligands and contain only a BH3 domain (Zhang 2004-2005). These proteins function as antagonists/agonists that determine the response of the cell to apoptotic death signals and they mostly function in the external mitochondrial membrane (Mita et al. 2006). Bcl-2 anti-apoptotic proteins inhibit Apaf-1 mediated activation of caspase-9 (Coultas and Strasser 2003). In addition, Bax/Bok proteins promote cytochrome c release from the mitochondria whereas Bcl-2 proteins prevent this (Green and Reed 1998; Gross et al. 1999; Wang 2001). Therefore, it has been suggested that these proteins control mitochondrial membrane integrity (Green and Reed 1998; Gross et al. 1999; Wang 2001).

3.1.3 Extrinsic pathway

The extrinsic pathway is controlled by a number of tumour death receptors and their ligands through induction of signal transduction pathways in order to activate apoptotic cascades (Mita et al. 2006; Logue and Martin 2008). The key regulator death receptors are TNF superfamily receptors including the functional death domain receptors DD, CD95 (Fas/APO-1) and the TNF-related apoptosis-inducing ligand receptor -1 (TRAIL-1), and TRAIL-2 receptors (Mita et al. 2006). Therefore, when a ligand binds to its death
receptor, the adaptor protein FADD recruits pro-caspase-8 and -10, forming the death-inducing signalling complex (DISC), and activates these two caspases (Ashkenazi and Dixit 1998; Schulze-Osthoff et al. 1998; Suliman et al. 2001). Consequently, caspase-8 and -10 result in activation of downstream executioner caspases leading to induction of apoptosis (Mita et al. 2006).

In addition, it has been reported that caspase-8 can amplify the intrinsic pathway by stimulating cleavage of Bid via permeabilization of the mitochondrial membrane and give rise to caspase-9 activation (Li et al. 1998; Fischer et al. 2003; Mita et al. 2006). Apoptosis induced by TRAIL, however, essentially requires the recruitment of FADD to the DISC which in turn activates caspases-8 and -10 (Bodmer et al. 2000; Kuang et al. 2000; Kischkel et al. 2001; Suliman et al. 2001). Any defect or mutation in caspase-8 or FADD will be associated with TRAIL resistance (Bodmer et al. 2000; Kuang et al. 2000; Kischkel et al. 2001; Suliman et al. 2001).

3.2 Deregulation of apoptotic pathways in cancer

Deregulation and inactivation of apoptotic pathways leads to cell survival and facilitates the progression and, therefore, the development of cancer (Taylor et al. 2008). When the cell cycle and cell division are not properly controlled, as in cancer cells, there is a resulting loss of balance between cell proliferation and cell death (PCD I/apoptosis) (Shao et al. 2004; Gerl and Vaux 2005; Tardy et al. 2005). Therefore, the lifespan of the cancer cell will be extended, promoting initiation of tumour formation (Shao et al. 2004; Gerl and Vaux 2005; Tardy et al. 2005). Deregulation in the intrinsic pathway involves overexpression of anti-apoptotic proteins such as Bcl-2 (Gross et al. 1999; Reed 1999). It
has been reported that cancers have low levels of expression of Apaf-1, a vital component in forming the apoptosome complex to activate caspase-9 (Soengas et al. 2001). Defects in overexpression of IAPs can result in inhibition of all intrinsic and extrinsic apoptotic pathways (Soengas et al. 2001). Hence, overexpression of survivin results in a major impact on resistance to treatment and carcinogenesis in a number of cancers (Deveraux and Reed 1999; Soengas et al. 1999; Green and Beere 2001). Also, other studies have shown an upregulation of the PI3K/Akt pathway, which may be caused by increased gene expression of PI3K or Akt and this can promote cell survival and inhibit apoptotic cell death (Datta et al. 1999; Shayesteh et al. 1999; Di Cristofano and Pandolfi 2000).

Defects in the extrinsic pathway may be caused by mutation or inhibition of death receptor expression or poor transport of TRAIL (Jin et al. 2004). Mutations or deletions in various caspases have been demonstrated in cancer cell lines. For example, in the MCF-7 breast cancer cell line there is a complete absence of the caspase-3 gene owing to a chromosome deletion (Kurokawa et al. 1999; Xue et al. 2003). Cancer progression and resistance to treatment may be caused by deficient expression of pro-caspase-8 (Ashkenazi 2002). Somatic mutations occur in different pro-apoptotic proteins such as the BH3 and Bax proteins, as determined in different solid tumours and haematological malignancies (Ashkenazi 2002).
3.3 Aim

In this first part of this project, I investigated the *in vitro* induction of non-caspase-dependent or apoptosis independent cell death in the breast carcinoma derived cells MCF-7 and MCF-7TaxR (with *in vitro* acquired resistance to paclitaxel). The response to anticancer drugs was examined in MCF-7 cells (lacking caspase-3) in terms of how they may activate apoptosis or other forms of cell death in response to cytotoxic insult. We hypothesized that MCF-7 cells show a moderate level of apoptotic cell death in response to anticancer drug treatments. However, for the MCF-7TaxR variant we would expect a severely compromised or reduced apoptotic response under the same conditions. We used the MDA-MB-231 and T47D breast cancer cell lines for comparison as they are caspase proficient (no apparent deletions of caspases reported) and, thus, were studied in the same manner as positive controls for apoptosis.

3.4 Methods

All methods carried out in this chapter are described in Chapter 2: Methods and Materials. I used the MTT assay, Annexin-V FITC/PI protocol and fluorescence microscopy using Hoechst nuclear staining.

3.5 Results

3.5.1 MTT assay for chemosensitivity testing

The MTT dye reduction assay (which assesses cell viability due to mitochondrial activity) was used as described (Chapter 2). Preliminary cytotoxicity studies were carried out to assess the activity of the anticancer drugs paclitaxel, cisplatin, carboplatin and doxorubicin.
on the MCF-7, MCF-7TaxR, T47D, T47DTaxR and MDA-MB-231 cell lines. Cells were treated with different concentrations of anticancer drugs (as described in section 2.4-Chapter 2). Cell viability was assessed at 72 h or 96 h (according to the cell doubling time of the cell line, in order to coincide with approximately a 10-20-fold increase in cell number) and compared with untreated controls.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cisplatin IC$_{50}$ μM</th>
<th>Carboplatin IC$_{50}$ μM</th>
<th>Doxorubicin IC$_{50}$ nM</th>
<th>Paclitaxel IC$_{50}$ nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>17 (± 2)</td>
<td>84 (± 8)</td>
<td>1000 (±225)</td>
<td>6.5 (± 4.5)</td>
</tr>
<tr>
<td>MCF-7TaxR</td>
<td>7.5 (± 1.12)</td>
<td>26 (± 8)</td>
<td>1080 (±229)</td>
<td>77.7 (± 6.1)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>5.6 (± 2.7)</td>
<td>15 (± 2)</td>
<td>-</td>
<td>4.5 (± 2.2)</td>
</tr>
<tr>
<td>T47D</td>
<td>45 (± 7)</td>
<td>-</td>
<td>-</td>
<td>1.6 (± 0.045)</td>
</tr>
<tr>
<td>T47DTaxR</td>
<td>75 (± 9.8)</td>
<td>-</td>
<td>-</td>
<td>10 (± 8)</td>
</tr>
</tbody>
</table>

Table 3-1: Determination of drug sensitivity of breast cancer cell lines to different anticancer drugs.

Data are the mean IC$_{50}$ values for at least 4 separate experiments (performed in quadruplicate) with standard deviation (±SD) in parentheses. IC$_{50}$ values were determined as the drug concentration necessary to cause a 50% reduction in cell viability compared to untreated controls.

By expressing the IC$_{50}$ value of the resistant cell line in terms of the drug sensitive parental line (i.e. as a ratio), the level of drug resistance can be calculated for the resistant cell line. Hence, the MCF-7TaxR line showed a 77.7/6.5 = 11.9 fold increase in resistance to paclitaxel. However, the MCF-7TaxR cell line showed significant sensitivity (collateral sensitivity) to cisplatin and carboplatin when compared to the parental MCF-7 cells. With doxorubicin, the level of drug sensitivity was similar in both MCF-7 and MCF-7TaxR cells. MDA-MB-231 cells showed the highest relative level of sensitivity to the anticancer drugs examined. The IC$_{50}$ of T47DTaxR cells indicates a decrease in sensitivity to cisplatin (approximately 1.6 fold increase in resistance) and an approximately 6 fold
increase in resistance to paclitaxel compared with the parent T47D cells.

### 3.5.2 Preliminary testing of Z-VAD and paclitaxel response in MCF-7 and MCF-7TaxR cells using the Trypan blue excision test

In this first phase of the work, I set out to investigate the overall death response in the selected human breast cancer cell lines. Preliminary testing was carried out as a guide for the design of the major experiments planned for subsequent stages of the work. Using the pan-caspase inhibitor Z-VAD, the contribution of apoptotic (caspase-mediated) cell death in response to paclitaxel treatment of the MCF-7 and MCF-7TaxR lines was examined. The testing was carried out using cells grown in 96 well plates incorporating paclitaxel treatment with or without Z-VAD. Cell death after 72 h of drug exposure was assessed with Trypan blue staining of cells and quantified using a haemocytometer.
Figure 3-2: Determination of the inhibitory effect of Z-VAD on apoptotic cell death in MCF-7 and MCF-7TaxR cells. Cells were harvested following treatment with 100 μM Z-VAD in the presence or absence of different paclitaxel concentrations for 72 h. Cells were stained with Trypan blue as described in Chapter 2, Materials and Methods. The percentage of cells staining with trypan blue represents the mean percentage of dead cells obtained from three independent experiments. Error bars are ± SD. C represents control untreated cells.

Figure 3-2 shows the effect of Z-VAD on MCF-7 and MCF-7TaxR cells in combination with paclitaxel treatment. The total average effect of Z-VAD treatment on paclitaxel-induced cell death was approximately 5.4% in MCF-7 cells. There was no apparent
protection of cell death in MCF-7TaxR cells following incubation with Z-VAD.

3.5.3 Effect of staurosporine and Z-VAD on apoptotic cell death induction using Annexin V-FITC/PI staining

Annexin V-FITC/PI staining with flow cytometric analysis was performed to investigate the induction of non-apoptotic cell death in MCF-7 and MCF-7TaxR cells. The MDA-MB-231 cell line (which does not possess any deletions in caspases according to the current literature) was used as a positive control for apoptosis induction in these experiments. We used staurosporine treatment, as previously used by many others, for the induction of cell death (Xue et al. 2003). Staurosporine is a protein kinase inhibitor which induces cell growth arrest leading to cell death (Tamaoki et al. 1986). In addition, we used 100 µM of Z-VAD (a pan caspase inhibitor) in order to assess the contribution of caspases in the cell death response in the different breast cancer cell lines. A concentration of 100 µM Z-VAD was used, based on previous studies involved MCF-7 cell line (Kirsch et al. 1999), and also on our results, to give a maximal effect without giving rise to significant cell death over the experimental period. The treatment protocol for MDA-MB-231 cells was only for 24h (as opposed to 48h for MCF-7 lines) as this cell line is very sensitive to drug treatment and a 48h exposure gave rise to very substantial cell death, making analysis difficult. The Annexin V-FITC/PI staining method is based on the use of two fluorescent probes, Annexin V conjugated to FITC and propidium iodide (PI) in order to distinguish between viable, early apoptotic, late apoptotic, and necrotic cells.
CHAPTER 3

MDA-MB-231 Cells

24h

- Control
- Annexin-V FITC +ve
- 100 µM Z-VAD

(a) 0.5 µM staurosporine
(b) 0.5 µM staurosporine + 100 µM Z-VAD

MCF-7 Cells

48h

- Control
- 100 µM Z-VAD

(b) 0.5 µM staurosporine
(b) 0.5 µM staurosporine + 100 µM Z-VAD
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Figure 3-3: Annexin V-FITC/PI flow cytometric analysis of apoptotic cell death induction in human breast cancer cell lines. The histograms are representative of typical data obtained. Cells were grown in T25 flasks and treated with 0.5 μM staurosporine or 100 μM Z-VAD or both for 24 h or 48 h. Cells were harvested and processed for Annexin V-FITC/PI staining analysis as described in Chapter 2, Materials and Methods, using flow cytometry. The data are presented in a four quadrant format: F1, necrotic or end stage apoptosis; F2, mid-phase apoptosis; F3, viable cell population; F4, early phase apoptosis.

Figure 3-3 depicts typical data obtained from the Annexin-V FITC/PI analysis. The results showed differences between staurosporine treatment versus staurosporine and Z-VAD treatments of 41.2 - 92.2%, 81.7 - 90.2% and 65.2 - 66% for MDA-MB-231, MCF-7 and MCF-7TaxR cells respectively.
Figure 3-4: Apoptosis inhibition by Z-VAD treatment in MDA-MB-231 but not in MCF-7 and MCF-7TaxR cells.
The graph shows a significant level of apoptosis inhibition in MDA-MB-231 cells in response to Z-VAD treatment. MCF-7 and MCF-7TaxR showed no significant inhibition of apoptosis. Data shown (obtained from Annexin V -ve/ PI -ve cells (%) quadrant -F3) are the means of 4 independent experiments. Error bars represent ±SD. * indicates p-value < 0.005 using Student’s paired T-test. (NS) non-significant.

Figure 3-4 summarises data obtained from the Annexin V-FITC/PI analysis of four independent experiments with the MDA-MB-231, MCF-7 and MCF-7TaxR cells using the Annexin V-FITC/PI method, as shown in Figure 3-3. The MDA-MB-231 cells, as a consequence of their caspase proficiency, showed significant differences between staurosporine treatments without and with Z-VAD treatment, as shown by the viable cell populations of 33.6% (±9.65) and 89.9% (±5.17), respectively p=0.004. MCF-7 cells showed no significant differences between staurosporine treatment without and with Z-VAD 73.2% (±25.69) versus 79.6% (±22.45), p=0.091. A modest effect of Z-VAD protection was seen but this contrasted with the effects seen for MDA-MB-231. MCF-7TaxR cells, showed no significant difference between staurosporine treatment without and with Z-VAD, 71.8% (±15.96) versus 75% (±19.36), p=0.234. Thus, the extent of caspase-mediated cell death (as assessed using Z-VAD protection) can be summarised as
MDA-MB-231 >> MCF-7 >> MCF-7TaxR.

3.5.4 Nuclear Hoechst staining for examination of cell death morphology in human breast cancer cells

Nuclear staining was carried out to check cell death morphology in MCF-7, MCF-7TaxR, and T47D cell lines with Hoechst 33258 dye (as described in Chapter 2, Materials and Methods) after treatment with 1 μM staurosporine for 48 h. Images were collected using fluorescence microscopy.
Figure 3-5: Fluorescence microscopy of the nuclear morphology changes in T47D, MCF-7 and MCF-7TaxR.
Control untreated cells are shown (a), (c), and (e) and cells treated with 1 μM staurosporine for 48 h shown in (b), (d) and (f). Cells were fixed and stained with Hoechst 33258. Arrows show dead cells with apoptotic or non-apoptotic features. Magnification, 40x. Images are representative of repeat experiments (n = 3).
In Figure 3-5 (b), T47D cells treated with 1 μM staurosporine for 48 h showed classical features of apoptotic cell death associated with nuclear fragmentation, chromatin condensation and formation of apoptotic bodies. Comparing image (b) with image (d), for staurosporine treated MCF-7 cells, features of cell death were apparent, with fragmented nuclei but no apoptotic body formation, not strictly consistent with apoptotic cell death. For MCF-7TaxR cells treated with staurosporine (f), the nuclei appeared intact with a relative reduction in cell number but no apoptotic cell death features as seen with staurosporine treated T47D cells.

3.5.5 Effect of anti-Fas induced apoptosis, detected using Annexin V- FITC/PI analysis

We went on to examine the effect of CD95 or anti-Fas in mediating apoptotic cell death in these cells. Cycloheximide (CHX) is an inhibitor of protein synthesis which can induce cell death by apoptosis (Tang et al. 1999). Many reports have indicated that different cells become sensitised to cell death by agents such as staurosporine in the presence of cycloheximide, possibly due to enhancement of DISC formation (Tang et al. 1999). HeLa cells were used as a positive control for the effects of FAS and cycloheximide, in accord with the previous literature (Wajant et al. 2000). The treatment protocol for HeLa cells was only for 48 h (as opposed to 72 h for MCF-7 lines) as this cell line is very sensitive to anti-Fas treatment and a 72 h exposure gave rise to very substantial cell death, making analysis difficult. The experiment used the Annexin V-FITC/PI method of analysis, as above.
Figure 3-6: Light microscopy images of MCF-7, MCF-7TaxR, and HeLa cells showing the effect of anti-Fas treatment. Cells were treated with 200 ng/ml anti-Fas in the absence or presence of 1 μg/ml CHX for 48 h for HeLa cells, or 72 h for MCF-7 and MCF-7TaxR. (Magnification, 100x).
Figure 3-6 shows the effect of anti-Fas on HeLa cells and served as a positive control for the experiment. The MCF-7 cell line showed some resistance to Fas-induced apoptotic cell death compared to the HeLa cell control. In the case of MCF-7TaxR cells, there was also resistance to cell death induction in the presence of anti-Fas. Thus, the MCF-7 and MCF-7TaxR cell lines appeared refractory to Fas-mediated apoptosis.
CHAPTER 3

48h

HeLa Cells

(a)

72h

MCF-7 Cells

(b)
Figure 3-7: Annexin V-FITC/PI analysis of HeLa, MCF-7 and MCF-7TaxR cells shows the effect of anti-Fas and CHX in induction of apoptotic cell death. The histograms are representative of typical data obtained. Cells were treated with 200 ng/ml anti-Fas or 1 µg/ml CHX, alone or in combination for 48 h or 72 h. Cells were harvested and processed for Annexin V-FITC/PI staining analysis using flow cytometry, as described in Chapter 2, Materials and Methods. The data are presented in a four quadrant format: F1, necrotic or end stage apoptosis; F2, mid-phase apoptosis; F3, viable cell population; F4, early phase apoptosis.

Figure 3-7 shows a representative of typical data obtained from the Annexin-V FITC/PI experiments testing the effect of anti-Fas to induce Fas receptor-mediated apoptotic cell death in MCF-7, MCF-7TaxR, and HeLa cell lines. HeLa cells were sensitive to Fas-induced apoptosis (viable cell population after 48 h = 71.3%), consistent with the findings of Wajant et al. (2000). In addition, cycloheximide amplified the sensitivity to apoptotic cell death (viable cell population = 9.7%, but significant cell death was shown with
cycloheximide alone with a viable cell population of only 16.5%). MCF-7 cells showed resistance to Fas-induced cell death with a viable cell population of 86.4%, which was reduced by a further 78.3% with the addition of cycloheximide. MCF-7 TaxR cells showed resistance to anti-Fas, with a viable cell population of 93%, and the combined treatment resulting in 84% viability.

Figure 3-8: Anti-Fas resistance in MCF-7 and MCF-7 TaxR cells.
Data shown (obtained from Annexin V -ve/ PI -ve cells (%)) quadrant -F3) are the mean of 3 independent experiments of Annexin V-FITC/PI analysis. Cells were treated with 200 ng/ml anti-Fas + 1 µg/ml CHX for 72 h. Error bars are the ±SD with levels of statistical significance designated by * = <0.005 using Student’s paired T-test. (NS) non-significant, for MCF-7 and MCF-7 TaxR, p=0.1 and p=0.14, respectively.

As seen in Figure 3-8, in the presence of cycloheximide and anti-Fas treatment, MCF-7 and MCF-7 TaxR cells showed no significant differences compared to control or single anti-Fas treatment, which indicates that neither cell line undergoes receptor-mediated cell death.
3.6 Discussion

There is now ample evidence to support the role of cell death mechanisms - particularly apoptosis - as important factors governing tumour cell responsiveness to chemotherapeutic agents (Yang et al. 2007). However, one of the major factors related to abnormalities in apoptosis is change in caspase expression in cancer cells. Yang et al. (2007) observed that defects in, or downregulation of, caspases may be potentially frequent events in breast cancer, thus leading to cancer cell progression and development of cancer resistance to chemotherapy.

This chapter focused on the use of in vitro drug resistant tumour cell line models to study apoptotic cell death pathways and associated mechanisms underlying the altered response to cytotoxic agents. We showed that the parental MCF-7 cells will die according to a mechanism that partially involves apoptosis, but a fraction of the cells will die from some other form of cell death. Importantly, we show here that the paclitaxel-resistant breast cancer cell line MCF-7TaxR exhibited the induction of an exclusively non-caspase dependent cell death in response to cytotoxic insult.

