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Cytotoxic activity of Fagonia cretica against human breast cancer cells

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Summary

In many parts of the world, plants are directly utilised for their medicinal properties. Traditional medicine from Pakistan, India and the Far East is well documented and its history is embedded in folklore. It has been documented that an aqueous extract of the desert shrub, *Fagonia cretica,* is a popular treatment for breast cancer in Pakistan. The administration of an aqueous extract of *Fagonia cretica* is reported effective at reducing tumour size and improving the quality of life of breast cancer patients, is well tolerated and does not exhibit adverse effects like vomiting, diarrhoea or alopecia which are common side effects of standard cytotoxic therapy. In the past, many pharmacologically active and chemotherapeutic compounds have been isolated from plants which subsequently have proven to be successful in clinical trials and been used as primary compounds in therapeutic regimes. *Fagonia cretica* has historical use as a treatment for breast cancer, yet there is little scientific evidence which shows chemotherapeutic potential towards breast tumours. Preparation and analysis of an aqueous extract of *Fagonia cretica* may reveal novel chemotherapeutic agents that can be used to effectively target cancer cells. An understanding of the mechanism of any activity may improve our understanding of cancer cell biology and reveal novel therapeutic targets. This thesis describes for the first time that an aqueous extract of *Fagonia cretica* shows potent *in vitro* cytotoxic activity towards breast cancer epithelial cell lines which was not seen towards normal mammary epithelial cells. Elucidation and characterisation of the cytotoxic mechanism was undertaken by analysing DNA damage, cell cycle status, apoptosis, metabolic state and expression of transcription factors and their targets. Finally, methods for the isolation and identification of active compound(s) were developed using various chromatographic techniques.

An aqueous extract of *Fagonia cretica* was able to reduce cell viability significantly in two phenotypically different breast cancer cell lines (MCF-7 and MDA-MB-231). This activity was markedly reduced in normal mammary epithelial cells (HMEpC). Further investigation into the mode of action revealed that extract treatment induced cell cycle arrest and apoptosis in both MCF-7 and MDA-MB-231 cell lines. This coincided with the formation of DNA double stranded breaks and the DNA repair marker γ-H2AX. In MCF-7 cells, ATM/ATR activation resulted in increased p53 expression and of its transcriptional targets p21 and bax, suggesting a role for a p53-mediated response. Furthermore, inhibition of extract-induced p53 expression with siRNA reduced the cytotoxic effect against MCF-7 cells. Extract treatment was also associated with increased FOXO3a expression in MCF-7 and MDA-MB-231 cells. In the absence of functional p53, siRNA knockdown of extract-induced FOXO3a expression was completely abrogated, suggesting that FOXO3a

plays a vital role in extract-induced cytotoxicity. Isolation and characterisation of the active compound(s) within the extract was attempted using liquid chromatography and mass spectrometry in conjunction with a cell viability assay. Multiple fractionations generated an active fraction that contained four major compounds as detected by mass spectrometry. However, none of these compounds were identified structurally or chemically due to constraints within the methodology.

Key Words: DNA damage, FOXO3a, breast cancer, p53, cancer metabolism

Dedication

I would like to dedicate this thesis to my parents for all their help and support during my life.

Acknowledgments

First and foremost I would like to thank Aston University and Midtech for funding this project. I would also like to thank Prof. Helen R. Griffiths and Amtul R. Carmichael for providing supervisory support, knowledge and teaching during my PhD. I would like to thank Dr. Michael Davis for help with compound extraction and TLC, Dr. Andrew Devitt for assistance with fluorescent microscopy and Dr. Marcus Cooke for assistance with the comet assay protocol. Again, I would like to thank Amtul R. Carmichael for providing the raw plant material. Finally, I would like to thank everyone in Lab 358 for thought provoking and entertaining discussion, scientific or otherwise.

Abbreviations

AMPK	5’ AMP-activated protein kinase
AMPKK	AMPK-kinase
APAF-1	Apoptotic protease activating factor 1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR-interacting protein
BAGF	Bio-assay guided fractionation
Bax	Bcl-2-associated X
BER	Base excision repair
BRCA1	Breast cancer type 1 susceptibility protein 1
CAK	CDK-activating kinase
CARD	Caspase activation and recruitment domain
CD95	Cluster of differentiation 95
CDC25	Cell division cycle 25
CDK	Cyclin-dependent kinase
DCFH-DA	2’-7’-Dichlorofluorescien diacetate
DDR	DNA damage response
DED	Death effector domain
DISC	Death-inducing signalling complex
DMF	Dimethylformamide
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FADD	Fas-associated protein with death domain
FasL	FAS ligand
FasR	FAS receptor
FOX	Forkhead box

FPG	Fapy DNA glycosylase
GADD45	Growth arrest and DNA damage-inducible protein 45
HER2/ neu	Human epidermal growth receptor 2
HIC-1	Hypermethylated in cancer 1
HIF	Hypoxia-inducible transcription factors
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis proteins
IGF-1	Insulin-like growth factor
JNK	c-Jun N-terminal kinase
LKB-1	Liver kinase B1
MAC	Mitochondrial apoptosis-induced channel
MCL-1	Myeloid leukaemia cell differentiation protein
MDM2	Murine double minute 2
MRN	Mre11, Rad 50, Nbs1
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
NAC	N-Acetyl-L-cysteine
Na_2.EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
NHEJ	Non-homologous end joining
NLS	Nuclear localisation signal
PARP-1	Poly [ADP-ribose] polymerase 1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDK-1	Phosphoinositide-dependent kinase-1
PERP	p53 apoptosis effector related to PMP-22

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Chapter 1: Introduction

1.1: Breast cancer

Malignant breast neoplasm is a category of cancers that originate from within the breast tissue and primarily arise from the lactiferous duct or connected lobules of the mammary gland. Breast cancer represents the third most frequent cancer worldwide and amongst females, is the most common malignancy, making up 21% of all new diagnoses. Overall, breast cancer is ranked fifth as the cause of death in cancer patients but in females, represents the most common cause of cancer mortality, making up 14.1% of female cancer deaths [1]. Although mortality as a result of breast cancer is still common, survival rates over the past 50 years have nevertheless been steadily increasing, primarily due to improvements in diagnosis and treatment. In the UK, five-year survival rates for patients diagnosed between 2001-2006 reached 82%, compared to 52% for patients diagnosed between 1971-1975 [2]. Breast cancer is a disease that primarily affects females but it can also presents in males, although rarely, contributing to about 1% of total breast cancer cases [3].

As with all cancers, tumorigenesis within the breast occurs as a result of a combination of dysregulated oncogenes and deactivation of tumour suppressors, resulting from functional mutations within specific genes [4]. This can occur not only as a consequence of spontaneous mutations from DNA replication or endogenous genomic insult during cellular energetics but also by exogenous agents such as UV-light, radiation and carcinogenic compounds [5]. Familial genetics can also increase the risk of neoplastic transformation. For example, hereditary mutations to the breast cancer type-1

and 2 susceptibility genes (BRCA1 and BRCA2), increases the risk of developing breast cancer by age 90 to 60% [6]. Other risk factors related to increased breast cancer incidence include age, diet, weight, alcohol intake, smoking, use of oral contraception and use of hormone replacement therapy [7].

Breast cancer is a complex, multi-factorial disease and thus, tumours are classified clinically based on various histological, molecular and phenotypic characteristics. The majority of breast cancers appear as mammary ductal carcinomas and are characterised as either being invasive or noninvasive [8]. Invasive mammary ductal carcinomas account for approximately 55% of all breast cancers and are characterised as a malignant proliferation of mammary epithelial cells with metastatic behaviour [9]. Non-invasive ductal carcinomas are neoplasms of the breast epithelium that have not yet infiltrated the surrounding tissue. In breast cancer classification, the standard TNM (Tumour, Nodes, Metastasis) malignant tumour staging system, is used to provide an in-depth physical description of the tumour type. It is based on three main parameters that take into account tumour size, presence of tumour cells in the lymph nodes and degree of metastasis, which combined, help to give an indication of patient prognosis [10].

The treatment options available for a patient diagnosed with breast cancer include surgery, radiotherapy, cytotoxic chemotherapy, hormone therapy or targeted therapy. The treatment choice is based on the staging and classification of the tumour. As well as the staging system mention above, breast cancer tissue is also characterised based on molecular phenotypes. In mammary epithelial cells, the presence of steroid hormone receptors such as estrogen receptor (ER) and progesterone receptor (PR), are vital for

normal mammary development [11]. However, either over-expression or over-activation of these receptors is implicated in tumorigenesis due to stimulation of cellular proliferation [12]. Activation of estrogen receptor signalling by estrogen modulates the activity of multiple signal transduction cascades involved in cell survival and proliferation [13]. Estrogen receptors are over-expressed in approximately 70% of breast cancers and thus represents a useful therapeutic target [14]. The most successful breast cancer therapy to-date for ER-positive tumours has been Tamoxifen. Metabolism of tamoxifen in the liver produces active metabolites (primarily afimoxifene and endoxifen) which act as ER-antagonists and suppress tumour cell growth [15]. However, even with its widespread use, around half of patients with ER-positive breast cancer in an advanced stage will fail to respond to tamoxifen. Furthermore, an initial response to tamoxifen treatment, is often followed by progression to a resistant phenotype [16]. There is currently little understanding of how this resistance occurs but analysis of cell transcriptome and proteome phosphorylation status revealed distinct signal transcription programs involved in tamoxifen resistance [17, 394]. For example, *Zhou et al*. demonstrated that tamoxifen resistance in breast cancer was associated with down-regulation of ER-regulated genes and activation of survival signalling coupled with an increased migratory capacity.

Human epidermal growth factor receptor 2 (HER2/neu) is a member of the epidermal growth factor receptor (EGFR) family and regulates multiple signalling pathways involved in cell proliferation, differentiation and survival [18]. The HER2/neu signalling pathway directs cell growth and division and its importance in tumorigenesis is demonstrated by the fact that over-expression of HER2/neu is prevalent in around 25% of early-stage breast cancers [19]. Targeted therapy

using trastuzumab (Herceptin) can be effective at slowing tumour growth for patients with tumours over-expressing HER2/neu [20]. Trastuzumab is a human monoclonal antibody with specificity towards an extracellular domain of HER2/neu. This blocks normal receptor signalling and inhibits cell proliferation regulated by the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signalling pathway [21]. Although trastuzumab has high affinity for HER2/neu, it has been reported that 70% of HER2-positive tumours do not respond to therapy, highlighting the complexity of breast cancer pathology. As well as this, nearly all of the patients treated with trastuzumab develop resistance during the treatment program [22]. It is not currently known why patients are or become resistant but genomic approaches have revealed the PI3K signalling pathway as major determinant of trastuzumab resistance [23]. In some tumours PI3K may be mutated, which prevents trastuzumab-mediated inhibition of Akt signalling and subsequent downstream effects [24]. It has also been postulated that receptor cross-talk with insulin-like growth factor-1 (IGF-1) may contribute to resistance [25].

There also exists a highly aggressive sub-type of breast cancer, which has been coined ‘triple negative’. This form of breast cancer is characterised as tumours that lack expression of ER, PR and HER2/neu. This sub-set comprises of around 15% of breast cancer cases and is associated with a poor clinical prognosis [26]. The lack of receptor expression in this sub-set render treatments such as tamoxifen and trastuzumab redundant. Currently there is no targeted treatment regime for this type of breast cancer and many patients experience relapse to cytotoxic chemotherapy. Recently, it was discovered that the triple-negative phenotype shares similarities with breast cancer type 1 susceptibility protein (BRCA1) mutant breast cancers,

particularly with deficiencies in the repair of DNA double strand breaks [27]. Therefore, it has been proposed that DNA-damaging agents are most likely to be effective against this aggressive sub-type. BRCA1 normally forms part of a DNA repair complex during homologous recombination of double strand breaks. In the absence of functional BRCA1, this role is carried out by poly [ADP-ribose] polymerase 1 (PARP-1), thus it has been predicted that the use of PARP-1 inhibitors in combination with DNA-damaging agents could provide a therapeutic option for triple negative breast cancers [28]. Indeed, it was recently demonstrated in a clinical trial that co-treatment of a PARP-1 inhibitor plus chemotherapy, was significantly more effective then chemotherapy alone [29]. The use of PARP inhibitors in cancer therapy has recently been proposed to be effective against any tumour that harbours defects within DNA repair pathways as a means of sensitising cells to classical cytotoxic agents [395].

1.2: Tumorigenesis

The development of a tumour results from the abnormal proliferation of any cell in the body. One of the principle features of tumours is that the population of proliferating cells show clonality. This means that tumour development occurs from a single progenitor that develops a survival advantage and demonstrates abnormal proliferation [30]. Tumour formation and progression can be divided into four main states which are:

1) Precursor neoplastic lesion
2) Intermediate lesion
3) Primary invasive cancer
4) Metastasis

The precursor lesion is the initial alteration to a cell that confers an advantage for cell survival over the surrounding tissue and promotes clonality [31]. These transformations are benign, cease growth after an initial phase and are often removed through natural progression and differentiation pathways [32]. Those precursor lesions which are not removed may go on to form intermediate lesions. This second group of neoplastic lesions is still contained to the tissue area where they originated but now begin to proliferate slowly without termination [33]. At this stage the probability of the intermediate lesion becoming a primary tumour are high and represents the most important transformation to a pathological condition [34]. Progression to primary invasive cancer is characterised by unrestricted proliferation, clonal expansion and growth outside of the initial tissue compartment [35]. At this stage many primary tumours do not exhibit metastatic ability and removal of the tumour by surgery often results in cure [36]. The final stage in tumour progression is metastasis of the cancer. Vascularisation of the tumour allows the expanding tumour to deposit malignant cells into the vascular network [37]. Metastasis enables tumour cells to infiltrate other areas of the body where their survival advantage can lead to clonal expansion in a secondary tumour site [38].

1.3: DNA damage

Generation of a neoplastic lesion occurs as a result of a genomic mutation that leads to cellular transformation and abnormal proliferation. This is caused by damage to DNA which alters the chemical structure of the double helix and effects gene transcription. DNA damage can be induced by a wide variety of exogenous and endogenous sources. Endogenous reactive oxygen species (ROS) and reactive nitrogen species (RNS) are able to directly modify nucleotide

bases and cause structural modifications to DNA [39]. It is well established that ROS such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals are cytotoxic and that oxidative DNA damage can lead to neoplastic mutations [40]. The most common oxidative base modification present in tumours is a guanine to thymidine transversion and when occurring in oncogenes (such as *Ras*) or tumour suppressor genes (such as *p53*) can promote tumorigenesis [41]. As well as this, natural replication errors are capable of inducing DNA damage and activating the DNA damage response [42].

Exogenous DNA damage can occur from a variety of sources including ultra-violet light, x-ray and gamma-ray radiation, plant toxins, viruses and mutagenic chemicals [43,44]. The most common DNA lesions associated with these agents include DNA cross-links, indirect oxidation of bases and single or double strand breaks.

It has been shown that DNA damage is associated with breast cancer risk [45]. This is not only a result of direct DNA damaging agents such as those mentioned previously but also because of common dysregulation of DNA repair pathways that are normally activated in response to DNA damage [46]. Defects to the DNA repair mechanism potentially allow accumulation of pathological mutations leading to tumorigenesis. An important protein involved in the repair of double strand breaks is BRCA1. The BRCA1 protein forms a complex with Rad51 during the repair of double strand breaks which binds DNA and inhibits the exonuclease activity of MRN (described in section 1.4) [47]. This helps to promote homologous recombination of DNA rather then non-homologous end joining which can result in chromosomal translocations [48]. It is thought that as many as 30% of all breast cancers have some degree of BRCA1 inactivation [49].

1.4: The DNA damage response

The DNA damage response (DDR) is a complex signalling network responsible for activating DNA repair mechanisms, cell cycle arrest and apoptosis, in response to DNA damage [50]. The network involves recognition of damaged sites by sensor proteins and transduction of the damage 'signal' to effector molecules to elicit a cellular response (figure 1.1) [51]. It is thought that members of the rad protein family are responsible for sensing DNA damage [52]. The proteins rad1 and rad9 have been shown to be directly involved in sensing aberrant DNA structures caused by DNA damage [53]. In fact, a heterotrimeric complex involving rad1, rad9 and a third checkpoint protein, Hus1, has been shown to interact at sites of single strand breaks [54]. It has also been demonstrated that upon DNA damage rad17 and rad24 are associated with DNA and that removal of both proteins can delay repair of damaged DNA [55,56]. In response to double strand breaks a heterotrimeric protein complex involving Mre11, Rad50 and Nbs1 (MRN) is rapidly localised to damaged sites to initiate a DNA repair response [57]. Recruitment of the MRN complex is essential for stabilisation of double strand breaks, initiation of cell cycle checkpoint signalling cascades and regulation of chromatin remodelling at sites of DNA damage [58].

Transduction of the DNA damage signal primarily occurs through the serine/threonine protein kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins. These transducers are members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family and are rapidly activated and recruited to sites of DNA damage. The function of these protein kinases is to relay the DNA damage signal to other transducers, often transcription factors, so that a cellular

response can be elicited [59,60]. Activation of cell cycle checkpoints, apoptosis and DNA repair as a result of DNA damage is required to maintain genomic integrity and suppress tumorigenesis.

Figure 1.1. ***Overview of ATM/ATR mediated response to DNA damage.*** In response to double strand breaks the sensor proteins Mre11, NBS1 and Rad50 are recruited to sites of damage which facilitates co-recruitment of the transducer ATM. Activation of ATM induces downstream effector cascades including p53, CHK2, BRCA1 and H2AX, resulting in transcriptional regulation of apoptosis cell cycle arrest and DNA repair. Single strand breaks to DNA recruits RPA and ATRIP and the trimeric Rad9-Rad1-Hus1 complex which both co-recruit ATR and TopB1. Interaction between TopB1 and ATR results in activation of ATR-mediated signalling cascades including CHK1-mediated cell cycle arrest.

The ATM kinase is primarily involved in the detection of double strand breaks and is dysregulated in ataxia-telangiectasia disorder. This disease is characterised by neurodegeneration, susceptibility to cancer and immunodeficiency brought on by genomic instability [61]. ATM is recruited to sites of DNA damage following recruitment of the MRN complex via conserved carboxy-terminal motifs within Nbs1 [62,63]. However, in order for ATM to remain associated with sites of DNA damage and sustain a response, auto-phosphorylation at serine-1981 on ATM, is also required [64]. This phosphorylation step is also required to initiate kinase activity against ATM substrates and activate the DDR [65]. Once activated, ATM causes the phosphorylation of many substrates, including the transcription factor p53, the protein kinase CHK2 and the DNA repair proteins BRCA1 and H2AX [66,67]. Phosphorylation and activation of p53 and CHK2 leads to cell cycle arrest following inhibition of cyclin-CDK complexes [68]. Activation of CHK2 occurs rapidly with persistent ATM signalling leading to secondary p53 activation [69]. Furthermore, CHK2 exhibits kinase activity towards p53 which can increase its stability, half-life and thereby amplify p53 signalling and lead to apoptosis in the face of lethal DNA damage [70]. CHK2 also phosphorylates CDC25 and BRCA1 to induce cell cycle arrest and DNA repair [71,72] (table 1.1).

Key ATM/ATR substrates	Targets (effect)
p53	*CDKN1A (cell cycle arrest)* *BAX (apoptosis)* *GADD45 (DNA repair)* *PUMA (apoptosis)* *FasL (apoptosis)*
CHK2	p53 (stabilisation/activation) CDC25 (cell cycle arrest) BRCA1 (DNA repair)
BRCA1	Rad51 (DNA repair) γ-H2AX (repair factor recruitment)
γ-H2AX	BRCA1 (repair factor recruitment) Nibrin (DDR)
CHK1	CDC25 (cell cycle arrest)

Table 1.1. ***Key substrates of ATM/ATR and their downstream targets and effects.*** ATM and ATR are protein kinases involved in transduction of DNA damage response signalling. Both kinases interact with various target proteins to initiate cell cycle arrest, DNA repair and apoptosis in response to DNA damage.

ATR is activated in response to persistent single-stand breaks such as those formed at stalled replication forks [73]. In a general mechanism, single-strand breaks become coated with replication protein A (RPA), which prevents the damaged strand from forming secondary structures [74]. This allows access for DNA repair proteins during nucleotide excision repair. ATR and its partner protein, ATR-interacting protein (ATRIP) are co-recruited to DNA-RPA sites and are both required to initiate a response to single-strand breaks [75]. As mentioned previously, ATR is also recruited to sites of single-stranded DNA damage by the Rad9-Rad1-Hus1 complex. Once recruited to sites of DNA damage, ATR becomes active after interacting with DNA-topoisomerase II binding protein 1 (TopBP1) [76]. ATR can then exert kinase activity on its many target substrates, including CHK1 and

TopBP1, which amplifies ATR-activity in a positive feedback loop [77]. The kinase activity of CHK1 blocks CDC25 resulting in cell cycle arrest and is required to activate the G2/M DNA damage checkpoint (table 1.1) [78].

The response of ATM and ATR to DNA damage is not entirely a parallel response and it has been shown that ATM functions upstream of ATR following exposure to ionising radiation [79]. It has also been demonstrated that ATR-ATRIP complexes are recruited to sites of double-strand breaks which is stimulated by ATM activity [80]. Both ATM and ATR also appear to exhibit kinase activity towards each other. In response to UV treatment ATM is phosphorylated by ATR resulting in ATM-dependent phosphorylation of CHK2 and induction of cell cycle arrest [81]. It is also apparent that ATM and ATR share kinase activity towards substrates. In particular, the tumour suppressor p53, is phosphorylated at serine-15, in response to DNA damage by both ATM and ATR [82,83]. Other post-translational modifications of p53, such as acetylation, cooperate in conjunction with phosphorylation to provide a stimulus-specific response [396].

Dysfunction of DNA damage pathways in cancer represents a double edged sword, where on one side loss of an effective response can drive tumorigenesis, and on the other hand provides a potential therapeutic target (figure 1.2). A complex network of DNA repair mechanisms ensures cellular survival by maintaining genomic integrity. Single-strand breaks can be repaired by base-excision repair involving PARP-1, XRCC1 and Ligase 3 interaction at the site of damage [84]. Double-stranded DNA breaks are repaired by homologous recombination or non-homologous end joining (NHEJ) involving ATM, BRCA1/2, CHK 1/2 and Rad51 [85,86]. As well as strand breaks, alterations to DNA

Figure 1.2. ***DNA repair pathways as drug targets for cancer therapy (Adapted from Lord 2012 [88]).*** Specific DNA repair mechanisms are activated in response to different types of DNA damage. Single strand breaks are repaired by base excision repair (BER) involving the repair proteins PARP1, XRCC1 and ligase 3. Double strand breaks are are repaired by homologous recombination or non-homologous end joining (NHEJ) involving ATM, BRCA1/2, DNA-PK and KU70/80. Dysregulation in these repair mechanisms are common and contribute to tumorigenesis. They also represent a therapeutic target as these cells cannot respond to DNA damage as effectively and are thus more susceptible to DNA damaging agents. For example PARP inhibitors can be effective at blocking DNA repair induced by double strand breaks in cells that lack functional BRCA1/2.

structure by alkylating agents, formation of bulky adducts or base mis-matches, can induce DNA repair by nucleotide excision repair (NER), mismatch repair or direct reversal [87]. Dysfunction within any of these DNA repair pathways can be exploited for cancer therapy and examples of drugs and the pathway they target are outlined in figure 1.2 [88].

The overall function of the DDR is to initiate cell cycle arrest, DNA repair and if required, cell death. This is controlled by the kinase activity of ATM/ATR, which initiates a response by directly or indirectly phosphorylating target proteins. As mentioned previously, p53 is phosphorylated by ATM/ATR in response to DNA damage. This site-specific phosphorylation is important for regulatory control of p53 and will be described in more detail later. As well as this, ATM can phosphorylate murine double minute 2 (MDM2) and destabilise the MDM2-p53 repressor complex [89,90]. These post-translational modifications serve to increase transcriptional activity of p53 and up-regulate target genes involved in DNA repair, cell cycle arrest and apoptosis. It is known that ATM dysfunction caused by mutations within the *ATM* gene is associated with breast cancer risk [91] and that *ATM* mutations that cause ataxia-telangiectasia are associated with breast cancer susceptibility alleles [397]. Furthermore, suppression of tumorigenesis by p53 is dependent on activation of functional ATM, highlighting the importance of the ATM-p53 pathway in tumour suppression [92].

1.5: The tumour suppressor p53

The tumour suppressor, p53, is encoded in human by the *TP53* gene. The gene itself is located on the short arm of chromosome 17 (locus: 17p13.1) and spans 20kb [93]. The *TP53* gene is evolutionarily conserved amongst mammals, but its existence predates the appearance of cancer, suggesting that the original functions of p53 may have been un-related to its tumour suppressive properties [94]. Structurally, p53 is a 393 amino acid long polypeptide with seven domains including an acidic N-terminus transactivation domain, a zinc DNA-binding core domain, and a homo-oligomerisation domain [95]. p53 also contains a proline-rich and a second transactivation domain which are involved in the pro-apoptotic activity of p53 and its interaction with other proteins [96]. The homo-oligomerisation domain is essential for tetramisation of p53 and subsequent *in vivo* activity [97]. p53 is involved in many cellular functions including apoptosis, cell cycle arrest, DNA repair and senescence. It is modulation of these functions that have led to p53 being labelled 'the 'guardian of the genome'. Furthermore, mutations to *TP53* which result in functionally deficient p53 expression, are present in over 50% of all tumours [98].

Under normal conditions, p53 is maintained at a low level by ubiquitination, which targets the protein for degradation by the proteasome [99]. This is achieved via interaction with the negative regulator of p53, MDM2, an E3 ubiquitin ligase which binds p53 protein and marks it for degradation [100]. MDM2 forms a stable complex with p53 by binding to the N-terminal domain [101]. The C-terminal RING domain of MDM2 is vital to its ability to mark p53 for degradation, as it recruits ubiquitin-conjugating enzyme E3, resulting in polyubiquitination of p53 [102]. The p53-MDM2 repressor complex is vital to organismal development as shown by the

fact that MDM2 knockout mice die early in embryonic development. Interestingly, this effect can be reversed by co-inactivation of p53, demonstrating that MDM2 is crucial to the repression of p53 [103]. As well as this, mutations within the proline-rich region of p53 increase MDM2 binding capability, suggesting that this region is important in protecting p53 from degradation [104]. Reducing p53 half-life is not the only effect MDM2 exerts on p53 and it has been demonstrated that MDM2 also directly inhibits p53 transcriptional activity [105]. MDM2 binds to the transactivation domain of p53, which shields it from interaction with other proteins associated with transcriptional activation [106]. The *MDM2* gene is a transcriptional target for p53, thus, p53 regulates itself in a negative feedback loop by increasing expression of its own inhibitor [107].

The p53 transcriptional programme can be activated by a variety of cellular stressors such as DNA damage, oxidative stress, osmotic shock and nucleotide depletion [108,109,110,111]. The first step in p53 activation involves destabilisation of the MDM2-p53 repressor complex, which increases p53 half-life, and induces a rapid accumulation of p53 protein. Secondly, p53 is subjected to post-translational modifications such as acetylation and phosphorylation, which activate its transcriptional activity by driving nuclear accumulation of p53 [112,113]. Activation of p53 plays a major role in coordinating cell cycle arrest, DNA repair and apoptosis in response to DNA damage. As described in section 1.4, the protein kinase ATM is rapidly activated upon detection of double strand breaks, resulting in activation of the DNA damage response. Activation of ATM plays a pivotal part in the p53-mediated effect following DNA damage [114]. It has been shown that loss of ATM prevents p53 accumulation following DNA damage and blocks p53-mediated cell cycle arrest [115]. In response to DNA

damage, the ATM kinase increases p53 protein levels by site-specific phosphorylation of serine-15 (figure 1.3) [116]. It has also been shown that ATM activates CHK2 which phosphorylates p53 at serine-20 [70]. These residues are situated within the MDM2 interacting region of the N-terminal domain of p53, suggesting that phosphorylation of p53 at serine-15/20 destabilises the p53-MDM2 complex [117]. However, mutation of these residues does not prevent p53 stabilisation *in vitro*, suggesting that other mechanisms are involved in p53 stabilisation in response to DNA damage [118]. ATM also demonstrates kinase activity towards MDM2. In particular, ATM-dependent phosphorylation of serine-395 results in p53 stabilisation following DNA damage [89]. Effective degradation of p53 is dependent on controlled nucleo-cytoplasmic shuttling. Both p53 and MDM2 are exported from the nucleus simultaneously by their nuclear localisation signal and nuclear export signal sequences [119]. Phosphorylation of p53 at serine-15 induced by DNA damage inhibits p53 nuclear export thus reducing p53 degradation and increasing p53 activation [120].

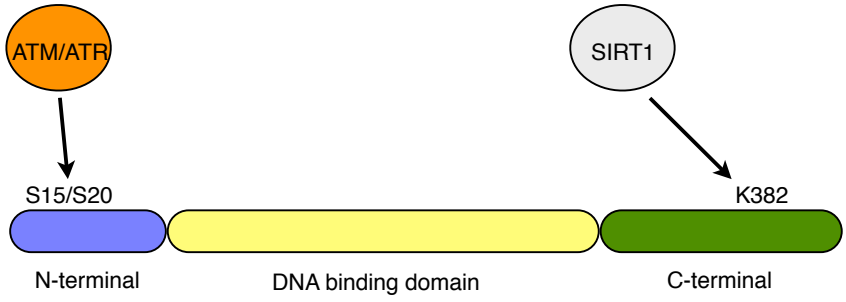


Figure 1.3. ***Post-translational modifications of p53 by ATM/ATR and SIRT1.*** ATM/ATR phosphorylate p53 at serine-15 and serine-20, which reduces binding affinity for MDM2 and increases transcriptional activity. SIRT1 de-acetylates p53 lysine-382 within the nuclear localisation signal and represses p53 transcriptional activity.

Acetylation is another important post-translational modification that regulates p53 activity [121]. The same lysine residues on p53 that are involved in ubiquitination are susceptible to acetylation and it has been shown that acetylation of these residues inhibits MDM2 binding [122]. Furthermore, acetylation potentiates transcriptional activity by promoting co-activator recruitment at p53-target genes, as well as enhancing DNA binding [123,124]. It has also been established that loss of p53 acetylation sites abrogates a p53-mediated response to DNA damage [125]. Sirtuin 1 (SIRT1), a NAD+ dependent deacetylase, is a p53 co-factor which inhibits p53 activity by site-specific deacetylation of lysine 382 within the nuclear localisation signal of p53 (figure 1.3) [126]. Up-regulated SIRT1 expression is associated with many cancers as a result of enhanced p53 suppression. Furthermore, functional loss of the tumour suppressor hypermethylated in cancer 1 (HIC-1) increases SIRT1 activity and attenuates p53-mediated cell cycle arrest and apoptosis, in response to DNA damage [127].

The pro-apoptotic features of p53 are key to its tumour suppressor ability [128]. Central to p53-mediated apoptosis in response to DNA damage is Bcl-2-associated X protein (bax). The bax protein is encoded by the p53-inducible *BAX* gene and is up-regulated in response to DNA damage [70]. Bax is a member of the Bcl-2 protein family containing multiple BH-domains, which are characteristic of the Bcl2 family, and provide sites for hetero- or homo-dimerisation [129]. During apoptosis, bax forms oligomeric complexes within the outer-mitochondrial membrane, resulting in cytochrome C release and induction of apoptosis. The role of p53 and bax in apoptosis will be discussed in detail later.

Although much data exists describing the role of p53 in coordinating cell cycle arrest and apoptosis, our knowledge

of how p53 mediates cell fate under different responses, is not as well understood [130]. It is known that cell fate decisions made by p53 are tissue and stimulus specific [131]. As well as this, specific post-translational modifications and co-factor interaction, are involved in determining p53 transcriptional programmes [132,133,134]. The proliferative rate of a cell is associated with apoptotic induction by p53 in response to DNA damage and it has been shown that pharmacological induction of quiescence protects cells from genotoxic stress [135]. As well as this, cycling cells demonstrate increased E2F activity, which is associated with cell priming towards p53-mediated apoptosis [136]. Recently, a model based on p53 pulses has been proposed, which states that p53 target genes are selectively induced based on an oscillatory signal relayed by ATM [137]. At low levels of DNA damage, cell cycle arrest and cell survival is promoted by transcription of p21, as a consequence of a low level of p53 pulses. In the presence of sustained p53 pulses, apoptosis is triggered and cell death occurs [138].

1.6: The eukaryotic cell cycle

The eukaryotic cell cycle is a sequential and temporal process which ultimately leads to cellular replication and division. Efficient and complete cell cycle is required for proper organismal development, from zygote maturation, through to tissue renewal and regeneration. Therefore, it is a critical that effective regulation and control of the cell cycle is maintained to avoid potentially catastrophic consequences such as neoplastic transformation [139]. In a developed organism, many cells are quiescent or senescent, meaning that they have exited the cell cycle and are in a resting phase. This phase is one of five cell cycle phases and is termed gap 0 or G0 phase [140]. In some cell types, such as heart muscle cells or neurones, this phase represents a

terminal differentiation step where complete cellular maturity is reached [141]. In the presence of growth factors and/or cytokine stimulation, cells become primed for proliferation and various signalling pathways are activated to initiate cell cycle progression from G0 [142]. Initiation of cell division begins with entry into gap 1 (G1) phase of the cell cycle. This is the first step within the pre-mitotic stage called interphase. This phase is commonly known as the growth phase as it is during this period that biosynthesis of proteins and enzymes required for successful replication and cell division occurs. Once conditions are optimal, the cell cycle progresses to S-, or 'synthesis' phase, where DNA content is doubled in preparation for cell division. Once the cell is ready it enters the final interphase stage called gap 2 (G2) phase. During this phase, further biosynthesis occurs, primarily of microtubules and other structures required for mitosis. Finally, the cell cycle progresses into M-, or 'mitotic' phase, where two daughter cells are generated from the parent (figure 1.4) [143,144].

Throughout the cell cycle the status of the cell is monitored periodically to ensure cell division occurs correctly and to avoid the replication of pathogenic genomic mutations. The transition between G1/S-phase, S/G2-phase and G2/M-phase is tightly controlled by cell cycle checkpoints [145]. These checkpoints are ultimately controlled by the sequential and temporal formation of cyclin-cyclin dependent kinase (CDK) complexes (figure 1.5) [146]. The cyclins are a family of proteins that activate CDKs to drive cell cycle progression. Their intracellular concentrations vary depending on the phase of the cell cycle and the expression of specific cyclins is required for complete cell cycle progression. The formation of specific cyclin/CDK complexes during distinct phases of the cell cycle is crucial to regulation of target proteins involved in cell cycle progression [147].

Figure 1.4. ***The eukaryotic cell cycle (Adapted from Freeman 1995 [144].*** The cell cycle consists of four distinct phases; Gap1 (G1), S-phase, Gap 2 (G2) and mitosis (M). Quiescent cells are held in GO phase until stimulated to enter the cell cycle at G1-phase. At this point conditions are monitored for effective cell cycle progression to occur and biosynthesis of proteins required for cell cycle progression occurs. During S-phase all genomic material is duplicated (2n) and cell components for replication are manufactured. Gap 2 phase allows for further biosynthesis of components for cellular replication. The cell then enters mitosis where two daughter cells are generated from the parent.

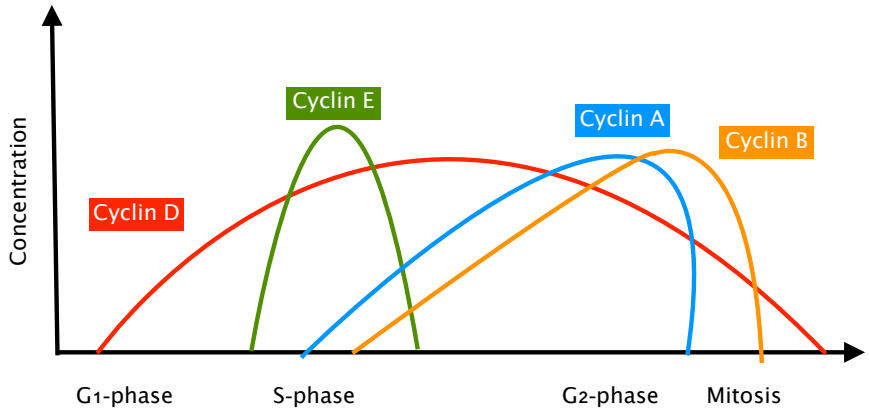


Figure 1.5. ***Expression of specific cyclins regulate cell cycle progression.*** Progression through the cell cycle is controlled by the formation of specific cyclin-CDK complexes at distinct cell cycle phases. The sequential expression of phase specific cyclins helps to control the cell cycle.

Cellular progression through the G1/S transition is controlled by temporal formation of cyclin D/CDK4/6 and cyclin E/CDK2 complexes driven by growth factor stimulation [148]. The formation of these complexes during G1-phase results in phosphorylation of the target protein, retinoblastoma protein (pRB), which controls progression to S-phase via its associating with E2F transcription factors (figure 1.6) [149]. E2F transcription factors exist within the cell as heterodimers comprised of E2F and DP subunits. Both DP and E2F contain DNA-binding motifs and synergistically act to promote transcription [150,151]. Under normal conditions i.e. cellular quiescence, E2F-DP is stably associated with pRB, which restricts E2F transactivation [152]. Stimulation of cell proliferation by growth factors and mitogenic signals results in activation of cyclin D/E and subsequent binding to pRB as a result of upstream signalling pathways including Ras/MAP kinase and PI3K. Following this, phosphorylation of pRB by CDK4/2 induces a conformational change to pRB, which results in E2F-DP dissociation from the repressor complex [153,154]. Activation of the E2F-transcriptional programme initiates cell cycle progression by up-regulating genes such as CDK2, which is required for progression from G1-phase to S-phase [155]. The importance of pRB in controlling cell cycle progression is highlighted by its role as a tumour suppressor. Consequently, pRB mutations and dysregulation of pRB activity is common in many cancers [156].

The progression of the cell cycle through S-phase is driven by the temporal increase in cyclin A-CDK2 complexes. These complexes remain elevated through G2-phase but are rapidly degraded as the cell enters M-phase [157]. However, it is the formation of cyclin B/CDK1 complexes that is required for complete G2/M-transition [158]. The regulation of CDK1 expression during the G2/M transition is complex

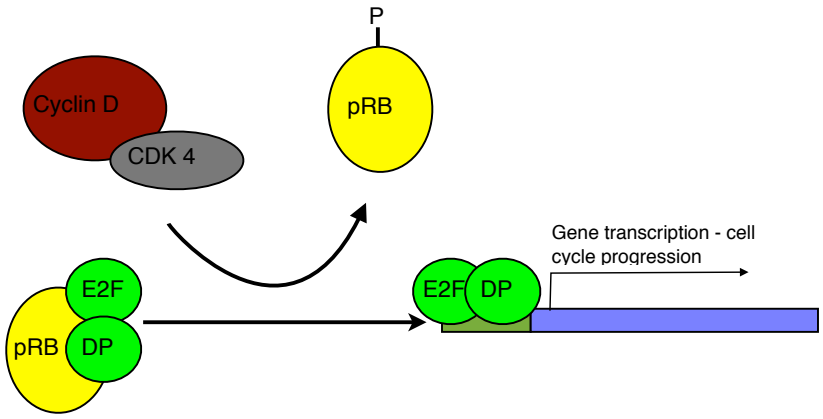


Figure 1.6. ***Regulation of E2F transcription by pRB and cyclin D-CDK4.*** In its hypo-phosphorylated state pRB is in complex with E2F and DP and represses E2F transcriptional activity. Formation of cyclin D/CDK4 complexes during G1-phase of the cell cycle results in phosphorylation of pRB and dissociation from the E2F-DP dimer. This allows E2F-DP to bind to E2F response elements and initiate gene transcription and cell cycle progression.

and involves tight regulation by a variety of proteins (figure 1.7). Newly formed complexes are imported into the nucleus where they are phosphorylated by CDK-activating kinase (CAK) and polo-like kinase (PLK) [159]. As this step occurs early in G2-phase, inactive cyclin B/CDK1 complexes are held in a hyper-phosphorylated state by Wee1 and Myt1. At the G2/M transition boundary, CDK1 is activated by an opposing de-phosphorylation step catalysed by the phosphatase, cell division cycle 25 (CDC25) [160,161]. The rapid activation of CDK1 is maintained by a positive feedback loop involving activation of CDC25 via phosphorylation from CDK1. It is no surprise that CDC25 is over-expressed in many tumours considering the regulatory function it plays in cell cycle progression [162].

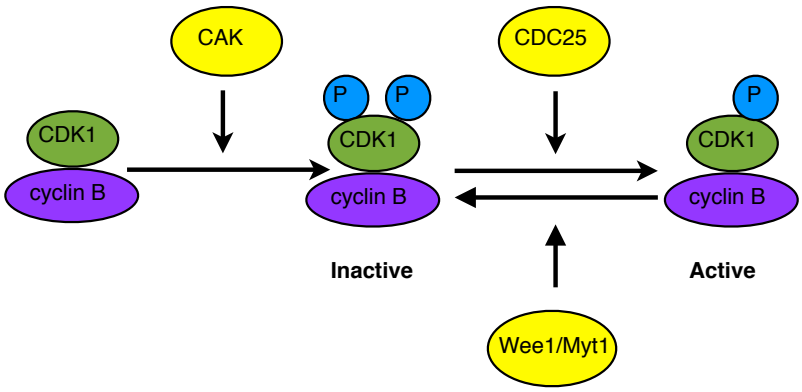


Figure 1.7. ***Regulation of CDK1/cyclin B1 complexes during the cell cycle.*** Early in G2-phase, CDK1/cyclin B complexes are imported into the nucleus, where they are activated following phosphorylation by CAK. Cyclin/CDK complexes are held in an inactivate state following hyper-phosphorylation by Wee1/Myt1. Late in G2-phase, complexes are rapidly activated as a result of de-phosphorylation by CDC25, which drives progression into M-phase.

The tight regulation of cell cycle control through these checkpoints ensures correct replication of cells and prevents tumorigenesis by suppressing cellular proliferation. A major dysfunction in tumour cells is activation of a hyper-proliferative state and the dysregulation of cell cycle checkpoints is common in many cancers [163,164]. It has been shown that cyclin E is over-expressed in around 70% of breast cancer cases and this has been associated with defects in pRB function in 40% of tumours compared to no pRB defects in tumours with normal cyclin E levels [165,166]. As well as this, over-expression of cyclin D is common in human cancers, particularly in mammary carcinogenesis [167]. Over-expression of cyclin D has been shown to be a neoplastic marker in breast tissue and its amplification is maintained throughout disease progression [168].

A major part of cell cycle checkpoints is to monitor genomic stability and remove damaged DNA bases prior to cell division. The G2/M transition is the primary checkpoint

activated in response to DNA damage and involves a multitude of proteins and signalling networks. Dysregulation of single proteins within the G2/M transition can drive tumorigenesis by allowing replication of damaged DNA [169]. In particular, the tumour suppressor p53, plays a central role in regulating the G2/M transition in response to DNA damage and is dysfunctional in over 50% of all human cancers. p53 is responsible for inducing cell cycle arrest in response to DNA damage. The cyclin-dependent kinase inhibitor $p21^{WAF/CIP1}$ is transcriptionally regulated by p53 and is vital for DNA damaged induced growth arrest [170]. Regulation of the cell cycle is controlled by p21-dependent inhibition of cyclin-CDK2/1 complexes and subsequent abrogation of pRB-dependent cell cycle progression [171]. In response to DNA damage, p53-mediated increases in p21 expression are required to induce cell cycle arrest in G1-phase [172]. In addition to this, p21 has been shown to interact with proliferating cell nuclear antigen (PCNA), a co-factor for DNA polymerase activity. Over-expression of p21 inhibits PCNA and DNA synthesis resulting in cell cycle arrest in G1 and G2-phase [173]. Furthermore, it has been shown that p53 and p21 are both essential, to sustain G2-arrest following DNA damage [174]. Other transcription factors such as those of the FOXO family are also involved in regulation of the cell cycle and in conjunction with p53, provide a signalling framework that ensures effective control of cell proliferation, cell metabolism and cell death.

1.7: Forkhead box class O3 transcription factor

The forkhead box (FOX) proteins are a family of transcription factors characterised by the presence of a fork head DNA-binding domain [175]. There are more than 100 members that have been discovered in humans and are categorised as FOXA to FOXR [176]. Members of the FOXO class of FOX

proteins share the characteristic of being regulated by the insulin/PI3K/protein kinase B (Akt) signalling pathway [177]. The four human FOXO isoforms have been designated FOXO1, FOXO3a, FOXO4 and FOXO6. All have been identified as important regulators of cellular proliferation, cell cycle arrest, apoptosis, autophagy and metabolism [178,179,180]. The FOXO proteins have been implicated as potential therapeutic targets in a wide range of diseases because of their control over angiogenesis, stem cell proliferation, neurodegeneration, tumorigenesis and cellular ageing [181]. Down-regulation of FOXO3a activity is often seen in cancers and ERK-mediated inhibition of FOXO3a has been shown to promote tumorigenesis [182]. Furthermore, inhibition of FOXO3a by IκB kinase, has been shown to promote breast cancer tumorigenesis [183]. Enhancing FOXO3a activity has recently emerged as an effective therapeutic target for cancer chemotherapy. In particular, FOXO3a regulation of genes involved in cell cycle and apoptosis, has been suggested to explain the cytotoxic effect of many anti-cancer agents [184]. As well as this, over-expression of FOXO3a, has been shown to inhibit breast tumour growth *in vitro* and *in vivo* [183]. It is uncommon that FOXO3a is mutated or dysfunctional in cancer but more often it is apparent that the problem lies with the regulatory pathways controlling FOXO3a activity [185]. Therefore, FOXO3a cannot be considered a direct tumour suppressor like p53, but rather acts as 'silent guardian' which is activated to preserve the cell under harsh conditions [186]. In fact, p53 has been shown to transactivate FOXO3a within a longevity intron of the FOXO3 gene, suggesting that p53 and FOXO3a form a regulatory network to guard against age-related diseases [187].

The PI3K/Akt signalling pathway is involved in cell growth, differentiation and development and as such is frequently

dysregulated in cancer (figure 1.8) [188]. PI3K are members of a lipid kinase family characterised by their ability to phosphorylate the hydroxyl group of inositol in phospholipids. PI3K exist as dimers compromised of an adaptor sub-unit (p85) and a catalytic sub-unit (p110) [189].

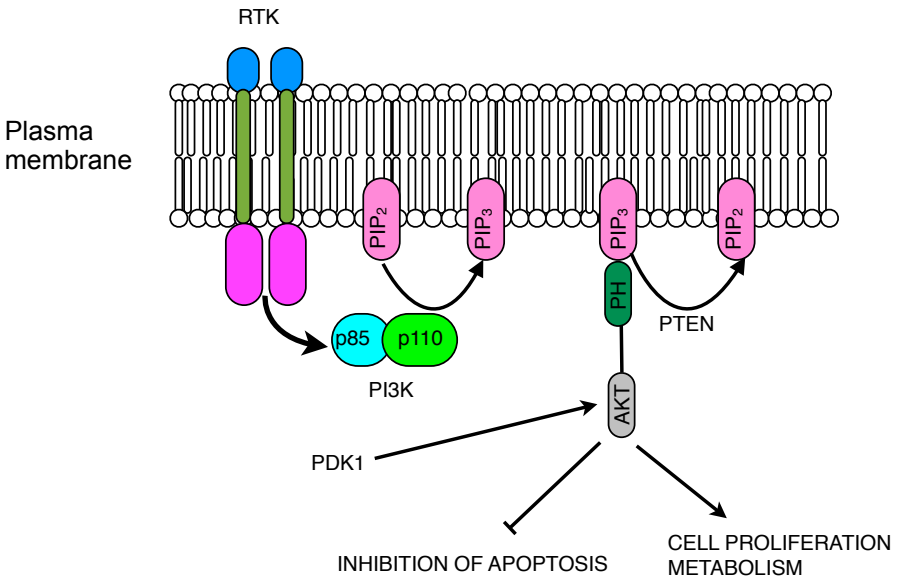


Figure 1.8. ***PI3K/Akt signalling regulated by receptor tyrosine kinase.*** Activation of membrane bound receptor tyrosine kinases recruits PI3K to the cytoplasmic domain. This in turn brings PI3K within range to phosphorylate PIP_2 to PIP_3. Akt interacts with PIP_3 via its PH-domain which exposes regulatory sites on Akt. Phosphorylation of these sites by PDK1 results in Akt activation and subsequent regulation or cell survival and cell proliferation.

PI3K signalling is activated by membrane bound receptor tyrosine kinases (RTKs). RTKs can be activated by a variety of extracellular substrates including growth factors, cytokines

and hormones [190]. Activation of receptor tyrosine kinases in the plasma membrane induces an association of PI3K with the cytosolic component of the receptor. This in turn generates production of phosphatidylionsitol-3,4,5-triphosphate (PIP_3) by phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2), mediated by RTK-mediated activation of p110 [191]. Proteins, such as Akt, contain a pleckstrin homology (PH) domain which enables binding to PIP_3 and subsequent Akt activation [192]. The interaction of PIP_3 with Akt induces a conformational change that exposes two regulatory phosphorylation sites [193]. The protein kinase, phosphoinositide-dependent kinase-1 (PDK1), phosphorylates Akt in a process that is crucial for effective Akt membrane dissociation and activation [194]. Akt is a serine/theronine protein kinase with multiple cellular targets that regulate cellular survival and metabolism [195]. It is not surprising then that alterations of the PI3K/Akt signalling pathway are associated with tumorigenesis and disease progression.