The cytotoxicity of paclitaxel was determined from data generated using the MTT assay. The level of paclitaxel resistance in the MCF-7TaxR cells was calculated as approximately 12-fold. Interestingly, MCF-7TaxR cells showed a collateral sensitivity to cisplatin and carboplatin compared to the parent MCF-7 cells. Levels of sensitivity for MCF-7 and MCF-7TaxR to doxorubicin are similar. The Trypan blue screening experiment led us to consider the nature of the negligible effect of Z-VAD and compare the differences between the MCF-7TaxR and MCF-7 cell lines when exposed to an
anticancer agent such as paclitaxel. Staurosporine is known as a potent and non-specific protein kinase inhibitor and a strong inducer of apoptosis (Tamaoki et al. 1986). Therefore, it was used as a tool for cell death induction in the Annexin V-FITC/PI analysis. It has been reported in the literature that MCF-7 possesses a genetically defective form of caspase-3, an important apoptosis inducing effector molecule (Janicke et al. 1998; Yang et al. 2001). Furthermore, a caspase-3-independent pathway for apoptosis in staurosporine treated MCF-7 cells has been demonstrated (Janicke et al. 1998). However, staurosporine has been shown to initiate death activation in MCF-7 cells mediated by caspase activation (Kirsch et al. 1999). Importantly, MCF-7TaxR cells showed no significant differences between staurosporine treatment alone or combined with 100 µM Z-VAD. The nuclear morphology appeared fragmented following staurosporine treatment in MCF-7 cells with atypical apoptotic features. It was reported that the lack of caspase-3 in MCF-7 cells may prevent DNA fragmentation following staurosporine treatment (Janicke et al. 1998). The MCF-7TaxR cells treated with staurosporine appeared to be undergoing a cell death process with no resemblance to apoptosis. The Fas/CD95 and its ligand FasL mediated cell death pathway requires the activation of FADD, leading to caspase-8 activation and triggering the protease cascade which activates the downstream effector caspases, caspase-3, -6, -7 (Kuida et al. 1996; Scaffidi et al. 1998). Different studies have been reported for the MCF-7 human breast cancer cell line and they indicate modest Fas expression, but the cells still possess resistance to Fas-mediated apoptosis (Keane et al. 1996; Naujokat et al. 1999). Moreover, Xue et al. (2003) showed that caspase-8 and the pro-apoptotic protein Bid were inactivated in MCF-7 cells treated with staurosporine upon their commitment to cell death. Our data obtained on MCF-7 cells indicating resistance to Fas also agrees with the work of Naujokat et al. 1999 and Keane et
1996. There was also marked resistance to Fas mediated cell death seen for MCF-7TaxR cells. A study by Lukyanova et al. (2009) showed that MCF-7 cells with resistance to cisplatin and doxorubicin are characterised by alterations in expression of apoptotic proteins (Lukyanova et al. 2009). However, in contrast, our results indicate that MCF-7TaxR cells may induce cell death in a wholly caspase independent manner in view of experiments involving use of Z-VAD.

Other studies, presented by Xue et al. (2003), revealed that the presence of caspase-3 in MCF-7 cells by caspase-3 transfection showed the importance of this enzyme in inducing rapid apoptosis, compared to the original MCF-7 line (Buckley et al. 1999; Friedrich et al. 2001; Yang et al. 2001; Xue et al. 2003). In addition, the paclitaxel resistant MCF-7TaxR cell line under study herein is caspase-3 deficient, but shows collateral sensitivity to platinum agents compared with the parent MCF-7 cells. Therefore, an important question may be asked: what is the mechanism of cell death in human breast cancer cells induced by chemotherapeutic agents when caspase-3 is absent? The activity of caspase-3 is important in mediating cell death in MCF-7; however, there is a possibility that caspase-7 can only partially compensate for this deletion, at least for MCF-7 cells. Consequently, more investigations determining the presence and level of caspases in MCF-7 and MCF-7TaxR cell lines are required in order to understand how the system of apoptotic cell death is being modulated in response to chemotherapeutic insult. These investigations will be carried out in the next chapters to confirm or clarify the apparent lack of caspase dependent cell death in MCF-7TaxR cells.
CHAPTER 4

CHARACTERISATION OF PACLITAXEL RESISTANT MCF-7 BREAST CANCER CELLS: EXPRESSION OF APOPTOTIC GENES
4 Characterisation of Paclitaxel Resistant MCF-7 Breast Cancer Cells: Expression of Apoptotic Genes

4.1 Gene Microarray

The evolution of gene microarray technology has revealed novel prospective therapeutic targets with the potential of producing new drugs (Jayapal and Melendez 2006). In addition, microarray technology has been important in determining genetic causes of various human diseases (Debouck and Goodfellow 1999). Gene microarrays—which refers to a gene array or DNA microarray—have been used in examining multiple types of cancers such as breast cancer, prostate cancer and colon cancer (Alon et al. 1999; Pérou et al. 1999; Pérou et al. 2000). This technology allows the examination of thousands of genes and their expression at the same time (Jayapal and Melendez 2006). Microarrays provide information on gene products that are expressed in cancer cells but not in normal cells, thus aiding in the detection of tumour markers for diagnostic purposes (Schulze and Downward 2001). Moreover, microarray technology is capable of differentiating between gene expression in clinically distinct subtypes of leukaemia, lymphoma, breast cancer and melanoma (Alon et al. 1999; Alizadeh et al. 2000; Bittner et al. 2000; Perou et al. 2000).

Microarray technology uses RNA extracted from cells or tissue or other biological source materials to determine the expression levels of many genes. This technology consists of a set of distinct gene-specific, nucleic acid probes immobilized on a solid support. Thus, during a microarray experiment, RNA is converted to labelled cDNA or RNA by enzymatic reactions and, subsequently, hybridized to the immobilized nucleic acid probe.
The labelled target binds to each gene-specific spot which is typically detected using chemiluminescent, fluorescent or radioactive methods. Each spot produces a signal that corresponds to the amount of a message in the original RNA sample.

4.2 Aim

According to data obtained in Chapter 3, caspase dependent apoptotic cell death is absent in the MCF-7TaxR cell line and a modest level of apoptosis is observed in MCF-7 cells. Therefore, we set out to clarify the specific genetic changes correlated with the lack of apoptosis in the MCF-7TaxR paclitaxel resistant cell line. We have obtained a specific 112 apoptosis gene microarray profile for the MCF-7 and MCF-7TaxR cell lines. In addition, Q-PCR and immunoblotting analyses were carried out for caspase detection as a confirmation of the microarray data in the cell lines under investigation.
CHAPTER 4

4.3 Results

4.3.1 Apoptosis GEArray analysis for MCF-7 and MCF-7TaxR cells

Here, we carried out RNA extraction from the MCF-7 and MCF-7TaxR cell lines as described in Chapter 2 (Materials and Methods), and assessed the quality and quantity of the samples, as previously described. RNA samples for microarray analysis were processed by the SuperArray Corp™ (Frederick, California, USA), a commercially available service, using the “Apoptosis GEArray DNA Microarray” system. This analysis allowed the identification and semi-quantitation of genes associated with the apoptosis pathway. The apoptosis array is designed to determine the expression profile of genes that encode key ligands, intracellular modulators, receptors, and transcription factors involved in the regulation of programmed cell death.

The gene list obtained from 3 separate analyses is shown in Table-4.1. The data show the most significantly altered gene expression levels comparing the MCF-7 parent cells with the paclitaxel resistant MCF-7TaxR cells. The data confirmed the absence of caspase-3 in both cell lines, as expected. The caspase-3, 4, 5, 6, 7, and 9 genes were absent from the MCF-7TaxR cell line; caspase-6, 7, and 9 were present in the parent MCF-7 cells. Moreover, other major genes in the apoptotic machinery, such as APAFl, Bel-2, BID, FADD and TNF were absent from the MCF-7TaxR cells, but present in MCF-7 cells. Fas gene was absent in MCF-7 and MCF-7TaxR cells.
(a) Figure showing microarray analysis of apoptotic genes spotted and assembled on an immobilising solid support. (b) Table showing the major apoptotic gene expression levels in MCF-7 and MCF-7TaxR cell lines (with gene function described), (P) = presence of gene expression; (A) = absence of gene expression. Data obtained from 3 separate analyses.
4.3.2 Demonstration of caspase-8 expression in MCF-7 and MCF-7TaxR cells by immunoblotting

Caspase-8 is a crucial factor for initiating the extrinsic apoptotic pathway (Mita et al. 2006). Therefore, it is important to demonstrate the expression of caspase-8 in MCF-7 and MCF-7TaxR, in order to verify the existence of the extrinsic apoptotic pathway in these model systems.

![Western blotting for caspase-8 expression in untreated MDA-MB-231, MCF-7 and MCF-7TaxR cells, or cells exposed to staurosporine 48 hours.](image)

Figure 4-1: Western blotting for caspase-8 expression in untreated MDA-MB-231, MCF-7 and MCF-7TaxR cells, or cells exposed to staurosporine 48 hours.

Earlier studies were carried out by Dr. Helen Coley for the detection of caspase-8 in the MCF-7 and MCF-7TaxR cell lines. The MDA-MB-231 cell line was included in this analysis as a positive control for caspase-8 expression. Figure 4-1, (data obtained from Dr. Helen Coley), shows clear constitutive caspase-8 expression in MCF-7 and MCF-7TaxR cells. The MDA-MB-231 cells constitutively expressed pro-caspase-8 (55kDa), the levels
of which were diminished following staurosporine treatment as a consequence of caspase activation (data not shown). Interestingly, the extent of caspase-8 activation for both MCF-7 and MCF-7TaxR cells appeared to be less than that seen with the caspase proficient line MDA-MB-231.

4.3.3 Detection of apoptotic caspases and Bcl-2 anti-apoptotic protein in MCF-7 and MCF-7TaxR cells

According to the microarray data, the genes for caspase-7, caspase-9 and Bcl-2 were not expressed in MCF-7TaxR cells, but were expressed in the parental MCF-7 cells. To verify these data, Western blotting analysis was carried out to confirm the levels of caspase-7, caspase-9 and Bcl-2. This analysis was also carried out following paclitaxel and staurosporine treatment of MCF-7 and MCF-7TaxR cells in order to give an indication of functionality. Also, other human breast cancer cell lines were used as positive controls in the Western blotting analysis, i.e. MDA-MB-231, MDA-MB-231VPR (etoposide resistant), T47D and the paclitaxel resistant T47DTaxR cell lines for the detection of caspase-7 and caspase-9.
Figure 4-2: Western blotting for caspase-7 and caspase-9 expression of whole cell lysates of MCF-7 and MCF-7TaxR cells with staurosporine or paclitaxel treatment. In (a) and (b), cell lysates were made following a 1 μM staurosporine treatment for 24 h, 48 h, or treatment with 100 nM paclitaxel for 48 h or 72 h, as described in the Materials and Methods. Samples (50μg protein) were electrophoresed on a 10% polyacrylamide gel, transferred to a PVDF membrane and exposed to monoclonal anti-mouse caspase-7, or -9 (Cell Signaling Technology®) at 1:1000 dilutions and exposed to anti-mouse alkaline phosphatase (Invitrogen™). Bands were detected with a chemiluminescent substrate (Invitrogen™). Data are representative of repeat experiments (n = 4).
As seen in Figure 4-2, MCF-7 cells expressed pro-caspase-7 and caspase-9 with partial activation, shown by the appearance of cleaved forms, following staurosporine treatments and lesser activation of pro-caspase-7 with paclitaxel treatment. In marked contrast, the MCF-7TaxR cells showed a complete lack of caspase-7 and caspase-9 expression, as seen for all samples analysed. These data are in agreement with the microarray data (Table 4-1). Absence of these proteins in MCF-7TaxR cells is highly suggestive of a major defect in the intrinsic apoptotic pathway.

Figure 4-3: Western blotting for caspase-7 and caspase-9 constitutive expression of MDA-MB-231, MDA-MB-231VP16R, T47D and T47DTaxR cells. Cells were lysed as described in the Materials and Methods. Samples (50μg protein) were electrophoresed on a 10% polyacrylamide gel, transferred to a PVDF membrane and exposed to monoclonal caspase-7, or -9 antibodies (Cell Signaling Technology®) at 1:1000 dilution and exposed to anti-mouse alkaline phosphatase (Invitrogen™). Bands were detected using a chemiluminescent substrate (Invitrogen™). Data are representative of repeat experiments (n = 3).
The Western blotting analysis in Figure 4-3, showed that T47D, T47DTaxR, MDA-MB-231 and MDA-MB-231VPR cells express caspase-7 and caspase-9 constitutively, and the levels do not appear to change with the acquisition of a drug resistant phenotype. The evidence for a small band of activated, cleaved caspase-7 is sometimes noted for cells in culture but is not considered to be of any great significance.

![Western blotting analysis for Bcl-2 expression in control and staurosporine or paclitaxel treated MCF-7 and MCF-7TaxR cells.](image)

**Figure 4-4: Western blotting analysis for Bcl-2 expression in control and staurosporine or paclitaxel treated MCF-7 and MCF-7TaxR cells.**

Whole cell lysates were made following 24 h or 48 h staurosporine treatment, as described in the Materials and Methods. Samples (50µg protein) were electrophoresed on a 10% polyacrylamide gel, transferred to a PVDF membrane and exposed to monoclonal anti-mouse Bcl-2 (Santa Cruz, Inc) at 1:1000 dilution and exposed to anti-mouse alkaline phosphatase (Invitrogen™). Bands were detected by chemiluminescent substrate (Invitrogen™). Data are representative of repeat experiments (n = 3).

Figure 4-4 shows a total absence of Bcl-2 expression in MCF-7TaxR cells compared to MCF-7 cells, in both control and staurosporine treated cells. These data are in agreement with the apoptosis microarray results shown above.
4.4 Quantitative PCR analysis of caspase-7 and -9 mRNA expression

As shown in previous sections of this chapter, there is no expression of caspase-7 or caspase-9 in the paclitaxel resistant MCF-7TaxR cell line. To confirm this observation, we performed quantitative PCR analysis for MCF-7 and MCF-7TaxR. RNA was extracted from MCF-7 and MCF-7TaxR cells with the Trizol reagent, as described previously in Chapter 2, section 2.12. The PCR method used the QIAGEN® One-step Quantitect® SYBR® Green RT-PCR reagents. The PCR reactions were carried out using a TaqMan® 700 ABI Prism instrument (Applied Biosystems). The △ct values were calculated as the relative differences between expression levels of test genes (caspase-7 and -9) and a housekeeping gene (β-actin). The fold change was equal to $2^{\Delta \Delta CT}$.

Human caspase-7 and caspase-9 primers were obtained from SuperArray® as 10 μM working solutions. The primers were designed for amplification of the NM_001227.3 fragment of the caspase-7 gene and the NM_001229.2 fragment of the caspase-9 gene.

Caspase-7 human gene sequence primer 5'-3': Forward, CCGTGGGAAAGTAAAGAAGA; Reverse, GTTGGATGGATCGCATG.

Caspase-9 human gene sequence primer 5'-3': Forward, CCTGCTTAGAGGACACAGGG; Reverse, TGGACAACTTTGCTGCTTG.
4.5 Discussion

A large body of evidence has been established that supports the notion that apoptotic defects can develop in a cancer cell (Jaattela 1999; Evan and Vousden 2001). Moreover, these defects occur following exposure to chemotherapeutic agents during the development of drug resistance in tumours (Jaattela 1999; Evan and Vousden 2001). These processes contribute to cancer progression and tumour proliferation (Jaattela 1999; Evan and Vousden 2001). In the present work, the paclitaxel resistant MCF-7TaxR cells showed major alterations and defects in the apoptosis pathway as a consequence of developing paclitaxel resistance. The absence of a number of effector and executioner caspases was shown in MCF-7TaxR cells. These results agree with the previous data in
Chapter 3, which showed the induction of non-caspase dependent cell death in response to treatment of these cells with cytotoxic agents. In contrast with the MCF-7TaxR line, death may be induced in the parental MCF-7 cells according to a mechanism that involves some apoptosis and potentially some other form of cell death. A number of studies have reported the significance of MCF-7 breast cancer cells as a perfect tool for studying apoptosis or other altered forms of cell death mechanisms due to their lack of caspase-3 expression (Simstein et al. 2003). The MCF-7 cell line has been proposed to be an ideal in vitro model for the study of caspase independent cell death (Kurokawa et al. 1999; Simstein et al. 2003).

The microarray analysis, Western blotting and qPCR analysis we carried out has provided us with ample evidence of a lack of caspases in our novel drug resistant breast cancer line MCF-7TaxR. The microarray analysis confirmed the absence of the caspase-3 gene from MCF-7 cells, consistent with previous work (Janicke et al. 1998; Buckley et al. 1999; Friedrich et al. 2001; Yang et al. 2001; Xue et al. 2003). In addition, and as we expected, caspase-3 is lacking in the MCF-7TaxR cell line. Our contributions to studies on breast cancers, along with the work of others, indicate that the lack of caspase-3 transcription and protein expression may be an important factor in the pathogenesis of breast cancer (Devarajan et al. 2002). Importantly, a lack of caspase expression has been cited as an important and frequent phenomena. Moreover, in the case of caspase-3, low or unmeasurable levels have been reported for approximately 75% of breast cancers (Devarajan et al. 2002). Considering other caspase forms, caspases -4 and -5 are involved in the maturation of cytokine function (Galluzzi et al. 2008). Microarray analysis showed that the caspase-4 and -5 genes were also lacking in MCF-7TaxR and MCF-7 cells, and,
based on their function, there is a suggestion that their role in apoptosis is not as crucial as other caspases (Marzo et al. 1998; Martinon and Tschopp 2004).

The MCF-7TaxR paclitaxel resistant cell line showed that the greater proportion of its apoptotic machinery was either lacking or markedly down-regulated. Caspase-7 may compensate for the lack of caspase-3 function in the case of MCF-7 cells (Twiddy et al. 2006). In fact, it has been reported that caspase-7 and caspase-3 are almost identical in their substrate specificity (Fernandes-Alnemri et al. 1995). In addition, cells that possess a defect in the caspase-7 gene are more resistant to apoptotic cell death-inducing drugs and cause a delay in the externalization of phosphatidylserine (as measured by the Annexin V-FITC/PI method) and in DNA fragmentation (Korfali et al. 2004). A previous study revealed that a loss in apoptotic activity may occur when caspase-7 is mutated in cancers (Soung et al. 2003). Thus, our data and previous studies suggest that somatic mutation, deletion, or silencing of caspase-7 may be involved in the apoptotic resistance of cancer cells (Soung et al. 2003). Therefore, the lack of caspases-6, and -9 in MCF-7TaxR cells suggests that the route of cell death in this cell line is predominantly non-caspase dependent.

The data obtained thus far prompt an important question: how does the MCF-7TaxR cell line commit to cell death in response to chemotherapeutic drug exposure? Both caspase-9 and Apaf-1 are required for initiating the mitochondrial pathway and DNA damage. A study showed that the absence of Apaf-1 expression or activity may impair formation of a functional apoptosome in numerous cancers, e.g. melanoma, leukaemia, glioblastoma, and gastric cancer (Pommier et al. 2004). Therefore, according to our data, the absence of
caspase-9 and Apaf-1 in MCF-7TaxR strongly suggests the lack of commitment to the intrinsic pathway of apoptosis.

The Fas-associated death domain (FADD) protein requires the TNF-receptor associated death domain (TRADD) protein, resulting in apoptosis via activation of caspase-8 through the extrinsic pathway (Ashkenazi and Dixit 1998; Lowe and Lin 2000). This leads to activation of the other caspases causing cleavage of cellular targets and, thus, apoptotic cell death results (Ashkenazi and Dixit 1998; Lowe and Lin 2000). As a consequence, defects in FADD can prevent the activation of caspase-8 and, consequently, weaken the receptor mediated cell death pathway or the apoptotic extrinsic pathway (Yeh et al. 1998; Lowe and Lin 2000). It has been widely reported that there is cross-talk between the two apoptotic pathways – extrinsic and intrinsic - since they share the executioner caspases, caspase-3, caspase-7 and caspase-6, in addition to the involvement of Bid cleavage (Mita et al. 2006). Furthermore, the mitochondrial regulators can be changed or disrupted in their function by the extrinsic pathway regulator (Mita et al. 2006). Hence, each pathway can interact and influence the other to induce cell death (Mita et al. 2006). Other studies have reported changes in the response of apoptosis to chemotherapy in breast cancer (Mita et al. 2006). According to all these different studies, TNF, Fas, and the death domain of FADD are vital elements in initiating extrinsic apoptosis (Bodmer et al. 2000; Kuang et al. 2000; Kischkel et al. 2001; Suliman et al. 2001). Importantly, in the present study, all these elements are absent from the MCF-7TaxR cells according to the microarray data, in addition to loss of executioner caspases (-3,-7,-6). Moreover, this result explains the resistance of MCF-7TaxR to anti-Fas (as shown in Chapter 3). Although earlier findings (by Dr Helen Coley) revealed the expression of caspase-8 in both MCF-7 and MCF-
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7TaxR cell lines, there was some indication that there was minimal activation of this caspase form following staurosporine treatment (see Figure 4.1). The microarray data revealed the presence of caspase-10 in MCF-7TaxR and MCF-7 cells, but it has been suggested that caspase-8 and -10 may not be activated due to major apoptosis defects in Fas, TNF, and FADD, as well as the absences of the executioner caspases-3,-6,-7, the effector caspase-9, and Apaf-1 in MCF-7TaxR cells.

Bcl-2 family members serve as pro- and anti-apoptotic regulators, each consisting of between one and four Bcl-2 homology (BH) domains. The anti-apoptotic members Bcl-2, Bcl-XL, Bcl-w, Bcl-b, Mcl-1, and A1 all contain four BH domains (Petros et al. 2004). The pro-apoptotic Bcl-2 members contain between one and three BH domains; the pro-apoptotic members Bax, Bak and Bok contain BH domains 1-3 (Petros et al. 2004). The other pro-apoptotic members, such as Noxa, PUMA, Bad, Bim, Bis and Bik, containing a single BH-3 domain (so-called BH3-only proteins) as well as other functional domains which may control protein stability or allow interactions with other regulators (Petros et al. 2004). Many earlier investigations concentrated on studying the interactions between these proteins and how the ratio of pro- to anti-apoptotic Bcl-2 family members controls the release of cytochrome c (Logue and Martin 2008). The microarray data showed that Bid (coding for a BH-3 only protein) is absent from MCF-7TaxR cells. This particular pro-apoptotic BH3-only protein may serve as an important link between the extrinsic and intrinsic pathways of apoptosis since it is cleaved to a more active form, tBid (truncated Bid), by caspase-8 which is itself activated following engagement of death receptors (Mita et al. 2006). In the absence of executioner caspases, the unavailability of the pro-apoptotic protein Bid, even though caspase-8 is available, would severely compromise any apoptotic
response — as is the case in MCF-7TaxR cells. These data confirm that the extrinsic and intrinsic pathways cannot function in response to cytotoxic insult. In addition, the microarray data and Western blotting analysis also revealed a complete absence of Bcl-2 from the MCF-7TaxR cells. Bcl-2 is an anti-apoptotic protein and its absence in the MCF-7TaxR cell line is supposed to promote the induction of apoptosis. However, the story is entirely different in this particular cell line, since the absence of Bcl-2 would indicate a pro-apoptotic effect, but, due to the substantial lack of the most crucial apoptosis machinery, caspase-3, -4, -5, -6, -7, -9, 10 and Apaf-1, Bid, Fas, TNF, FADD, TRADD, apoptosis is a very unlikely event for these cells.