FOXO transcription factors are directly regulated by Akt in response to RTK activation by insulin or growth factors [196]. Phosphorylation of FOXO3 at three conserved sites (threonine-32, serine-253 and serine-315) by Akt triggers a rapid localisation from the nucleus to the cytoplasm [197]. This re-localisation of FOXO3a protein negatively regulates its transcriptional activity, thus, Akt activation in the presence of growth factors effectively suppresses FOXO3a activity [198]. This is in part mediated by FOXO3a association with the nuclear export protein 14-3-3, which has affinity for phosphorylated FOXO3a within the nucleus [199]. Binding of 14-3-3 to FOXO3a induces a conformational change within the nuclear localisation signal (NLS) and promotes nuclear export (figure 1.9) [185]. Phosphorylation of FOXO3a by Akt is directly associated with 14-3-3 binding and the opposing

dephosphorylation of FOXO3a by PP2A is required for its dissociation from 14-3-3 [200]. Nuclear export of FOXO3a is also required for proteasomal degradation following ubiquitination promoted by Akt-mediated cytoplasmic localisation of FOXO3a [201]. However, stress-induced phosphorylation of 14-3-3 by c-Jun N-terminal kinase (JNK) reduces its FOXO3a binding capacity and directly inhibits nuclear export of FOXO3a thereby increasing its transcriptional activity.

FOXO3a is also regulated by acetylation and the deacetylase SIRT1 is a critical regulator of FOXO3a activity [202]. Deacetylation of FOXO3a by SIRT1 produces a dual effect which inhibits FOXO3a promotion of cell death but increases FOXO3a-dependent cell cycle arrest and stress resistance [203]. In this way, SIRT1 can directly modulate the cellular response under different conditions by determining cell fate orchestrated by FOXO3a [204]. It is thought that this is responsible for longevity associated with increased SIRT1 activity. However, in tumour cells, over-active SIRT1 can inhibit apoptosis and promote cell survival in the face of cellular stress [205]. This can not only cause tumorigenesis but also lead to chemotherapeutic drug resistance [206]. FOXO transcription factors are activated in response to oxidative stress, metabolic dysregulation, growth factor withdrawal and DNA damage. Removal of growth factors inhibits PI3K/Akt signalling and increases nuclear localisation of FOXO3a leading to gene transcription. In the nucleus, FOXO3a interacts with target gene promoters via the FOX DNA-binding domain, to promote transcription. Nuclear FOXO3a can induce cell cycle arrest through expression of the CDK-inhibitor $p27^{kip1}$ [207]. Activation of p27 induces cell

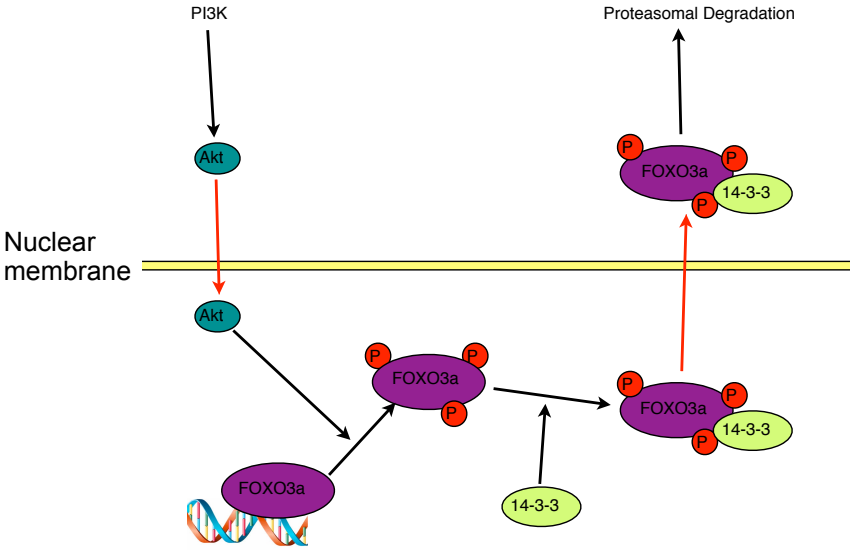


Figure 1.9. ***PI3K/Akt regulated nuclear-cytoplasmic shuttling of FOXO3a.*** In the presence of growth signals PI3K.Akt signalling phosphorylates nuclear FOXO3a at three conserved residues and promotes its binding to the nuclear export protein 14-3-3. This induces cytoplasmic localisation of FOXO3a and switches off FOXO3a transcription.

cycle arrest at the G1/S-phase checkpoint and it has been shown that p27 expression is vital for FOXO3a induced cell cycle arrest [179]. As well as this, FOXO3a can also promote expression of p21 and repress expression of cyclin D, contributing to G1-phase arrest [208]. It has also been shown that FOXO3a can induce arrest in G2-phase by promoting expression of growth arrest and DNA damage-inducible protein 45 (GADD45) [209]. One of the important functions of FOXO3a-driven transcription is to regulate cellular stress resistance by increasing cellular defence to stress. In the presence of excess cellular oxidants FOXO3a increases cellular levels of the anti-oxidant enzymes, manganese superoxide disumutase and catalase, which remove specific reactive oxygen species [210].

Cellular energy homeostasis is tightly regulated and FOXO3a has been identified as a key transcription factor that controls cellular responses to energy depletion [211]. FOXO3a can be activated by an increased AMP/ATP ratio mediated by 5'

AMP-activated protein kinase (AMPK) [212]. In the presence of increased AMP concentrations, AMPK undergoes a conformational change, which exposes an active site within its α-subunit [213]. This allows an AMPK-kinase (AMPKK), such as liver kinase B1 (LKB1), to phosphorylate threonine-172 and activate AMPK [214]. Activation of AMPK due to energy depletion directly results in phosphorylation of FOXO3a and promotion of FOXO3a nuclear translocation [186]. It has been shown that FOXO3a is phosphorylated by AMPK at six conserved sites, five of which are located within the transactivation domain [215]. The role of the AMPK-FOXO3a axis in longevity was shown to be vital to the effects of dietary restriction on healthy ageing and lifespan [216]. Interestingly. the AMPK-FOXO3a pathway is still inducible in cancer, providing a potential therapeutic target for cancer chemotherapy [186]. Decreasing energy levels through glycolysis inhibition effectively activates FOXO3a transcriptional programmes associated with cell cycle arrest, autophagy and apoptosis [217,218]. In response to a reduced energy supply FOXO3a drives cell cycle arrest and autophagy in an attempt to retain energy and produce more ATP. This is mediated by the transcription of autophagy genes such as *ATG6, 7, 12* and up-regulation of catabolic enzyme expression [219].

Under certain conditions, FOXO3a activation can induce an apoptotic transcriptional programme, including transcription of TRAIL, Fas-L and bim [220,221]. This suggests FOXO3a as a potential anti-cancer drug target and indeed the chemotherapeutic drug, paclitaxel, has been shown to induce apoptosis in breast cancer cells via nuclear localisation of FOXO3a and transcription of bim [222,223]. Furthermore, cytoplasmic localisation of FOXO3a has been shown to correlate with poor survival in breast cancer patients [224]. As well as this, persistent metabolic stress leads to a

FOXO3a transcriptional switch from cell survival to cell death. This is characterised by an increase in apoptotic gene transcription associated with autophagic cell death [225,226].

1.8: Cancer cell metabolism - the 'Warburg effect'

A definitive feature that distinguishes cancer cells from healthy cells is an alteration in their metabolic programming. Under normal conditions cells undergo aerobic respiration to produce ATP via oxidative phosphorylation and the electron transport chain in the mitochondrial membrane. In contrast to this, cancer cells undergo high rates of glycolysis in the cytosol, which is coupled with lactic acid fermentation and a comparatively low ATP output compared to oxidative phosphorylation [227]. This phenomenon was termed the Warburg effect after Otto Warburg, who discovered that even in the presence of ample oxygen, cancer cells will preferentially metabolise glucose by glycolysis [228]. The Warburg effect is associated with an increased cellular uptake of glucose in order to maintain energy homeostasis in the absence of efficient ATP production [229].

The main theory behind the Warburg effect dictates that clonal selection favours glycolytic respiration. This means that natural selection at a cellular level results in the emergence of clones that are able to survive in low oxygen conditions [230]. This theory is highly probable due to the fact that the early tumour microenvironment is hypoxic as access to local blood supplies are restricted [231]. This is regulated by the hypoxia-inducible transcription factors (HIF) which respond to changes in oxygen availability. Under normoxic conditions, HIF becomes hydroxylated at conserved proline residues, which marks then for proteasomal degradation following ubiquitination [232]. The

hydroxylation of HIF is dependent on the presence of oxygen, thus, in hypoxic conditions HIF is stabilised and transcriptionally active [233]. HIF activation results in increased expression of enzymes and proteins associated with glycolysis, including glucose transporters and inhibitors of mitochondrial metabolism [234]. Interestingly, in tumours, HIF remains dependent on oxygen levels and therefore cannot be entirely responsible for the Warburg effect [235].

As mentioned in section 1.7, the PI3K/Akt signalling pathway plays a role in inhibiting FOXO3a-mediated regulation of metabolism. As well as this, Akt signalling is associated with increased glucose uptake and HIF-independent glycolysis [236,237]. Mutations within the tumour suppressor PTEN, a negative regulator of Akt activation, can result in constitutively active Akt and alterations in cellular metabolism [238]. It has been shown that Akt over-activation can increase membrane translocation of glucose receptors, activate rate-limiting enzymes such as phosphofructokinase-1 (PFK1) and inhibit FOXO3a [239]. In turn, FOXO3a inhibition prevents suppression of glycolytic gene expression, which could contribute to the Warburg effect [240].

The metabolic regulation of AMPK by the tumour suppressor LKB1 links cell metabolism to tumorigenesis [241]. LKB-1 is an upstream activator of AMPK and is often mutated in human lung and cervical cancers [242,243]. The protein kinase mTOR is a suppressed by AMPK activity, thus, dysregulation in AMPK signalling could enhance mTOR-mediated gene expression [244]. Consequently, one of the mTOR targets is HIF-1, which provides a link between AMPK dysregulation and cancer cell metabolism [245]. Recently, the use of AMPK modulators, has been proposed as potential anti-cancer therapy [246]. Reducing glycolysis in

cancer cells using p38α MAPK inhibitors has been shown to induce FOXO3a-dependent changes in metabolic programming and push tumour cells towards autophagic cell death [218]. This highlights the potential for targeting the AMPK-FOXO3a axis in cancer chemotherapy.

1.9: Cell death - Apoptosis, necrosis and autophagy

Apoptosis is a process of controlled or programmed cell death resulting in elimination of a cell. It is an energy-dependent process which leads to characteristic morphological changes including membrane blebbing, reduction in cellular volume, nuclear fragmentation, chromatin condensation and DNA fragmentation [247]. Apoptosis is an evolutionarily conserved process and several of the key enzymes involved in apoptosis have shared homology with bacteria [248]. Apoptosis is an essential process for organism development and homeostasis. For example, apoptosis is required for effective tissue remodelling, such as generation of digits by selective killing of cells in the intervening webbing and also the shaping of organs [249]. Apoptosis is also a vital part of immune system development as it is essential for removal of self-reactive T and B cells [250]. Furthermore, apoptosis is the process by which infected cells are induced to die by cytotoxic T-lymphocytes [251]. Defects in apoptosis are associated with a variety of pathologies including atrophy, cancer, HIV progression, autoimmunity, developmental defects and result in increased cell survival [252,253,254].

The process of apoptosis can be divided into two effector arms termed the intrinsic and extrinsic apoptotic pathways. The intrinsic pathway, also known as the mitochondrial pathway, of apoptosis is dependent on mitochondrial

permeabilisation by Bcl-2 family proteins and subsequent release of pro-apoptotic factors (figure 1.10) [255,256]. Intracellular stress from DNA damage, heat-shock, radiation, nutrient deprivation, hypoxia and viral infection results in activation of pro-apoptotic Bcl-2 family proteins including bax, bad, bim, bid and bak [257]. Oligomerisation of these proteins is responsible for formation of the mitochondrial apoptosis-induced channel (MAC) and subsequent release of pro-apoptotic mediators including cytochrome C and Smac/ Diablo [258]. Permeabilisation of the mitochondria outer membrane via MAC formation represents the commitment step in apoptosis. It has been shown that expression of bax and/or bax is essential for MAC formation and induction of apoptosis [259]. Under normal conditions, bak is associated with the mitochondrial outer membrane, but is prevented from oligomerisation by the presence of voltage-dependent anion-selective channel protein 2 (VDAC2) [260]. Bak oligomerisation is induced during apoptosis following disruption of the bak-VDAC2 interaction by a truncated(t) form of bid [261]. In comparison, bax is located in the cytosol, until mitochondrial membrane translocation is stimulated by apoptotic signals. Translocation of bax to the outer mitochondrial membrane is associated with a rapid loss of mitochondrial membrane potential and release of cytochrome C [262,263].

Formation of the MAC results in depolarisation of the outer membrane and release of mediators of apoptosis including cytochrome C and second mitochondria-derived activator of caspases (Smac/diablo) [264]. Cytochrome C is a direct mediator of apoptosis through activation of the caspase

Figure 1.10. ***Intrinsic apoptosis pathway (Adapted from Testa 2004 [256]).*** The intrinsic apoptosis pathway relies on mitochondrial permeabilisation following formation of MACs in the outer membrane by Bcl-2 family proteins such as bax and bak. Depolarisation of the outer membrane releases cytochrome C and Smac/Diablo which promote caspase cleavage and induction of apoptosis. Cytochrome C interacts with APAF-1 to form the apoptosome which initiates a caspase cascade following proteolytic cleavage of pro-caspase 9. Smac/ Diablo inhibits IAPs which promotes pro-caspase cleavage.

cascade [265]. The caspase cascade is the final stage in the initiation of apoptosis and is responsible for the characteristic morphological changes associated with cell death by apoptosis. Caspases are a group of aspartate-specific cysteine proteases that are sequentially activated to promote apoptosis [266]. Activation of the intrinsic apoptotic pathway triggers autocatalytic cleavage of the initiator caspase 9 from the pro-caspase isoform to generate a catalytic sub-unit. Activated caspase 9 directly cleaves pro-caspase 3 and 7 to form active caspases that induce the morphological changes of apoptosis [267]. Caspase 9 is activated by self-cleavage upon formation of the apoptosome, a large multi-protein complex which is formed in the presence of cytochrome C

and the adaptor protein, apoptotic protease activating factor 1 (APAF-1) [268]. The apoptosome structure is a heptameric wheel-shaped complex with APAF-1 at its core. Oligomerisation of APAF-1 is dependent on cytochrome C binding within the C-terminus of APAF-1 and binding of dATP [269,270]. This then allows the synchronised recruitment of pro-caspase 9 to the caspase activation and recruitment domain (CARD) of APAF-1 [271]. The active apoptosome requires seven APAF-1 molecules in a 1:1 ratio with cytochrome C before cleavage of the caspase 9 zymogen is initiated [272]. Although APAF-1 is required for mitochondrial dependent apoptosis, it has also been demonstrated that the process can be initiated in a cytochrome C/apoptosome-independent manner [273].

The release of Smac/DIABLO from mitochondria after formation of the MAC can also facilitate caspase activation. Smac is an inhibitor of inhibitor of apoptosis proteins (IAPs), a family of proteins, which bind to and inactivate caspases [274]. Smac contains an IAP-binding motif within the N-terminus, which is exposed upon mitochondrial export, following proteolytic cleavage of the mitochondrial localisation signal of Smac. Cytosolic Smac forms homodimers which bind IAPs and prevent inhibition of caspase activity [275]. Within the intracellular environment, the balance of IAPs and pro-apoptotic signals from cytochrome C and Smac, are in a dynamic equilibrium [276]. This means that the presence of IAPs can block an apoptotic response to small amounts of cytochrome C release but increased mitochondrial damage can cause leakage of Smac, inhibition of IAPs, and amplification of a pro-apoptotic signal. Consequently, tumour cells have been shown to express high levels of IAPs, which may contribute to chemotherapy resistance and tumorigenesis [277].

The second method of apoptosis induction is known as the extrinsic pathway (figure 1.11) [278]. Activation of this pathway is initiated by membrane-associated death receptors, which belong to the tumour necrosis factor (TNF) receptor family, and are characterised by the presence of a cytoplasmic death domain [279,280]. The most commonly studied of these receptors is the FAS receptor (FasR) also known as cluster of differentiation 95 (CD95). Activation of the FasR is mediated by trimerisation of the type-II transmembrane protein Fas ligand (FasL), which in turn induces trimerisation of the FasR [281]. This produces aggregation of the FasR death domains and induces internalisation of the receptor complex allowing the adaptor molecule, Fas-associated protein with death domain (FADD), to bind to the death domains of FasR [282]. FADD contains a death effector domain (DED) which is able to bind and induce self-cleavage of the zymogen of initiator caspase 8 [283]. Oligomerisation of active caspase 8 causes its release from the death-inducing signalling complex (DISC) where it cleaves effector caspases and initiates apoptosis [284].

The tumour suppressor, p53, plays an important role in regulating apoptosis. Activation of p53 by cellular stress has been shown to induce apoptosis via transcription dependent and independent mechanisms [285]. p53 has been implicated in the activation of both the extrinsic and the intrinsic apoptotic pathways [286]. Transcriptionally active p53 up-regulates genes for FasL, death receptor 5 (DR5) and p53 apoptosis effector related to PMP-22 (PERP) proteins which are involved in activation of the extrinsic

Figure 1.11. ***Extrinsic apoptosis pathway (Adapted from Bazzoni 1996 [278]).*** The extrinsic apoptosis pathway is initiated by membrane-associated death receptors such as FasR. Trimerisation of FasR recruits FADD and pro-caspase 8 to the intracellular death domain of FasR. Proteolytic cleavage of pro-caspase 8 initiates a caspase cascade that results in apoptosis.

apoptosis pathway. DR5 and PERP are up-regulated in response to DNA damage and contribute to apoptosis induced by genotoxic stress [285]. The intrinsic pathway is mediated by pro-apoptotic members of the Bcl-2 family of proteins. The *BAX* gene is a transcriptional target of p53 and the requirement for bax in apoptosis appears to be cell-type specific [287]. In response to DNA damage, p53-mediated transcription of p53 up-regulated modulator of apoptosis (PUMA), induces apoptosis via bax activation [288]. PUMA dissociates bax and bak from their repression complex with Bcl-2 and other anti-apoptotic Bcl-2 family proteins. The

APAF-1 promoter also contains p53-responsive elements and transactivation of p53 has been shown to directly promote apoptosome formation [289].

Transactivation of p53 is not essential for it to carry out its pro-apoptotic functions and mechanisms of transcription independent apoptosis have been described [290,291]. In the presence of stress stimuli, p53 is localised to the mitochondrial membrane, where it promotes membrane permeabilisation by inhibiting Bcl-2 [292]. *In vitro* experiments have demonstrated that purified p53 directly induces mitochondrial outer membrane permeabilisation and cytochrome C release from isolated mitochondria [293]. It is thought that cytosolic p53 acts as a BH3-only protein and interacts with other Bcl-2 family proteins containing multiple BH-domains to form the MAC [294]. The effect of transcription-independent apoptosis is present in cell lines carrying mutations generating non-transcriptional forms of p53 [289,295]. As discussed in section 1.5, activation of p53 induces nuclear translocation and transcriptional activation, suggesting that transcription-independent apoptosis must be part of a wider regulatory programme influenced by other co-factors. It has been proposed that specific post-translational modifications could regulate nuclear shuttling of p53 and drive transcription-independent apoptosis [294]. Acetylation of p53 at lysine-120 has been shown to promote mitochondrial association of p53 during apoptosis. Acetylation of p53 at lysine-120 displaces the bak-inhibitory protein, induced myeloid leukaemia cell differentiation protein (MCL-1), from bak allowing oligomerisation and MAC formation [296]. Furthermore, it has been demonstrated that acetylation at lysine-320, 373 and 382 is required for transcription-independent apoptosis mediated by p53 [297]. As well as this, up-regulation of the transcription factor, forkhead box O3 (FOXO3a), has been shown to induce

cytoplasmic accumulation of p53 and induce apoptosis upon serum starvation [298].

Necrosis is defined as premature cell death in an un-progammed and inflammatory manner [299]. In contrast to apoptosis, necrosis does not result in cytokine release or exposure of phosphatidylserine which signals apoptotic cells for phagosome removal. This consequently results in a localised inflammatory build-up of cell debris. As well as this, membrane integrity and apoptotic bodies are not formed during necrosis, which can lead to leakage of cellular lysosomes and proteases that may damage near-by cells causing contiguous tissue necrosis [300]. Necrosis is characterised by distinct morphological features including cell swelling, chromatin degradation, loss of membrane integrity and DNA hydrolysis [301]. Necrosis is thought of as accidental cell death but it is known that in the absence of phagocytosis, apoptotic bodies may undergo a type of necrosis induced by the apoptotic programme [302]. Effective cancer chemotherapy should ideally induce apoptotic, not necrotic, cell death in cancer cells.

Autophagy, also known as macroautophagy, is a catabolic process characterised by self-degradation of cellular components through the lysosomal machinery [303]. It is involved in cell growth, development and homeostasis. It is also a means by which a cell can increase energy levels during metabolic stress to coordinate cell survival [304]. De-regulation of autophagy is involved pathologically in neurodegeneration, cancer, cardiomyopathy, liver diseases and gastrointestinal disorders [305]. There are several types of autophagy, which are distinguished by the selectivity of the autophagic programme, but all converge on the same cellular outcome [303]. The process first involves the vacuolation of organelles or proteins in a double-membraned autophagic

vesicle. This is followed by fusion of the vesicle with the membrane of a lysosome to form an autophagosome. Within the lysosome the vesicle contents are degraded by lysosomal enzymes [306]. During starvation this process can be used to catabolically break down of non-vital components into free energy and nutrients. It has been shown that the PI3K product, PIP_3, is required for autophagy [307]. PIP_3 appears to localise at membranes of autophagosomes and their inhibition prevents effective autophagosome formation [308]. Other important proteins in the autophagy process include Beclin-1 and LC3. LC3 is recruited to autophagosomes and converted to LC3 II by site-specific proteolysis. The presence of autophagosomes containing LC3 II are a hallmark of autophagy activation [309]. Beclin-1 is involved in the formation of autophagosomes following interaction with PI3K [308]. Furthermore, PI3K/Akt signalling is linked to mammalian target of rapamycin (mTOR), which represses autophagy in the presence of insulin and growth factors [310].

Although autophagy is associated with cellular survival, it has become evident that the autophagic machinery can be recruited to kill cells using a caspase-independent form of programmed cell death, also termed autophagic cell death [311]. Autophagic cell death is morphologically distinct from apoptosis and is characterised by early organelle degradation and very late DNA fragmentation. Similar to apoptosis but in contrast to necrosis, autophagic death is a non-inflammatory form of cell death. It was thought that autophagy triggers cell death by inducing apoptosis but removal of apoptotic machinery showed that non-necrotic cell death could still be induced by an autophagic response. Interestingly, removal of the autophagy genes beclin-1 and atg5, prevented cell death in bax and bak double knockout murine embryonic fibroblasts [312]. This suggests that

autophagic cell death may be involved when apoptosis is compromised. This has positive implications for the treatment of cancers, where mutations in apoptotic pathways are commonplace. Beclin-1 deficient mice show reduced levels of autophagy and hyper-proliferation of mammary epithelial cells suggesting a role for autophagy in tumour suppression [313]. However, beclin-1 null cells are not resistant to UV-induced cell death, suggesting that the tumour suppressor function of beclin-1 is not attributed to autophagic cell death [305]. Autophagic cell death plays a crucial role to organismal survival as it is able to prevent survival of metabolically stressed cells. Extensive activation of autophagic pathways contributes to autophagic cell death in cancer cells and provides a potential therapeutic target for chemotherapy.

Our increased understanding of cancer cell metabolism, evasion of apoptosis and the importance of autophagy has been important in identifying novel drug targets for cancer therapy. It is important to expand on current ideas and theories in order to better understand how these processes interact with each other depending on the conditions presented. This could provide routes to effective combination therapies that can selectively target cancer cells based on their neoplastic and tumorigenic properties. High-throughput synthetic chemistry has been the main method of drug discovery in recent years due to rapid technological advances and the ability to screen large compound libraries [314]. It has become apparent that this method has not produced the results that were originally expected and so the industry is returning to a ‘back-to-basics’ approach involving drug discovery from natural products [315,316].

1.10: Fagonia cretica

Fagonia cretica is a herbaceous plant of the Zygophyllaceae family. It is widely distributed throughout the worlds desert regions and can be found growing near dry calcareous rocks [317]. In Pakistan *Fagonia cretica* is part of the indigenous system of medicine and its uses as a treatment for many ailments is well documented. It has been reported that it can be used to treat fever, dysentery, asthma, urinary discharges, liver troubles, typhoid and skin diseases [318]. It is also believed that an aqueous decoction is used to treat breast cancer. However, no study has ever been conducted that verifies the chemotherapeutic activity of an aqueous extract of *Fagonia cretica*, or attempts to understand the mechanism by which the treatment may be effective. An in-depth investigation into the anti-cancer characteristics of *Fagonia cretica* could unveil new and exciting therapeutic targets as well as potentially revealing new compounds with anti-cancer activity.

Fagonia cretica has been shown to elevate GSH levels as well as having strong free radical scavenging properties [319]. Compounds isolated from *Fagonia cretica* also demonstrate cytotoxic activity towards erythrocytes and leukocytes [317]. As well as this, *Fagonia cretica* has been shown to have anti-inflammatory properties including platelet aggregation, down-regulation of COX-2 and up-regulation of VEGF [319]. Antibacterial effects of *Fagonia cretica* have also been reported [320]. A saponin isolated from another species of *Fagonia* plant, *Fagonia indica*, has been shown to selectively induce apo-necrosis in cancer cells. Furthermore, an aqueous extract of *Fagonia indica*, was able to significantly increase survival time in mice with induced tumours.

1.11: Aim and hypothesis

The overall aim of this project is to understand the cytotoxic mechanism of an aqueous extract of *Fagonia cretica* on human breast cancer cells and to elucidate the active component(s) of the extract. The hypothesis is that an aqueous extract will demonstrate selective bioactivity towards human breast cancer cells by inducing cell death or growth inhibition. Furthermore, it can be hypothesised that the extract may contain multiple bioactive compounds that could work alone or in combination to restrict cell survival.

1.12: Objectives

The specific objectives of this project are:

1) To develop methods for the preparation of an aqueous *Fagonia cretica* extract.

2) To determine the specific cytotoxicity of the extract towards human breast cancer epithelial cells.

3) To determine the mechanism(s) by which the extract treatment inhibits cell proliferation and/or induces cell death.

4) Isolate and identify bio-active compounds from the extract for a possible patent.

Chapter 2: Materials and Methods

2.1: Reagents, buffers and solutions

2.1.1: Cell culture

MCF-7 and HMEpC cells were obtained frozen from *HPA cultures*. MDA-MB-231 cells were a gift from Dr. Stephane Gross (*Aston University*). For experiments, cells were used between passage numbers 8-60. Cell doubling time for MCF-7 and MDA-MB-231 cells was approximately 24 hours.

RPMI 1640 cell culture media with stable glutamine (*PAA, UK*) was supplemented with 10% v/v foetal calf serum (*Lonza, UK*) and 1% v/v penicillin/streptomycin (*Lonza, UK*) (50 U/ml). Mammary epithelial growth media *(Invitrogen, UK)* was supplemented with mammary epithelial cell growth supplements *(Invitrogen, UK)* (bovine pituitary extract, 0.4% v/v, bovine insulin, 0.5 µg/ml, hydrocortisone, 0.5 µg/ml, human epidermal growth factor, 3 ng/ml) and 1% penicillin/ streptomycin (50 U/ml). Cell culture media was stored at 4˚C and discarded after 2 months.

2.1.2: General reagents

Phosphate buffered saline (PBS) stock solution was prepared by dissolving 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.46 mM KH_2PO_4 in distilled H_2O.

Caffeine stock solution was prepared fresh just before use by dissolving 300 mM caffeine in DMSO. For experiments, stock solution was diluted in culture media to a final test concentration of 3 mM.

N-Acetyl-L-cysteine (NAC) stock solution was made fresh just before use by dissolving 30 mM NAC in culture media. For experiments, stock solution was diluted in culture media to a final test concentration of 3 mM.

Hydrogen peroxide stock solution (8.8 M) was stored at 4˚C at diluted immediately before use to 1 M in culture media.

Resveratrol stock solution was made fresh just before use by dissolving 3 mM resveratrol in DMSO.

All chemicals were from Sigma unless stated otherwise.

2.1.3: MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution was prepared to give a concentration of 12 mM in PBS and stored at 4˚C in the dark.

Cell lysis buffer stock solution was prepared by dissolving 0.7 M sodium dodecyl sulphate (SDS) in 50% dimethylformamide (DMF) with the pH adjusted to pH 4.7 by addition of anhydrous glacial acetic acid.

2.1.4: DCF-DA assay

Phenol red-free RPMI 1640 cell culture media *(PAA, UK)* was supplemented with penicillin/streptomycin (50 U/ml) and stored at 4˚C.

2',7'-Dichlorofluorescien diacetate (DCFH-DA) stock solution was made by dissolving in DMSO to a concentration of 10 mM and was stored at -20˚C in the dark.

2.1.5: Cell cycle analysis

Cell fixation stock buffer was prepared with 1% v/v formaldehyde in PBS. Buffer was stored at room temperature for up to 1 month.

Cell permeabilisation buffer was prepared just before use by dissolving 0.5% w/v bovine serum albumin (BSA) in PBS with 0.25% v/v Triton X-100.

Mouse anti-human cyclin A-FITC antibody (*BD Biosciences, UK*) was supplied ready to use by manufacturer. Further details are specified in chapter 2.6.

Propidium iodide stock solution was made by dissolving 20 µg/ml propidium iodide in PBS and stored at 4°C in the dark for up to 1 month.

2.1.6: Annexin V apoptosis assay

Annexin V apoptosis detection kit (*Abcam, UK*) was supplied with binding buffer, annexin V-FITC and propidium iodide solution ready to use.

2.1.7: Comet assay

Cell lysis buffer was prepared just before use by dissolving 100 mM ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2.EDTA), 2.5 M NaCl, 10 mM tris-HCL and 1% v/v Triton X-100 in distilled H_2O, adjusted to pH 10.0 with 10 M sodium hydroxide.

Fapy DNA glycosylase (FPG) stock buffer was prepared at 10 times concentration with 40 mM HEPES, 0.1 M potassium chloride, 0.5 mM EDTA and 0.2 mg/ml BSA in distilled H_2O,

adjusted to pH 8.0 with potassium hydroxide and stored at -20˚C for up to 6 months.

Alkaline electrophoresis buffer was prepared just before use by dissolving 0.3 M sodium hydroxide and 1 mM EDTA in distilled H_2O.

Propidium iodide stock solution was prepared by dissolving 150 µM propidium iodide, 10 mM potassium phosphate and 150 mM NaCl in distilled H_2O and stored at 4˚C in the dark for up to 1 month.

2.1.8: SDS-PAGE and western blot

Lysate stock buffer was prepared by dissolving 150 mM NaCl, 1 % v/v Triton X-100, 0.5 % w/v SDS and 50 mM tris-base in distilled H_2O. Aliquots of 500 µl were stored at 4˚C for up to 3 months Protease inhibitor cocktail (*Sigma, UK)* (AEBSF 104 mM, Aprotinin 80 µM, Bestatin 4 mM, E-64 1.4 mM, Leupeptin 2 mM and Pepstatin A 1.5 mM) was added at 0.1% v/v immediately before use.

Resolving gel stock buffer was prepared by dissolving 1.5 M tris-base and 0.4% w/v SDS in distilled H_2O, adjusted to pH 8.4 with 10 M concentrated HCl and stored at room temperature for up to 3 months.

Stacking gel stock buffer was prepared by dissolving 0.5 M tris-base and 0.4% w/v SDS in distilled H_2O, adjusted to pH 6.8 with concentrated HCl and stored at room temperature for up to 3 months.

SDS-PAGE running buffer was prepared just before use by dissolving 25 mM tris-base, 190 mM glycine and 0.1% w/v SDS in distilled H_2O.

Transfer buffer was prepared just before use by dissolving 25 mM tris-base, 190 mM glycine and 20% v/v methanol in distilled H_2O.

TBS-Tween (TBST) was prepared just before use by dissolving 50 mM tris-base, 200 mM NaCl and 0.05% v/v Tween-20 and adjusted to pH 7.5 with concentrated HCl.

2.1.9: Nuclear extraction

Buffer A stock solution was prepared by dissolving 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.05% v/v Triton X-100 and adjusted to pH 7.9 with concentrated HCl. Aliquots of 500 µl were stored at -20˚C for up to 3 months.

Buffer B stock solution was prepared by dissolving 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0. 5mM DTT and 26% v/v glycerol and adjusted to pH 7.9 with concentrated HCl. Aliquots of 500 µl were stored at -20˚C for up to 3 months.

2.1.10: Enliten ATP assay

ATP extraction stock solution was made by dissolving 0.02 M glycine, 0.05 M MgCl and 0.004 M EDTA. Aliquots of 1 ml were stored at -20°C for up to 3 months.

2.1.11: *Fagonia cretica*

Dried *Fagonia cretica* originating from Pakistan was a gift from Amtul Carmichael (*Dudley Group Hospitals*).

2.2 Generation of *Fagonia cretica* extracts

2.2.1: Principles

Extracts of Fagonia cretica were obtained using a Soxhlet reflux extraction method (figure 2.2.1) [321]. The Soxhlet extraction represents a relatively straight forward method of solid-liquid extraction by treatment of the solid with a solvent. This method uses the partition coefficient of individual compounds to isolate species from a mixture of compounds. The process involves heating a desired solvent in a still pot attached to a Soxhlet chamber containing the sample of interest to reflux. As the condenser cools the evaporated solvent it begins to submerge the sample and extract specific compounds. When the Soxhlet chamber reaches maximum capacity the solvent is siphoned back to the still pot. Over a matter of hours or days the recycled solvent in the still pot becomes concentrated with the extracted compounds.

2.2.2: Method

In a soxhlet extraction, dried *Fagonia cretica* plant material was extracted under reflux using a range of solvents based on their polarity index (table. 2.2.1). The solvents used were PET-Ether 40-60, toluene, diethyl ether, dichloromethane, methanol and water. Fresh plant material was used for extraction in each solvent. A total of 25g dry weight of plant material was extracted in 500 ml of solvent for either 2 hours, 5 hours or 24 hours. After the extractions had completed, solvents were removed under reduced pressure in a rotary evaporator at a temperature not exceeding 50°C before desiccating under vacuum overnight. Powder extracts were stored at 4^0C until use. Plant material for cell viability assays and aqueous extracts used in experiments outlined in this thesis was extracted as above for 5 hours.

Figure 2.2.1: ***Soxhlet extraction apparatus set-up (Adapted from AM Glassware.com/soxhletextraction [321].*** Plant material is placed in the soxhlet chamber and compounds are extracted by heating solvent in the still-pot to boiling point. The evaporated solvent is condensed and collected in the soxhlet chamber, submerging the plant material and extracting compounds of interest. The solvent is then siphoned back to the still post and the cycle restarted.

Solvent	Polarity Index
PET-ether (40-60)	0.1
Toluene	2.4
Diethyl ether	2.8
Dichloromethane	3.1
Methanol	5.1
Water	9

Table 2.2.1. ***Polarity index of solvents used in extraction process.*** Several solvents spanning a range of polarities were selected for extraction of compounds from *Fagonia cretica*. The greater the polarity index the more polar the solvent.

Solvent	% Recovery - 2 hours	% Recovery - 5 hours	% Recovery - 24 hours
PET-ether (40-60)	11.6%	10.43%	14.21%
Toluene	2.4%	5.32%	6.1%
Diethyl ether	5.2%	5.89%	5.01%
Dichloromethane	16.34%	12.73%	19.43%
Methanol	9.87%	13.24%	12.9%

Table 2.2.2. ***Percentage recovery of dry weight following Soxhlet extraction.*** Several solvents spanning a range of polarities were selected for extraction of compounds from *Fagonia cretica*. 25g of plant material was extracted in 500 ml of solvent by Soxhlet extraction. Extracts were dried under reduced pressure and desiccated under vacuum overnight.

2.3 Thin layer chromatography of *Fagonia cretica* extracts

2.3.1: Principles

Thin layer chromatography (TLC) is a a standard chromatographic technique for the separation of compounds in a mixture. It is most commonly carried out by blotting a small amount of a mixture onto a silica gel (stationary phase) plate and applying a solvent or solvent mixture (mobile phase) to be drawn up the plate by capillary action. Compounds in a mixture that demonstrate different solubility properties in the mobile phase will migrate to different positions on the stationary phase allowing the separation of compounds. The separated compounds can then be visualised using a wide range of different methods, including vanillin or iodine staining and UV detection.

2.3.2: Method

A small amount of dried extract produced in chapter 2.2 was solubilised in its extraction solvent and applied 1.5 cm from the bottom of a silica gel, aluminium backed TLC plate (*Fisher Scientific*), cut to 6cm by 10cm (W x H). Ethyl acetate:methanol (90:10) mobile phase was used to separate the compound mixtures. After the solvent front had migrated to within 2 cm of the top of the plate the solvent was removed by air drying and the compounds stained by submerging the plate in vanillin solution and heating on a hot plate at 60˚C until compounds were visible.

2.4 Cell culture

2.4.1: Background of cell lines

MCF-7 cells are a human breast adenocarcinoma cell line derived from the pleural effusion of a 69 year old caucasian female. The adherent cell line expresses both wild-type and variant oestrogen receptors as well as the progesterone receptor and have been shown to express functional wild-type p53.

MDA-MB-231 cells are a human breast adenocarcinoma cell line derived from the pleural effusion of a caucasian female. The cell line is considered basal-type or triple negative as it does not express either oestrogen, progesterone or HER2/neu receptors. The cell line also carries a mis-sense mutation in codon 280 resulting in an arginine-lysine residue change which is responsible for expression of a non-functional form of p53.

HMEpC cells are derived from human mammary glands. They are an epithelial cell line and demonstrate high levels of cyclin-dependent kinase (CDK) 6 activity which is responsible for restraining cell proliferation.

2.4.2: Method

MCF-7 and MDA-MB-231 cells were cultured in RPMI 1640 with stable glutamine, supplemented with FCS (10%) and penicillin/streptomycin (50 U/ml) and incubated at 37°C under 5% CO_2. Cells were passaged with trypsin-EDTA *(PAA, UK)* when 90% confluent and seeded at a density of 5 x 10^5 cells per 75 cm^2.

HMEpC cells were cultured in mammary epithelial growth medium supplemented with growth supplements and penicillin/streptomycin (50 U/ml) and incubated at 37°C under 5% CO_2. Cells were passaged with trypsin-EDTA when 90% confluent and seeded at a density of 5 x 10^5 cells per 75 cm^2.

2.5: MTT assay

2.5.1: Principle

Changes in cell metabolic activity as a marker of viability after treatment with chemotherapeutic drugs can be measured using the colorimetric MTT assay. The assay is based on the principle that MTT, a yellow tetrazole, will be reduced by mitochondrial reductase in viable cells to produce a purple formazan product. Due to the nature of the assay, it is not possible to differentiate between cell death (apoptosis/ necrosis/autophagy) or an arrest of cellular growth, as the chemical reduction of MTT is based on the metabolic output of the cell population.

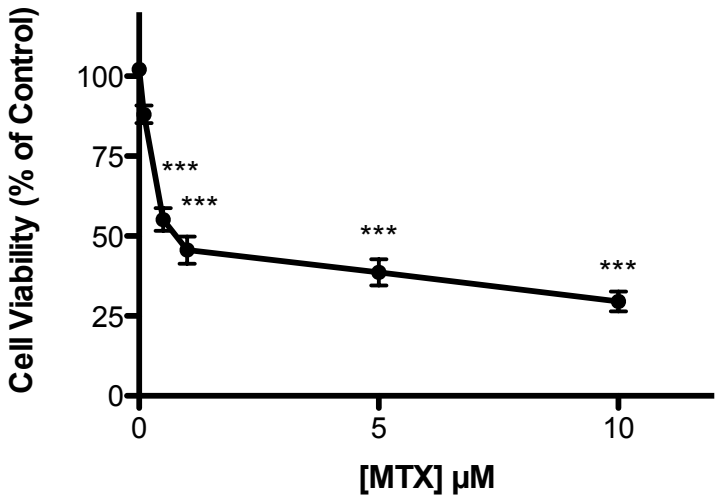
2.5.2: Method

Cultured cells were seeded in 96-well plates at a density of 2 x 10^5 cells per ml in a volume of 100 µl and allowed to adhere overnight. The media was then refreshed with 100 µl fresh media, followed by treatment as indicated in the relevant chapters. Hydrogen peroxide at a final concentration of 160 µM was used as a positive control. Post-treatment, the media was refreshed with 100 µl fresh media and 10 µl stock MTT solution was added to each well. After 4 hours incubation at 37˚C the formazan product was solubilised with 50 µl of MTT lysis buffer. Culture plates were left overnight for formazan solubilisation before absorbances were recorded spectrophometrically at 570nm. Data was expressed as a percentage of cell viability, calculated as a percentage of the mean of untreated controls for each independent experiment.

2.5.3: Assay validation

In order to assess the reliability of the MTT assay to detect changes in MCF-7 cell viability post-treatment with a cytotoxic agent, cultured cells were treated with the chemotherapeutic agent methotrexate or hydrogen peroxide. MCF-7 cells were seeded into 96-well culture plates as described in 2.5.1 and were treated with up to 10 µM methotrexate (MTX) or 320 µM H_2O_2 for 24 hours prior to MTT analysis. Figure 2.5.1 shows the effects of methotrexate (MTX) and hydrogen peroxide treatment on MCF-7 cell viability after 24 hours as measured by MTT reduction. Both agents significantly reduce MCF-7 cell viability at micro-molar concentrations with MTX and H_2O_2 demonstrating an IC_{50} of 0.67 µM and 163.11 µM, respectively (figure 2.5.1a and b). Cytotoxicity of MTX to MCF-7 cells in this model agrees with the work of *Hattangadi et al* [322]. who report an IC_{50} of 0.3 µM towards MCF-7 cells.

a)



b)

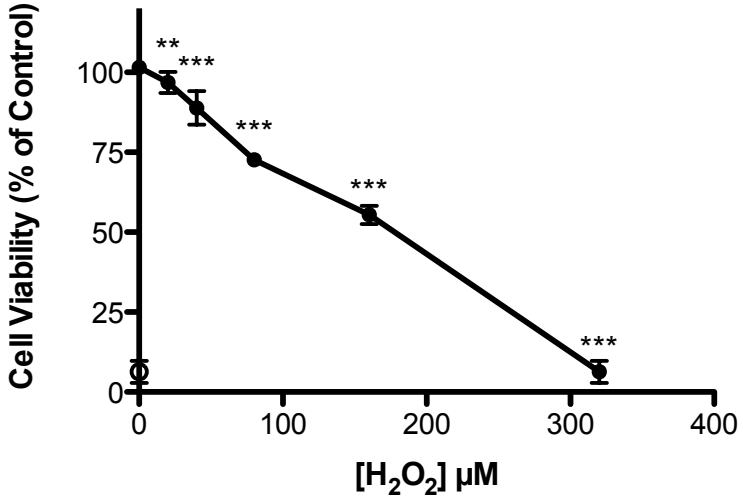


Figure 2.5.1. ***Methotrexate and H_2O_2 reduce MCF-7 cell viability.*** MCF-7 cells were treated with indicated concentrations of (a) methotrexate (MTX) or (b) H_2O_2 for 24 hours prior to MTT analysis. Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to untreated controls analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is from at least three independent experiments performed in triplicate and presented as mean ± standard deviation.

2.6: Cell Cycle Analysis

2.6.1: Principles

The cell cycle status of individual cells within a population can be analysed using dual fluorescent staining coupled to flow cytometry. The cell cycle is controlled in a temporal manner by the formation of active cyclin-CDK complexes that drive the transitions between cell cycle phases. It is known that at different stages of the cell cycle, expression of specific cyclins reach a peak. Utilising this principle, it is possible to stain for cyclin A, which is expressed at low levels during G1 phase before rising through S-phase, peaking during G2 phase and finally declining into M-phase. Using a FITC-conjugated mouse IgE antibody raised against human cyclin A, it is possible to measure fluorescence emission by flow cytometry. Co-staining with the DNA dye propidium iodide allows the user to separate cell populations into G0/G1, S, G2 or M-phase. Cell cycle phases can be gated based on their fluorescent profiles. Cells in G0/G1 phase of the cell cycle show low levels of cyclin A staining and low, or N=1 level of PI staining, associated with normal diploid DNA content (figure 2.6.1 A). Cells in S-phase demonstrate low to high levels of cyclin A staining depending how far through S-phase they are committed (figure 2.6.1 B). Cells in G2-phase show high levels of cyclin A staining and high, or N=2 levels of PI staining (figure 2.6.1 C). In M-phase, levels of cyclin A are in decline, thus M-phase populations show a mid-level of cyclin A staining but retain a N=2 level of PI staining (figure 2.6.1 D).

2.6.2: Method

Cultured cells were seeded into a six well plate at a density of 2 x 10^5 cells per ml in 2 ml and allowed to adhere

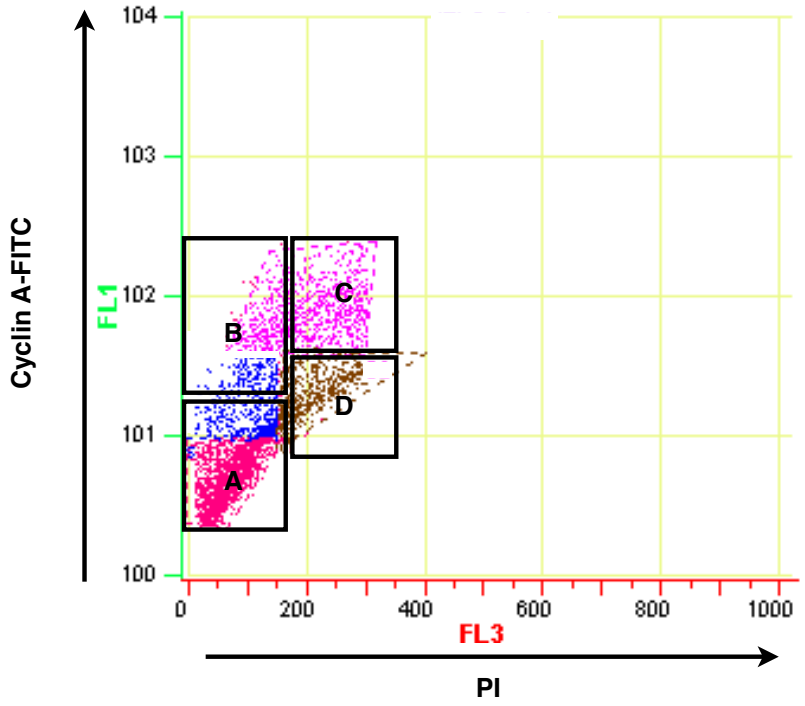


Figure 2.6.1. ***Characteristic staining of untreated MCF-7 cells with anti-cyclin A FITC and propidium iodide.*** MCF-7 cells were stained with anti-cyclin FITC A and propidium iodide prior to analysis by flow cytometry. FITC fluorescence was detected using FL1 (Em: 670nm) and propidium iodide was detected on FL3 (Em: 525nm). Cell populations were gated based on fluorescent profiles (A = G0/G1, B = S, C = G2, D = M).

overnight. Cells were treated with a final concentration of 2 mg/ml of extract for up to 24 hours. After treatment, cells were collected by scraping the cells in 1ml of fresh media and counted on a haemocytometer. A total of $5x10^5$ cells were centrifuged at *300 g* for 5 minutes, the supernatant discarded and the cells washed in 1 ml PBS. Cells were fixed in 1 ml 1% v/v formaldehyde in PBS for 15 minutes. Cells were centrifuged at *300 g* for 5 minutes, the supernatant discarded and the cells permeabilised in 100 µl of permeabilisation buffer for 15 minutes. Permeabilised cells were then incubated with 20 µl anti-cyclin A FITC for 60

minutes in the dark at room temperature with 20 µl propidium iodide solution added for the final 10 minutes. Stained cells were diluted with 400 µl permeabilisation buffer and centrifuged at *300 g* for 5 minutes. The supernatant was discarded and the pellet resuspended in 500 µl permeabilisation buffer before analysis. Stained cells were analysed on a Beckman-Coulter Cell Lab Quanta-SC flow cytometer measuring FITC fluorescence (FL1; Em: 670nm) and PI fluorescence (FL3; Em: 525nm) for 20,000 events per sample and gated for non-specific antibody binding using an isotype negative control mouse IgE. Log FL1 was plotted against linear FL3 to generate histograms. Cell cycle populations were gated according to their fluorescent profiles and data expressed as percentage of cells compared to untreated control population.

2.6.3: Assay validation

Cell cycle analysis was validated by inducing growth arrest in MCF-7 cells by serum starvation for 24 hours or incubation for 24 hours with 0.1 µM colchicine. Figure 2.6.2b shows that serum starving MCF-7 cells for 24 hours results in a shift in cell populations towards G0/G1 and S-phase of the cell cycle with a 45.9% increase in G0/G1 cells (figure 2.6.3b). This is expected as it is known that serum starvation of cells can synchronise cells to G0/G1 phase of the cell cycle [323]. Treatment with the microtubule disrupter colchicine for 24 hours resulted in a significant shift in cell population to M-phase (figure 2.6.2c) with a 100% increase in M-phase cells (figure 2.6.3d). These results demonstrate that the dual stain anti-cyclin A FITC and PI can be used to analyse the cell cycle by flow cytometry.