On the one hand, the executioner caspases, caspase-6, -7, are present in MCF-7 cells, suggesting that they may compensate for the absence of caspase-3 as the executioner caspase (Tang et al. 2000). These executioner caspases have been shown to promote apoptosis by cleaving cellular substrates that induce the morphological and biochemical features of apoptosis (Wolf and Green 1999). Blanc et al. (2000) studied the influence of caspase-3 and how it is necessary in regulating the mitochondrial pathway through caspase-9 activation. Cytochrome c release from mitochondria occurs following cisplatin treatment and was controlled by transfected caspase-3 in MCF-7 cells (Blanc et al. 2000). The data also showed that cytochrome c release may be partially regulated or affected by the caspase-3 substrate by causing a delay in cytochrome c release in caspase-3 deleted MCF-7 cells (Blanc et al. 2000). The effector caspases-8, -9, -10 are still present in the MCF-7 cell line, as confirmed in the microarray data. Caspase-8 expression in MCF-7 cells was confirmed earlier by Dr. Helen Coley. A study by Xue et al. (2003), showed that caspase-8 and Bid were not activated in staurosporine treated MCF-7 cells (lacking
caspase-3), but were activated in MCF-7 cells transfected with caspase-3 (Xue et al. 2003). Moreover, our Western blotting analysis confirmed that caspase-7 and caspase-9 were constitutively expressed in untreated MCF-7 control cells and became partially activated in cells treated with staurosporine. However, there was lesser activation of caspase-7 by MCF-7 cells following paclitaxel treatment. According to our study, MCF-7 cells successfully activate the mitochondrial apoptotic cell death pathway in response to treatment with anticancer drugs. In agreement, a study by Twiddy et al. (2006), showed that MCF-7 cells may undergo mitochondrial dependent apoptosis via caspase-7 activation (Twiddy et al. 2006). This result agrees with our finding that caspase-7 may compensate for caspase-3 absence as an executioner caspase in MCF-7 cells. However, in the present study only a modest activation of caspase-7 was evident following cell stress induction via paclitaxel treatment of MCF-7 cells. This occurred in spite of very marked morphological changes of the cultures which were suggestive of loss of cell viability (data not shown).

In our study, the microarray data indicated an absence of Fas gene expression, which indicated that the extrinsic pathway is partially compromised, in the MCF-7 cell line. This finding is also consistent with our previous findings from Chapter 3, when we carried out the Annexin V-FITC/PI experiment in order to examine the induction of apoptosis by Fas. MCF-7 cells showed resistance to anti-Fas treatment, as shown previously (Natjokat et al. 1999). Although MCF-7 cells are proficient in TNF and FADD gene expression (according to our apoptosis microarray analysis), Fas is essential for caspase-8 activation leading to cytochrome c release (Blanc et al. 2000; Twiddy et al. 2006). Thus, our observation in MCF-7 cells suggests that the apoptosis pathway mediated by caspase-8 is non-functional in response to anticancer drugs due to the lack of Fas, and this observation
is consistent with previous findings (Naujokat et al. 1999; Xue et al. 2003). Moreover, cytochrome c release from the mitochondria and caspase-8 activation by Fas have both been implicated in chemotherapy-induced apoptosis (Friesen et al. 1996; Boesen-de Cock et al. 1999; Fulda et al. 1999; Blanc et al. 2000). MCF-7 cells may undergo apoptosis in response to chemotherapy through the intrinsic pathway, involving sequential activation of caspase-9 and -7 (Twiddy et al. 2006; Coley 2008). One study showed that activation of both the intrinsic and extrinsic apoptotic pathways in MCF-7 cells treated with selenadizole derivatives, which is inconsistent with our finding and previous studies (Chen et al. 2008).

The pro- and anti-apoptotic Bcl-2 family proteins are key regulators in the apoptotic pathway. Different studies of the Bcl-2 protein, which examined induction of apoptotic cell death by chemotherapeutic agents or ionizing radiation, demonstrated that overexpression of Bcl-2, localized in the mitochondrial membrane, may prevent cell death (Vaux et al. 1988; Nunez et al. 1989; Hockenbery et al. 1990). The Bcl-2 protein was shown to be constitutively expressed in MCF-7 cells in control and drug treated cells. A previous study showed that overexpression of Bcl-2 can inhibit apoptosis induced by most chemotherapies such as alkylating agents and topoisomerase inhibitors (Kamesaki et al. 1993; Walton et al. 1993; Pommier et al. 2004). These previous reports provide evidence that apoptosis could, under some circumstances, be inhibited in MCF-7 cells. In addition, MCF-7 cells have more recently been considered as a model of non-caspase-mediated cell death, or as a representative model of autophagy (Ogier-Denis and Codogno 2003). Different studies have explored the possibility of autophagic cell death in MCF-7 cells when treated with different chemotherapies. A study by Fazi et al. (2008), showed the
induction of autophagic cell death in response to the drug Fenretinide in the MCF-7 cells and also showed the induction of apoptosis in caspase-3 reconstituted MCF-7 cells (Fazi et al. 2008). This means that when apoptosis is defective, autophagic cell death may take over (Lefranc et al. 2007; Fazi et al. 2008). We and others have suggested that the MCF-7 cell line can commit to cell death by apoptosis and autophagic cell death.

In summary, the MCF-7TaxR cell line is a novel cell line due to the profound absence of apoptotic genes (Figure 4-5). The microarray data presented in this chapter gave us an insight into the complexity of the modifications occurring in the MCF-7TaxR cell line as a consequence of the drug resistant phenotype and are consistent with earlier data in Chapter 3. Our data confirmed, for the first time, that drug resistant cancer cells, such as the MCF-7TaxR cell line, may fail to undergo apoptotic cell death. All the apoptotic defects that may be present result in a cancer cell that, although compromised in its ability to carry out apoptosis, may commit caspase-independent cell death. Therefore, our data are strongly suggestive of the presence of an alternative form of cell death for MCF-7TaxR cells. At this stage, I hypothesized this to be the second type of programmed cell death: autophagy.
Figure 4-5: Major alterations in apoptosis, PCD I, occur in the MCF-7TaxR cell line. All the main components of executioner caspases are absent in MCF-7TaxR cells, in addition to caspase-9, Apaf-1, Fas, Bid, TNF, FADD and TRADD.
CHAPTER 5

CHARACTERISATION OF PACLITAXEL RESISTANT MCF-7 BREAST CANCER CELLS: CHANGES IN EXPRESSION OF PROTEINS DURING THE EVOLUTION OF DRUG RESISTANCE
5 Characterisation of Paclitaxel Resistant MCF-7 Breast Cancer Cells: Changes in Expression of Proteins During The Evolution of Drug Resistance

5.1 Multiple drug resistance (MDR)

One of the major problems that faces breast cancer patients undergoing chemotherapeutic treatment is the development of multi-drug resistance (MDR), resulting in a failure to cure the disease (Gottesman and Ling 2006; Higgins 2007; Coley 2008; McGrogan et al. 2008). Multi-drug resistance is a phenomenon in which cancer cells, exposed to one anticancer drug, simultaneously show resistance (cross-resistance) to various anticancer drugs that are structurally and functionally different from the initial anticancer drug, resulting in an acquired resistance termed multi-drug resistance (MDR) (Gottesman et al. 2002; Yusuf et al. 2003; Choi 2005; McGrogan et al. 2008).

The mechanism of MDR is the amplification of the multiple drug resistance gene (MDR1), which is associated with over-expression of a transporter protein called P-glycoprotein (P-gp) (Gottesman et al. 2002; Coley 2008). P-gp belongs to a class of the ATP-dependent transporter family, also called the ATP-binding cassette (ABC) family (Gottesman et al. 2002; Coley 2008). P-gp is a phospho-glycoprotein with a molecular weight of 170 kDa, consisting of two ATP-binding cassette energy and two transmembrane regions (Chen et al. 1986; Coley 2008). Each transmembrane region contains six transmembrane domains (Chen et al. 1986; Coley 2008). The P-gp transporter's role is to bind to a wide range of hydrophobic drugs and natural products such as doxorubicin and paclitaxel (Table 5-1) (Schinkel et al. 1997; McGrogan et al. 2008). When a drug binds to P-gp, the resulting
conformational change gives rise to the activation of one of the ATP domains resulting in hydrolysis of ATP (Ramachandra et al. 1998; McGrogan et al. 2008). This action results in efflux of the drug from the intracellular to the extracellular compartment (Figure 5-1), thus preventing adequate accumulation of the drug inside the cell and, hence the basis for drug resistance (Ramachandra et al. 1998; McGrogan et al. 2008).

<table>
<thead>
<tr>
<th>Substrates of P-gp (MDR1)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Anthracyclines</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td></td>
<td>Daunorubicin</td>
</tr>
<tr>
<td></td>
<td>Epirubicin</td>
</tr>
<tr>
<td></td>
<td>Mitoxantrone</td>
</tr>
<tr>
<td>Topoisomerase Inhibitors</td>
<td>Etoposide</td>
</tr>
<tr>
<td></td>
<td>Teniposide</td>
</tr>
<tr>
<td>Vinca alkaloids</td>
<td>Vinblastine</td>
</tr>
<tr>
<td></td>
<td>Vincristine</td>
</tr>
<tr>
<td>Taxanes</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td></td>
<td>Docetaxel</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Cepharanthine</td>
</tr>
<tr>
<td></td>
<td>Homoharringtonine</td>
</tr>
<tr>
<td>Antitumor antibiotics</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td></td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>Cytarabine</td>
</tr>
<tr>
<td>Acridines</td>
<td>Amscarbione</td>
</tr>
<tr>
<td>Anthracenes</td>
<td>Bisantrene</td>
</tr>
</tbody>
</table>

Table 5-1: The most common anticancer drugs which bind to the P-gp drug transporter. Adapted from (Coley 2008).
Figure 5-1: Anticancer drug transport in the absence and presence of P-glycoprotein. (a) The passive diffusion of an anticancer drug into a cancer cell results in subsequent induction of cell death (absence of P-gp). (b) The effects on drug transport due to expression of P-gp transporter in a multi-drug resistant cancer cell results in subsequent cell survival. (Gottesman et al. 2002)
P-gp expression may be detected in untreated breast cancers (Leonessa and Clarke 2003; Sacki et al. 2005). Moreover, P-gp expression was examined by a meta-analysis which found that 40% of all breast cancer tumours express P-gp, although there were some wide differences (heterogeneity) reported between different studies and evidence of inter-individual heterogeneity (Trock et al. 1997; Coley 2008). The levels of P-gp were not different between metastatic and non-metastatic breast cancer disease (Trock et al. 1997; Coley 2008). However, the levels of P-gp (MDR1) were higher after treatment with chemotherapy or hormonal therapy, increasing by up to 1.8 fold (Trock et al. 1997; Coley 2008). These data suggest that tumour resistance due to P-gp (MDR1) expression can be induced after exposure to a single anticancer drug (Trock et al. 1997; Coley 2008).

As mentioned previously, failure in curing cancer is at least in part due to the failure to accumulate enough chemotherapeutic agent intra-tumorally (Choi 2005; Coley 2008). Therefore, with high expression levels of MDR transporters, cancer cell sensitivity to drugs will be particularly affected (Choi 2005). There are a large number of research papers that have focused on P-gp inhibitors/modulators which reverse the action of P-gp when used in conjunction with chemotherapy (reviewed in Choi 2005; Coley 2008). Early clinical trials studied P-gp modulators in acute leukaemia; cancerous cells surviving chemotherapy treatment with modulators gave rise to a clinical relapse, but had reduced P-gp expression (List et al. 1993; Marie et al. 1993). Other studies on P-gp modulators have shown evidence of inhibition in P-gp expression, albeit rather limited (Gottesman 2002). Some of the early first generation modulators included calcium blockers such as verapamil and the immunosuppressant cyclosporine A (Gottesman 2002). A study performed with second generation modulators, showed a great increase in efficacy of the cytotoxicity of
the compound but very high toxicity was observed (Gottesman 2002). The newest generation of modulators (third generation) could be promising and less toxic with high specificity (Gottesman 2002). These compounds include XR9756 (Tariquidar), R101933, and LY335979 (Gottesman 2002).

5.2 Taxane resistance

Paclitaxel and docetaxel belong to the family of taxane anticancer agents. This class of anticancer drugs is used in the treatment of breast, ovarian, non-small cell lung cancer and prostate cancers. Taxanes are microtubule stabilizing agents; microtubules normally function in maintaining the cell shape, cell signalling, division and mitosis (Jordan and Wilson 2004). Therefore, microtubules are an excellent cancer target for a number of available microtubules inhibitors such as taxanes (Jordan and Wilson 2004). Taxanes specifically target the spindle microtubule dynamics of the β-subunit in tubulin by interfering with and suppressing the metaphase/anaphase transition, causing inhibition of mitosis and inducing apoptosis (Jordan and Wilson 2004; Kamath et al. 2005). The cytotoxic effect of paclitaxel and docetaxel were studied in vitro and are time and drug concentration dependent (Braakhuis et al. 1994; Torres and Horwitz 1998; Hernandez-Vargas et al. 2007). Their IC\(_{50}\) value for cytotoxicity is in the nanomolar range for a number of cell lines (Braakhuis et al. 1994; Torres and Horwitz 1998; Hernandez-Vargas et al. 2007). Elevated concentrations of paclitaxel inhibit mitosis and result in G2/M cell cycle arrest followed by apoptosis (Jordan et al. 1996).

The development of taxane resistance in cancer cell lines has been extensively studied and different mechanisms of drug resistance have been described (Cabral 2000; Dumontet
2000; Orr et al. 2003). Paclitaxel was shown to be a substrate for P-gp (Schinkel et al. 1997; McGrogan et al. 2008). Expression of MDRI was assessed by the National Cancer Institute in 60 cancer cell lines using a quantitative real time-polymerase chain reaction (qPCR) analysis (Alvarez et al. 1995; McGrogan et al. 2008). They found low levels of MDRI expression, with elevated sensitivity to paclitaxel in the cell lines (Alvarez et al. 1995; McGrogan et al. 2008). The efflux of taxanes to the extracellular compartment by P-gp can lead to a reduction in drug efficacy (Ehrlichova et al. 2005). Development of taxane resistance is complex, and may involve defects in tubulin (Kavallaris et al. 1999; Kamath et al. 2005). Over-expression of the β-III tubulin isotype has been described as a factor underlying paclitaxel resistance (Kavallaris et al. 1999; Kamath et al. 2005). Also, it has been reported that resistance to docetaxel is associated with over-expression of β-tubulin isotypes I-IV (Hasegawa et al. 2003; Paradiso et al. 2005). Conversely, reduced levels of tubulin have been associated with taxane resistance (Wang and Cabral 2005). The multi-resistance associated protein (MRP) is another class of drug efflux pump implicated in developing taxane resistance (Borst et al. 2000; Hopper-Borge et al. 2004). MRP1, a 190 kDa protein, was first shown to be overexpressed in a doxorubicin resistant lung cancer cell line in the absence of MDRI/P-gp expression (Cole et al. 1992). Moreover, human epidermal growth factor receptor 2 (HER2), encoded by the proto-oncogene HER2/neu, was shown to be overexpressed in many breast cancer cell lines and leads to resistance to paclitaxel (Yu et al. 1998). Taxane acquired resistance involves alterations in apoptotic programmed cell death signalling (Kutuk and Letai 2008). Hence, paclitaxel utilizes the mitochondrial apoptotic pathway to mediate apoptosis in breast cancer cell lines (Tudor et al. 2000; Sunters et al. 2003). According to another study (Kutuk and Letai 2008) involving paclitaxel resistant breast cancer cell lines, the key
mediator in development of resistance was an alteration in mitochondrial apoptotic pathway control.

5.3 Aim

After studying the induction of caspase-independent cell death in MCF-7TaxR cells (Chapter 3 and 4), and from the microarray data in Chapter 4, it is clear that many important apoptotic genes are absent from the MCF-7TaxR cells. In this study, we focused on detecting changes in levels of P-glycoprotein (MDRI gene product), caspase-7, caspase-9, the anti-apoptotic protein Bcl-2, and the pro-apoptotic proteins Bax, Bim and Bad, during the evolution of paclitaxel resistance. By selecting various cultures of MCF-7TaxR cells at different passages, we were able to see the sequence of events that contributed to the final resistant phenotype.

5.4 Methods

The methods used in this study were the MTT assay, Western blotting analysis and quantitative PCR analysis. All methods are explained in Chapter 2, Methods and Materials.
5.5 Results

5.6 Development of the paclitaxel resistant MCF-7TaxR cell line

To develop a resistant cell line model, MCF-7 cells were made resistant to paclitaxel and resulted in the MCF-7TaxR cell line. The sensitivity of the developing variant MCF-7TaxR cell line to the anticancer drugs paclitaxel, doxorubicin, and cisplatin, was tested at passage 8, passage 18, and passage 23 using the MTT assay (Table-5-2) (Figure 5-2). IC\text{50} values (calculated as the dose necessary to cause a 50% reduction in cell viability, relative to untreated control cells) were calculated from the dose response curves.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Paclitaxel IC\text{50} nM</th>
<th>Doxorubicin IC\text{50} nM</th>
<th>Cisplatin IC\text{50} \mu M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>5.5 (±2.6)</td>
<td>1000 (±225)</td>
<td>12.7 (±2.2)</td>
</tr>
<tr>
<td>MCF-7TaxR 8</td>
<td>9.0 (±1.1)</td>
<td>1080 (±229)</td>
<td>12.3 (±3.5)</td>
</tr>
<tr>
<td>MCF-7TaxR 18</td>
<td>162.7 (±21.7)</td>
<td>3296 (±828)</td>
<td>5.6 (±2.8)</td>
</tr>
<tr>
<td>MCF-7TaxR 23</td>
<td>220 (±33)</td>
<td>2346 (±736)</td>
<td>6.1 (±1.1)</td>
</tr>
</tbody>
</table>

Table 5-2: Sensitivity of the breast cancer cell line MCF-7 and its paclitaxel resistant derivative MCF-7TaxR to anticancer drugs at different cell passage numbers. The IC\text{50} values are the means for at least 4 separate experiments (performed in triplicate) with paclitaxel (nM), doxorubicin (nM) and cisplatin (\mu M) as determined by the MTT assay. The ± SD values are shown in parentheses. The numbers in the left hand column after MCF-7TaxR indicate the cell passage number. Cells were incubated with each drug for 72 h. The IC\text{50} values were calculated as the dose necessary to cause a 50% reduction in cell viability, relative to untreated control cells.
Figure 5-2: Changes in drug sensitivity in developing paclitaxel resistant MCF-7TaxR cells.

IC₅₀ values are the means for at least 4 separate experiments (performed in triplicate), and were determined by the MTT assay for the parent MCF-7 and MCF-7TaxR passage 8, 18 and 23 cells. Cells were incubated for 72 h, with paclitaxel (nM), doxorubicin (nM), and cisplatin (µM). Error bars represent the ±SD.
As shown in Table 5-2 and Figure 5-2, the original MCF-7 cells were highly sensitive to paclitaxel ($IC_{50} = 5.5$ nM). However, as the number of cell passages in paclitaxel containing medium increased, the paclitaxel $IC_{50}$ values gradually increased, showing that a paclitaxel resistant cell population was emerging. The paclitaxel resistant MCF-7TaxR cell line showed a relatively low level of resistance after 8 passages ($IC_{50} = 9$ nM) in paclitaxel containing medium, but this increased with further cell passages. The paclitaxel $IC_{50}$ values were 162.7 nM and 220 nM at passage 18 and 23, respectively. The increase in paclitaxel resistance was paralleled by a modest but transient increase in doxorubicin resistance. However, MCF-7TaxR cells failed to become resistant to cisplatin, even after 23 cell passages in paclitaxel. On the contrary, collateral sensitivity to the agent developed.

### 5.7 P-gp (MDR1) expression

We examined the levels of the MDR1 drug transporter P-gp in the human breast cancer cell line MCF-7 during treatment with the anticancer drug paclitaxel. MCF-7TaxR cells were harvested at different cell passages 8, 23, 53, and MCF-7TaxRREV (MCF-7TaxR cells that had been grown in the absence of drug for several passages to ascertain whether the phenotype was reversible) were also examined. The expression of P-gp was determined at each of the passages by immunoblotting analysis as described in section 2.9 in Chapter 2 (Materials and Methods).
Figure 5-3: Overexpression of P-gp (MDR1) in paclitaxel resistant MCF-7TaxR cells.

Cells were lysed as described in Materials and Methods. Samples (50 µg protein) were electrophoresed on a 7% SDS-polyacrylamide gel, transferred to a PVDF membrane, and blotted with an MDR1 (Santa Cruz Biotechnology, Inc.) rabbit polyclonal antibody at a dilution of 1:200 before being exposed to anti-rabbit alkaline phosphatase (Invitrogen™). Bands were detected using a chemiluminescent substrate (Invitrogen™). Data are representative of repeat experiments (n = 3).

The data in Figure 5.3 shows that P-gp expression increased in MCF-7TaxR cells treated with paclitaxel. The MCF-7 parental cells and the MCF-7TaxR cells at cell passage 8 did not show detectable levels of P-gp. However, P-gp expression gradually increased with further passages of the MCF-7TaxR cell line. At passage 18, a modest increase in P-gp expression was observed, followed by an even greater increase at passage 23, as indicated by the intensity of the band at 170 kDa. P-gp expression was also observed in MCF-7TaxR passage 53 and in MCF-7TaxRREV cells, suggesting that the drug resistant phenotype was likely to be stable in the latter case.
5.8 Caspase-7 and caspase-9 expression

We looked for changes in the expression of caspases during the acquisition of paclitaxel resistance in MCF-7 cells. Caspase-7 and caspase-9 were detected in MCF-7 and MCF-7TaxR cells at selected passage numbers in paclitaxel-containing medium and MCF-7TaxRREV cells by Western blot analysis as described previously.

Figure 5-4: Caspase-7 expression in MCF-7TaxR cells decreases with cell passage in paclitaxel-containing medium.
Cells were lysed as described in Materials and Methods. Samples (50 µg protein) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, and then exposed to a monoclonal anti-mouse caspase-7 (Cell Signaling Technology®) at a 1:1000 dilution before being exposed to anti-mouse alkaline phosphatase (Invitrogen™). Bands were detected using a chemiluminescent substrate (Invitrogen™). Data are representative of repeat experiments (n = 3).
Figure 5-5: Caspase-9 expression in MCF-7TaxR cells decreases with cell passage number in paclitaxel containing medium.

Cells were lysed as described in Materials and Methods. Samples (50 µg protein) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, and exposed to a monoclonal anti-mouse caspase-9 antibody (Cell Signaling Technology®) at a 1:1000 dilution before being exposed to anti-mouse alkaline phosphatase (Invitrogen™). Bands were detected using a chemiluminescent substrate (Invitrogen™). Experiments were repeated more than three times. Data are representative of repeat experiments (n = 3).

The Western blot data shown in Figures 5-4 and 5-5 show that caspase expression decreased as the MCF-7TaxR cells became more resistant to paclitaxel. Caspase-7 and caspase-9 were present in the MCF-7 parental cells and the MCF-7TaxR cell line at passage 8 and passage 18, indicating that a substantial change in the apoptotic mechanism occurred in these cells as they developed resistance to paclitaxel. Significant changes in caspase expression occurred around passage 23 when caspases-7 and -9 could no longer be detected. The MCF-7TaxR cells at passage 53 and the MCF-7TaxRREV cells continued to show a complete lack of caspase-7 and-9 expression, further indicating the stability of the drug resistance phenotype.
5.9 Quantitative PCR analysis of caspase-7 and -9 mRNA expression

As established in previous section, there is no expression of caspase-7 and caspase-9 in MCF-7TaxR with acquisition paclitaxel resistance. To confirm this, we examined the detection of caspase-7 and caspase-9 genes expression in these cells. We performed quantitative PCR analysis with MCF-7 and MCF-7TaxR cells at selected passages and also with MCF-7TaxRREV cells. RNA was extracted with the Trizol reagent from MCF-7 and MCF-7TaxR cells, at pre-determined cell passages as described previously in Chapter 2, section 2.12. The QIAGEN® One-step QuantiTect® SYBR® Green RT-PCR method was used. The PCR reactions were carried out using TaqMan® 700 ABI Prism (Applied Biosystems). The ΔΔct values were calculated as the relative differences between expression levels of test genes (caspase-7 and -9) and a house keeping gene (β-actin). The fold change was equal to $2^{(-\Delta\Delta C_t)}$. Human caspase-7 and caspase-9 primers were obtained from SuperArray® as 10 μM working solutions. The primers were designed for amplification of the NM_001227.3 fragment of the caspase-7 gene and the NM_001229.2 fragment of the caspase-9 gene.

**Caspase-7 human gene sequence primer 5’-3’:** Forward,
CCGTGGGAACGTAAGAAGAA; Reverse, GTCTTGATGGATCGCATGG.
**Caspase-9 human gene sequence primer 5’-3’:** Forward,
CCTGCTTAGAGGACACAGGC; Reverse, TTCGACAACTTTGCTGCTCGTG.
Figure 5-6: qPCR analysis of caspase-7 gene expression in MCF-7 and MCF-7TaxR cells.

(a) △ct levels of caspase-7 mRNA decreased with the emergence of paclitaxel resistance in the MCF-7TaxR cell line. Data are the mean of 3 independent experiments (performed in duplicate); error bars, ±SD. (b) The table shows the △ct values in MCF-7 and MCF-7TaxR cell lines at different passage numbers.
The data in Figure 5-6 and 5-7 quantified the levels of caspase-7 and caspase-9 gene expression in human breast cancer cell lines during the acquisition of paclitaxel resistance. Caspase-7 and caspase-9 were detected in the MCF-7 parent cell line and the MCF-7TaxR cell line at passage 8. In MCF-7TaxR passage 18 cells, the expression levels of both genes (caspase-7 and caspase-9) began to decrease. When the cell line started to
become resistant at passage 23, the MCF-7TaxR cells had lost nearly all detectable caspase-7 and caspase-9 gene expression. At passage 53, caspase gene expression could no longer be detected in the MCF-7TaxR cell line or in the MCF-7TaxRREV cell line. These data indicate that the MCF-7TaxR cell line undergoes reductions in the transcription of apoptotic genes concomitant with the emergence of paclitaxel resistance.

5.10 Changes in anti-apoptotic and pro-apoptotic protein expression

As established previously, significant deficiencies in the main apoptotic regulators, caspase-7 and caspase-9, were observed in MCF-7TaxR cells during the acquisition of paclitaxel resistance. We also examined the expression of other apoptotic regulators such as the pro-apoptotic proteins Bax, Bim, Bad, and the anti-apoptotic Bcl-2, in the parental MCF-7 and the MCF-7TaxR cell lines at predetermined cell passage numbers of cells grown in paclitaxel containing medium using immunoblotting analysis.
Figure 5-7: Changes in pro- and anti-apoptotic protein expression in human breast cancer cell lines during the acquisition of resistance to paclitaxel.

Cells were lysed as described in Materials and Methods. Samples (50 μg protein) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, and then, exposed to an anti-mouse Bax antibody, anti-mouse Bcl-2 antibody, anti-rabbit Bim antibody, anti-rabbit Bad or P-Bad (Ser112) antibodies (obtained from Cell Signaling Technology® or Santa Cruz Biotechnology, Inc.) at 1:200 or 1:1000 dilution before being exposed to anti-rabbit or anti-mouse alkaline phosphatase (Invitrogen™). Bands were detected using a chemiluminescent substrate (Invitrogen™). Data are representative of repeat experiments (n = 3).

Figure 5-8 shows that Bax (a pro-apoptotic protein) was expressed in all cell lines examined, irrespective of the cell passage number. The expression of Bcl-2 (anti-apoptotic) and the BimL isoform (pro-apoptotic) were readily detected up to cell passage 18, after which the expression of both proteins underwent a drastic reduction at passage 23. As a result of alternative splicing, Bim exists in three isoforms, BimL, BimL and BimL (O'Connor et al. 1998). Other forms of Bim (BimL 23 kDa and BimL 12 kDa) were not
detected in the MCF-7 parental cells. Looking at approximate ratios of P-Bad: Bad (judged by eye) there are small fluctuations seen throughout the sample series. However, there are no marked changes for the levels of Bad and P-Bad during the emergence of paclitaxel resistance in this cell line model.

5.11 Discussion

The development of acquired resistance to chemotherapy has a negative impact on the effective treatment of human tumours (Pommier et al. 2004). Many cancers initially show good clinical response to chemotherapy and some will actually be completely cured of the disease (Pommier et al. 2004). Therefore, understanding the mechanism of anticancer drug resistance is still a major obstacle for the successful treatment of cancer (reviewed by Coley 2008).

In this study, we showed that the breast cancer cell line MCF-7 become resistant to paclitaxel after a short duration of exposure (3 weeks). Many studies of paclitaxel resistance in cell lines models have been conducted and various mechanisms have been identified (Cabral 2000; Dumontet 2000; Orr et al. 2003). One of the reported mechanisms is the overexpression of ABC transporters, such as P-glycoprotein/MDR1/ABCB1 and ABCB4 (MDR2) (Hopper-Borge et al. 2004). It was surprising to find that MCF-7TaxR cells developed collateral sensitivity to cisplatin. Overall, the MCF-7TaxR cell line did not show a phenotype that was consistent with the classical P-gp theory, i.e. development of cross resistance to other structurally unrelated natural product anticancer drugs (Gottesman 2002; Gottesman et al. 2002). Expression of
P-gp was reported in a number of tumours with resistance to a variety of microtubules agents such as paclitaxel and vinca alkaloids (Penson et al. 2004; Hari et al. 2006). Here, we confirmed the expression of P-gp by Western blotting analysis in the MCF-7TaxR cell line, indicating that the P-gp transporter is a potential mechanism of resistance to paclitaxel in this cell line. MCF-7TaxR cells at passage 18 to passage 23 appeared to be the point at which cells developed the drug resistant phenotype. When the MCF-7TaxR cell line reached passage 23, P-gp protein expression was strongly upregulated, which is generally associated with the MDR phenotype. Previous studies showed that different cell lines with acquired resistance to paclitaxel were derived using either a single, high dose of paclitaxel or by step-wise increments of increasing paclitaxel (Dumontet et al. 1996; Cabral 2000). However, in some studies, paclitaxel resistance was shown to come about as a consequence of multiple factors many of which had an unknown basis (Hari et al. 2006).

A study by Li et al. (2009) demonstrated the overexpression of P-gp in MCF-7 cells with acquired resistance to doxorubicin and similar results have been reported in gastric cancer cells (Du et al. 2005; Li et al. 2009). Some of the mechanisms are mutations in tubulin, overexpression of P-gp, changes in expression of microtubule-associated proteins, or alteration in apoptosis proteins (Poruchynsky et al. 2001; Martello et al. 2003; Hari et al. 2006).

The presence of drug resistance can affect the accumulation of drug intracellularly and, further, this process may be exacerbated by alterations in other factors such as apoptotic proteins (Johnstone et al. 2002). The two main pathways in apoptosis (programmed cell death I) are the intrinsic pathway through the mitochondria and the extrinsic pathway, as discussed earlier in Chapters 1 and 3. A large body of evidence has indicated that
inactivation of apoptosis leads to tumour progression (Jaattela 1999; Evan and Vousden 2001). Therefore, human tumours may possess defective or mutated pro-apoptotic genes or may over-express anti-apoptotic proteins to confer chemoresistance (Mathiasen and Jaattela 2002). Here, we assessed caspase-7 and caspase-9 expression by Western blotting and qPCR in MCF-7 cells over the time course of acquisition of resistance to chronic paclitaxel exposure. These data were consistent with our previous data in Chapters 3 and 4, which indicated that the MCF-7TaxR cell line is resistant to apoptosis and, thus, the cell line may undergo caspase-independent cell death. It was suggested that P-gp is involved in inhibition of the apoptosis cascade in Chinese hamster ovary fibroblasts (Robinson et al. 1997). Also, it has been reported that a major cause of blocking apoptotic cell death is the presence of P-gp overexpression (Pawlik et al. 2005).

Paclitaxel utilizes the mitochondrial apoptotic pathway to mediate apoptosis in breast cancer cell lines (Tudor et al. 2000; Sunters et al. 2003). Defects in apoptotic cell death mechanisms include alterations in the ratio of expression of pro- and anti-apoptotic proteins of the Bcl-2 family associated with acquired paclitaxel resistance (Kutuk and Letai 2008). The Bcl-2 family members classified into pro-apoptotic proteins that promote apoptosis include Bax and Bak (BH1-3 domain of Bcl-2 homology) (reviewed by Strasser et al. 2000). The BH3 subfamily of pro-apoptotic proteins includes Bid, Bim, Bad and PUMA (reviewed by Strasser et al. 2000). In addition, the anti-apoptotic subfamily includes Bcl-2, Bcl-xL and Mcl-1 (which share BH1-4 domains); this group inhibits apoptosis (reviewed by Strasser et al. 2000). In our investigation of the expression of different anti-and pro-apoptotic proteins, a gradual loss in expression was seen in MCF-7TaxR cells whilst developing paclitaxel resistance. For example, the Bax pro-apoptotic
protein was expressed constitutively in the MCF-7 cell line and all MCF-7TaxR sublines, which is consistent with our previous microarray data. The BH3-only protein Bim induces Bax activation which undergoes conformational change and oligomerization causing permeabilization of the outer mitochondrial membrane allowing cytochrome c release from the mitochondria, which initiates the intrinsic apoptotic pathway (Kutuk and Letai 2008). Thus, the expression of Bax in the parental MCF-7 cells indicates an activation of the intrinsic apoptotic pathway (MCF-7 cells possess caspase-9, -7), in agreement with our previous finding and those of others (Kutuk and Letai 2008). However, although paclitaxel resistant MCF-7 cells at passages 18, 23 and 53 cells express Bax, MCF-7TaxR cells failed to initiate the intrinsic pathway due to the lack of caspase-7, 9 and Apaf-1 and other factors, according to our previous findings. It has been reported that the pro-apoptotic protein Bax can be activated by Bid (Kutuk and Letai 2008) and this was absent from MCF-7TaxR cells. Considering other Bcl-2 family members, Bim, a pro-apoptotic protein, consists of a three isoforms, BimEL, BimL and Bims (Bim extra long, Bim long and Bim short) as a result of alternative splicing (O'Connor et al. 1998). These isoforms are cytotoxic and have different forms of regulation by various pro-death and pro-survival signalling pathways (Ley et al. 2005). The expression of these isoforms varies in different cell types and tissues (O'Reilly et al. 2000). Bim, located in other sub-cellular compartments, translocates to the mitochondria to activate Bax and Bak (O'Connor et al. 1998). BimL and Bims are more potent inducers of apoptosis than BimEL (O'Connor et al. 1998). However, the long isoforms of BimL and BimEL are regulated by phosphorylation by c-Jun N-terminal kinase in response to different stress which stimulates apoptotic cell death (Lei and Davis 2003; Hubner et al. 2008). In this study, we observed that BimL was expressed in the MCF-7 parental line and the MCF-7TaxR cells at passage 8. According
to the literature, MCF-7 cells may express BimL form (Suyama et al. 2007). However, the antibody used for Western blotting analysis in that work was not the same as the one used in my study. Hence, given the very variable nature of the performance of different antibodies it is difficult to draw conclusions. However, the difference seen between MCF-7 and MCF-7TaxR for BimL expression is very marked - with total absence in the paclitaxel resistant line. The apoptosis microarray platform did not include Bim, which might have given more support to our findings. Decreased BimL expression was detected at passage 18 in MCF-7TaxR cells when the resistance to paclitaxel was becoming established. Subsequently, loss of BimL expression was detected in MCF-7TaxR cells at passage 23. Bim functions to promote death by interacting with pro-survival Bcl-2 proteins at the mitochondria; however, this function may be restrained by interactions between Bim and the microtubule-associated dynein complex, specifically via binding to dynein light chain 1 (DLC1, or LC8). This in turn may implicate Bim in death arising from disruption of the microtubule or cytoskeletal network (Puthalakath et al. 1999). Therefore, it has been reported that Bim is involved in mediating apoptosis by paclitaxel (Bouillet et al. 1999; Sunters et al. 2003). In addition, it was demonstrated in in vivo studies that apoptotic induction in paclitaxel treated cells is essentially dependent on the action of Bim (Tan et al. 2005). A study by Kutuk and Letai (2008), showed that the MDA-MB-468 breast cancer cell line with acquired resistance to paclitaxel was associated with decreased Bim levels (Kutuk and Letai 2008). This is consistent with our findings showing deficient levels of Bim in our paclitaxel resistant MCF-7TaxR cells. The pro-apoptotic factor Bad regulates apoptosis induction through inhibiting the anti-apoptosis Bcl-2 family members Bcl-2, Bcl-xL and Mcl-1 (reviewed by Strasser et al. 2000). Bad activation stimulates the action of Bak and Bax, pro-apoptosis members leading
cytochrome c release, followed by mitochondrial apoptotic pathway induction (Cheng et al. 2001). Thus, survival factors such as IL-3 can inhibit the apoptotic activity of Bad by stimulating intracellular signalling pathways that result in the phosphorylation of Bad at Ser112 and Ser136 (Zha et al. 1996). Phosphorylation of Bad at these sites results in binding of Bad to 14-3-3 proteins and inhibition of Bad binding to Bcl-2 and Bcl-xI, thus mediating apoptosis induction (Zha et al. 1996). Bad and phospho-Bad, a pro-apoptotic protein, showed low to moderate expression in parent MCF-7 cells. A study by (Berndtsson et al. 2005) showed phosphorylation of Bad at Ser128 in paclitaxel treated fibroblasts. Considering other Bcl-2 members, a high level of Bcl-2 phosphorylation was shown clinically in breast tumours (Shitashige et al. 2001). In addition, increased resistance to taxanes is also associated with inhibition of Bcl-2 phosphorylation (Shitashige et al. 2001). Detection of the anti-apoptotic factor Bcl-2, as seen in the MCF-7 parental cell line, was consistent with the previous apoptosis microarray findings. At passage 18 of the MCF-7TaxR cell line, a significant changing point in the cell line development, the Bcl-2 levels were reduced. Moreover, complete loss in Bcl-2 expression was detected at passage 23 in the MCF-7TaxR cell line. Thus, the loss of Bcl-2 expression in MCF-7TaxR may lead to inhibition of the binding of pro-apoptotic Bad to Bcl-2 (due to their absence) (Zha et al. 1996). Taken together, these results indicate that alterations in the Bcl-2 family of proteins - whether a decrease or increase- are associated with acquired paclitaxel resistance in breast cancer cells.

In summary, the data shown in the present chapter suggest that acquisition of paclitaxel resistance is a multifactorial process. In the case of the MCF-7TaxR cell line, this acquisition of resistance involved overexpression of P-gp and extraordinary alterations in
the expression of apoptotic proteins. Interestingly, the differences in passage number from 18 to 23 passages means that over a period of only 2 weeks drastic changes occurred in apoptotic protein expression. The parental MCF-7 cells have already been identified as being deficient in caspase-3 according to previous studies and our findings further indicate that other apoptotic genes may also be absent (Kurokawa et al. 1999; Simstein et al. 2003). The apoptotic defect in caspase-3 in MCF-7 cells appears to contribute to a major modification in the way these cells develop paclitaxel resistance. Some studies of human breast cancers have shown that caspase-3 deficiency at the mRNA and protein levels may account for approximately 75% of cases of breast cancer (Devarajan et al. 2002). However, MCF-7TaxR cells appear to retain sensitivity to many types of chemotherapies and this provides strong - albeit indirect - evidence that these cells have become proficient in recruiting a non-caspase mediated form of cell death. Identification of altered apoptotic genes may be useful in predicting the response to paclitaxel in tumours of breast cancer pati
CHAPTER 6

INVESTIGATION OF ALTERNATIVE CELL DEATH MECHANISMS: AUTOPHAGIC CELL DEATH IN HUMAN BREAST CANCER CELLS
6 Investigation of Alternative Cell Death Mechanisms: Autophagic Cell Death in Human Breast Cancer Cells

6.1 Overview of autophagic cell death

Autophagy is an ancient physiological process which has an essential role in cell physiology and pathology (Yang et al. 2005). Autophagy was originally described by de Duve and Wattiaux in 1966. They demonstrated the development of lysosomes and showed that autophagy was involved in the turnover of long-lived proteins in mammalian cells (de Duve C. & Wattiaux 1966; Dunn 1994; Klionsky and Ohsumi 1999). Autophagy has a crucial function in cellular degradation of long-lived proteins and cytoplasmic organelles and, therefore, increased catabolism will increase autophagic activity, which may be important in different stress conditions (Gozuacik and Kimchi 2004). The autophagic process plays a role in cell survival during starvation by providing a source of energy (Moretti et al. 2007). In addition, autophagy may induce programmed cell death type II (PCD II), termed autophagic cell death, under extreme stress circumstances as an alternative non-apoptotic cell death (Yang et al. 2005). Therefore, autophagy may be either a cell survival mechanism during starvation or a cell death mechanism under stress conditions when, for example, apoptotic cell death (PCD I) may be inhibited (Moretti et al. 2007). Defects in autophagy have been demonstrated for diseases such as cancer and neurodegenerative disease such as Alzheimer's and Parkinson's disease (Edinger and Thompson 2004).
Autoophagy literally means, in Greek, “to eat oneself” whereby the cell switches to a catabolic metabolic programme to degrade cell constituents in order to provide energy for cell survival during nutrient stress (Edinger and Thompson 2004). Basically, cells undergo autodigestion following deprivation of nutrients and this prolongs cell survival (Tsujimoto and Shimizu 2005). Thus, autophagy processes change from catabolic survival to cell death characterised by the engulfment of cytoplasm and cytoplasmic constituents such as organelles including mitochondria and endoplasmic reticulum by a double or multimerbrane sac or vesicles (Edinger and Thompson 2004; Gozuacik and Kimchi 2004). When the membraneous sacs are formed in a double membrane structure called the autophagosome (Edinger and Thompson 2004; Gozuacik and Kimchi 2004), the autophagolysosomes are then produced by a fusion of the outer membrane of the autophagosomes and lysosomes, where contents are then sequestered and degraded (Figure 6-1) (Gozuacik and Kimchi 2004; Yorimitsu and Klionsky 2005). Therefore, cells digest themselves to induce death in the form of autophagic cell death (Edinger and Thompson 2004).
CHAPTER 6

Figure 6-1: Autophagy as a cell survival process in mammalian cells or as a cell death pathway in cancer cells and other diseases.

The sequential recruitment of autophagy proteins (Atg12-Atg5) and the conjugation of LC-3 (Atg8) occurs at the isolation membrane after the activation of the Beclin 1 complex. Then, after the formation of the autophagosome, LC-3 II binds to the luminal side of the autophagosome membrane and is recycled in the cytosol. However, the Atg 1 complex may be involved in individual steps of autophagosome development. The ultimate step is the fusion between the autophagosome and lysosome, and degradation of sequestered materials including LC3-II. Adapted from (Lefranc et al. 2007; Pattingre et al. 2008).