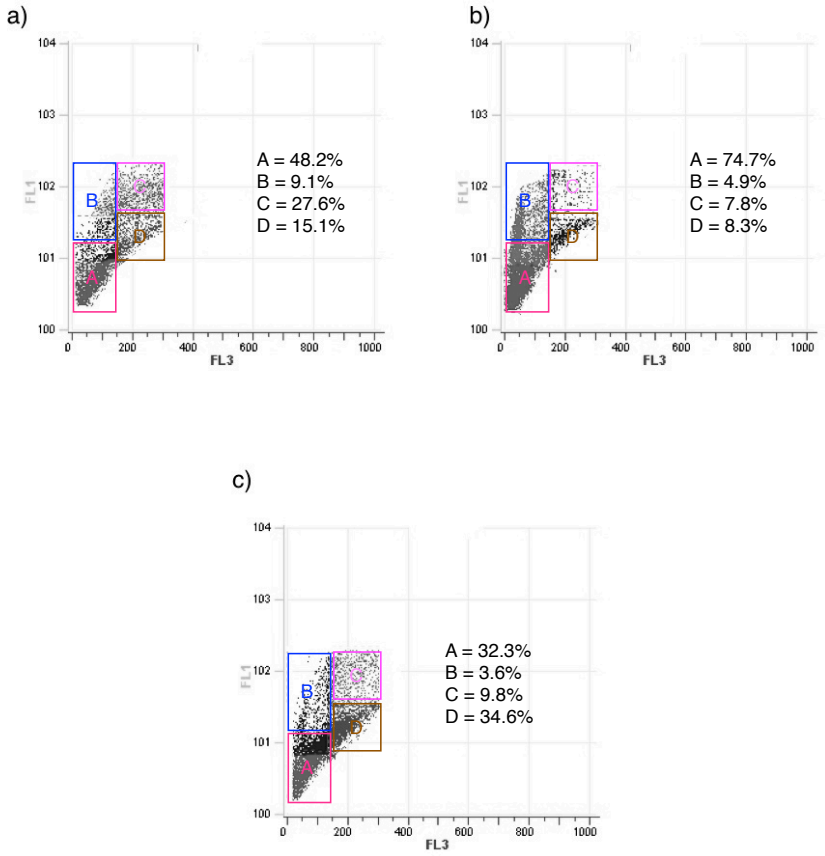


Figure 2.6.2. ***Serum starvation and colchicine treatment induces growth arrest in MCF-7 cells.*** MCF-7 cells, (a) untreated, (b) serum starved or treated with (c) 0.1µM colchicine for 24 hours were subjected to cell cycle analysis by flow cytometry. Histograms were generated by plotting log levels of FITC (FL1) against linear propidium iodide (FL3). A = G0/G1, B = S-phase, C = G2, D = M-phase Data is representative of three independent experiments performed in duplicate.

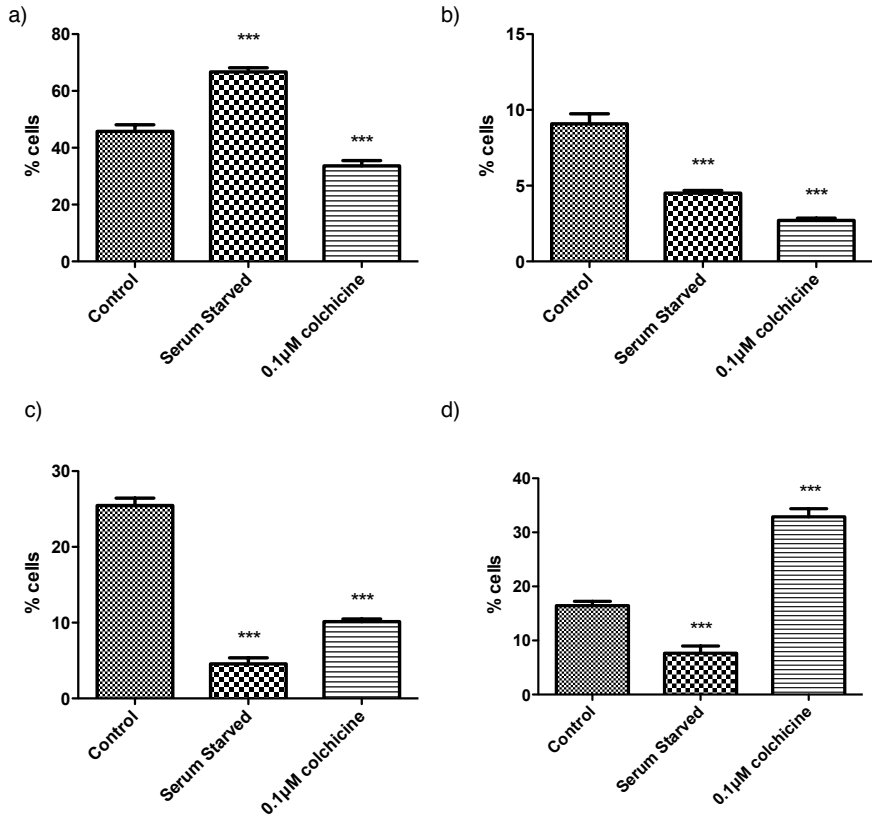


Figure 2.6.3. ***Effect of serum starvation and colchicine treatment on cell cycle.*** MCF-7 cells were either untreated, serum starved or treated with 0.1μM colchicine for 24 hours prior to cell cycle analysis by flow cytometry. (a) G0/G1, (b) S, (c) G2, and (d) M populations were gated according to fluorescence profile and expressed as percentage of total cell population. Data denoted *** ($p<0.001$) and ** ($p<0.01$) are statistically significant compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test (mean ± standard deviation). Data is from three independent experiments performed in duplicate.

2.7: Detection of apoptosis

2.7.1: Principles

Apoptosis is a form of programmed cell death that is vital to normal organism development, the immune response to infection and removal of oncogenic cells. It is a process characterised by distinct morphological changes such as membrane blebbing, nuclear fragmentation and chromatin condensation [128]. Apoptosis also induces plasma membrane changes such as the translocation of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the cell membrane [324]. Under normal conditions, PS is held on the inner leaflet of the plasma membrane by the enzyme flippase until it is cleaved by caspases following apoptotic activation, which causes the 'flip' of PS to the outer cell membrane [325]. Phosphatidylserine expressed on the cell surface can be detected by staining with a fluorophore and detection using flow cytometry. The protein annexin V is part of the annexin group of proteins and has high affinity for PS binding, *in vitro,* in the presence of calcium ions. Thus, using FITC-conjugated annexin-V protein, it is possible to detect PS present on the surface of apoptotic cells [324]. Co-staining with the DNA dye propidium iodide allows the user to isolate cell populations that are live, apoptotic or necrotic as propidium iodide is not cell permeable. Flow histograms generated by plotting FITC against PI fluorescence can be quadrant gated according to the fluorescent profile of untreated controls (figure 2.7.1). Quadrant 2 (Q2) represents necrotic cells displaying high levels of FITC and PI, while quadrant 4 (Q4) represents apoptotic cells with high FITC and low PI fluorescence. Quadrant 3 (Q3) is representative of the live cell population (low FITC, low PI).

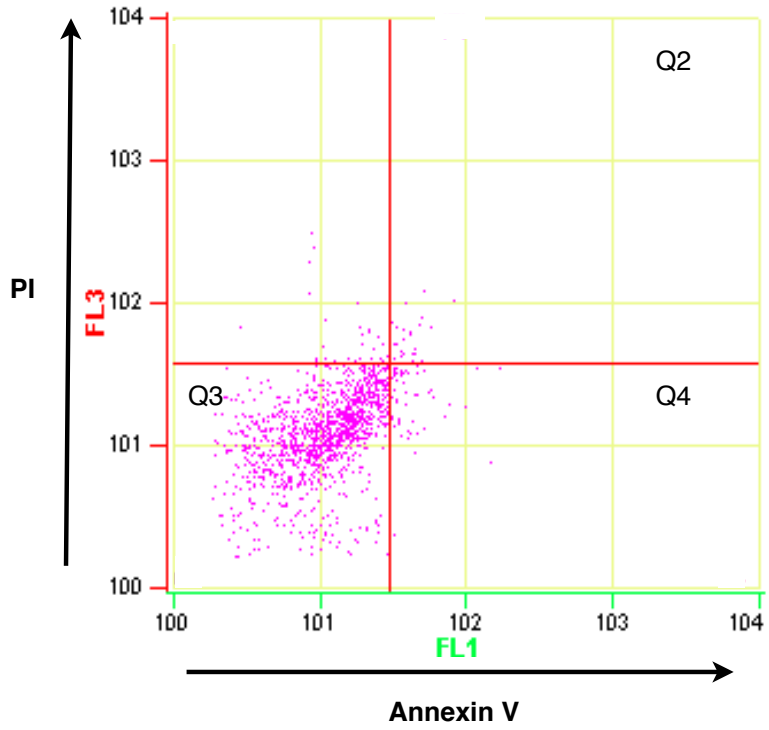


Figure 2.7.1. ***Typical flow cytometric dot-plot of untreated MCF-7 cells stained for annexin V (FL1) and propidium iodide (FL3).*** MCF-7 cells were stained with annexin V-FITC and propidium iodide prior to analysis by flow cytometry. Cells were gated so Q3 (live cells) > 95% total cells. Q2: necrotic cells, Q3: live cells, Q4: apoptotic cells. Data is representative of three independent experiments performed in triplicate.

2.7.2: Method

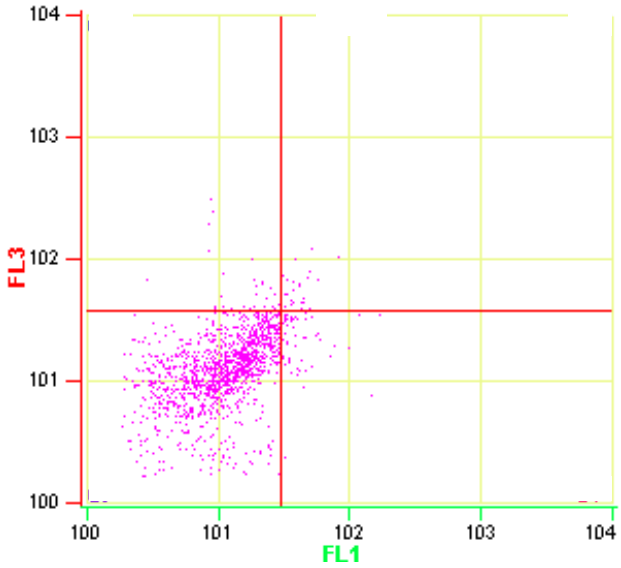
MCF-7 cells were seeded into a six well plate at a density of $2x10^5$ cells per ml in a volume of 2 ml and allowed to adhere overnight. Cells were refreshed with 1 ml of fresh media and treated with a final concentration of 2 mg/ml extract for up to 72 hours. At indicated time points, cells were scraped into 1ml PBS and centrifuged at *300 g* for 5 minutes. The supernatant was discarded and the cells resuspended in 100 µl PBS. Cell number was determined by trypan blue staining and $1x10^5$ cells transferred to an eppendorf in a total volume

of 500 µl PBS. Cells were centrifuged at *300 g* for 5 minutes and resuspended in 500 µl of annexin V binding buffer. Cells were incubated with 5 µl of annexin V-FITC and 5 µl propidium iodide solution for 5 minutes in the dark at room temperature. Stained cells were analysed on a Beckman-Coulter Cell Lab Quanta-SC flow cytometer measuring FITC fluorescence (FL1; Em: 670) and PI fluorescence (FL3; Em: 525) for 20,000 events per sample. Log FL3 was plotted against log FL1 to generate histograms. Cells were gated using quadrant gating dependent on fluorescent profile of untreated controls (Q3 > 95% cells).

2.7.3: Assay Validation

To validate the detection of apoptosis using the supplier's kit, MCF-7 cells were treated with 160 µM H_2O_2 for 5 hours, prior to analysis by flow cytometry. Figure 2.7.2 and 2.7.3 demonstrates that treatment with 160 µM H_2O_2 induced apoptotic cell death in MCF-7 cells after 5 hours that was detectable using the annexin V apoptosis detection kit from Abcam.

a)



b)

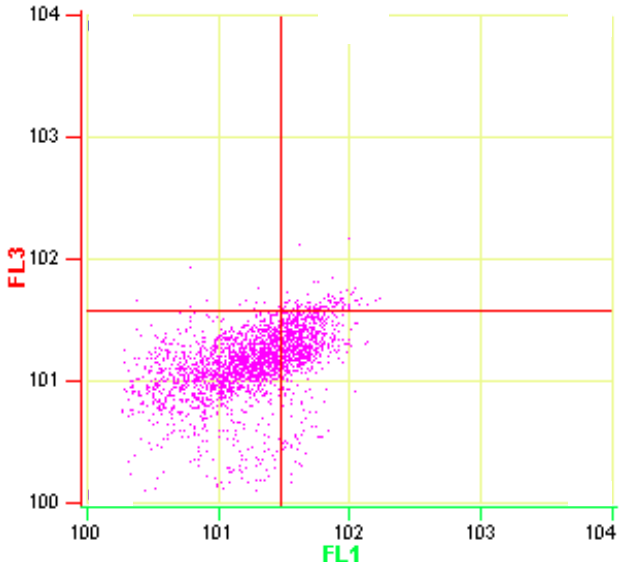


Figure 2.7.2. ***Hydrogen peroxide treatment induces apoptosis in MCF-7 cells.*** MCF-7 cells (a) untreated or treated with (b) 160μM H_2O_2 for 5 hours were subjected to an apoptosis detection assay using annexin V-FITC. Dot-plots were generated as a plot of log FL3 (propidium iodide) against log FL1 (FITC). Data is representative of three independent experiments performed in triplicate.

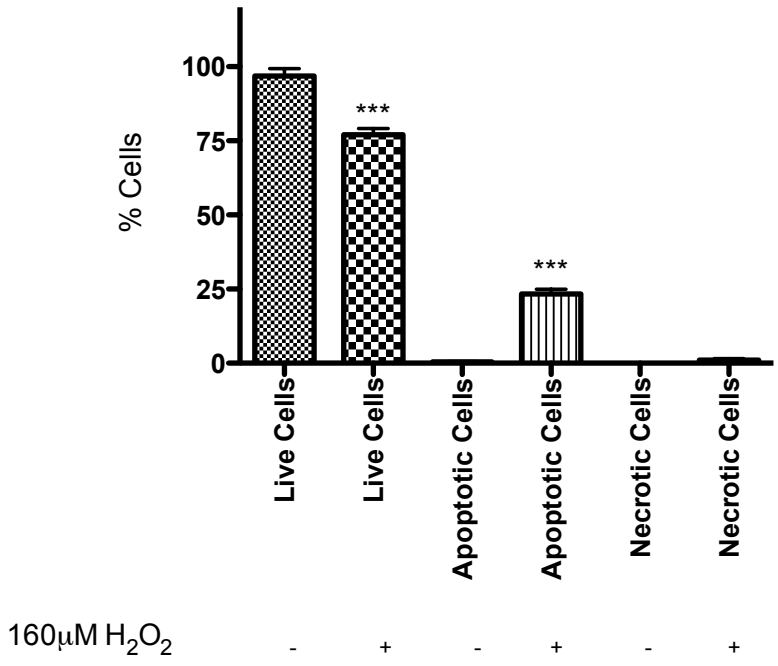


Figure 2.7.3. ***Hydrogen peroxide treatment induces apoptotic cell death in MCF-7 cells.*** MCF-7 cells were either untreated or treated with 160μM H_2O_2 for 5 hours prior to staining with annexin V-FITC and detection of apoptosis using flow cytometry. Data denoted *** ($p<0.001$) is significant compared to untreated as indicated by capped bar analysed by one-way ANOVA with Bonferroni's multiple comparison post-test (mean ± standard deviation). Data is representative of three independent experiments performed in triplicate.

2.8 Measurement of intracellular reactive oxygen species

2.8.1: Principles

Alterations in the levels of reactive oxygen species (ROS) in cultured cells can be measured fluorometrically using the probe 2'.7'-dichlorofluorescien diacetate (DCFH-DA). The non-fluorescent probe is readily taken up by cells where local esterases hydrolyse it to DCFH, which in turn can be oxidised to highly fluorescent DCF by cellular oxidants such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). The use of DCFH-DA as a probe for oxidative stress is not recommended if one is trying to study specific radical species, but due to its indiscriminate nature, it allows the assay to measure the overall oxidative stress in a cell population [326].

2.8.1: Method

Cultured cells were seeded into a 24-well plate at a density of 1 x 10^5 cells per ml in 500 µl and allowed to adhere overnight. The cells were then washed with 500 µl of sterile PBS before being refreshed in phenol red free/serum free RPMI 1640 and incubated for 2 hours to equilibrate. At this stage cells were treated. One hour before the end of treatment, the cells were probed with 50 µl of 100 µM DCFH-DA (10 mM stock diluted in phenol red free/serum free RPMI 1640) to give a final concentration of 10 µM. After incubation with the dye for 60 minutes, the media was removed and the cells were washed in sterile PBS. Finally, the cells were refreshed with 500 µl PBS and fluorescence was detected immediately on a Molecular Devices SpectraMAX Gemini EM plate reader (Ex: 504 nm; Em: 524 nm). Data was

expressed as a fold change of DCF-fluorescence (DCF-FL) compared to untreated control.

2.8.2: Assay validation

To assess the suitability of the DCF assay to measuring intracellular ROS production, the level of DCF fluorescence generated by H_2O_2 treatment in the presence of NAC was determined in MCF-7 cells. Cells were pre-treated with 3 mM NAC or vehicle for 60 minutes prior to treatment with and without 160 μM H_2O_2 for 3 hours. Treatment with hydrogen peroxide induced a significant 2.0 fold increase in DCF fluorescence which was significantly attenuated by pretreatment with 3 mM NAC (figure 2.8.1).

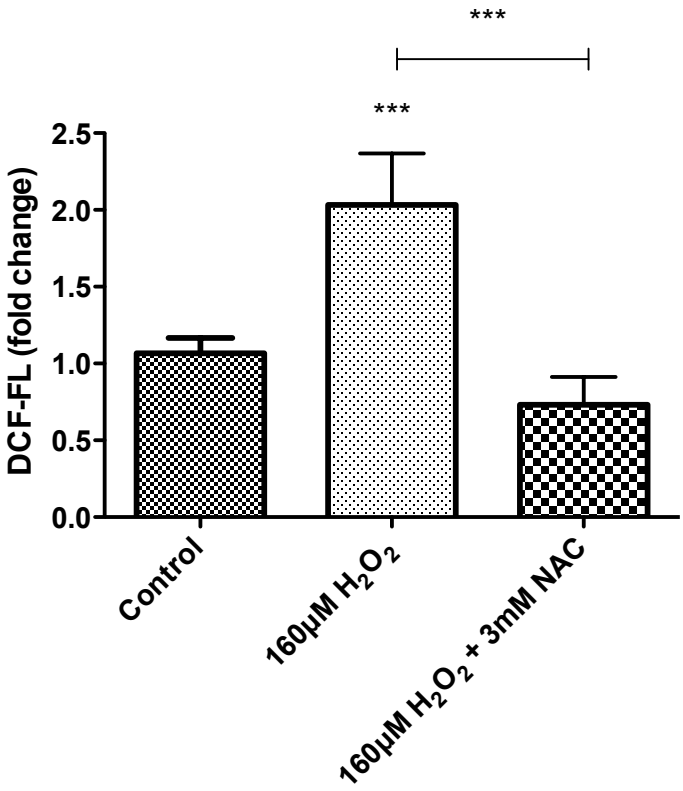


Figure 2.8.1. ***H_2O_2 treatment of MCF-7 cells increases DCF fluorescence which can be attenuated with NAC.*** MCF-7 cells were pretreated with 3mM NAC for 60 minutes prior to treatment with 160μM H_2O_2 for 3 hours. Intracellular reactive oxygen species were detected using the DCFH-DA probe and measured fluorometrically (Ex: 504nm, Em: 524nm). Data denoted *** ($p<0.001$) are significant compared to control or as indicated analysed by one-way ANOVA with Bonferroni's multiple comparison post test (mean ± standard deviation). Data is from three independent experiments performed in triplicate.

2.9: Detection of the DNA damage lesion 8-oxo-guanine

2.9.1: Principles

The presence of DNA damage in individual cells can be detected using the comet assay. The comet assay is a single cell gel electrophoretic method based on the principle that smaller fragmented DNA will migrate in an electric field easier than larger intact DNA. Cells are first lysed in a detergent and high salt buffer, which disrupts cellular proteins including RNA and removes the cell membrane. The exposed DNA is denatured with an alkaline pH that causes strand separation leaving a single stranded nucleoid [327]. An electric field facilitates separation of nucleoid content to produce the characteristic comet that can be visualised with a DNA stain such as propidium iodide or ethidium bromide.

Using the DNA glycosylase protein, FPG, it is possible to modify the comet assay to detect the presence of the most common oxidative DNA lesion, 8-oxo-guanine. FPG protein recognises the oxidised guanine base of 8-0xo-guanine and is involved in its removal during base excision repair creating a single strand break at the site of mutation. This means that in the presence of FPG protein, more strand breaks will be generated if 8-oxo-guanine is present, thus generating a larger comet tail. It should be noted that FPG also has specificity for other oxidative DNA lesion and so is not a single measure of 8-oxo-guanine but of oxidative DNA damage in general.

2.9.2: Method

Cultured cells were seeded into a 6-well plate at a density of 2 x 10^5 cells per ml in a volume of 3 ml and incubated

overnight to allow adherence. The medium was removed and replaced with 1 ml fresh media, followed by treatment with either vehicle control, 0.25, 0.5, 1 or 2 mg/ml of extract for up to 3 hours. Hydrogen peroxide at a final concentration of 80 µM for 60 minutes was used as a positive control to induce strand breaks. After treatment, the media was removed and the cells washed in 1 ml PBS before being scraped into 1 ml fresh PBS. The cells were centrifuged at *400 g* for 5 mins and the supernatant discarded. The pellets were resuspended in 300 µl of 0.6% low melting point (LMP) agarose in PBS and 50 µl was applied to a microscope slide pre-coated with 1% normal melting point (NMP) agarose in water with the aid of a coverslip. The LMP gel was allowed to set at 4˚C for 10 minutes before the cover slip was removed, the slide was submerged in ice cold lysis buffer and left for 1 hour at 4˚C in the dark. For the detection of 8-oxo-guanine, slides were washed 3 times with ice cold FPG buffer before either 50 µl of FPG protein (20 U/ml) diluted in FPG buffer or 50 µl of FPG buffer alone was applied to the LMP gel with the aid of a coverslip. The slides were then incubated at 37˚C for 30 minutes. The slides were then submerged in ice cold electrophoresis buffer for 40 minutes in order for alkaline unwinding to take place before electrophoresis in a flat bed tank for 30 minutes (25V, 300mA) at 4˚C in the dark. The slides were removed and washed with neutralisation buffer (0.4 M tris-HCL, pH 7.5) three times, followed by three washes in distilled H_2O. The slides were stained with 1 ml of propidium iodide solution for 20 minutes in the dark at room temperature, washed three times with distilled H_2O and allowed to dry at 37˚C in the dark. Comets were visualised using an Axiovert 200M (*Carl Zeiss Microimaging Inc.*) microscope equipped with a digital CCD camera (*Hamamatsu Photonics*) and Metamorph software (*Universal Imaging Corp.*). Photos were analysed using COMETscore *(autocomet.com)*. For each experiment

ten photos were taken and all comets in view were analysed totalling at least 50 comets per slide. Data was expressed as percentage DNA present in the tail.

2.9.3: Assay validation

The comet assay was validated by treating MCF-7 cells with a known DNA damaging agent H_2O_2 and measuring the change in DNA damage compared to untreated control. MCF-7 cells were treated with 160 µM H_2O_2 for 30 minutes prior to comet assay as shown in 2.9.2. Samples were incubated with and without FPG protein for the assessment of oxidative DNA damage. Treatment of MCF-7 cells with 160 µM H_2O_2 for 30 minutes significantly increased the percentage of DNA in comet tails, which could be significantly increased by incubation with FPG protein. In response to hydrogen peroxide treatment, DNA damage increased by approximately 135% which was increased by a further 52% in the presence of FPG (figure 2.9.1). These results demonstrate that the comet assay can detect significant changes in DNA damage and is sensitive to specific detection of oxidative lesions induced by a known oxidative DNA damaging agent.

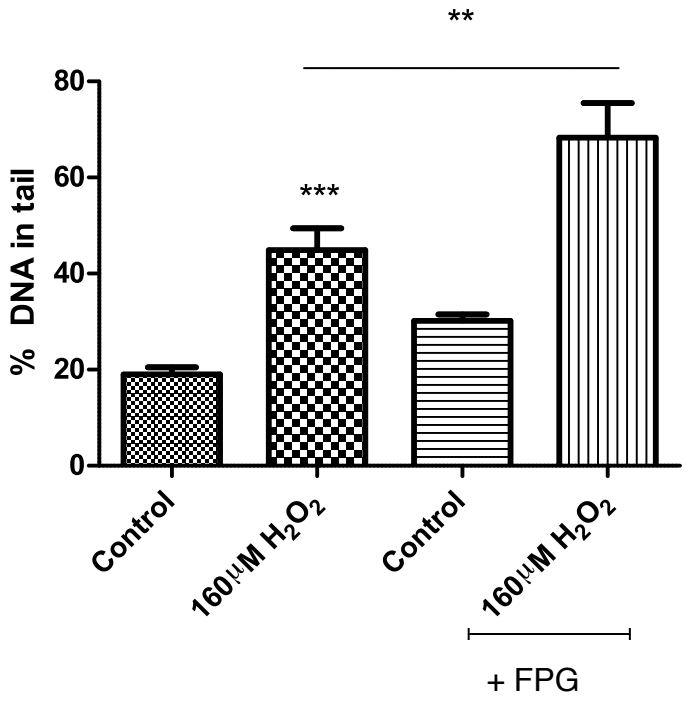


Figure 2.9.1. ***Hydrogen peroxide treatment induces DNA damage detectable by comet assay.*** MCF-7 a were treated with 160μM H_2O_2 for 30 minutes prior to analysis by comet assay with and without FPG protein incubation. Data is expressed as % DNA in tail as analysed by COMETscore software. Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to untreated control or as indicated. Data was analysed by one-way ANOVA with Bonferroni's multiple comparison post test (mean ± standard deviation). Data is from three independent experiments performed in duplicate.

2.10 Protein characterisation by western blot

2.10.1: Principles

Western blotting is an analytical technique used primarily for the identification and semi-quantitation of proteins. The technique is based on antibody antigen interactions between the protein to be characterised and the probing antibody. Gel electrophoresis of a sample is undertaken to separate the proteins before they are transferred to a suitable membrane which can be probed with an antibody against the protein of interest. A secondary antibody labelled with a detection enzyme, such as horseradish-peroxidase (HRP), against an antigenic site on the primary antibody can be used to visualise the presence of the bound protein. Analysis of protein expression by western blot allows quantification of specific protein levels in a sample by measuring band intensity using densitometry. Densities can be normalised to a standard that is unaffected by the experimental conditions and is in high abundance such as β-actin or GAPDH.

2.10.2: Method

Cultured cells were seeded into a 6-well plate at a density of $2x10^5$ cells per ml in a volume of 2 ml. After treatment, as described in relevant chapters, the media was removed and the cells washed with 1 ml of sterile PBS before scraping into 1 ml of fresh PBS. The cell suspension was centrifuged at *400 g* for 5 minutes and the supernatant discarded. The cell pellet was resuspended in 1 ml of PBS before centrifuging again at *400 g* for 5 minutes. The cell pellet was then resuspended in 100 µl of cell lysis buffer and heated at 90˚C for 10 minutes followed by storage on ice for 15 minutes. The total protein content of the cell lysate was determined by absorbance at 560nm using a bicinchonic acid protein kit

(*Sigma*). Total protein, 20 µg, was loaded onto a SDS-PAGE gel and subjected to electrophoresis (constant 115V) for 120 minutes. The migrated proteins were transferred to a PVDF membrane (*Bio-Rad)* and electro-blotted (constant 240 mA) in transfer buffer for 65 minutes. After the transfer, the membrane was blocked with 3% BSA in TBST overnight at 4^0C. The membrane was then briefly washed with TBST for 5 minutes before incubation with a primary antibody diluted to an optimised concentration in 0.2% BSA in TBST for 2 hours at room temperature. The primary antibody was then removed and the membrane was washed six times in TBST for 5 minutes each. This was followed by incubation with a HRP-conjugated secondary antibody diluted 1/20,000 in TBST containing 0.2% BSA for 2 hours at room temperature. The membrane was then washed six times in TBST for 5 minutes each before HRP-conjugated secondary antibody was detected using ECL detection reagent (*CalBioChem).* Each gel was controlled for equal loading by stripping the membrane with Restore *(Thermo Scientific)* stripping buffer for 15 minutes before washing in TBST three times for 10 minutes each. The membrane was then blocked with 3% BSA in TBST for 60 minutes before probing with a mouse anti-human β-actin primary (*Sigma*) at a 1/10,000 dilution in 0.1% BSA in TBST for 1 hour at room temperature. The primary antibody was removed and the membrane washed six times in TBST for 5 minutes each before probing with a relevant HRP conjugated secondary at a dilution of 1/20,000 in 0.1% BSA in TBST for 1 hour at room temperature. The antibody as then removed and the membrane was washed 6 times in TBST for 5 minutes each before HRP was detected using ECL detection reagent. Primary antibody species and concentrations are listed in table 2.10.1.

Antibody	Dilution
rabbit anti-human p53	1/1000
mouse anti-human p21	1/100
rabbit anti-human λH2AX	1/1000
rabbit anti-human Bax	1/1000
rabbit anti-human SIRT1	1/1000
rabbit anti-human FOXO3a	1/1000
mouse anti-β-actin	1/10000

Table 2.10.1. ***Primary antibody dilutions used for western blot.***

2.11: siRNA interference

2.11.1: Principles

Small interfering RNA or siRNA are double stranded RNA molecules that use RNA interference to influence target gene expression. Sequences of specific siRNA can be generated that are complementary to the mRNA of a particular gene product. Thus, through the RNA interference pathway, siRNA are capable of degrading target mRNA and reducing gene expression. This tool is useful for understanding the importance of specific gene products involved in disease pathology or treatment efficiency. The siRNA is incubated with a transfection reagent that acts as a delivery platform for the siRNA to the target cells. The silencer plasmid is then transfected into the cells where the siRNA is released in the nucleus. The free siRNA can then interact with target mRNA and mark it for degradation, thus, inhibiting protein translation. This process is transient and does not inhibit protein translation permanently.

2.11.2: Methods

Validated siRNA for *TP53* (*Ambion*) and *FOXO3* (*Ambion*) were used to transiently silence p53 and FOXO3a expression in MCF-7 cells or MDA-MB231 cells. Sequences of siRNA strands are detailed in table 2.11.1. Silencer siRNA supplied as a powder was diluted to 3 pmol/µl with RNAase free water and mixed 1:50 with Opti-MEM in either a 24-well (MTT assay) or 6-well (western blot) plate. After addition of 1% lipofectamine RNAiMAX plates were incubated at room temperature for 20 minutes. Cells were diluted to $1x10^5$ cells per ml in cell culture media free from antibiotics. Either $5x10^4$ (24 well plate) or $2x10^6$ (6 well plate) cells in suspension were added to siRNA-lipofectamine

complexes and incubated for 24 hours. Cells were then treated with up to 2 mg/ml of aqueous extract for 24 hours prior to analysis of cell viability by MTT assay or determination of protein expression by western blot.

Target	Strand	Sequence (5'--> 3')
TP53	sense	GGGUUAGUUUACAAUCAGC(dtdt)
	anti-sense	GCUGAUUGUAAACUAACCC(dtdt)
FOXO3	sense	GGCUCCUCCUUGUACUCAAtt
	anti-sense	UUGAGUACAAGGAGGAGCCtg

Table 2.11.1. ***Validated Silencer siRNA sequences from Ambion.*** Validated siRNA oligonucleotides were obtained from Ambion and used to transiently knockdown p53 and FOXO3a expression in MCF-7 or MDA-MB-231 cells.

2.11.3: Assay validation

To validate the use of Ambion *TP53* silencer siRNA to successfully knockdown p53 expression, MCF-7 cells were transfected with siRNA for 24 hours using lipofectamine RNAiMAX according to the manufacturer's protocol. MCF-7 cells were transfected with either 2.5 nM, 5 nM, 10 nM siRNA, lipofectamine alone or a validated negative control (Ambion) for 24 hours prior to analysis of cell viability by MTT assay and protein quantification by western blot. Figure 2.11.1 shows the effects of TP53 siRNA incubation on MCF-7 cell viability after 24 hours. None of the tested siRNA concentrations induced a significant reduction in cell viability indicating that *TP53* siRNA up to 10nM can be used for siRNA interference assays (figure 2.11.1). Analysis of

p53 expression by western blot showed that *TP53* siRNA inhibited p53 expression in a dose dependent manner (figure 2.11.2). Treatment of MCF-7 cells with 10 nM *TP53* siRNA resulted in a near complete depletion in basal p53 expression as detectable by western blot. Therefore, all following experiments were performed with transfection of 10 nM *TP53* siRNA for 24 hours prior to treatments.

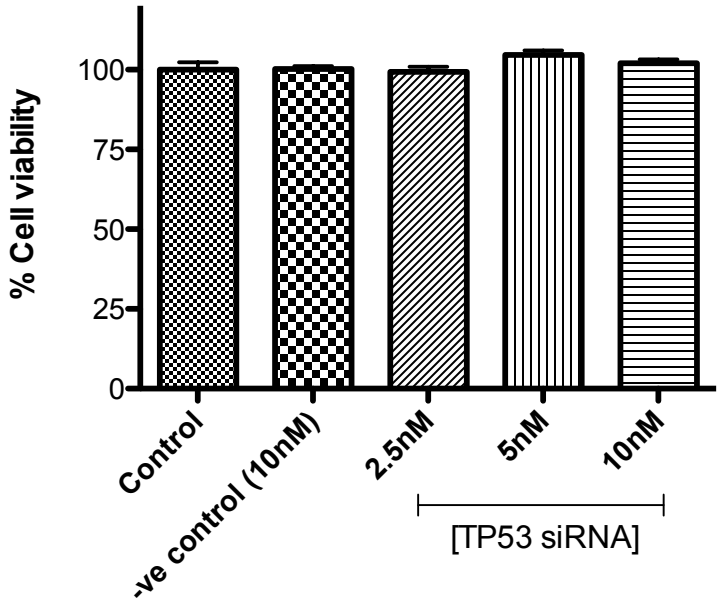


Figure 2.11.1. ***Silencer TP53 siRNA transfection does not effect MCF-7 cell viability.*** MCF-7 cells were transfected with *TP53* siRNA plus lipofectamine RNAiMAX for 24 hours prior to analysis of cell viability by MTT assay. Negative control was a validated non-reactive siRNA sequence (Ambion). Data is from three independent experiments performed in triplicate and is representative of the mean ± standard deviation.

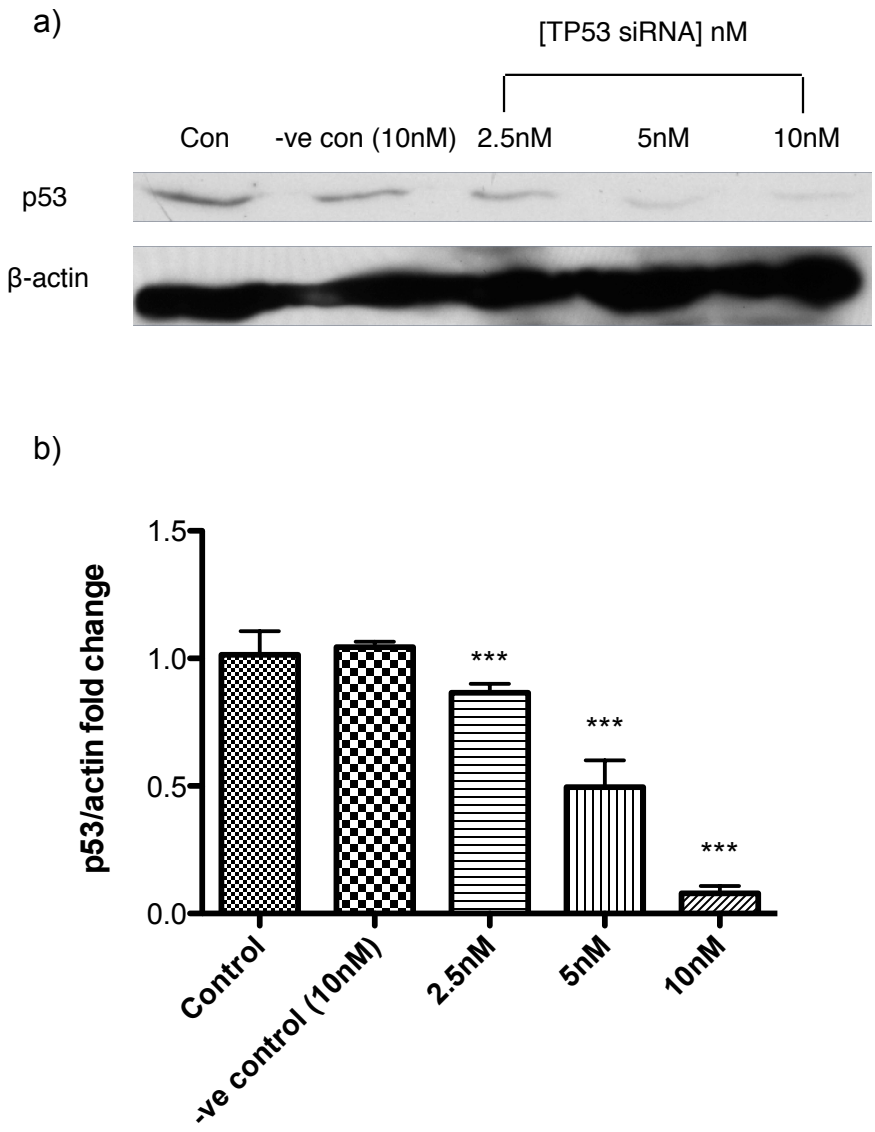
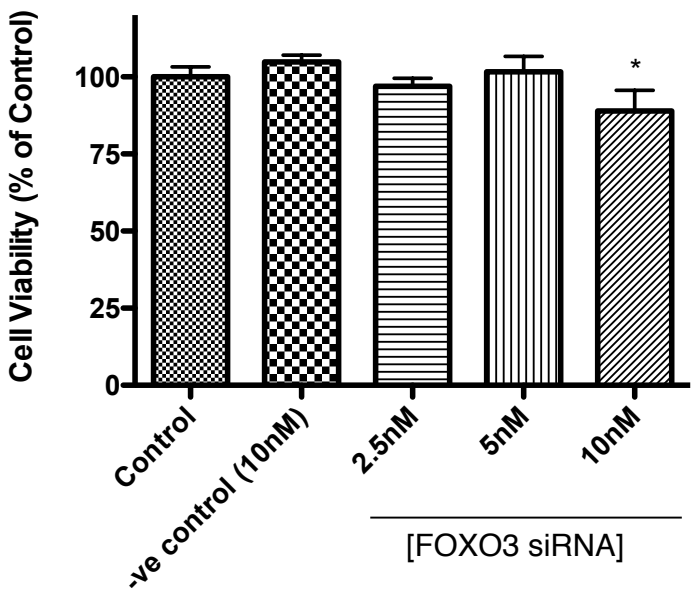


Figure 2.11.2. ***Silencer siRNA TP53 knockdown in MCF-7 cells.*** MCF-7 cells were transfected with *TP53* or negative control siRNA plus lipofectamine RNAiMAX for 24 hours. (a) Characterisation of p53 protein expression was determined by western blot. (b) Densitometry for was calculated as a ratio to β-actin and expressed as fold change compared to control (*time=0 hours*). Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments. Negative control was a validated non-reactive sequence from Ambion. β-actin was used as a loading control. Blot is representative of three independent experiments.

To validate the use of Ambion *FOXO3* silencer siRNA to successfully knockdown FOXO3a expression, MCF-7 and MDA-MB-231 cells were transfected with siRNA for 24 hours using lipofectamine RNAiMAX according to the manufacturer's protocol. Cells were transfected with either 2.5 nM, 5 nM, 10 nM siRNA, lipofectamine alone or a validated negative control (Ambion) for 24 hours prior to analysis of cell viability by MTT assay and protein quantification by western blot. Figure 2.11.3 shows the effects of *FOXO3* siRNA incubation on MCF-7 and MDA-MB-231 cell viability after 24 hours. Transfection with 10nM siRNA induced a significant reduction in cell viability in both cell lines indicating that *FOXO3* siRNA up to 5nM can be used for siRNA interference assays (figure 2.11.3). Analysis of FOXO3a expression by western blot showed that *FOXO3* siRNA inhibited FOXO3a expression in a dose dependent manner (figure 2.11.4). Treatment of MCF-7 and MDA-MB-231 cells with *FOXO3* siRNA resulted in a dose dependent decrease in basal FOXO3a expression as detectable by western blot. As shown in figure 2.11.3, treatment of 10 nM siRNA is cytotoxic to both cell lines, therefore all following experiments were carried out with transfection of 5nM *FOXO3* siRNA for 24 hours prior to treatments.

a)



b)

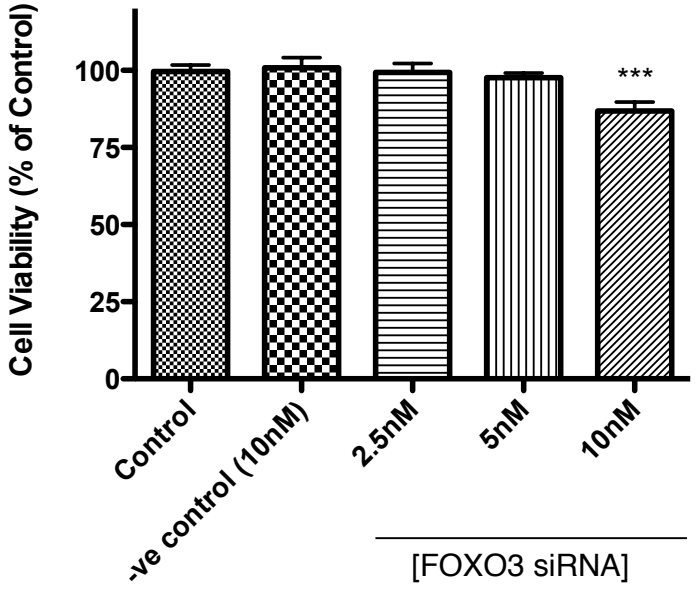


Figure 2.11.3. ***Effects of Silencer FOXO3 siRNA transfection on human breast cancer cell viability.*** (a) MCF-7 and (b) MDA-MB-231 cells were transfected with *FOXO3* siRNA plus lipofectamine RNAiMAX for 24 hours prior to analysis of cell viability by MTT assay. Negative control was a validated non-reactive siRNA sequence (Ambion). Data denoted * ($p<0.05$) and *** ($p<0.001$) are significant compared to control (mean ± standard deviation). Data is from three independent experiments performed in triplicate.

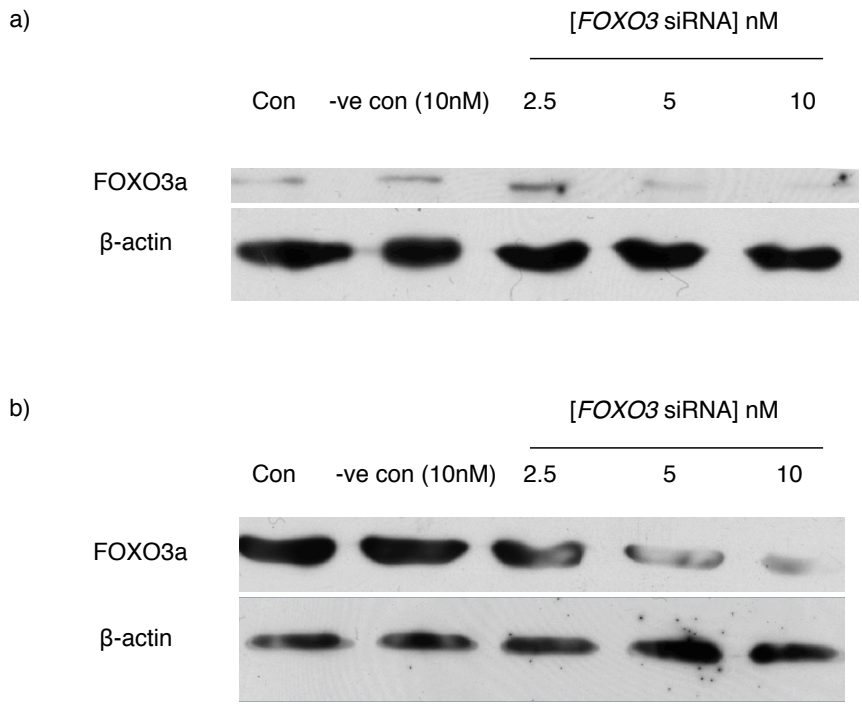


Figure 2.11.4. ***Silencer siRNA FOXO3 knockdown in human breast cancer cells.*** (a) MCF-7 and (b) MDA-MB-231 cells were transfected with *FOXO3* or negative control siRNA plus lipofectamine RNAiMAX for 24 hours. Characterisation of FOXO3a protein expression was determined by western blot. Negative control was a validated non-reactive sequence from Ambion. β-actin was used as a loading control. Blot is representative of three independent experiments.

2.12: SIRT1 activity assay

2.12.1: Principles

SIRT1 is a NAD^+ dependent histone/protein deacetylase involved in chromatin dynamics, gene repression and transcription factor regulation. The SIRT1 assay kit (*Sigma*) is based on a two-step enzymatic reaction that utilises a novel SIRT1 substrate with an acetylated lysine side chain. Cleavage of the deacetylated substrate by a developer solution releases a highly fluorescent group which can be measured fluorometrically. The measured fluorescence is directly proportional to the deacetylation activity of SIRT1 present in the sample. The SIRT1 activity assay can be carried out cell-free with purified SIRT1 enzyme for screening of inhibitor/activators and as part of *in vitro* experiments using cellular nuclear extracts to measure enzymatic activity.

2.12.2: Methods

Nuclear extracts were isolated from MCF-7 cells treated for 24 hours with 2 mg/ml extract with and without 50 µM resveratrol (SIRT1 activator) pre-treatment for 60 minutes. MCF-7 cells were cultured in a T75 until 80% confluent before treatments. After treatment, cell were washed with 5 ml ice-cold PBS before scraping into 5ml PBS and centrifugation at *300g* for 5 minutes. Cell pellets were washed with 1 ml ice-cold PBS before re-suspending in 500 µl of buffer A containing 0.1% protease inhibitor cocktail (AEBSF 104 mM, Aprotinin 80 µM, Bestatin 4 mM, E-64 1.2 mM, Leupeptin 2 mM, Pepstatin A, 1.5 mM) and retaining on ice for 10 minutes. Cells were centrifuged at *800 g* for 10 minutes at 4˚C. Cell pellets were resuspended in 374 µl of buffer B with 26 µl 4.6 M NaCl. Cell pellets were homogenised by passing through a 25-gauge needle 20

times and stored on ice for 30 minutes. Cell extracts were centrifuged at *16000 g* for 20 minutes at 4˚C and the supernatant retained for BCA protein assay and SIRT1 activity assay. SIRT1 activity assay was carried out according to the manufacturer's protocol (*Sigma CS1040*) using 15 µg total protein from nuclear extracts.

2.13: ATP assay

2.13.1: Principles

The ENLITEN® ATP assay system (Promega, FF2000) is a bioluminescence detection kit for the measurement of ATP. It uses a recombinant luciferase reagent which is used to catalyse the following reaction:

$$ATP + {}_{D}\text{-Luciferin} + O_2 \longrightarrow \text{Oxyluciferin} + AMP + PPi + CO_2 + \text{light (560nm)}$$

where ATP is the rate limiting component of the reaction. The amount of light generated is directly proportional to the amount of ATP present in the sample.

2.13.2: Method

MCF-7 cells at a density of 2 x 10^5 were seeded into a 6-well tissue culture plate in 2 ml media and allowed to adhere overnight. Media was refreshed and cells were treated with 2 mg/ml aqueous extract or serum-free media for up to 24 hours. After treatment, cells were washed in ice cold PBS, scraped into 1 ml fresh PBS and centrifuged at *300 g* for 5 minutes. Cells were washed with 1 ml ice-cold PBS and centrifuged at *300 g* for 5 minutes. Cell pellet was resuspended in 100 µl ATP extraction buffer and incubated at 90°C for 45 seconds. 10 µl of cell extract was used to assay for ATP content using the ENLITEN ATP assay kit according to the manufacturers instructions.

2.14 Bio-assay guided fractionation

2.14.1 Principles

In order to isolate and characterise potential chemotherapeutic compounds from an aqueous extract of *Fagonia cretica* a bio-assay guided fractionation (BAGF) method was employed. BAGF is a common method in research development of new drug compounds from natural sources. It involves multiple step fractionations using analytical chemistry techniques such as solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) to separate compounds of interest. At each step the isolated fractions, or pure compounds, can be tested with a bioassay, such as the MTT assay, for activity before identifying candidate leads. Compounds of interest can be identified and/or characterised using analytical methods such as mass-spectrometry and nuclear magnetic resonance (NMR).

In this investigation BAGF was carried out using SPE and HPLC methods. Solid-phase extraction is an extraction method involving separation of compounds, based on their physical or chemical properties, in a liquid mobile phase on a solid stationary phase such as a silica gel matrix. For example, a C18 column contains a stationary phase packed with inert silica particles attached to non-polar C18 carbon chains. This generates a hydrophobic surface on the column allowing more polar compounds to be eluted first.

HPLC is a common chromatographic technique for the separation of a mixture of compounds. It has wide applications covering compound isolation, quantification and identification. Generally a column containing a similar matrix to that seen in a C18 SPE column is densely packed to

provide a stationary phase tightly packed with straight carbon chains. The dense packing of the stationary phase creates a surface area with high hydrophobicity that provides sensitive separation of compounds based on their polarity. As with SPE, a polar liquid mobile phase applied to a C18 HPLC column will elute the most polar compounds first. Increasing the level of organic solvent present in the mobile phase will allow compounds with high hydrophobicity to be eluted. Employing a gradient of organic solvent as a mobile phase allows fine separation of compounds in a mixture.