The molecular mechanism of autophagy is genetically controlled by autophagic genes called APG (autophagy) and AUT (auto-phagocytosis) in the yeast Saccharomyces cerevisiae which are conserved in mammalian cells (Tsukada and Ohsumi 1993; Thumm et al. 1994; Ohsumi 2001; Huang and Klionsky 2002). As a result of these studies, Apg and Aut have finally been named in the unified nomenclature ‘Atg’ (autophagy-related) genes (Tsukada and Ohsumi 1993; Thumm et al. 1994; Ohsumi 2001; Huang and
Klionsky 2002; Klionsky et al. 2003). The Atg proteins are involved in the formation of autophagosomes that include the Atg1 complex which integrates signalling from TOR (Target Of Rapamycin) kinase (Klionsky et al. 2003; Pattingre et al. 2008). This complex is involved in several steps during the nucleation and expansion steps of autophagosome formation (Shintani and Klionsky 2004). In addition, the Beclin 1 (a yeast Atg6 orthologue)/class III PI3K (hVps34) complex is involved in the nucleation phase (Kondo et al. 2005). Another marker for autophagic cell death is LC-3 (the microtubule-associated protein 1 light chain 3, MAP1 LC-3), the mammalian homolog of yeast Atg8, which is involved in autophagosome formation and is located on the autophagosome membrane (Kabeya et al. 2000; Mizushima et al. 2004). LC-3 and Atg12 are both two ubiquitin-like conjugating systems; Atg12 conjugates Atg5 during the initiation step and promotes the conjugation of LC-3 to phosphatidylethanolamine for the elongation step (Pattingre et al. 2008). The recycling pathway is controlled by Atg1 and Atg9, which mediates the shuttling of Atg proteins (Reggiori et al. 2005; Young et al. 2006). The maturation of autophagosomes requires the activity of Rab GTPases (Gutierrez et al. 2004; Jager et al. 2004).

6.2 Regulation of autophagic cell death

Autophagic cell death is regulated by different signalling pathways implicated in tumorigensis (Moretti et al. 2007). These signalling processes include phosphoinositide 3-kinases (PI3K), Akt, and the mammalian target of rapamycin (mTOR) pathway (Moretti et al. 2007) (Figure 6-2). In the presence of nutrients and growth factors, the PI3K/Akt/mTOR pathway is activated, causing autophagy suppression and activation of cell proliferation (Chen and Karantza-Wadsorth 2009). Thus, inhibition of the
PI3K/Akt/mTOR axis may lead to stimulation of autophagy (Chen and Karantza-Wadsworth 2009).

The PI3K family enzymes confer an essential function in the autophagic pathway (Lefranc et al. 2007; Moretti et al. 2007). The PI3K family also functions in cell growth and differentiation, apoptosis, cytoskeletal organization, and membrane trafficking (Toker and Cantley 1997; Cantley 2002). Class III PI3K is involved in the early stages of autophagosome production (Kondo et al. 2005; Lefranc et al. 2007). Class I PI3Ks produces PI(3,4)P₂ and PI(3,4,5)P₃ which bind to Akt (Meijer and Codogno 2004; Ng and Huang 2005; Moretti et al. 2007). The class III PI3Ks have been shown to phosphorylate phosphatidylinositol (PI) to produce phosphatidylinositol-3,4,5-triphosphate PI(3)P (Moretti et al. 2007). This mechanism is involved in the transfer of lysosomal enzymes of the trans-Golgi network to lysosomes (Kondo et al. 2005; Linder and Shoshan 2005). Dysregulation in the PI3K pathway can lead to increases in tumorigenesis and promote chemotherapy resistance, as reported in cancers such as prostate, breast, pancreatic, ovarian, and stomach cancers (Nicholson and Anderson 2002). The tumour suppressor phosphatase and tensin homologue deleted from chromosome 10 (PTEN), which is a lipid/protein phosphatase, dephosphorylates PI at the 3 position and hence downregulates the PI3K/Akt cascade (Wu et al. 1998). Thus, PTEN is an antagonist of the Akt/PI3K pathway and positively regulates autophagy (Ng and Huang 2005). The occurrence of frequent somatic mutations or deletion of PTEN has been reported for many types of cancers (Steck et al. 1997; Freeman et al. 2006). PTEN regulates different products generated from PI3K class I activity (Arico et al. 2001). Dysregulation or mutations in PTEN result in activation of the Akt cascade and inhibition of autophagy (Arico et al. 2001).
Importantly, there is a central protein in PI3K class III pathways called Beclin 1 (Atg 6 or BECN1) (Petiot et al. 2000; Boya et al. 2005). Beclin 1 is an essential protein required for binding to PI3K class III and forms a complex to stimulate vacuolar formation and induction of autophagic cell death (Kondo et al. 2005). The Beclin 1/PI3K class III complex found on the trans-Golgi network is involved in the isolation membrane that controls autophagy (Motyl et al. 2006). It was reported the Beclin 1 gene shows monoallelic deletion in 40%-70% of breast, ovarian, and prostate cancers (Aita et al. 1999; Moretti et al. 2007). Beclin 1 is expressed in MCF-7 breast cancer cells (Liang et al. 1999). According to Liang et al. (1999), Beclin 1 expression was absent in 8 of 11 tested human breast carcinoma cell lines (Liang et al. 1999). There are different compounds that may be used as tools for the investigation of autophagy, such as 3-methyladenine (3-MA), which inhibits autophagosome formation by inhibiting PI3K class III activity (Moretti et al. 2007; Chen and Karantza-Wadswoith 2009) (Figure 6-2). It was demonstrated that the addition of 3-MA to MCF-7 cells led to a reduction in autophagy via Beclin 1 inhibition (Liang et al. 1999). LY294002 and wortmannin are both PI3K inhibitors that inhibit kinase activity through different mechanisms (Blommaart et al. 1997; Aki et al. 2003; Chen and Karantza-Wadswoith 2009). LY294002 is derived from the naturally occurring bioflavonoid quercetin and it inhibits PI3K by competitive inhibition of the ATP binding site on the p85α subunit (Vlahos et al. 1994). Thus, LY294002 has been used as an autophagy inhibitor, via inhibition of PI3K (Moretti et al. 2007; Yang et al. 2008; Chen and Karantza-Wadswoith 2009). Wortmannin (derived from a fungal metabolite) inhibits PI3K via targeting the p110 subunit of PI3K (Powis et al. 1994; Wymann et al.
The mTOR signalling pathway has been closely associated with autophagy regulation (Pattingre et al. 2008). The TOR protein is a Ser/Thr protein kinase that has been identified in all eukaryotic cells as being derived from a single gene (Pattingre et al. 2008). TOR was first found in yeast as a target for the antifungal drug rapamycin produced by *Streptomyces hygroscopicus* (reviewed in Ng and Huang 2005). Thus, rapamycin inhibits TOR activity and induces autophagy in yeast and mammalian cells (Ng and Huang 2005). mTOR is a large protein of 289 kDa that is a kinase (Yang et al. 2005) and a gatekeeper for autophagy, inducing its inhibition (Yang et al. 2005). The mTOR pathway is regulated by two mechanisms. Firstly, mTOR controls autophagy by downstream effectors such as the S6K substrate and 4E-BP1 (Cardenas et al. 1999). Secondly, mTOR acts directly or indirectly to affect Atg proteins leading to interference with autophagosome formation (Levine and Klionsky 2004). Proteins in the mTOR pathway involve phosphorylation of S6K and its target ribosomal protein S6, causing inhibition of autophagy (Scott et al. 2004). The Akt pathway works in concert with mTOR, since Akt activates mTOR leading to autophagy inhibition (Ng and Huang 2005).

The family of death associated kinases includes the death associated protein kinase (DAPK) and DAPK-related protein-1 (Ng and Huang 2005). Overexpression of these proteins induces autophagy in different cancer cell lines (Inbal et al. 2002). A dominant-negative construct of DAPK-related protein -1 in MCF-7 breast cancer cells produced a decrease in both starvation and autophagy induction following tamoxifen treatment (Ng and Huang 2005). Moreover, reduced expression levels of DAPK in HeLa cells led to ...
decreased interferon-γ-induced autophagy (Ng and Huang 2005). In addition, the Ras pathway regulates autophagy in two ways. Firstly, Ras inhibits autophagy by activating class I PI3K and the Akt/mTOR pathway (Budovskaya et al. 2004; Furuta et al. 2004; Li et al. 2007). Secondly, the Ras pathway induces autophagy via the RAF1/MEK1/2/ERK1/2 pathway (Pattingre et al. 2003) (see Figure 6-2).

Figure 6-2: Summary of autophagy regulation, showing the important signalling pathways involved. Adapted from (Chen and Karantza-Wadsworth 2009).

6.3 Defects in autophagic cell death in cancer cells

Different studies have recently reported that a defect in autophagic pathways may correlate with cancer cell progression and proliferation (Okada and Mak 2004). The earliest link between autophagy and cancer was found in the late 1970s (Lefranc et al. 2007). It was found that the extent of autophagy activity is lower in cancer and was correlated with cancer cell survival (Ng and Huang 2005). However, different studies
showed that autophagy activity possesses a tumour suppressor function during the early stages of tumour development (Kondo et al. 2005). A study by Toth et al. (2002), on pancreatic adenocarcinoma revealed a reduction in autophagy activity (Toth et al. 2002). This study suggested that the level of autophagy first increases at an early stage of cancer or in the pre-malignant stage with a reduction in autophagy activity during the transition of adenoma to adenocarcinoma (Ng and Huang 2005). Therefore, under certain conditions a deficiency or defect in autophagy may contribute to the development of cancer (Ng and Huang 2005).

The autophagic mechanism is a major pathway for recycling long-lived proteins and increases their degradation rate, which leads to a reduction in cellular protein mass (Ng and Huang 2005). For cancer cells with a high level of cellular proliferation, induction of a high level of autophagy may inhibit proliferation (Ng and Huang 2005). Another vital function of autophagy is the elimination of damaged organelles and, therefore, when the autophagic mechanism is dysregulated, it causes accumulation of these organelles in cells, leading to an increase in genotoxic related mutations (Ng and Huang 2005). A study on endothelial cells showed that autophagic cell death features occur in the presence of endostatin, an angiogenesis inhibitor (Chau et al. 2003; Ng and Huang 2005). These findings indicate that autophagic cell death causes inhibition of angiogenesis, leading to the suppression of tumour progression (Chau et al. 2003; Ng and Huang 2005).

There are other defects occurring in a cancer cell that affect the autophagic process, and some of these defects are in oncogenes and tumour suppressor genes that control and influence the autophagy pathway (Ng and Huang 2005). These genes include PTEN, class
I PI3K, Akt and Ras (Ng and Huang 2005). Dysregulation of the Akt/PKB pathway is involved in tumorigenesis and resistance to therapy in breast, prostate, pancreatic, ovarian, and stomach cancers (Nicholson and Anderson 2002).

6.4 Autophagic cell death is an alternative to apoptosis in cancer

A large body of evidence in the literature has demonstrated a correlation between loss of apoptotic cell death responses in cancer cells and additional decreases in autophagic activity as a contributory process leading to carcinogenesis (Hanahan and Weinberg 2000; Green and Evan 2002; Gozuacik and Kimchi 2004). The autophagic mechanism may have dual functions in cultured cancer cells, delaying apoptosis pathways in one cell type and at the same time promoting cell death in another cell (Yang et al. 2005). Cells may undergo caspase-independent autophagic cell death, which may or may not be accompanied by signs of apoptosis (Assuncao Guimaraes and Linden 2004). However, in other situations, cells may have options to select between autophagic cell death and apoptotic cell death (Assuncao Guimaraes and Linden 2004). An IL-3-dependent cell line induced to undergo cell death by IL-3 deficiency led to activation of autophagy, but not autophagic cell death, because autophagy in this case was a survival mechanism (Lum et al. 2005). However, a study on murine embryonic fibroblast (MEFs) that were deficient in Bax/Bak (pro-apoptotic proteins), showed non-apoptotic cell death when exposed to different cytotoxic agents, such as etoposide, staurosporine and thapsigargin, even though all these agents are apoptosis inducers (Tsujimoto and Shimizu 2005). The features of cell death in MEFs revealed a loss in clonogenicity and positive propidium iodide staining of the plasma membrane with no signs of membrane disruption, and electron microscopy analysis
demonstrated evidence of a number of double-membrane vesicles (Shimizu et al. 2004). The vesicles in MEF cells were later confirmed to be autophagosomes by the presence of LC-3, a marker of autophagic cell death, and Atg8, which was concentrated on the autophagosomes but present throughout the cytoplasm (Shimizu et al. 2004). According to these earlier data, autophagic cell death involves association of autophagosome formation with autophagy proteins, which leads to cell death after exposure to cytotoxic insult (Shimizu et al. 2004). This established the existence of alternative cell death mechanisms (Shimizu et al. 2004). However, the field is still developing, but, in general, prior studies on autophagic cell death have revealed that autophagic cell death follows cytotoxic insult when apoptotic cell death is compromised for whatever reason (Tsujimoto and Shimizu 2005).

6.5 Aim

This chapter focuses on investigating the alternative cell death mechanism in paclitaxel resistant MCF-7TaxR cells following a line of enquiry directed towards understanding autophagic cell death.

6.6 Methods

Methods used in this chapter are Western immuno-blotting analysis, cytotoxic MTT assay, acridine orange staining experiment by flow cytometry analysis, confocal microscopy and electron microscopy imaging. All methods were described in Chapter 2 (Methods and Materials).
6.7 Results

6.7.1 Detection of Beclin 1 and LC-3 expression in paclitaxel resistant MCF-7TaxR cells

Beclin 1 is known to interact with class III PI3Ks and to induce autophagy (Aita et al. 1999; Moretti et al. 2007). As human Beclin 1 is deleted in 40-70% of breast and ovarian cancers (Aita et al. 1999; Liang et al. 1999), it is, therefore, important to assess the molecular mechanism of autophagic cell death in MCF-7TaxR cells by examining the expression of Beclin 1. In addition, we detected LC-3 expression in parental MCF-7 and MCF-7TaxR cells treated with staurosporine using Western blot analysis. LC-3I was localized to the cytosol, suggesting that, during the autophagy process, LC-3I is cleaved and conjugated to phosphatidylethanolamine to form LC-3II, which is important for autophagosome formation (Kondo et al. 2005). LC-3 detection has been used to estimate the abundance of autophagosomes in previous studies (Kondo et al. 2005).
Figure 6-3: Expression of Beclin 1 and LC-3 in staurosporine treated MCF-7 and MCF-7TaxR cells.
Whole cell lysates were made following 24 h or 48 h 0.5 μM staurosporine treatment, as described in the Materials and Methods. Samples (50 μg protein) were electrophoresed on a 10%-12% SDS-PAGE gel. The proteins were then transferred to a PVDF membrane and probed with a polyclonal ant-rabbit BECN1 antibody (Santa Cruz Biotechnology Inc) or a monoclonal anti-rabbit LC3B antibody (Cell Signaling Corp), at a 1:200 or 1:1000 dilution, respectively. The immunoreactive bands were revealed by further incubation of the immunoblot with an anti-rabbit alkaline phosphatase antibody (Invitrogen™) followed by incubation with a chemiluminescent substrate (Invitrogen™). The densitometric ratios of LC-3II were calculated with respect to LC-3I. Data shown are representative of repeat experiments (n = 3).
In Figure 6-3, Western blot analysis showed that Beclin 1 was expressed in untreated and staurosporine treated MCF-7 and MCF-7TaxR cells. The level of Beclin 1 was increased in MCF-7TaxR cells following treatment with staurosporine, an effect not seen for MCF-7 cells. An important autophagic marker, LC-3 was also examined. The two forms, LC-3 I and II, were expressed in MCF-7 and MCF-7TaxR cells in the presence and absence of 0.5 µM staurosporine.

![Western blot analysis of Beclin 1 and LC-3](image)

Relative densities of bands on Western blots

<table>
<thead>
<tr>
<th>Relative ratio</th>
<th>MCF-7 C</th>
<th>MCF-7 5 µM Ver. 24 h</th>
<th>MCF-7 5 µM Ver. 48 h</th>
<th>MCF-7TaxR C</th>
<th>MCF-7TaxR 5 µM Ver. 24 h</th>
<th>MCF-7TaxR 5 µM Ver. 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-3II/LC-3I</td>
<td>1.4</td>
<td>1.6</td>
<td>1.5</td>
<td>1</td>
<td>2.2</td>
<td>2.1</td>
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**Figure 6-4: Expression of LC-3 in MCF-7 and MCF-7TaxR following treatment with verapamil.**

Whole cell lysates were made following 24 h or 48 h 5 µM verapamil treatment, as described in the Materials and Methods. Samples (50µg protein) were electrophoresed on a 12% SDS-PAGE gel. The proteins were then transferred to a PVDF membrane and probed with a monoclonal anti-rabbit LC3B antibody (Cell Signaling Corp) at a 1:1000 dilution. The immunoreactive bands were revealed by further incubation with an anti-rabbit alkaline phosphatase antibody (Invitrogen™) followed by incubation with a chemiluminescent substrate (Invitrogen™). The densitometric ratios of LC-3II were calculated with respect to LC-3I. Data shown are representative of repeat experiments (n = 3).
It can be seen from Figure 6-4 that MCF-7TaxR cells had higher levels of LC-3II than MCF-7 parental cells, following treatment with 5 μM verapamil. Since verapamil is a Ca^{2+} channel antagonist, these results suggest that verapamil may stimulate autophagic cell death (Williams et al. 2008). During the autophagic process, LC-3I is converted to LC-3II which is involved in autophagosome formation. Therefore, the high ratio of LC-3II to LC-3I, indicates that verapamil treatment enhanced the induction of autophagic cell death. The MCF-7 cells showed higher levels of LC-3I than the converted form LC-3II, suggesting a higher level of autophagic activity in MCF-7TaxR cells. These data were confirmed and explored in more detail in the following sections.
6.7.2 Autophagic vacuole detection by acridine orange staining using flow cytometric analysis

To detect autophagic cell death in the MCF-7 and paclitaxel resistant MCF-7TaxR cell lines, we performed acridine orange staining with flow cytometry, which has been widely used for the identification of autophagy, offering a semiquantitative assessment of the process (Klionsky and Ohsumi 1999). Acridine orange was used to stain the acidic vesicles associated with autophagic vacuoles in MCF-7 and MCF-7TaxR cells. MDA-MB-231 cells were used as a negative control. The treatment period for MDA-MB-231 cells was 24 h (as opposed to 48 h for the MCF-7 cell lines), as this cell line is very sensitive to drug treatment and a 48 h exposure gives rise to very substantial amounts of cell death, making analysis difficult. Cells were treated with staurosporine and bafilomycin (an autophagy inhibitor). Bafilomycin inhibits the vacuolar H⁺-ATPase, the enzyme that mediates acidification of autophagic vacuoles (Paglin et al. 2001). Samples were analysed using flow cytometric analysis and acridine orange staining was measured at 630 nm for red fluorescence and at 525 nm for green fluorescence (Figure 6-5).
CHAPTER 6

**Acridine Orange Staining +ve**

### 24h

**MDA-MB-231 Cells**

- **Control**
- **5 μM bafilomycin**
- **1 μM staurosporine**
- **1 μM staurosporine + 5 μM bafilomycin**

### 48h

**MCF-7 Cells**

- **Cdh1/β1**
- **5 μM bafilomycin**
- **1 μM staurosporine**
- **1 μM staurosporine + 5 μM bafilomycin**

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Figure 6-5: Quantitation of acidic vesicles in MDA-MB-231, MCF-7, and MCF-7TaxR cells by acridine orange staining using flow cytometric analysis.
The histograms are representative of typical data obtained. Cells were treated with 5 μM bafilomycin or 1 μM staurosporine, or both drugs for 24 h or 48 h. Cells were harvested and processed for acridine orange staining as described in the Materials and Methods. Samples were then analysed using flow cytometric analysis of acridine orange stained cells at 630 nm for red fluorescence and at 525 nm for green fluorescence.

The data presented in Figure 6-5 are representative of the acridine orange staining analysis for MDA-MB-231, MCF-7, and MCF-7TaxR cells. In (a), treatment of MDA-MB-231 cells with staurosporine resulted in a decrease in the percentage of living cells (F3), and the decrease was similar to that in cells treated with bafilomycin in the presence of staurosporine. These results indicate that no inhibitory effect of bafilomycin on autophagy was present in these cells and most likely indicates an absence of this form of cell death. In (b), MCF-7 cells treated with staurosporine showed a decrease in the live cell percentage (F3). In (c), staurosporine treatment resulted in a drop in the number of (F3)
live MCF-7TaxR cells. The cell death of MCF-7TaxR cells was suppressed when the cells were treated with staurosporine and bafilomycin, again indicating the presence of autophagic cell death in the presence of cellular stress.

Figure 6-6: Inhibition of autophagy in MCF-7TaxR cells treated with bafilomycin. The graph shows a significant level of autophagic cell death inhibition in MCF-7 and MCF-7TaxR cells treated with bafilomycin. Data (obtained from the low acridine orange staining, F3 quadrant) are means of 3 independent experiments; error bars show ±SD. ** p = 0.038, and *** p = 0.012, using a paired Student’s T-test.

As shown in Figure 6-6, there was no significant difference in red fluorescence between MDA-MB-231 staurosporine treated cells and those treated with both staurosporine and bafilomycin (p= 0.921). These data suggest that MDA-MB-231 cells are devoid of autophagic cell death. However, there was a significant difference between MCF-7 cells treated with staurosporine and MCF-7 cells treated with both staurosporine and bafilomycin (p= 0.038). Additionally, there was a significant difference between MCF-7TaxR cells treated with staurosporine and those treated with both staurosporine and bafilomycin (p=0.012). These data indicate an inhibitory effect of bafilomycin on autophagic cell death in MCF-7 cells, with an even more pronounced effect in MCF-
7 TaxR cells. The level of autophagy seen in the breast cancer cell lines examined can be ascribed the following order: MDA-MB-231<< MCF-7< MCF-7TaxR.

6.7.3 Detection of the autophagic cell death marker LC-3 by immunostaining

The localization of LC-3, a subunit of microtubule-associated proteins 1A and 1B, was investigated in MCF-7 and MCF-7TaxR cells. LC-3 has been described as a reliable marker for autophagic activity (Kabeya et al. 2000; Mizushima et al. 2004). MDA-MB-231 cells were used as a negative control for LC-3 detection as we failed to see any evidence of autophagy in this model. In order to make valid comparisons between the various cell lines, it was necessary to use the same instrument imaging settings for MCF-7TaxR cells as for MCF-7 and MDA-MB-231 cells.
MDA-MB-231 Cells

Figures 6-7

(a) Control  
(b) 0.25 μM St.
(c) 0.5 μM St.
(d) 0.5 μM St.
MCF-7 Cells

Figure 6-7
MCF-7TaxR Cells

Figure 6-7
Figure 6-7: Confocal microscopy imaging of LC-3 in MCF-7 and MCF-7TaxR cell lines.

Cells were incubated with 0.5 μM or 0.25 μM staurosporine for 24 h, harvested, and processed as described in Materials and Methods. Imaging of the cells was carried out by confocal microscopy (Carl Zeiss Laser Scanning System LSM510 (multi scan mode, 2 channels, wavelengths 488 nm and 633 nm). The MCF-7TaxR cells treated with staurosporine in (j) and (k) revealed a high level of LC-3 staining, as did the controls (i). In the enlarged image (L2), a higher level of LC-3 staining was observed in the MCF-7TaxR cells than in the MCF-7 cells (h1 and h2). Data are representative of repeat experiments (n = 3).

In Figure 6-7, the MDA-MB-231 untreated staurosporine treated cells showed no evidence of LC-3 staining, indicating the absence of autophagy in these cells. LC-3 was clearly detectable in the control untreated MCF-7TaxR cells as well as in the treated MCF-7TaxR cells, where the LC-3 level was higher and more intense. The changes in the intracellular localization of LC-3, as seen in the MCF-7TaxR images (Figure 6-7) and in the enlarged image (L2), indicates a substantial recruitment of LC-3 to autophagic membranes, indicating autophagic activity. In the MCF-7 untreated control, LC-3 localization was also detectable and was the same as in MCF-7 cells treated with staurosporine, although the LC-3 level was lower than in MCF-7TaxR cells.

6.7.4 Electron microscopy analysis of autophagic vacuole formation

For the detection of autophagosomes in MCF-7TaxR cells and MCF-7 cells, we performed an electron microscopy analysis. Electron microscopy is one of the methods of choice for the investigation of autophagic cell death, and its use has been established in numerous studies (Klionsky et al. 2007). The electron microscopy analysis was carried out as described in Chapter 2 under Materials and Methods. The processing and embedding of
cells was performed in collaboration with Brunel University, Department of Biosciences. Samples (on copper grids) were analysed by scanning transmission electron microscopy (STEM, Philips CM200 TEM) at the MSSU, University of Surrey. Imaging was performed by Dr Vladimir Stolojan (Figure 6-8).

**Figure 6-8: Electron microscopy imaging for MCF-7 cells.**
Electron microscopy was carried out as described in Chapter 2, Materials and Methods. Images of MCF-7 control cells, and treated with 0.5 μM staurosporine for 48 h. The images were obtained using a Philips CM200 Transmission Electron Microscope (power 200kV x 13.0k). The bar indicates 5 μm or 2 μm magnification. A+B= artefact, C=possible burning by E.M source (shown by arrows), D=membrane blebbing, N=nucleus.
Figure 6-9: Electron microscopy imaging for in MCF-7TaxR cells.
Electron microscopy was carried out as described in Chapter 2, Materials and Methods. Images of MCF-7TaxR control cells, and treated with 1 μM staurosporine for 48 h. The images were obtained using a Philips CM200 Transmission Electron Microscope (power 200kV x 13.0k). The bar indicates 2 μm magnification. A= possible artefact effecting nuclear morphology, B= marked membrane blebbing, C= multiple small vesicle formation (shown by arrows), D= artefact, N=nucleus.

In Figure 6-8 and Figure 6-9, artefacts include problems with condition of cells prior to embedding (excessive membrane blebbing in untreated cells 6.9.B). Shrinkage of nucleus and cytoplasm seen in 6.8.A; evidence of problems with section cutting (possible
problems with embedding resin consistency) seen in 6.8.B and 6.9.D.

The electron microscopy images in Figure 6-8 and Figure 6-9, were taken for possible autophagosome detection in MCF-7 and MCF-7TaxR cells. However, due to the technical artefacts, images were difficult to interpret. However, the cytoplasm in MCF-7TaxR treated with staurosporine (6.9 bottom left and right) indicated a high number of small vesicle formation (possibly lysosomal). Control and staurosporine treated MCF-7 cells showed more intact cytoplasm with less vesicular structures apparent. Evidence of autophagolytosome structures was inconclusive due to technical difficulties e.g. artefact, resolution used and limited number of clear images showing cellular structures.

6.7.5 Inhibition of autophagy by the inhibitors 3-MA and LY294002 detected by the MTT assay

Our previous observations showed some evidence of autophagosome formation in MCF-7TaxR cells (based on LC3 examination). Therefore, we decided to explore this further using different PI3K inhibitors that have been widely used to cause autophagy inhibition in prior studies (Liang et al. 1999; El-Kholy et al. 2003; Moretti et al. 2007). We used 3-methyladenine (3-MA), which has been used to inhibit vacuole formation via PI3K class III inhibition during autophagy (Moretti et al. 2007). Also, we used LY294002 which was demonstrated in a previous study to inhibit the kinase activity of PI3K (Blommaart et al. 1997; Chen and Karantza-Wadsworth 2009). The experiment was carried out using the MTT cell viability assay (Figure 6-9 and 6-10). Doses of 300 nM 3-MA or 10 μM LY294002 were administered concomitantly with a cytotoxic drug dose of cisplatin for a
72 h period to the MCF-7 and MCF-7TaxR cells in 96 well plates. Results were expressed as IC\textsubscript{50} values, which were calculated as the drug doses that caused 50% cell death relative to the untreated controls, as described in the Materials and Methods.

![Graph showing IC\textsubscript{50} values for MCF-7 and MCF-7TaxR cells.]

**Figure 6-10:** A significant loss of cisplatin cytotoxicity in MCF-7 and MCF-7TaxR cells in response to 3-methyladenine co-administration.

MTT assay, MCF-7 and MCF-7TaxR cells were grown in 96 well plates at a cell density of 2x10\textsuperscript{4} - 3x10\textsuperscript{4}/ml for 24 h. After treatment with cisplatin or a combination of cisplatin and 300 nM 3-MA, the cells were incubated for a further 72 h. Each data point is the mean of 3 independent experiments (performed in triplicate); error bars are ±SD.
Figure 6-11: A significant loss of cisplatin cytotoxicity in MCF-7TaxR cells in response to LY294002. MTT assay, MCF-7 and MCF-7TaxR breast cancer cells were grown in 96 well plates to a cell density of $2 \times 10^4 - 3 \times 10^5$ ml for 24 h. After treatment with cisplatin or a combination of cisplatin and 10 µM LY294002, the cells were incubated for 72 h. Each data point is the mean of 3 independent experiments (performed in triplicate); error bars are ±SD.
Table 6-1: The IC_{50} values for MCF-7 and MCF-7TaxR cells treated with cisplatin, cisplatin +3-MA, or cisplatin + LY294002.
The IC_{50} values are the means of 3 independent MTT assays (performed in triplicate); the ±SD values are shown in parentheses. p values were calculated using a paired Student's test of cisplatin IC_{50} versus the IC_{50} of cisplatin with 3-MA or LY294002. p** < 0.05, p*** < 0.02.

As shown in Figures 6-10 and 6-11, 300 nM 3-MA or 10 μM LY294002 was administered concomitantly with a cytotoxic drug dose of cisplatin for 72 h to MCF-7 and MCF-7TaxR cells. The statistical significance in the differences in cisplatin cytotoxicity with co-administration of either 3-MA or LY294002 is shown in Table 6-1. The LY294002 inhibitor was more efficient in reducing drug sensitivity than 3-MA in MCF-7TaxR cells.

6.7.6 Detection of Akt/mTOR expression by Western blot analysis

The PI3K/Akt/mTOR pathway axis is a prototypic survival mechanism, and constitutive activation of this pathway is commonly encountered in human cancers (LoPiccolo et al. 2008). Abnormal activation of this pathway has been shown to cause inhibition of autophagy (Arico et al. 2001; Ueno et al. 2008). Therefore, it was relevant to investigate the expression of Akt/mTOR and its downstream effector S6K in MCF-7 and MCF-7TaxR cells in response to staurosporine or the mTOR inhibitor rapamycin. Cells treated with 0.5 μM staurosporine were subjected to Western blot analysis to detect the expression of Akt, mTOR, and S6/P-S6K. In addition, cells were treated with a dose of
rapamycin (40 μM), which was chosen after testing of different doses of rapamycin in the MTT assay (data not shown) with MCF-7 and MCF-7TaxR cells. The data we obtained indicated that 20 μM rapamycin had no effect on cell viability. Moreover, we note that rapamycin analogues such as RAD001 are many fold more potent in vitro than rapamycin (based on other studies carried out in our laboratory, unpublished). Thus, we treated cells with 40 μM rapamycin and, then, performed Western blot analysis for mTOR expression.

Relative densities of bands on Western blots

<table>
<thead>
<tr>
<th>Relative ratio</th>
<th>MCF-7 C</th>
<th>MCF-7 0.5 μM St. 24 h</th>
<th>MCF-7 0.5 μM St. 48 h</th>
<th>MCF-7TaxR C</th>
<th>MCF-7TaxR 0.5 μM St. 24 h</th>
<th>MCF-7TaxR 0.5 μM St. 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-Akt/Akt</td>
<td>0.72</td>
<td>0.3</td>
<td>0.3</td>
<td>0.74</td>
<td>0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Figure 6-12: Detection of Akt/P-Akt expression in MCF-7 and MCF-7TaxR cells following staurosporine treatment.
Whole cell lysates were made following 24 h or 48 h 0.5 μM staurosporine treatment, as described in the Materials and Methods. Samples (50 μg protein) were electrophoresed on a 10% gel. After transfer of the proteins to a PVDF membrane, the membrane was probed with a polyclonal anti-rabbit Akt antibody or a monoclonal anti-rabbit P-Akt [pS^473] antibody (Biosource) at dilutions of 1:200 for both. Then, the blot was exposed to an anti-rabbit alkaline phosphatase antibody (Invitrogen™). Bands were detected using a chemiluminescent substrate (Invitrogen™). The densitometric ratios of P-Akt were calculated with respect to Akt. Data are representative of repeat experiments (n = 3).
As shown in Figure 6-12, the ratio of phosphorylated Akt:Akt was similar for both control MCF-7 parental and paclitaxel resistant lines. After treatment with staurosporine for 24 h or 48 h, MCF-7 cells expressed Akt, whereas MCF-7TaxR cells showed only a weak signal for Akt protein. Phosphorylated-Akt [pS\textsuperscript{473}] was reduced in staurosporine treated MCF-7 cells, whereas it was completely absent in MCF-7TaxR cells under the same conditions.
Relative densities of bands on Western blots

<table>
<thead>
<tr>
<th>Relative ratio</th>
<th>MCF-7 C</th>
<th>MCF-7 0.5 μM St. 24 h</th>
<th>MCF-7 0.5 μM St. 48 h</th>
<th>MCF-7TaxR C</th>
<th>MCF-7TaxR 0.5 μM St. 24 h</th>
<th>MCF-7TaxR 0.5 μM St. 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-mTOR/mTOR</td>
<td>1</td>
<td>0.7</td>
<td>0.7</td>
<td>0.48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P-S6K-S6K</td>
<td>1.4</td>
<td>0.73</td>
<td>0.45</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 6-13: Expression of mTOR and 70S6K in MCF-7 and MCF-7TaxR cells following staurosporine treatment.

Whole cell lysates were made following 24 h or 48 h 0.5 μM staurosporine treatment, as described in the Materials and Methods. Samples (50 μg protein) were electrophoresed on a 7% or a 12% gel. After transfer to a PVDF membrane, the proteins were probed with polyclonal anti-rabbit mTOR/P-mTOR (Ser2448) antibodies or S6K/P-S6K (Ser371) anti-rabbit antibodies (obtained from Cell Signaling Corp) at a dilution of 1:1000. The membrane was then exposed to an anti-rabbit alkaline phosphatase antibody (Invitrogen™). Bands were detected by using a chemiluminescent substrate (Invitrogen™). The densitometric ratios of P-mTOR and P-S6K were calculated with respect to mTOR and S6K respectively. Data shown are representative of repeat experiments (n = 3).

In Figure 6-13, mTOR protein was expressed in untreated MCF-7 and MCF-7TaxR cells as well as in MCF-7 and MCF-7TaxR cells treated with staurosporine. However, phosphorylated mTOR was only expressed in the control and staurosporine-treated MCF-7 cells. The MCF-7TaxR control and treated cells showed some expression of mTOR, but
the treated cells showed no evidence of phosphorylated mTOR (Ser 2448). S6K and its phosphorylated form, P-S6K (Ser371), were detected in both untreated and staurosporine-treated MCF-7 cells. Untreated MCF-7TaxR cells showed a low but detectable level of S6K protein expression, but S6K and P-S6K (Ser371) expression was abolished following staurosporine treatment.

Relative band density ratios on Western blots.

<table>
<thead>
<tr>
<th>Relative ratio</th>
<th>MCF-7 C</th>
<th>MCF-7 40 μM rapamycin 48h</th>
<th>MCF-7TaxR C</th>
<th>MCF-7TaxR 40 μM rapamycin 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-mTOR/mTOR</td>
<td>0.36</td>
<td>0</td>
<td>0.47</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 6-14: Expression of mTOR and P-mTOR in MCF-7 and MCF-7TaxR cells following rapamycin treatment.

Whole cell lysates were made following 24 h or 48 h 40 μM rapamycin treatment, as described in the Materials and Methods. Samples (50 μg protein) were electrophoresed on a 7% gel, and the proteins were transferred to a PVDF membrane. After probing with poly-clonal anti-rabbit mTOR/P-mTOR antibodies (Ser2448) (obtained from Cell Signaling Corp) at a dilution 1:1000, the blot was exposed to an anti-rabbit alkaline phosphatase antibody (Invitrogen™). Bands were detected using a chemiluminescent substrate (Invitrogen™). The densitometric ratios of P-mTOR were calculated with respect to mTOR. Data shown are representative of repeat experiments (n = 3).
Figure 6-14 shows that MCF-7 and MCF-7TaxR cells treated with rapamycin expressed varying amounts of mTOR. In control untreated MCF-7 and MCF-7TaxR cells, mTOR and P-mTOR (Ser2448) were expressed. When MCF-7 and MCF-7TaxR were treated with rapamycin, there was a complete abolition of phosphorylated mTOR (Ser2448), indicating that rapamycin inhibits the phosphorylation of mTOR.

6.7.7 Serum free medium deprivation test for autophagy inhibition in MCF-7 and MCF-7TaxR cells.

To further evaluate the inhibition of autophagy in MCF-7 and MCF-7TaxR cells, we grew MCF-7 and MCF-7TaxR cells in serum free medium (absence of essential growth factors), harvested them, and then further processed them for Annexin V-FITC/PI analysis to reveal the presence of an apoptotic population. This approach was used because the removal of serum growth factors from the culture medium switches off the autophagy-suppressive pathway dependent on PI3K class I (Petiot et al. 2000). This analysis was carried out by seeding cells in standard medium (containing essential growth factors) in parallel with cells seeded in 1% serum containing medium and serum free medium for 72 h. Cells were then harvested and processed for Annexin V-FITC/PI labelling.
Figure 6-15: Annexin V-FITC/PI analysis of MCF-7 and MCF-7TaxR cells in the presence of 1% serum medium or serum free medium. The histograms are representative of typical data obtained. Cells were seeded in standard culture medium containing 10% FCS, 1% FCS, or in serum free medium for 72 h and harvested and processed for Annexin V-FITC/PI staining as described in Chapter 2, Materials and Methods. The percentages of cells in each of the quadrants represent the mean of three experiments.

The data shown in Figure 6-15 indicate that both MCF-7 and MCF-7TaxR cells respond to serum deficiency with an increased level of apoptosis. MCF-7 and MCF-7TaxR cells grown in 1% medium showed no alteration in cell viability compared to control cells. Using serum free medium, 16.6% early apoptotic cells were detected in MCF-7 cells and 8.8% were detected in MCF-7TaxR cells.
**Figure 6-16: Data analysis of Annexin V-FITC/PI staining of MCF-7 and MCF-7TaxR cells.**
The graph shows the differences in cell death inhibition in serum free medium and in 1% serum medium compared to controls (cells with -ve Annexin V-FITC/ -ve PI staining quadrant, F3). MCF-7 and MCF-7TaxR cells were no significantly different in control versus serum free medium. (#) p>0.05, using a paired Student’s t-test. Error bars, ±SD. Data shown are representative of repeat experiments (n = 3).

**6.8 Discussion**

In this chapter, we provided evidence for upregulation of the autophagic cell death pathway in paclitaxel resistant MCF-7 breast cancer cells. We used the most widely used methods for autophagic cell death detection, including confocal microscopy analysis of LC-3, detection of Beclin 1, LC-3 and Akt/mTOR by Western blotting, acridine orange staining and flow-cytometry analysis, and detection of autophagy inhibition using different agents (3-MA and LY294002), with cell viability readouts.

MCF-7 cells have already been established as models for the investigation of alternative cell death responses due to the constitutive absence of caspase-3 in these cells (Kurokawa et al. 1999; Simstein et al. 2003; Lamparska-Przybysz et al. 2006). This major apoptotic
defect gives rise to a number of changes in the routing of cell death signals in MCF-7 cells, and in the development of drug resistance, as we observed for the paclitaxel resistant MCF-7TaxR model. A previous study demonstrated induction of autophagic cell death in MCF-7 cells with tamoxifen treatment (Bursch et al. 1996). Another study, by Cui et al. (2007), showed induction of autophagy in MCF-7 cells treated by oridonin, a Chinese herbal extract that is widely used in the clinical setting (Cui et al. 2007). Our screen for apoptotic genes in paclitaxel resistant MCF-7TaxR cells demonstrated a complete absence of most major apoptosis regulators, such as caspase-3, -6, -7, and caspase-9, which led us to hypothesize that a non-apoptotic process such as autophagy is likely to be the predominant cell death mechanism operating in these cells.

Beclin 1 is an essential and major regulator of autophagy; it is alternatively referred to as the mammalian autophagy gene Atg6, which is required for autophagosome formation in conjunction with class III PI3Ks (Kondo et al. 2005). Different studies have reported that frequent loss of Beclin-1 protein expression in breast cancers correlates with the loss of tumour suppressor functions of Beclin-1 (Liang et al. 1999). Beclin-1 is an important autophagy regulator, since human Beclin-1 is mono-allelically deleted in approximately 75% of ovarian cancers, 50% of breast cancers, and 40% of prostate cancers (Aita et al. 1999). In addition, the level of Beclin 1 protein was significantly decreased in 18 of 32 breast cancer cases using immunohistochemical analysis, suggesting a carcinoma cell-specific loss of Beclin 1 expression (Gozuacik and Kimchi 2004). Here, we demonstrated that expression of Beclin-1 increased in MCF-7TaxR staurosporine treated cells. The expression of Beclin 1 in MCF-7TaxR cells suggests that the cells are potentially autophagy positive. The tumour suppressor properties of Beclin 1 were demonstrated in
vitro by clonogenic assays and in vivo by assessing tumorigenicity in nude mice (Liang et al. 1999; Liang et al. 2001). In subsequent studies, Beclin 1 expression was detected in MCF-7 breast cancer cells and associated with increased formation of autophagic vesicles, which is consistent with our current findings (Gozuacik and Kimchi 2004). Also, in our investigation, Beclin 1 was expressed in MCF-7 control and staurosporine treated cells, and this result agrees with previous studies involving the MCF-7 cell line (Liang et al. 1999).

Another major marker for the presence of autophagosome formation is LC-3, as described in various studies (Kondo et al. 2005). LC-3 is the microtubule-associated protein 1 light chain 3 (MAP1 LC-3), the mammalian homolog of yeast Atg8, which participates in autophagosome formation and is an important element of autophagosome membranes (Kondo et al. 2005). LC-3I is a marker for autophagy induction and is subsequently converted to the lipid conjugated form, LC-3II, which is associated with acidic autophagic vacuoles, the so-called autophagolysosomes (Kabeya et al. 2000; Mizushima et al. 2004). Formation of LC-3II leads to upregulation of the major autophagy proteins Atg5 and Beclin 1 (Figure 6-1) (Kabeya et al. 2000; Mizushima et al. 2004). In addition, different studies have shown that the LC-3II form is mainly localised to the autophagosome membranes (Kabeya et al. 2000). A study of PC-3 human prostate cancer cells, showed the presence of the processed LC-3II form, which is indicative of the redistribution of LC-3II to autophagosomes, by immunoblotting cell lysates from sulforaphane treated cells (Herman-Antosiewicz et al. 2006). Here, we demonstrated by Western blotting, the constitutive expression of LC-3/Atg8 (LC-3 I and II) in both MCF-7 and MCF-7TaxR cells, and in staurosporine treated samples. Furthermore, the MDR inhibitor verapamil has
been suggested to stimulate autophagic cell death (Williams et al. 2008), and treatment of MCF-7 and MCF-7TaxR cells with verapamil increased expression of LC-3I and II in both cell types. However, LC-3II was expressed at a much higher level in MCF-7TaxR cells. These data were further corroborated by confocal microscopy which detected the localisation of LC-3 in the cytoplasm of the cells. MCF-7TaxR cells showed a higher LC-3 level than MCF-7 cells. In a study of MCF-7 cells administered fenretinide, cells died via autophagic cell death (Fazi et al. 2008). The MCF-7 cell response to fenretinide treatment was associated with an increase in Beclin 1 expression with conversion of LC-3 to the autophagic vesicle-associated LC-3II form with a shift from a uniform distribution to a more punctuate staining pattern and an increase in lysosome/autophagosome formation (Fazi et al. 2008). The positive detection of LC-3 was very important, since this protein is widely recognized as a marker for autophagic cell death (Yang et al. 2008). The detection of LC-3 is considered proof of the development of the early stages of autophagy (Yang et al. 2008) and, hence, positive detection of LC3 in MCF-7TaxR cells is a further indication of the presence of autophagy in these cells.