Mass-spectrometry is a powerful analytical tool that measures the mass to charge ratio of charged particles, thus, providing accurate mass calculations of observed compounds. Commonly, electrospray-ionisation is used to generate the ions needed for mass spectrometry and is referred to as ESI-MS. In the electrospray method, the compound of interest is suspended in an appropriate solvent and acidified to increase the conductivity of the compound. The sample is fed into the vacuum stage where the solvent is evaporated under heat producing charged droplets that eventually deform to produce an aerosol by Coulomb fission [328]. The ions produced can then be observed by mass spectrometry. Tandem mass-spectrometry (MS/MS) involves fragmentation of the precursor ion to generate a fragmentation profile of product ions. In analytical chemistry it can be used to identify the particular structure of a precursor ion by analysing the fragmentation profile. Fragmentation of precursor ions is induced by collision-induced dissociation where gas-phase particles are accelerated by and electrical potential. This shifts the charged particles to a higher state of kinetic energy and enables collision with neutral molecules resulting in bond breakage and fragmentation [329].

2.14.2 Methods

In this study, SPE was carried out using a 1.5 ml Bond Elut C18 reverse phase column (*Analytichem International*). The SPE column was first equilibrated with 7.5 ml of 0.5% acetic acid before applying 20 mg of aqueous extract dissolved in 1 ml of 0.5% acetic acid. The sample volume was allowed to fully enter the column before being washed through with 5 ml of 0.5% acetic acid and collected. The column was then eluted with 5 ml of 100% ethanol and collected. The flow-through and elution fractions were then dried in a vacuum centrifuge before being used in a MTT cell viability assay or subjected to further purification techniques.

HPLC was carried out using a 1 ml C18 RP-HPLC column (150 x 4.6 mm, 5 micron) (*Phenomenex*) in conjunction with an AKTA Prime system (*GE Healthcare*) coupled to a UV detector (254 nm). The mobile phase was a mixture of 0.5% acetic acid and methanol. Fractions of 1 ml were collected periodically throughout the method. The flow rate was set to 0.8 ml/min. The column was first equilibrated with 5 ml of 0.5% acetic acid before a developed automatic method was applied to the system (table 2.14.1). Samples for loading were prepared by drying SPE fractions to under reduced pressure before resuspending in 500 µl of 0.5% v/v acetic acid for injection.

Time (mins)	Action
0	0% MeOH
2.5	Sample injection
12.5	25% MeOH
17.5	50% MeOH
27.5	75% MeOH
37.5	100% MeOH
47.5	0% MeOH
50	End method

Table 2.14.1. ***Method for HPLC separation of Fagonia cretica using C18 column.*** Isolation of compounds from a solid-phase extraction purified aqueous extract was performed on an AKTA Prime system. The column used was a 1 ml C18-HPLC equilibrated in 0.5% acetic acid. A methanol elution gradient was employed which increased step-wise by 25% over time. Flow rate was 0.8 ml/min, the eluate was monitored at 254 nm and fractions were collected at 1 ml intervals.

After the fractions had been collected, they were pooled according to their retention times and dried using a vacuum centrifuge before being used in a MTT cell viability assay or subjected to further purification and analytical techniques. Fractions from SPE or HPLC purification that were subjected to testing with the MTT assay were resuspended in 200 µl of fresh media before using as a treatment.

Composition of fractions was determined by ESI-MS using a Thermo Finnigan LCQ quadropole ion-trap mass spectrometer. Fractions were resuspended in 100 µl 0.5% formic acid (aq.) and applied to the ESI system by direct infusion with a hamilton syringe. Stable spray was

established and precursor ion profiles were generated by mass spectrometry in negative ion mode.

2.14.3: Validation of mass-spectrometry as a method of identifying natural products from a mixture.

In order to show that ESI-MS/MS could be used to identify natural compounds from a mixture, a mixture of several plant polyphenol standards was subjected to ESI-MS and precursor ions fragmented to validate MS/MS as a method for characterising compounds. The polyphenols selected to make up the standard mixture represent various classes of plant polyphenols and are outlined in figure 2.14.1. The mixture of polyphenols was solubilised in methanol with 0.5% v/v formic acid prior to direct infusion with the ESI system. Precursor ions were resolved in negative ion mode and fragmentation profiles generated with a CID energy of 30%. Figure 2.14.2a shows single stage mass spectral analysis of the polyphenol standard mixture with standard molecular weights highlights. ESI-MS/MS of gallic acid ($[M-H]^-$ 168.9) produced a product ion of 124.8 due to loss of the carboxyl group (fig. 2.14.2b). Caffeic acid ($[M-H]^-$ 179.0) also lost a carboxyl group, generating a product ion of 134.8, in response to fragmentation (fig. 2.14.2c). Fragmentation of phloretin ($[M-H]^-$ 273.0) generated a product ion of 166.9 owing to the loss of ring A from the conjugate system (fig 2.14.2d). Quercetin ($[M-H]^-$ 301.0) generated a more complex fragmentation profile with product ions forming at 193.0, 178.9, 150.9 ad 106.8 (fig. 2.12.3e). Product ion 193.0 corresponds with the loss of the benzoyl system (ring A) while the remaining product ions represent dissociation ring B which has become unstable. Fragmentation of quercetin 3-β-glucoside generated a product ion of 301.0 due to the loss of the sugar motif from the parent ion (fig. 2.14.2f). Fragmentation of product ion 301.0 generated the

characteristic profile seen with fragmentation of quercetin (fig. 2.14.2e). This data demonstrates that ESI-MS/MS can be used to detect polyphenols in a mixture. Furthermore, product ions can be dissociated to generate product ions that are characteristic to the compounds structural characteristics.

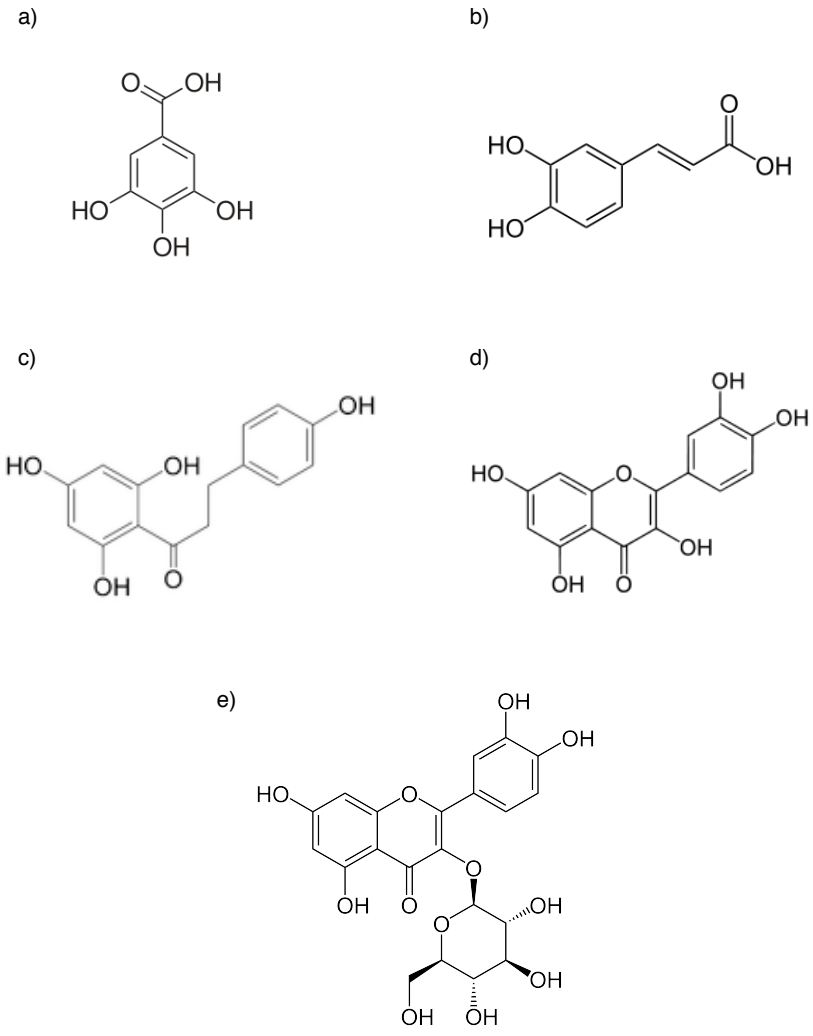
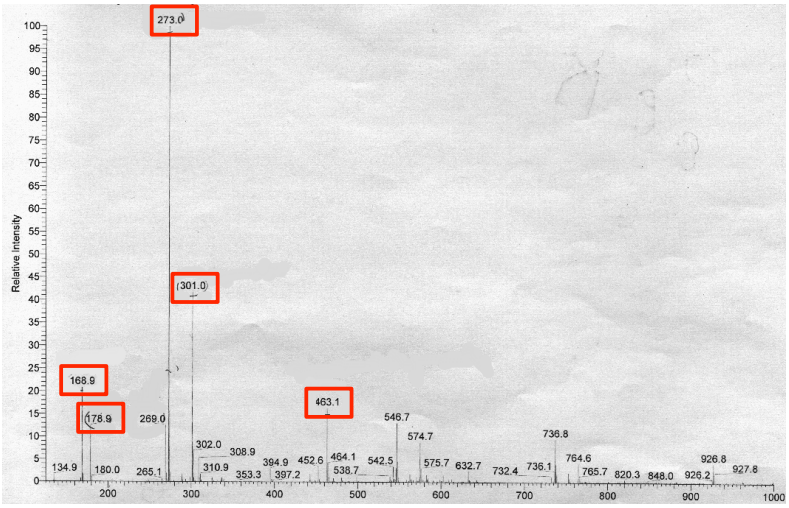
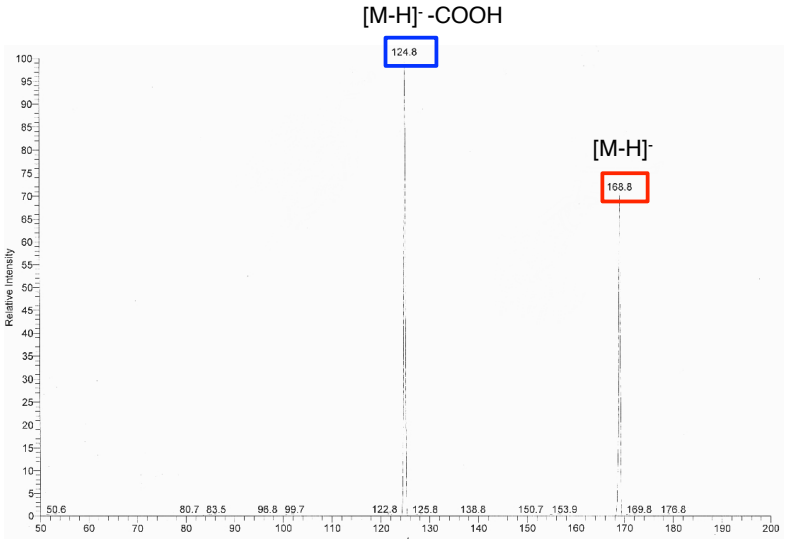


Figure 2.14.1. ***Polyphenol standards used to validate ESI-MS.*** a) Gallic acid [hydroxybenzoic acid], b) Caffeic acid [hydroxycinnamic acid], c) Phloretin [dihydrochalcone], d) Quercetin [Flavonoid]. e) Quercetin-3-β-glucoside [Flavanoid glycoside]

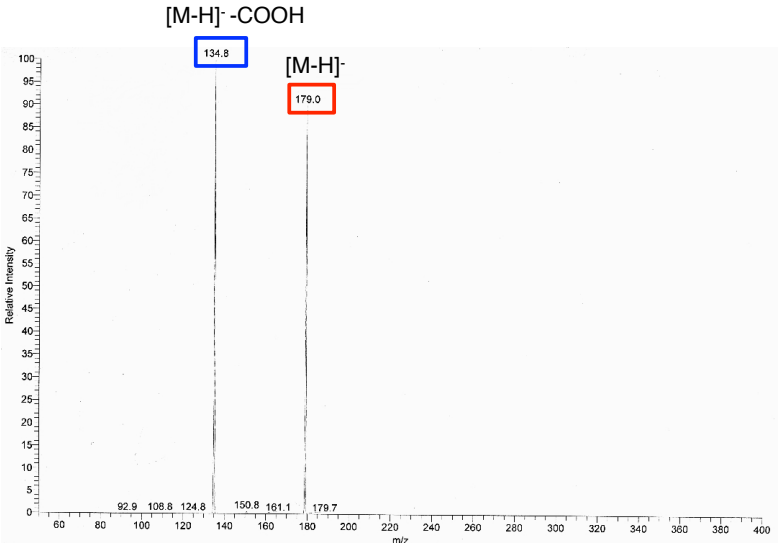
a)


Relative Intensity
273.0
301.0
168.9
178.9
269.0
463.1
546.7
574.7
736.8
764.6
926.8
927.8

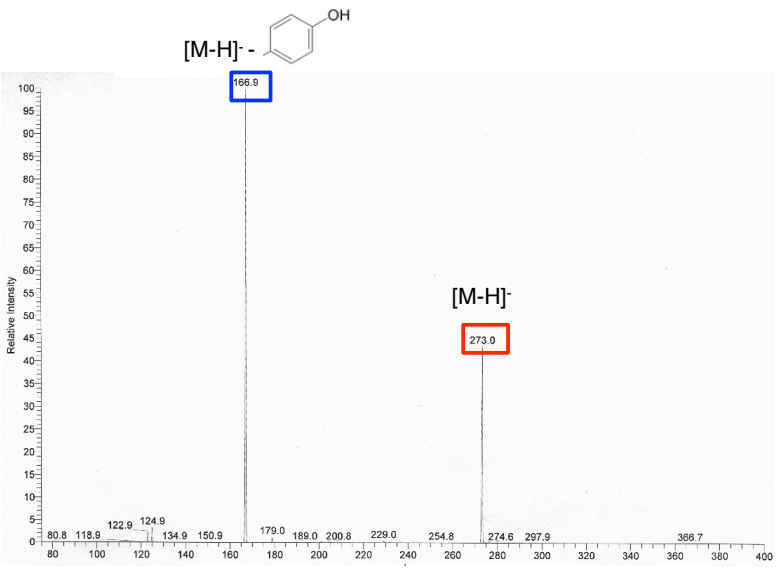
b)


[M-H]- -COOH
124.8
[M-H]-
168.8
Relative Intensity

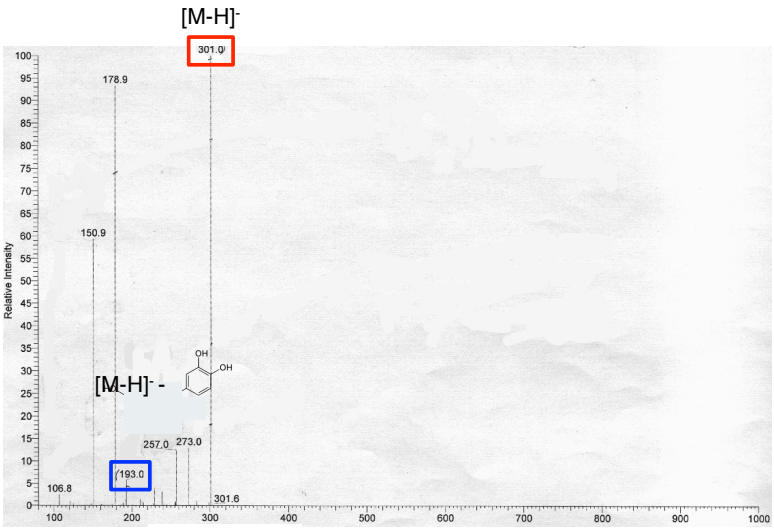
c)


[M-H]- -COOH
134.8
[M-H]-
179.0
Relative Intensity
92.9
108.8
124.8
150.8
161.1
179.7
m/z

d)


[M-H]- -
OH
166.9
[M-H]-
273.0
Relative Intensity
80.8
118.9
122.9
124.9
134.9
150.9
179.0
189.0
200.8
229.0
254.8
274.6
297.9
366.7

e)



f)

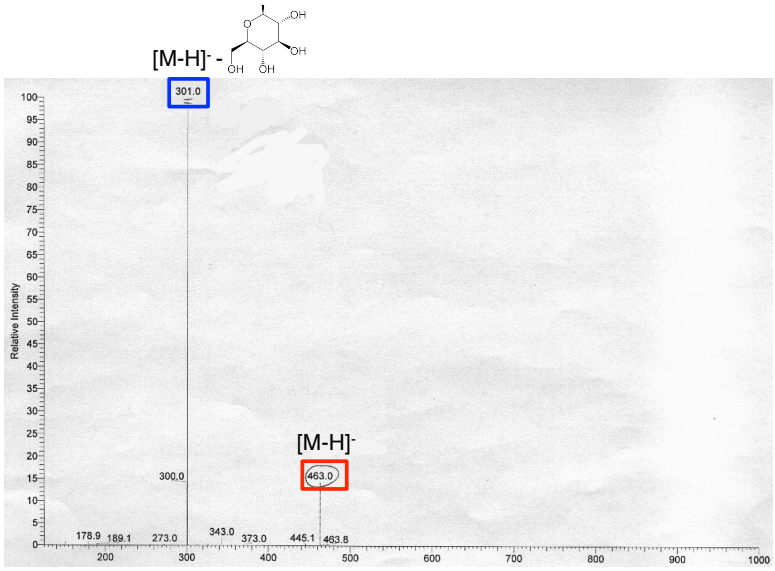


Figure 2.14.2. ***ESI-MS-MS of polyphenol standards.*** Polyphenol standards were subjected to ESI-MS/MS in negative ion mode. Parent ions $[M-H]^-$ are highlighted in red and precursor ions highlighted in blue. a) Full ESI-MS scan, b) Gallic acid, c) Caffeic acid, d) Phloretin, e) Quercetin, f) Quercetin-3-β-glucoside.

Chapter 3: Extract production and effects on breast cancer cells

3.1: Rationale

Traditionally, an aqueous extract of *Fagonia cretica*, prepared as a tea, is administered to breast cancer patients in the expectation that it can aid in reducing tumour size and improving the patients' quality of life. However, as no scientific evidence exists linking the tea to treatment effect, it is not known if *Fagonia cretica* has any chemotherapeutic potential. Many current chemotherapeutic agents have been developed based on bioactive molecules obtained by extraction and purification from natural products. Therefore, providing scientific evidence for the cytotoxicity activity of *Fagonia cretica* will demonstrate its chemotherapeutic potential as described in traditional medicine. The methods by which these compounds are obtained within a laboratory are common analytical techniques and include thin layer chromatography, liquid-liquid partitions and high-performance-liquid chromatography.

The bioactivity of plant compounds can be determined by any assay that tests the effects on biological function. For the development of novel anti-cancer compounds, a bioassay that evaluates the effects of treatment on cancer cell viability is often used. Bioactive candidates can then be implemented into the drug discovery pipeline that involves further purification and characterisation of active compounds as well as understanding the biological mechanisms that the compounds interfere with.

Many anti-cancer agents currently in clinical use are either directly isolated or are derived from the structures of bioactive molecules discovered in natural products.

Advances in synthetic chemistry and high-throughput screening of chemical libraries have become popular methods for drug development in recent years. However, there has been a resurgence in a 'back-to-basics' approach in drug discovery based on natural product chemistry, due to the successes enjoyed in the past.

This chapter aims to describe methods for extraction of dried *Fagonia cretica* plant material that can be used *in vitro* to investigate the effects on breast cancer and normal breast epithelial cells. The bioactivity of the various extracts in terms of their ability to reduce breast cancer cell viability and modulate cellular oxidant levels has also been investigated.

3.2: Introduction

Interpreting ethno-pharmacological information accurately, constitutes the primary step in determining the medicinal potential of a plant, and whether or not one should consider it for further scientific investigation. It is well documented in traditional medicine of the Eastern world that *Fagonia cretica* is used as a treatment for many ailments including fever, thirst, vomiting, dysentery and typhoid [317,318]. It is also documented that a tea or aqueous preparation of *Fagonia cretica* is a popular remedy for cancer. This substance is well tolerated and does not exhibit adverse effects like vomiting, diarrhoea or alopecia, which are common side effects of standard cytotoxic therapy. However, there is no scientific evidence to back up these claims and so it is not known if *Fagonia cretica* is at all useful therapeutically.

Isolation of natural products from plants has plays an important role in the discovery of novel chemotherapeutic agents. For example, the group of drugs called Vinca alkaloids are derived from *Catharanthus roseus,* also known

as the Madagascar Periwinkle [330]. They include drugs such as vinblastine, a microtubule inhibitor that suppresses microtubule dynamics and induces cell cycle arrest and apoptosis in tumour cells [331]. Vinca alkaloids have been used to treat a wide variety of cancer types, notably, in the combination regime ABVD (Adriamycin, Bleomycin, Vinblastine, Dacarbazine) for Hodgkin's lymphoma. Another microtubule inhibitor, paclitaxel, is widely used to treat patients with lung, ovarian and breast cancer. The original taxol compound was isolated from the Pacific yew tree in 1967 and since 1992 has been used clinically in chemotherapy regimes [332].

Secondary metabolites produced by plants provide a rich source of bioactive molecules and many extraction and purification methods have been developed for their isolation [333]. Commonly in a laboratory setting, soxhlet extraction is employed to extract plant material with different solvents. Extracts can be analysed using a simple TLC technique and applied directly to a bioassay after removal of the extraction solvent. This is a common first step in bioassay guided fractionation of plant material and many novel chemotherapeutic agents have been discovered using this method [334].

The primary goal of a successful chemotherapeutic drug is inhibition of tumour growth or reduction in tumour cell viability while minimising damage to healthy tissue that may also come into contact with the cytotoxic agent. A bioassay that measures treatment effects on cell viability is commonly employed to test the cancer-specific activity of chemotherapeutic compounds *in vitro*. The results can help with identifying novel compounds as candidates for drug discovery as it is possible to determine early on if the alleged medicinal source has any anti-cancer potential.

The cellular redox state is vital to the maintenance of signalling pathways that are altered by reactive oxygen species (ROS). Extensive work has provided evidence that excessive ROS production contributes to neoplasia, tumour growth and drug resistance [335]. Free radicals interact with DNA bases and can generate oxidative lesions that can result in tumorigenic mutations [336]. In contrast, chemotherapy-induced generation of ROS can specifically target cancer cells as they demonstrate elevated basal levels of ROS and so are more susceptible to lethal oxidative insult [337]. It is thought that higher ROS generation in cancer cells occurs as a result of increased metabolism and intrinsic generation of ROS as a by-product. As well as this, cancer cells exhibit elevated levels of intrinsic anti-oxidants and it has been proposed that reduction of these antioxidant systems pharmacologically can have a positive impact on treatment efficacy [338]. Furthermore, reductions in intracellular oxidants as a result of superoxide dismutase inhibition, has been shown to induce apoptosis in leukaemia cells [339]. On the contrary, anti-oxidant therapy is effective at reducing cancer initiation by lowering the risk of genomic mutations by free radicals. Secondary metabolites of plants contain redox modulating compounds in high abundance. They commonly occur as low molecular weight anti-oxidants and as such are able to sequester damaging free radicals and alter the cellular redox state. This can have widespread cellular consequences including potentiating cellular survival in response to oxidative stress, preventing tumorigenesis and modulating important signalling networks.

Little research has been conducted to investigate the chemotherapeutic potential of *Fagonia cretica* although its use as a cancer treatment are well documented [317, 318]. Interest lies mainly with an aqueous decoction, however, extraction with a range of solvents will allow isolation of

different compound species based on their polarity and a greater understanding of the bioactivity of the plant material. The primary aims described in this chapter are to develop a suitable extraction and preparation method to generate extracts that can be used to test bioactivity in a cell viability assay (MTT). The initial effects of extract treatment on viability and intracellular ROS in several breast cancer cell lines was also investigated.

3.3: Methods

3.3.1: Thin-layer chromatography

Dried *Fagonia cretica* (25 g) was subjected to Soxhlet extraction for 2, 5 or 24 hours as detailed in section 2.2. Extracts were dried under vacuum in a rotary evaporator and desiccated under vacuum for 24 hours. Thin layer chromatography of the extracts was carried out as detailed in section 2.3.

3.3.2: MTT assay

The effect of compounds extracted by different solvents on MCF-7, MDA-MB-231 and HMEpC cell viability was determined using the MTT assay as detailed in section 2.5. Cells were treated with up to 2 mg/ml of various extracts for up to 72 hours prior to analysis of cell viability using the MTT assay. The protective capability of NAC against extract treatment was determined by pre-treating MCF-7 cells for 60 minutes with 3 mM NAC prior to extract treatment.

3.3.3: DCF-DA assay

The level of intracellular ROS produced in MCF-7 cells was detected using the DCF assay as detailed in section 2.8.