The formation of autophagic vacuoles is a characteristic feature of autophagic cell death in cells responding to different cytotoxic treatments (Paglin et al. 2001; Daido et al. 2004). Acridine orange is a dye that moves freely across biological membranes in an uncharged state, and is characterised by green fluorescence (Arvan et al. 1984; Mains and May 1988; Traganos and Darzynkiewicz 1994; Herman-Antosiewicz et al. 2006). However, the protonated form of acridine orange accumulates in acidic compartments and forms aggregates, which are characterised by red fluorescence (Arvan et al. 1984; Mains and May 1988; Traganos and Darzynkiewicz 1994; Herman-Antosiewicz et al. 2006).
Acridine orange stains lysosomes, which are involved in autophagosome formation, since the number of lysosomes is usually higher in autophagic cells (Kanzawa et al. 2004). Lysosomes are normally acidic and, therefore, acridine orange stains the acidic vesicles or autophagolysosomes (Kanzawa et al. 2004). Our data generated from acridine orange staining experiments with flow cytometry revealed a positive detection of acidic vesicles in both MCF-7 and MCF-7TaxR cells, compared to MDA-MB-231 cells. The use of the autophagy inhibitor bafilomycin produced a significant inhibition in MCF-7 cells, and even more so in MCF-7TaxR cells. Bafilomycin inhibits the vacuolar H^+-ATPase, the enzyme that mediates acidification of autophagic vacuoles (Paglin et al. 2001). From these experiments, the MCF-7TaxR cell line was shown to undergo the greatest amount of autophagy in response to staurosporine treatment. These data agree with the results of the confocal microscopy experiments. Therefore, the levels of autophagic cell death activity can be expressed in following the order: MCF-7TaxR >> MCF-7 >> MDA-MB-231. A previous study showed that autophagy could be induced in MCF-7 cells using plumbagin, an anticancer drug that induces G2-M arrest and blocks cell proliferation (Kuo et al. 2006). However, according to our data, electron microscopy imaging did not show evidence of autophagosomes formation in MCF-7TaxR or MCF-7 cells due to a technical artefact. However, a previous study with MCF-7 cells showed evidence of autophagosome formation by electron microscopy imaging (Kuo et al. 2006). However, our electron microscopy imaging showed an altered cytoplasm in staurosporine treated MCF-7TaxR compared to control but no obvious double membrane structures characteristic of autophagosome formation.
The use of class III PI3K inhibitors, such as 3-MA, has been shown to block autophagic sequestration in cancer cells (Klionsky et al. 2007; Chen and Karantza-Wadsworth 2009). The drug 3-MA inhibits autophagic sequestration by inhibiting the PI3K class III mVps34 (Petiot et al. 2000). LY294002, also a class I and III PI3K inhibitor (Blommaart et al. 1997; Aki et al. 2003; Chen and Karantza-Wadsworth 2009). We investigated the extent of autophagy taking place in MCF-7 versus MCF-7TaxR cells. Our data demonstrated a significant inhibition in cell viability in response to 3-MA in MCF-7 and MCF-7TaxR cells. Also, LY294002 produced a marked inhibitory effect on cell death which was more significant for MCF-7TaxR cells. Taken together, our data are consistent with previous studies indicating the autophagy suppressive effects of LY294002 and 3-MA (Blommaart et al. 1997; Aki et al. 2003; Chen and Karantza-Wadsworth 2009).

A number of earlier studies reported that the Akt pathway is deregulated in different malignancies, leading to increased cancer cell proliferation and survival (Huang and Hung 2009). Activation of the class I PI3K/Akt pathway has been shown in numerous cancers, such as breast cancers, since receptor tyrosine kinases (RTKs) and the epidermal growth factor receptor (EGFR) are widely overexpressed in many cancers, leading to activation of the class I PI3K/Akt pathway (Huang and Hung 2009). In addition, activation of class I PI3K/Akt confers acquired resistance to chemotherapies in cancers and is associated with apoptosis resistance by its phosphorylation of downstream targets (Huang and Hung 2009). Constitutive activation of the PI3K/Akt/mTOR pathway is a common occurrence in human cancers, such as ovarian, breast, and gastric cancers (Lee et al. 2005). Akt and phosphorylated-Akt proteins are slightly inhibited in MCF-7 cells, but are even more so in MCF-7TaxR cells. Thus, inhibition of these proteins in MCF-7TaxR cells is likely to be
associated with autophagic cell death activation, which is consistent with previous studies (Nair and Klionsky 2005; Arsham and Neufeld 2006; Suzuki and Ohsumi 2007). A study on glioma xenografts used class I PI3K and mTOR inhibitors which inhibit Akt phosphorylation and block cell proliferation without inducing apoptosis (Fan et al. 2006). We observed an inhibition of PI3K/Akt in response to staurosporine treatment in MCF-7TaxR cells, but not in MCF-7 cells, which indicated autophagic cell death in the former.

mTOR is another recognised downstream target of the Akt pathway and the gatekeeper of autophagy (Nair and Klionsky 2005; Yang et al. 2005; Arsham and Neufeld 2006; Suzuki and Ohsumi 2007). In response to staurosporine treatment, mTOR expression was observed in MCF-7TaxR cells, where P-mTOR was completely absent compared to MCF-7 cells. Staurosporine is a protein kinase C (PKC) inhibitor that inhibits P-mTOR in MCF-7TaxR cells. Thus, our data may indicate that MCF-7TaxR cells are highly sensitive to autophagic cell death. S6K (p70S6K) is another important downstream effector in the mTOR pathway, and its expression correlates with autophagic activity (Codogno and Meijer 2005; Klionsky et al. 2005). Inhibition of mTOR function can occur as a result of a block to phosphorylation of downstream molecules such as P-S6 kinase (P-p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), leading to G1-arrest and induction of autophagy (Huang and Houghton 2003; Sawyers 2003; Takeuchi et al. 2005).

The expression of S6K and P-S6K were examined in the MCF-7 and paclitaxel resistant cell lines following staurosporine treatment. S6K and P-S6K were not expressed in MCF-7TaxR cells, indicating inhibition of the expression of these proteins in these cells. This result was expected, since mTOR inhibition was previously observed in the MCF-7TaxR cell line. Since S6K inhibition was due to inhibition of its principal regulator mTOR, this downstream substrate should also be inhibited, as observed in MCF-7TaxR cells.
Rapamycin, one of the main mTOR inhibitors, has chemotherapeutic antitumor activity in various models (Aoki et al. 2001; Faivre et al. 2006). Rapamycin is a highly specific inhibitor of mTOR activity which binds to the cytosolic FK-rapamycin binding protein (FKBP-12) (Kurmasheva et al. 2006). The FKBP-rapamycin complex binds to the FK-rapamycin binding (FRB) domain of mTOR, resulting in inhibition of mTOR function (Kurmasheva et al. 2006). According to the results of Western blot analysis, the P-mTOR level was reduced following a 40 μM rapamycin treatment, in both MCF-7 and MCF-7TaxR cells. We tested 20 μM rapamycin on the expression of P-mTOR in MCF-7TaxR and MCF-7 cells, but it did not inhibit mTOR activity (data not shown); this reflects differences in the responses of cancers cells to anticancer drugs. However, the use of both staurosporine and rapamycin helped us to delineate the differences between the MCF-7 and MCF-7TaxR cell autophagic processes. The activity of autophagy is increased in response to nutrient deficiency (Codogno and Meijer 2005). In addition, the removal of serum growth factors from culture medium switches off the autophagy-suppressive pathway dependent on PI3K class I (Petiot et al. 2000). Our results using serum free medium and the Annexin V-FITC/PI analysis showed the presence of apoptotic cells among the MCF-7 cell population, whereas MCF-7TaxR cells exhibited no apoptotic cell death.

Our findings are the first to show that the paclitaxel resistant breast cancer cell line MCF-7TaxR readily commits to an autophagic cell death mechanism in response to various cytotoxic insults. MCF-7 cells undergo autophagic cell death, but also maintain some ability to undergo apoptosis. MCF-7TaxR cells responded markedly to autophagic inhibitors such as bafilomycin, as shown by acridine orange staining, and showed
response to PI3K inhibitors, including 3-MA and LY294002, with an autophagy-suppressive effect resulting in reduced sensitivity to cisplatin. In conclusion, paclitaxel resistant MCF-7TaxR breast cancer cells commit to cell death in response to a cytotoxic insult via autophagic cell death-PCD II, but not via apoptosis mechanism or PCDI.
CHAPTER 7

EVIDENCE OF EPIGENETIC SILENCING OF APOPTOTIC GENES IN AN MCF-7 PACLITAXEL RESISTANT CELL LINE
CHAPTER 7

7 Evidence for Epigenetic Silencing of Apoptotic Genes in an MCF-7 Paclitaxel Resistant Cell Line

7.1 Overview of epigenetics in breast cancer

The term epigenetic is defined as the changes occurring between genes and environment with the development of a specific phenotype without changes in the DNA nucleotide sequence (Teodoridis et al. 2004). Different alterations may occur in the DNA, including chemical modification of proteins that are associated with DNA (Herman and Baylin 2003). Other changes occur in DNA, such as promoter methylation, which involves cytosine located 5' to guanosine, named CpG islands or CpG dinucleotides (Herman and Baylin 2003). Another type of epigenetic event includes histone modification (Chekhun et al. 2007). Hyper-methylation commonly occurs in nearly every type of cancer resulting in loss of gene expression; however, methylation may also occur in normal cells or tissues (Visvanathan et al. 2006). Different studies have implicated the existence of DNA methylation and histone modifications in cancer-drug resistant cells (Chekhun et al. 2007). The existence of epigenetic silencing of genes such as BRCA1 may occur during tumour progression (Esteller et al. 2000; Umbricht et al. 2001). In addition, DNA methylation of the BRCA1 gene has been demonstrated in breast hyperplasia and carcinoma (Esteller et al. 2000; Umbricht et al. 2001). DNA modifications caused by germ-line mutations are more stable and interfere with the DNA sequence, in contrast to epigenetic modification which may possibly be reversed using different demethylating agents and histone deacetylation inhibitor compounds (HDAC inhibitors), making them a potential target for cancer therapy (Herman and Baylin 2003). Recently, application of epigenetic detection in breast cancer was considered for early diagnosis, prevention and
treatment, but is still under investigation by clinicians and researchers (Visvanathan et al. 2006).

The mechanism of DNA methylation involves the transfer of a methyl group to carbon 5 of cytosine (Teodoridis et al. 2004). In a cancer cell, this mechanism is increased, leading to a block in gene transcription (Figure 1-8) (Teodoridis et al. 2004). The group of enzymes involved in this specific mechanism is the family of DNA methyltransferases (DNMT), DNTM1, DNTMT3a and DNTMT3b (Bird and Wolffe 1999; Liu et al. 2003). DNMT1 functions within the DNA replication complex which in turn binds to hemi-methylated DNA in order to maintain the methylation pattern after DNA replication (Vertino et al. 2002). Defects in DNMT1 function, or DNMT1 overexpression, lead to changes in genetic imprinting causing embryonic lethality - this is termed de novo methylation (Li et al. 1993; Biniszkiwicz et al. 2002; Teodoridis et al. 2004). DNA methylation usually inhibits binding of transcription factors to gene promoters leading to suppression of gene transcription (Teodoridis et al. 2004). The DNA methylation mechanism causes binding of a family of methyl-binding domain (MBD) proteins that bind specifically to methylated CpG sites on the DNA (Teodoridis et al. 2004). The MBD family members all share a common methyl-binding domain and three of these proteins are associated with large protein complexes containing the histone deacetylase HDAC1 (Teodoridis et al. 2004).

The other type of epigenetic alteration occurring in cancer is histone modifications which include acetylation, methylation, and phosphorylation (Teodoridis et al. 2004). In a normal cell, DNA is tightly compacted to prevent transcription factors from accessing a
gene (de Ruijter et al. 2003). The DNA is tightly packed by the chromatin and organised into protein-DNA complexes (Wade 2001; de Ruijter et al. 2003). The chromatin basic structure is composed of an octamer of four core histones (H2/H4 tetramer) and two H2A/H2B dimers surrounded by 146 bp of DNA (Ito et al. 2000; Strahl and Allis 2000). Therefore, the DNA is highly organised and capable of facilitating control of gene expression (Ito et al. 2000; Strahl and Allis 2000). Prior to transcription, the compact DNA is inaccessible, and it is available for DNA binding proteins by modification of the nucleosome (Ito et al. 2000; Strahl and Allis 2000). However, the acetylation and deacetylation processes are kept in balance via an equilibrium between the activities of histone acetyltransferases and histone deacetylases (HDACs) (Wade 2001). Nevertheless, increased deacetylation activity can lead to a reduction in the level of gene transcription, as may be seen in cancer cells (Figure 7-1) (Ito et al. 2000; Wade 2001). This form of histone deacetylase activity is responsible for epigenetic histone modification (de Ruijter et al. 2003; Teodoridis et al. 2004). Histones deacetylases function to remove the acetyl groups from lysine in the N-terminal tails of histones, yielding a positively charged amino group and making a strong interaction between the histone N-terminus and the negatively charged DNA backbone allowing the DNA to be less accessible for transcription factors (Struhl 1998; Strahl and Allis 2000; Teodoridis et al. 2004). In vitro studies with histone deacetylase inhibitors showed reduced tumour cell line proliferation and increased apoptosis in some cases (Marks et al. 2000; Marks et al. 2001).
CHAPTER 7

In Normal cell (chromatin packed) Normal transcription

Increased DNA Methylation and Deacetylation

In Cancer cell

Suppression of Gene Transcription

Figure 7-1: Epigenetic modification of DNA in cancer cells.

Levels of methylation (Meth) and histone deacetylation (HDAC) are increased, leading to a block gene transcription in cancer cells resulting cancer cell proliferation. (AC) acetyl group. (Teodoridis et al. 2004).

Overexpression of a DNA methyltransferase (MGMT) has been correlated with the magnitude of cyclophosphamide resistance in human lung tumour xenografts (Mattern et al. 1998). MGMT is an O6-methyl guanine methyltransferase, a DNA repair enzyme that functions to remove a potentially mutagenic alkyl-group from the O6 position of guanine (Mattern et al. 1998). The breast cancer resistant protein gene (BCRP) is associated with epigenetic regulation which may be relevant to the development of drug resistance (Doyle and Ross 2003). BCRP is regulated by cytosine methylation in drug resistant cells (Doyle and Ross 2003). FancF is important for activation of a DNA repair complex containing
 CHAPTER 7

BRCA1 and BRCA2, since a defect in this pathway results in inhibition of DNA damage repair and increased risk for cancer development (Olopade and Wei 2003). Methylation of the FancF gene was observed in ovarian cancer cell lines and in acute myeloid leukaemia (Taniguchi et al. 2003; Tischkowitz et al. 2003).

However, other methylation reactions, such as defects in methylation in pro-apoptotic genes, can lead to drug resistance (Teodoridis et al. 2004). Different pro-apoptotic genes that are susceptible to methylation include Apaf-1, caspase-8, Fas, TRAIL and others (Santouridis et al. 2001; Hopkins-Donaldson et al. 2003). The Apaf-1 gene was shown to be methylated in melanoma cells but the process may be reversed by administration of DNMT inhibitors leading to increased Apaf-1 transcription and increased apoptosis induction by doxorubicin, correlated with increased drug sensitivity (Soengas et al. 2001). As mentioned earlier, Apaf-1 is a crucial protein in the intrinsic apoptotic pathway which binds to and promotes pro-caspase-9 activation in the presence of cytochrome c triggering downstream executioner caspase cascades. Methylation of other apoptotic genes, for example, the gene encoding caspase-8, has been observed in tumours; however, using demethylating agents resulted in a reversal of gene methylation and re-expression of the gene leading to increased apoptosis and chemosensitivity (Fulda et al. 2001).

7.1.1 DNA methylation inhibitors

There are a number of demethylating agents that have been used for reversing methylation via targeting DNA methyltransferases (DNMTs) to restore gene expression in cancer cells (Teodoridis et al. 2004). These agents are small molecule inhibitors including 5-aza-2-deoxycytidine (decitabine), 5-azacytidine, dihydro-5-azacytidine (DHAC) and procaine,
hydralazine and zebularine. Moreover, it has been reported that demethylating agents are able to restore sensitivity to a broad range of chemotherapies such as cisplatin, epirubicin and temozolomide (Strathdee et al. 1999; Plumb et al. 2000). The commonly used small molecule inhibitor of the DNMTs is 5-aza-2-deoxycytidine which causes demethylation and re-expression of silenced genes (Bender et al. 1998; Rush et al. 2004). Additionally, 5-aza-2-deoxycytidine has been shown to induce apoptosis via DNMT inhibition (Dowell and Minna 2004; Oka et al. 2005; Schneider-Stock et al. 2005). A study using prostate cancer cells showed that following treatment with 5-aza-2-deoxycytidine, the cells exhibited increased sensitivity to apoptosis induction following cisplatin treatment (Fang et al. 2004).

7.1.2 Histone deacetylase inhibitors

Histone deacetylase inhibitors can activate expression of many genes that have been suppressed such as those involved in apoptotic signalling and cell cycle control (Donadelli et al. 2003). HDAC inhibitors act to prevent removal of acetyl groups from histones residues (Teodoridis et al. 2004). The commonly used HDAC inhibitors in cancer treatment are trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (Smith and Workman 2009). HDAC inhibitors are effective in treatment of haematological malignancies in vivo, as well as for use in in vitro studies (Teodoridis et al. 2004).
7.2 Aim

This chapter focuses on finding evidence of any epigenetic changes that may have occurred in the paclitaxel resistant breast cancer cells MCF-7TaxR. This aim has been facilitated by treatment with demethylating agents, including 5-aza-cytidine and zebularine, or with the HDAC inhibitor trichostatin A, in order to identify possible masked apoptotic genes, e.g. caspases-7 and -9.

7.3 Methods

Quantitative PCR analysis was carried out for detection of different apoptotic genes following treatment with demethylating agents (DNMT inhibitors) and HDAC inhibitors of cultured cells over a number of in vitro passages. The qPCR method is described in Chapter 2.

7.4 Results

7.4.1 Detection of caspase-7 and -9 in breast cancer cells following treatment with epigenetic reversal agents using quantitative PCR analysis

We carried out qPCR analysis in order to identify changes in apoptotic regulator genes, such as caspase-7, -9, following treatment of drug sensitive and drug resistant breast cancer cells with a combination of demethylating agents and HDAC inhibitors. The breast cancer cells were MCF-7, MCF-7TaxR, MCF-7TaxRREV, and they were treated with DNMT and HDAC inhibitors (DHcocktail). The treatment groups were named MCF-7DHcocktail and MCF-7TaxRDHcocktail, respectively. Also, we tested the detection of
the pl6 (CDKN2A) gene as an internal control to verify the efficiency of the demethylating agents and HDAC inhibitors treatments. The pl6 gene, as previously published, is silenced in MCF-7 cells (Bachman et al. 2003; D'Amico et al. 2004) and we therefore looked for this transcript in our qPCR analysis to indicate the effectiveness of the cocktail in unmasking gene expression. The analysis was carried out on MCF-7, MCF-7DHcocktail, MCF-7TaxR, and MCF-7TaxRDHcocktail cells. Cells were treated with a combination of 100 μM zebularine, 2.5 μM 5-azacytidine and 150-300 μM TSA and incubated with the drugs for approximately 72 h. Cells were treated every other passage, and the number of treatments was four (MCF-7 cells) or five (MCF-7TaxR cells). The MCF-7 cells were more sensitive to the treatment, as indicated by an MTT assay used to check cellular sensitivity to these agents (data not shown). The MCF-7TaxR cells tolerated the demethylation treatments and histone deacetylase inhibitor very well (data not shown). In addition, we included in the qPCR analysis the MDA-MB-231, T47D and paclitaxel resistant T47DTaxR breast cancer cell lines to check the gene expression levels of caspase-7 and -9. The qPCR method was performed as a two step analysis, as described in Chapter 2, Materials and Methods, using a reaction mixture ImProm-II™ Reverse Transcription System from Promega®, (following the manufacturer's instructions) and SYBR Green reagent obtained from Stratagene®. Primers were obtained from SuperArray® and reconstituted to give a 10 μM working solution. Δct values were determined as fold change in gene expression; thus, the difference calculated between experimental genes and the housekeeping gene β-actin was equal to $2^{(ΔΔCt)}$. 
Primers used were human CDKN2A (p16), caspase-7 and caspase-9 obtained from the SuperArray Bioscience Corporation. The primers were designed for amplification of the NM_00007.3 fragment of the CDKN2A (p16) gene, the NM_001227.3 fragment of the caspase-7 gene, and the NM_001229.2 fragment of the caspase-9 gene.

\[
\text{(p16) human gene sequence primer 5'}^{\prime}\text{-}3'': \text{Forward CAGAGGATTTGAGGGACAGG}
\]
\[
\text{Reverse CTCCTCTTTCTTCCCTCCGTT}
\]
\[
\text{Caspase-7 human gene sequence primer 5'}^{\prime}\text{-}3'': \text{Forward CCGTGGGAACGTAAGAAGAA}
\]
\[
\text{Reverse GTCTTGATGGATCGCATGG}
\]
\[
\text{Caspase-9 human gene sequence primer 5'}^{\prime}\text{-}3'': \text{Forward CCTGCTTAGAGGACACAGGC}
\]
\[
\text{Reverse TTCGACAACTTTGCTGCTGCTTG}
\]
Figure 7-2: qPCR analysis of p16 gene expression in MCF-7DHcocktail and MCF-7TaxRDHcocktail cell lines.
(a) Levels of p16 expression were increased in MCF-7DHcocktail and MCF-7TaxRDHcocktail cells. The MCF-7DHcocktail and MCF-7TaxRDHcocktail cell cultures were treated at every passage with 100 μM zebularine, 2.5 μM 5-azacytidine and 150-300 μM TSA and incubated for 72 h. Data are the means of 3 independent experiments (performed in duplicate), error bars indicate ±SD. (b) Table showing the Δct values of p16 expression in cells.

As seen in Figure 7-2, the qPCR analysis of p16 shows the effectiveness of demethylation and HDAC inhibitor treatments, which reversed p16 gene silencing (Bachman et al. 2003). The p16 gene (tumour suppressor gene) was shown to be weakly expressed in MCF-7 and MCF-7TaxR cells, as previously described in the literature (Bachman et al. 2003; D'Amico et al. 2004). The expression of p16 increased in MCF-7DHcocktail and MCF-7TaxRDHcocktail cells.
CHAPTER 7

Figure 7-3: qPCR analysis of caspase-7 and caspase-9 genes in breast cancer cells and cells treated with DNMT and HDAC inhibitors.
(a) Levels of caspase-7 expression were increased in MCF-7DHcocktail and MCF-7TaxRDHcocktail cells. The MCF-7DHcocktail and MCF-7TaxRDHcocktail cells were treated every passage with 100 μM zebularine, 2.5 μM 5-azacytidine and 150-300 μM TSA and incubated for 72 h. Data are the means of 5 independent experiments (performed in duplicate), error bars indicate ±SD. (b) Table showing the Δct values of caspase-7 and caspase-9 expression in cell lines.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Δct Caspase-7</th>
<th>Δct Caspase-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>0.00033</td>
<td>0.00024</td>
</tr>
<tr>
<td>MCF-7TaxR</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MCF-7TaxREV</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MCF-7DHcocktail</td>
<td>0.00047</td>
<td>0.00028</td>
</tr>
<tr>
<td>MCF-7TaxRDHcocktail</td>
<td>0.0010</td>
<td>0.0</td>
</tr>
<tr>
<td>T47D</td>
<td>0.00064</td>
<td>0.00016</td>
</tr>
<tr>
<td>T47DTaxR</td>
<td>0.00041</td>
<td>0.00027</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.00030</td>
<td>0.00041</td>
</tr>
</tbody>
</table>
Figure 7-3 shows the analysis of caspase-7 and -9 gene expression in breast cancer cell lines. MCF-7 cells showed moderate expression of caspase-7-9, as expected. The MCF-7TaxR cells showed no expression of these caspases, as expected, consistent with other data obtained in various sections of this thesis. Also, MCF-7TaxRRREV, a cell line that had been grown in the absence of paclitaxel treatment for several passages, showed a total absence of caspases—indicative of a stable resistance phenotype. However, treatment with the DNMT and HDAC inhibitors elicited an increase in caspase-7 expression in MCF-7DHcoctail and MCF-7TaxRDHcoctail cells compared to their parent MCF-7 and MCF-7TaxR cells, respectively. The Act value for caspase-7 was particularly increased in MCF-7TaxRDHcoctail cells, compared to MCF-7DHcoctail cells, and to parent MCF-7 and MCF-7TaxR cells. These data are evidence for there being epigenetic modifications to the caspase-7 gene in the MCF-7TaxR line. However, caspase-9 expression remained undetectable in MCF-7TaxRDHcoctail cells. Caspase-7, -9 was detectable in T47D, T47DTaxR and MDA-MB-231 breast cancer cells, as expected, and consistent with our previous findings (Western blotting data, Chapter 4).

7.5 Discussion

Epigenetic defects are frequently associated with the molecular pathology of cancer and its development, DNA hypermethylation being one of major changes that correlates with the severity and progression of disease (Feinberg and Tycko 2004). A small number of published studies have demonstrated that the development of a cancer-drug resistance phenotype may also be associated with DNA methylation (Nyce 1989).

In this study, we examined the possibility of epigenetic alterations in MCF-7 and
paclitaxel resistant MCF-7TaxR cells by treatment with different demethylating agents and HDAC inhibitors, allowing for possible reversal of epigenetic alterations. Moreover, according to our findings, MCF-7 cells contain a moderate number of apoptotic gene defects, e.g. caspase-3 and FAS receptor deficiencies (Janicke et al. 1998; Yang et al. 2001), whereas MCF-7TaxR cells are highly modified with regard to apoptosis (some caspases and some Bcl-2 family proteins being deleted). Therefore, we considered that potential hypermethylation of apoptotic genes might explain the reduction in caspase-7 and caspase-9 proteins and that their expression might be upregulated by the administration of DNMT/HDAC inhibitors. We first tested the validation of the DNMT/HDAC treatment method in our study by testing the expression of p16 (as an internal control, since this is known to be silenced in MCF-7 cells) following treatment with DNMT and HDAC inhibitors (Attri et al. 2005). The p16 gene (CDKN2A) is a tumour suppressor gene and had been shown to be methylated in a wide range of malignancies (Bachman et al. 2003; Attri et al. 2005). The p16 qPCR test was successful, since p16 expression levels were shown to increase in MCF-7DHcocktail treated cells, and more so for MCF-7TaxRDHcocktail cells, consistent with earlier studies. This result confirmed that our protocol for reversing epigenetic gene silencing was effective.

Our successful qPCR analysis revealed a switch-on of caspase-7 expression in MCF-7TaxRDHcocktail cells compared to MCF-7TaxR cells. Also, in MCF-7DHcocktail cells, caspase-7 was increased compared to the MCF-7 parent cells. The level of caspase-7 was higher in MCF-7TaxRDHcocktail cells compared to MCF-7DHcocktail cells. In support of our findings, one study on MCF-7 cells that developed resistance to doxorubicin and cisplatin showed that DNA hypermethylation is associated with acquired resistance
(Chekhun et al. 2007). The MCF-7 cells treated with doxorubicin developed resistance and the MDR phenotype; both MCF-7 cells resistant to doxorubicin and cisplatin showed hypermethylation of the \textit{Bad} gene, which possibly confers resistance to apoptosis (Chekhun et al. 2007). Thus, our results indicate that MCF-7TaxR cells may be subjected to genetic hypermethylation (as a consequence of resistance acquisition) and that the DNMT/HDAC inhibitor protocol is able to reverse this methylation, thereby increasing \textit{caspase}-7 expression. In addition, we have also run a Western blotting analysis to detect \textit{caspase}-7 protein (data not shown). This analysis revealed that \textit{caspase}-7 protein expression remained at an undetectable level in MCF-7TaxRDHcocktail cells. However, the demethylation protocol we used does not consistently lead to gene re-expression – as we saw for the expression levels of \textit{caspase}-9. \textit{Caspase}-9 expression was not induced by DNMT/HDAC inhibitors in MCF-7DHcocktail or MCF-7TaxRDHcocktail cells and the reasons for this could be inefficiency of the drug cocktail – or gene deletion. These data indicate that the DNA methylation process can play an important role in cancer cell development and progression, but this may not be the entire reason for the marked down regulation of \textit{caspase} genes in our cell line models. The technique of pyro-sequencing may be useful for future investigation of the apoptotic changes for MCF-7TaxR cell line. Moreover, our previous cytotoxicity assay on the MCF-7DHcocktail and MCF-7TaxRDHcocktail cells, testing their sensitivity to cisplatin (data not shown), showed that their sensitivities (IC_{50}s) towards cisplatin were not changed compared to the respective parent cells. It is well established that hypermethylation is strongly associated with cancer resistance, suggesting that methylation is critical in maintaining cancer cell growth (Segura-Pacheco et al. 2006). A study on a doxorubicin resistant MCF-7 cell line revealed that cells require DNA hypermethylation to generate the MDR phenotype leading to
apoptosis resistance (Segura-Pacheco et al. 2006). In MCF-7 cells resistant to
doxorubicin, treatment with hydralazine (a demethylating agent) reversed the drug
resistance (Segura-Pacheco et al. 2006).

MCF-7TaxR and MCF-7TaxRREV cells were shown to be devoid of caspase-7 and -9 as a
consequence of paclitaxel resistance, as first indicated in our microarray analysis. Screening
for caspase expression levels in other breast cancer cells that showed T47D, T47DTaxR and
MDA-MB-231 cells showed detectable constitutive expression of caspase-7 and -9 genes,
consistent with the Western blotting data (Chapter 4). These data are consistent with
previous published findings showing that T47D and MDA-MB-231 cells showed activation
of caspase-9 following treatment with the isothiocyanate compound sulforaphane (Pledgie-
Tracy et al. 2007).

In conclusion, the data provided in the present chapter indicate that some epigenetic
modifications are present in the MCF-7TaxR cell line but that other mechanisms of gene
modification such as gene deletion may be implied, based on findings from other aspects
of the work described throughout this thesis.
CHAPTER 8

OVERALL CONCLUSIONS AND FUTURE WORK
CHAPTER 8

8 Overall Conclusions and Future Work

According to the literature, there is more than one type of cell death that may be activated under specific conditions - particularly when the caspase-dependent apoptotic pathway is inactivated (Mathiasen and Jaattela 2002; Moretti et al. 2007). Defective apoptosis can occur following exposure to chemotherapeutic agents during development of the multiple drug resistance (MDR) phenomena in tumours, leading to tumour cancer progression and proliferation (Jaattela 1999; Evan and Vousden 2001; Gottesman 2002).

Apoptosis and autophagy are both forms of programmed cell death (PCD I and II, respectively) and are potential mechanisms for the elimination of cancer cells in response to radiation or chemotherapy (Motyl et al. 2006). The apoptotic process is rapid and controlled by series of caspase cascades, Bcl-2 proteins, and other regulators (Lockshin et al. 2000; Cuervo 2004; Meijer and Codogno 2004). The autophagic mechanism is an ancient and conserved process considered to be a catabolic mechanism for protein degradation (Lockshin et al. 2000; Cuervo 2004; Meijer and Codogno 2004). More importantly, autophagy functions in the elimination of cancer cells by triggering non-apoptotic cell death (PCD II) (Lockshin et al. 2000; Cuervo 2004; Meijer and Codogno 2004). The link between apoptosis and autophagy as part of the response to chemotherapeutic agents may depend on several factors such as mechanism of action of the drug and the presence (or absence) and function of regulators involved in apoptotic mechanisms (Motyl et al. 2006). For example, it was observed in a study on MCF-7 cells treated with camptothecin (an inhibitor of DNA topoisomerase I) that both apoptosis and autophagy were induced (Lamparska-Przybysz et al. 2005). Apoptosis was induced in a
subpopulation of cells that were set to initiate type I PCD. (Lamparska-Przybysz et al. 2005). Autophagy may be triggered in cells showing resistance to apoptotic cell death (Lamparska-Przybysz et al. 2005). These data agree with our finding that, in MCF-7 cells that showed a partial induction of apoptosis, a number of cells undergo autophagic cell death. Therefore, an autophagic response is a potential mechanism for cancer cells resistant to apoptosis to employ an alternative cell death process under conditions of persistent stress (Motyl et al. 2006). Thus, autophagic cell death is used by resistant cells incapable of undergoing apoptosis due to gene mutations and is an alternative to apoptotic cell death (Motyl et al. 2006). This hypothesis is consistent with our findings on the MCF-7TaxR paclitaxel resistant variant of MCF-7 cells which are sensitive to PCD II autophagic cell death due to apoptosis gene defects. In the present study, we showed that MCF-7 cells undergo both apoptosis and autophagic cell death whereas paclitaxel resistant MCF-7TaxR cells appear to exclusively commit to autophagic cell death under conditions involving chemotherapeutic drug induced stress.

The different apoptosis analysis methods carried out in the present study have provided evidence that MCF-7 cells undergo moderate levels of apoptotic cell death via the intrinsic pathway, using caspase-7 as the executioner caspase instead of caspase-3. Previous studies demonstrated that caspase-7 and caspase-3 are almost identical in their substrate specificity, thus caspase-7 may compensate for a lack of caspase-3 function (Fernandes-Alnemri et al. 1995). Our cell line characterisation studies indicated that caspase-7, caspase-9 and Apaf-1 were present in MCF-7 cells (apoptosis array, western blotting and qPCR data). We confirmed that the extrinsic pathway is not functioning in MCF-7 cells, consistent with previous studies. Even though caspase-8 is available, it is unable to
interact with the Fas receptor (Annexin V-FITC/PI and apoptosis array data) for inducing signalling leading to extrinsic pathway activation. Our data are consistent with those of Gorka et al. (2005) who reported that, in MCF-7 cells treated with different analogues of paclitaxel, induction of autophagic cell death with a subpopulation of cells committing to intrinsic apoptotic cell death via the mitochondrial route was seen (Gorka et al. 2005).

On the other hand, our observations provided clear evidence of apoptosis resistance (Annexin V-FITC/PI data) in MCF-7TaxR cells in spite of their lack of cross-resistance to many cancer drugs apart from paclitaxel. Our Apoptotic Gene Microarray work revealed major and significant changes in apoptosis genes in MCF-7TaxR cells. A large number of apoptosis genes (caspase-3, -4, -5, -6, -7, -9, -10 and Apaf-1, Bid, Fas, TNF, FADD, TRADD) were shown to be absent from MCF-7TaxR cells during the development of paclitaxel resistance which would significantly contribute to apoptosis resistance in these cells. This result highlights the utility of microarray analysis as a potential tool for disease diagnosis and classification (Stears et al. 2003). Our Western blotting and qPCR data confirmed the absence of expression of caspase-7 and caspase-9 in MCF-7TaxR cells but not in MCF-7 parental cells. In addition, it has been reported that cells that possess a defect in the caspase-7 gene are more resistant to apoptotic cell death-inducing drugs with a delay in apoptotic cell death induction (Korfiati et al. 2004). Another study showed that absence of expression or activity of Apaf-1 impaired the interaction of the apoptosome components (Apaf-1, pro-caspase-9, and cytochrome c) leading to inactivation of the intrinsic pathway, as observed in many cancers including melanoma, leukaemia, glioblastoma, and gastric cancer (Pommier et al. 2004). This may be caused by promoter hypermethylation or chromosome deletion (Pommier et al. 2004). The lack of apoptotic
genes in MCF-7TaxR is possibly only partially due to epigenetic silencing, as obtained via indirect evidence. The major mechanism underlying the absence of multiple apoptosis genes would appear to be via deletion.

In this present study, we demonstrated that the development of paclitaxel resistance is a multifactorial process. The characterisation of the MCF-7TaxR model was confirmed with our Western blotting data, which revealed overexpression of P-gp and the concomitant extraordinary alterations in the expression of apoptotic proteins, preventing apoptotic function which explains the apoptosis resistance developed in this cell line. Previous studies on paclitaxel in cancer cells have indicated that it may confer multifactorial resistance mechanisms which are still not fully understood (Hari et al. 2006). Mechanisms involved in paclitaxel resistance include overexpression of the P-gp transporter, mutations in tubulin genes and alterations in expression of microtubule-associated proteins or apoptosis proteins (Hari et al. 2006). In this part of the study wherein MCF-7TaxR developed resistance to paclitaxel, an approximately 18-fold increase in resistance resulting from the major genetic changes under discussion became apparent. By passage 18, MCF-7TaxR cells had acquired genetic alterations producing resistance to apoptotic cell death and overexpression of P-gp/MDR1 transporter. The beta III tubulin protein is not upregulated in our MCF-7TaxR cell line (data not shown, Dr. Helen Coley personal communication), but it is a commonly associated, albeit not universal, feature of paclitaxel resistance. Defects in apoptotic cell death mechanisms include alterations in the ratio of expression of pro-and anti-apoptotic proteins of the Bcl-2 family and this may also be associated with acquired paclitaxel resistance (Kutuk and Letai 2008). Our investigation of the expression of different anti-and pro-apoptotic proteins revealed a gradual loss in
expression seen in MCF-7TaxR cells whilst developing paclitaxel resistance. The pro-apoptotic Bax, Bad and P-Bad proteins showed to be expressed in MCF-7TaxR cells at passages 18, 23 and 53. However, the BH-3 only domain factor BimL was decreased in expression at passage 18 in MCF-7TaxR cells when the resistance to paclitaxel was becoming established. Subsequently, loss of BimL expression was detected in MCF-7TaxR cells at passage 23. Bim is involved in mediating apoptosis induced by paclitaxel (Bouillet et al. 1999; Sunters et al. 2003). In addition, it was demonstrated in in vivo studies that apoptotic induction in paclitaxel treated cells is essentially dependent on the action of Bim (Tan et al. 2005), consistent with our findings showing deficient levels of Bim in our paclitaxel resistant MCF-7TaxR cells. It would be interesting to look at siRNA against Bim in MCF-7 parental cells and see how this modulates sensitivity to paclitaxel, in the light of our findings.

Our study using methylation reversal agents on breast cancer cell lines provided indirect evidence for caspase-7 gene methylation in MCF-7TaxR cells, based on modest unmasking of this genotype following several treatments with demethylating and HDAC inhibiting agents. Our various autophagy detection analyses have confirmed the induction of autophagic cell death in MCF-7TaxR cells at a higher level than MCF-7 cells. Autophagic cell death (PCD II) appears to be a major death system operative in MCF-7TaxR cells according to our data. In the present work, detection of autophagy associated protein expression, showed strong positive expression of LC-3II and Beclin 1 in MCF-7TaxR cells following staurosporine treatment. Different anticancer agents that induce autophagic cell death include tumour necrosis factor (TNF), staurosporine, and tamoxifen (Janicke et al. 1998; Scarlatti et al. 2004); in the present study we used staurosporine.
Further, confocal microscopy for the localization of LC-3 activity provided evidence for autophagic cell death in response to staurosporine treatment in MCF-7TaxR and lesser so in MCF-7 cells. The inhibition caused by bafilomycin, a well known inhibitor of autophagy (Paglin et al. 2001), was used in this present work in conjunction with acridine orange staining. The results revealed a significant difference in autophagy inhibition in MCF-7TaxR and MCF-7 cells compared to MDA-MB-231 cells. MCF-7TaxR cells were sensitive to different inhibitors of PI3K (3-MA and LY294002) (Blommaart et al. 1997; Petiot et al. 2000; Aki et al. 2003). Moreover, investigations of the Akt/mTOR pathway, one of the main pathways involved in regulating autophagic cell death, were performed (Huang and Hung 2009). Western blotting analysis for Akt, mTOR and S6K expression showed an inhibition in expression in MCF-7TaxR cells following staurosporine or rapamycin treatment. Hence the inhibitory effects of these drugs contributed to the promotion of autophagic cell death in MCF-7TaxR cells (Figure 1-8).
Figure 8-1: Summary of the cell death mechanisms induced in MCF-7 and MCF-7TaxR cells in response to chemotherapeutic drug treatment.
Future work that could be carried out on MCF-7 cells, such as knockdown experiments using siRNA silencing of the caspase-9 gene, would be interesting to provide an understanding of the mechanisms of cell death that might be used by MCF-7 cells. The mechanism of resistance requires further exploration to determine any mutations possible in the paclitaxel binding region of β-tubulin. Since overexpression of tubulin has been shown to correlate with paclitaxel resistance (Kavallaris et al. 1997; He et al. 2001), it may be worthwhile to see if this influences the apoptotic pathway in any way. In addition, important further work can be performed on MCF-7TaxR cells using siRNA silencing of autophagic genes such as Beclin 1 and LC-3, in order to investigate different mechanisms of cell death in MCF-7TaxR cells when apoptosis and autophagic cell death are not functioning. Other possible experiments that might be carried out in MCF-7TaxR cells could involve more in depth use of PI3K inhibitors for treating cells and analysis of the expression of Akt and mTOR by Western blotting. In addition, more in depth electron microscopy analysis should be carried out on MCF-7TaxR cells for the detection of the autophagosomes and other morphological changes.

Our findings provided a significant insight into possible molecular pathways and effective biomarkers relevant to clinical responses to anticancer agents in breast cancer patients. Moreover, the MCF-7TaxR cells have been shown to be a good positive model for autophagic cell death, being more efficient in this process than MCF-7 cells. We have identified gene changes associated with resistance to paclitaxel in MCF-7 breast cancer cells. The present study, along with other previous studies, raises the possibility of using methylation profiling to identify patients who may benefit from established treatments or for whom alternative therapies may be more appropriate, e.g. novel agents that target
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epigenetic mechanisms (Teodoridis et al. 2004). These advances may help to identify highly effective biomarkers for chemo-response in breast cancer patients and, indeed, for other types of tumours. Identification of altered apoptotic genes is useful in predicting the response to the chemotherapy agent paclitaxel in tumours of breast cancer patients. However, an analysis of autophagic cell death processes is equally important and, hence, apoptotic cell death should not be considered in isolation. In addition, caspase-dependent and non-caspase dependent pathways and genes play a major role in clinical drug resistance and their changes in expression or activity may prove useful in determining patient prognosis post-treatment.
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