3.4: Results

3.4.1: Extracted *Fagonia cretica* compound profiles differ depending on solvent used

Extracts of *Fagonia cretica* obtained by Soxhlet extraction were analysed by TLC. The compound profiles of all extractions, except the PET-Ether extraction, separated by TLC contained similar bands although were not 100% identical indicating some cross over in compound extraction (figure. 3.4.1). Comparing individual extractions taken at the various time points revealed a uniform extraction with no degradation of compounds over the extraction process for any of the solvent extractions. It is important to note concentration differences in compound profiles which can be attributed to loading of samples not being equal as this was not controlled.

3.4.2: All extracts reduce MCF-7 cell viability

All the solvent extracts were tested for activity against MCF-7 cells using the MTT assay. After 24 hours treatment all extracts reduced MCF-7 cell viability in a dose dependent manner, albeit with varying effectiveness (figure 3.4.2). The PET-ether 40-60 extract demonstrated to be the most effective with an IC_{25} of 0.35 mg/ml and a reduction in cell viability of 87% with a 2 mg/ml extract treatment after 24 hours (figure 3.4.2f). Importantly for this study, the aqueous extract showed dose dependent activity against MCF-7 cells with an IC_{25} of 0.43 mg/ml and a 47% reduction in cell viability with 2 mg/ml treatment at 24 hours (figure 3.4.2a). Our knowledge of *Fagonia cretica* as a therapeutic option for breast cancer patients claims that is is administered as a tea.

Therefore, subsequent experiments have been performed with an aqueous extract of *Fagonia cretica.*

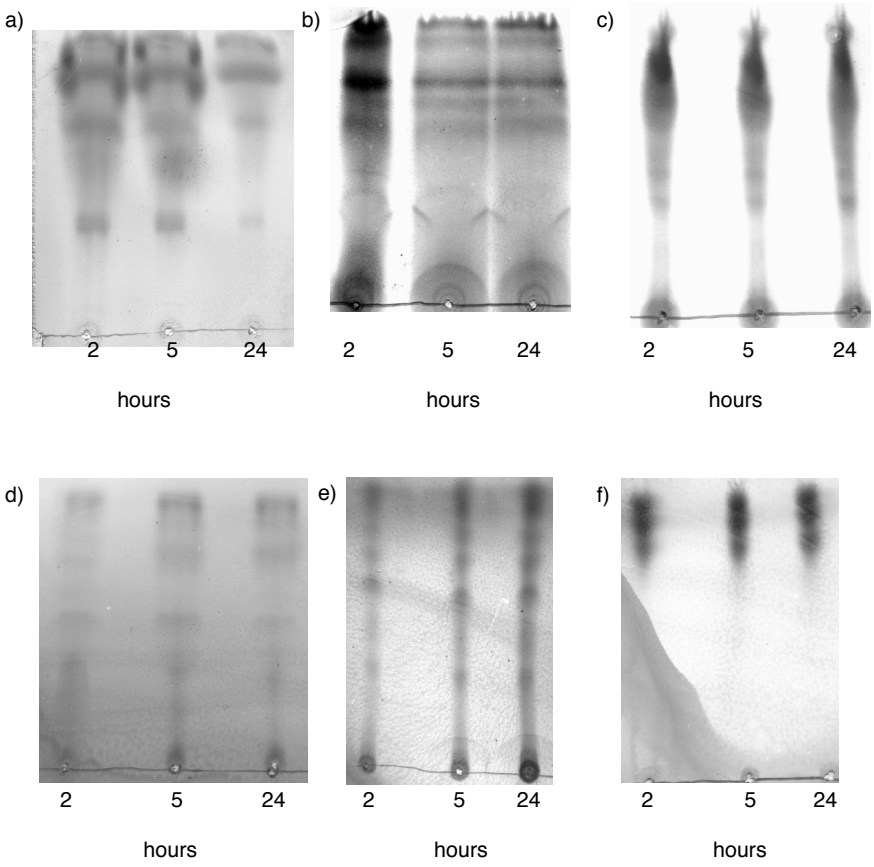


Figure 3.4.1. ***TLC analysis of Fagonia cretica solvent extractions.*** Dried *Fagonia cretica* was subjected to Soxhlet extraction in either (a) water, (b) methanol, (c) dichloromethane, (d) diethylether, (e) toluene or (f) pet-ether 40-60 for up to 24 hours. Extractions were evaporated in a rotary evaporator and desiccated under vacuum before analysis by TLC. TLC was carried out with a mobile phase of ethyl acetate:methanol (90:10) and visualised with vanillin stain. Stains are representative of three independent experiments.

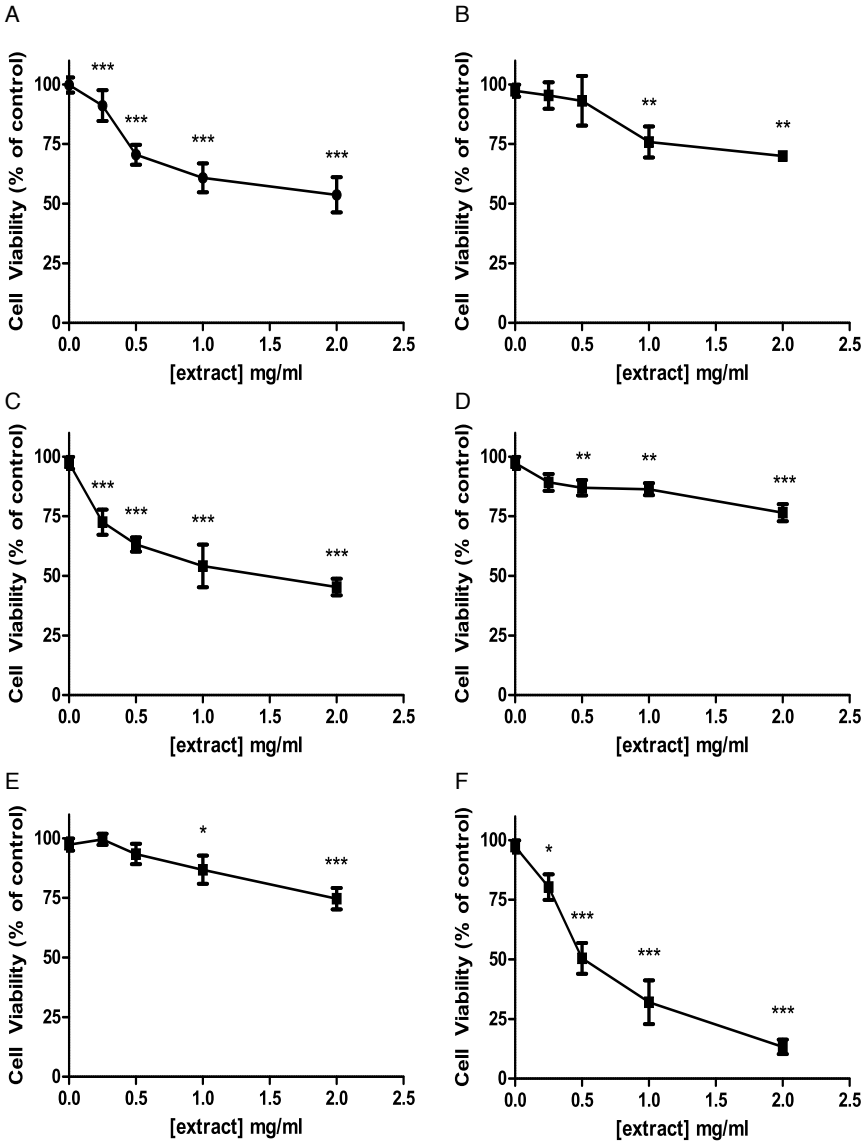


Figure 3.4.2. ***The effect of extracts of Fagonia cretica on MCF-7 cell viability.*** MCF-7 cells were treated for 24 hours with up to 2mg/ml of (a) water, (b) methanol, (c) dichloromethane, (d) diethyl ether, (e) toluene or (f) PET-ether (40-60) extracts of *Fagonia cretica* prior to analysis of cell viability using MTT assay. Data denoted * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) are significant compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test (mean ± standard deviation). All data is representative of at least three independent experiments performed in triplicate.

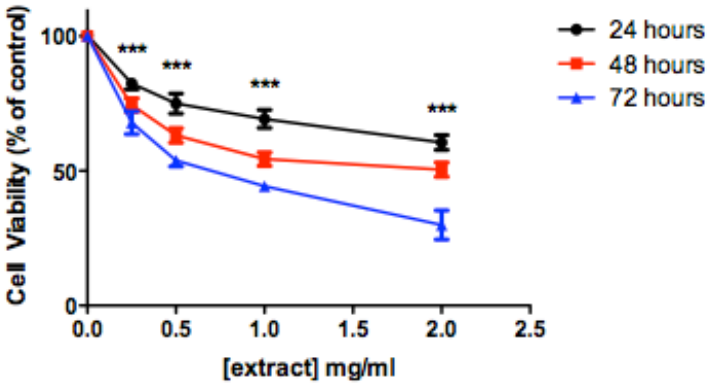
3.4.3: An aqueous extract reduces MCF-7 and MDA-MB-231 viability, but not HMEpC

An aqueous extract of *Fagonia cretica* was tested for its ability to specifically reduce breast cancer cell viability while not affecting human mammary epithelial cells. The aqueous extract was able to reduce cell viability in a time and dose dependent manner in both MCF-7 (figure 3.4.3a) and MDA-MB-231 (figure 3.4.3b). After 24 hours treatment with 2mg/ml extract, MCF-7 cell viability was significantly decreased by 39.6%, while MDA-MB-231 cell viability was decreased by 30.7%. After 72 hours of extract treatment, MCF-7 cell viability had decreased by 70.1% and MDA-MB-231 cell viability had decreased by 66.7%. Treatment was more effective against MCF-7 cells, which were sensitive to an extract IC_{25} of 0.4 mg/ml compared to an IC_{25} of 1.0 mg/ml against MDA-MB-231 cells after 24 hours. In comparison, extract treatment could only induce a loss of cell viability in HMEpC after 48 hours treatment with 2mg/ml (figure 3.4.3c). HMEpC cell viability was reduced 11.6% after 48 hours and 16.3% after 72 hours treatment with 2 mg/ml extract.

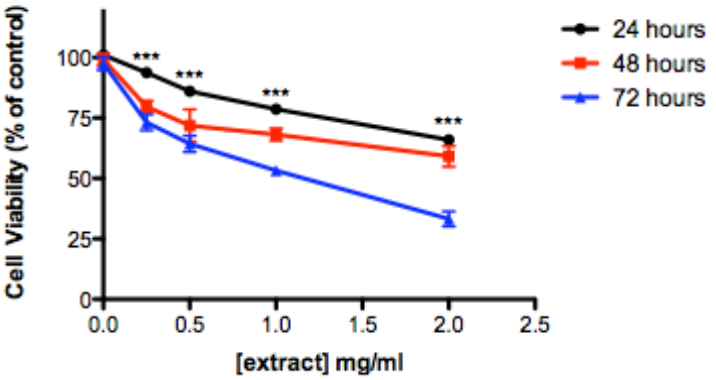
3.4.4: Aqueous extract treatment increases intracellular ROS levels in MCF-7 cells.

Plant extracts contain redox modulating molecules in high abundance. Alterations in the cellular redox state can have a wide range of cellular consequences including modulation of cell survival responses. Levels of intracellular ROS in response to extract treatment were assessed using the DCF-DA probe. Extract treatment of 1 or 2 mg/ml significantly increased the level of intracellular ROS in MCF-7 cells treated for 3 hours. All increases could be attenuated to basal level by pre-treatment with 3 mM NAC for 60 minutes before extract treatment (figure 3.4.4). Treatment with 1 mg/

a)



b)



c)

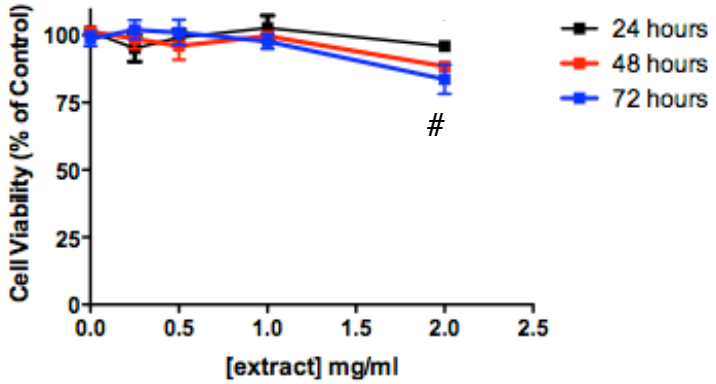


Figure 3.4.3. ***Aqueous extract reduces breast cancer cell but not human mammary epithelial cell viability.*** (a) MCF-7, (b) MDA-MB-231 and (c) HMEpC cells were treated with up to 2mg/ml aqueous extract for up to 72 hours prior to analysis of cell viability by MTT assay. Data denoted *** ($p<0.001$) is significant at all time points compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data denoted # ($p<0.001$) is significant at 72 hours compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test (mean ± standard deviation). All data is representative of at least three independent experiments performed in triplicate.

ml extract induced a 70.1% increase in intracellular ROS, while 2 mg/ml treatment induced a 76.3% increase, which was not significantly greater than the effect of 1 mg/ml extract. In order to determine the role of ROS in extract induced-cytotoxicity, MCF-7 cells were pre-treated with 3 mM NAC, prior to extract treatment and analysis of cell viability using the MTT assay. Cellular pretreatment with NAC did not significantly alter loss of cell viability in MCF-7 cells following extract treatment (figure 3.4.5).

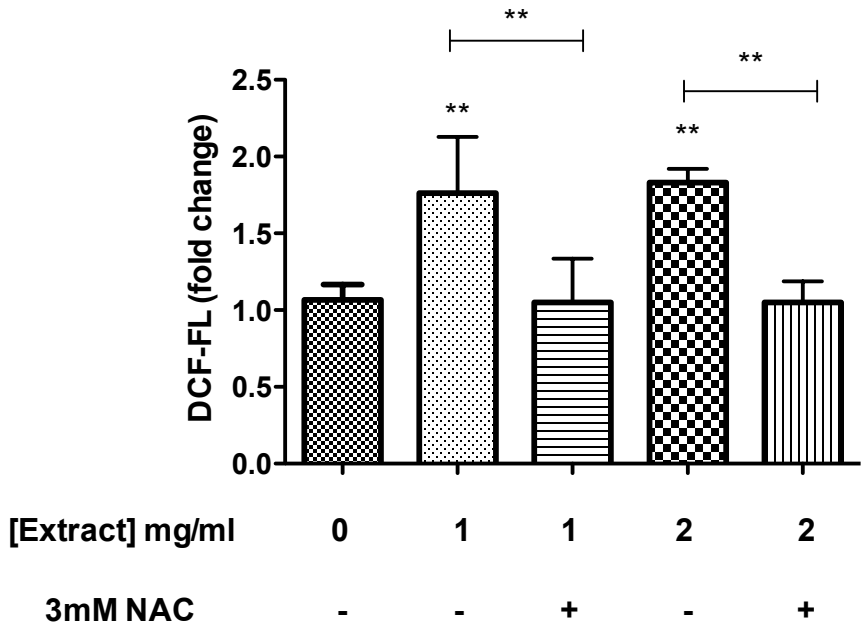


Figure 3.4.4: ***Aqueous Extract treatment increases intracellular ROS levels in MCF-7 cells.*** MCF-7 cells were treated with and without 3mM NAC for 60 minutes prior to extract treatment of indicated concentrations for 3 hours. Intracellular ROS was detected using the DCF-DA probe. Data denoted ** ($p<0.01$) is significant to untreated control or as indicate analysed by one-way ANOVA with Bonferroni's multiple comparison post test (mean ± standard deviation). Data is from three independent experiments performed in triplicate.

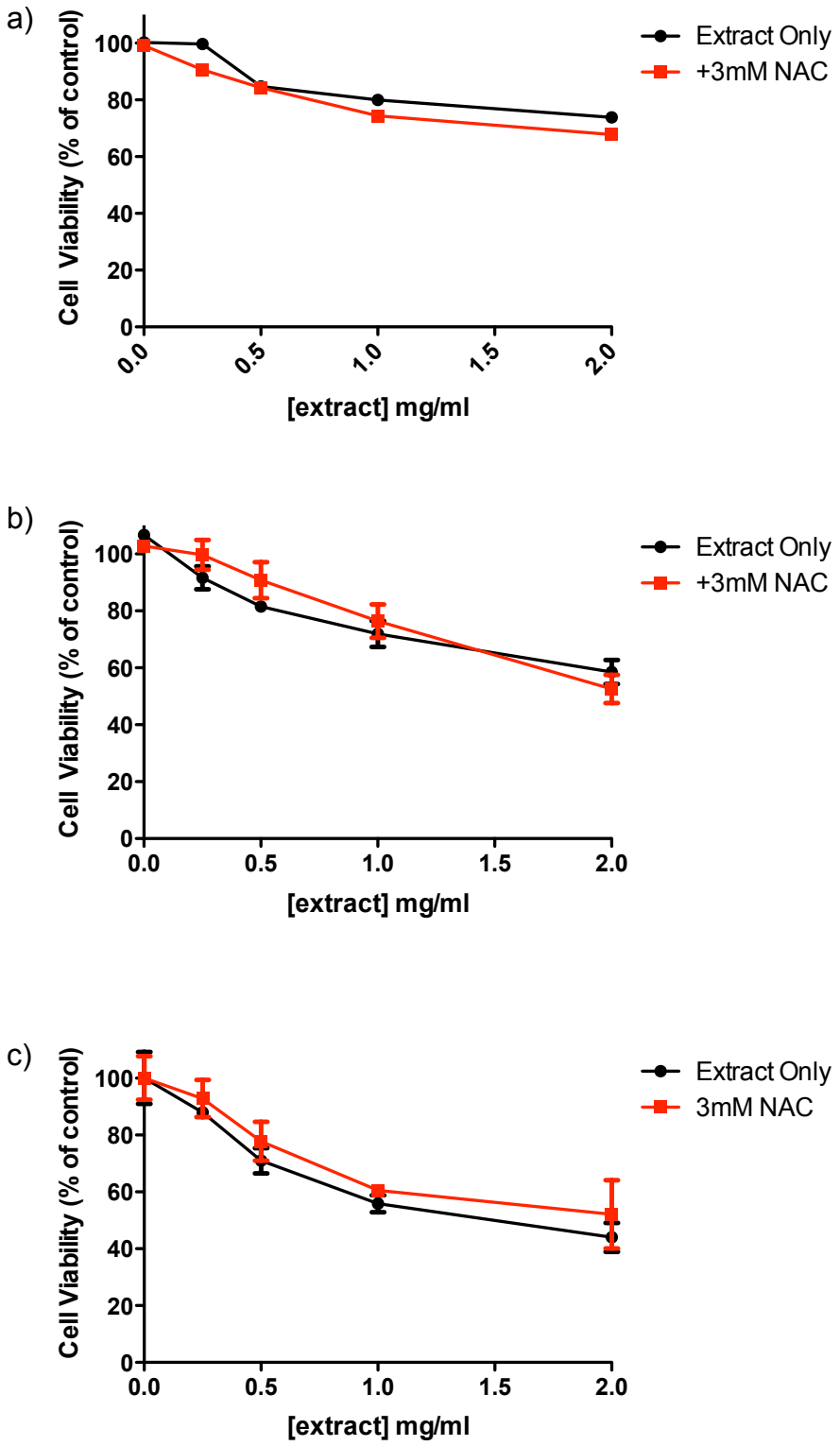


Figure 3.4.5. NAC does not effect *extract-induced loss of cell viability in MCF-7 cells.* MCF-7 cells were treated with and without 3mM NAC for 60 minutes prior to 2mg/ml extract treatment for (a) 24, (b) 48 or (c) 72 hours. Cell viability was determined by MTT assay. Data from three independent experiments performed in triplicate and representative of the mean ± standard deviation.

3.5: Discussion

Traditional eastern medicine documents that *Fagonia cretica* is administered as a herbal tea for the treatment of breast cancer [317, 318], however very little literature exists describing its anti-cancer activity in detail. The present chapter aimed to describe the development of methods for efficient extraction of compounds from *Fagonia cretica* using various solvents and test these extracts, with particular interest on the aqueous extract, for potential bioactivity towards breast cancer cells.

Results from the current study showed that extraction of *Fagonia cretica* using the Soxhlet method extracted compounds detectable by TLC and vanillin staining for all the solvents selected for extraction. Most of the TLC profiles were similar in banding patterns suggesting crossover between solvents and the compound species isolated. The PET-ether extract had a more individual TLC profile which may be reflected in its ability to reduce MCF-7 cell viability more then the other extracts. Precise differences in compound profiles between extracts could be determined by calculating the retardation factor of individual bands. However, in this case it is not necessary as this study is interested in developing suitable extraction methods to produce extracts suitable for testing the bioactivity of *Fagonia cretica* against cancer cells. Although the primary focus is to elucidate the chemotherapeutic potential of an aqueous extract, it is important to note that the other extracts could all reduce MCF-7 cell viability in a dose dependent manner after 24 hours. However, the limitations of the MTT assay mean it is not clear if the reduction is due to cell death, growth inhibition or a change in the metabolic state of the cells. *Hussain et al.* showed previously that a methanolic extract of *Fagonia cretica* was cytotoxic in a shrimp brine assay as well

as exhibiting anti-tumour activity against *Agrobacterium* in a potato disc assay [318]. This chapter shows that indeed a methanolic extract is cytotoxic (to MCF-7 cells), and for the first time, demonstrates that other solvent extracts including an aqueous extract are cytotoxic to MCF-7 cells. It is also shown here that an aqueous extract is cytotoxic to MDA-MB-231 cells but not HMEpC, highlighting the extracts potential to kill or inhibit the growth of cancer cells but not their normal counterparts. Furthermore, extract treatment increased intracellular ROS levels in MCF-7 cells, but attenuation with NAC did not alter cell survival. The anti-oxidant molecule NAC is readily taken up by MCF-7 cells in culture and is capable of reducing a chemically induced oxidative load as shown in figure 2.8.1.

The MCF-7 cell line represents a phenotypically different breast cancer cell line to MDA-MB-231 cells. Both cell lines were derived from pleural effusions of metastatic mammary carcinoma patients and are tumorigenic *in vivo* but MDA-MB-231 cells are considered to have a more aggressive phenotype [340]. This is due to changes in markers associated with epithelial to mensenchymal transition that are responsible for cell migration, invasion and mobility [341]. The MDA-MB-231 cell line also lacks expression of estrogen and Her2/neu receptors, a phenotype representative of the notorious 'triple negative' breast cancers, for which effective treatment is rare. One other important difference between the two cell lines is that MDA-MB-231 cells express a mutant non-functional form of the tumour suppressor p53 while MCF-7 p53 status is intact. This has wide reaching consequences on the cellular response to chemotherapy as p53 is a critical regulator of apoptosis and cell cycle arrest. In light of these differences, it is unsurprising that extract treatment exerts a varying degree of cytotoxic potential against the different breast cancer cell lines.

Modulation of intrinsic redox systems, in particular generation of excessive ROS or inhibition of conserved and adaptive anti-oxidant systems, can be detrimental to cellular survival. Excess ROS are a major cause of genomic damage and thus are implicated in tumorigenesis and other pathologies [342]. The oxidative DNA lesion, 8-oxo-dG, is the most mutagenic and is a prevalent mutation within tumour genetics. Reactive oxygen species are a byproduct of normal cellular metabolism and are effectively regulated by intrinsic anti-oxidant systems. However, exogenous sources of ROS such as ionising radiation and tobacco smoke, as well as deregulation of anti-oxidant systems, can create an environment of oxidative stress that results in pathology. In contrast to the tumorigenic properties of ROS, established tumours exhibit a higher basal level of ROS and are therefore, more susceptible to lethal damage by oxidative stress [338]. It is well documented that plant extracts contain a high level of redox modulating compounds. Indeed an extract of *Fagonia cretica* has previously been shown to protect against oxidative stress in rat hippocampal slices by increasing GSH levels and demonstrating free radical scavenging properties [319]. In this investigation, extract treatment of MCF-7 cells resulted in an increase in intracellular ROS after 3 hours, suggesting that excess generation of ROS may contribute to loss of cell viability. However, upon attenuation of extract-induced ROS production, no improvement on cell survival was shown. This suggests that increased ROS does not play a role in extract-induced cytotoxicity of MCF-7 cells. It is probable that the extract contains a complex mixture of compounds and that changes in ROS may be associated with treatment but not with efficacy.

3.6: Conclusion

This chapter has demonstrated that bioactive extracts of *Fagonia cretica* can be generated using the soxhlet extraction method and importantly, that an aqueous extract of *Fagonia cretica* is able to reduce cell viability of two phenotypically different breast cancer cell lines (MCF-7 and MDA-MB-231), without affecting normal breast epithelial cells (HMEpC). It has also been shown that aqueous extract treatment increase intracellular ROS levels in MCF-7 cells.

Although it is clear that extract treatment reduces cell viability in breast cancer cells, the limitations of the MTT assay mean that it is not possible to distinguish growth arrest from cell death, as the output measurement is based solely on mitochondrial activity. In order to determine if treatment induces cell death or growth inhibition, the effect of extract treatment on cell cycle and apoptosis, must first be investigated. Increasing ROS levels in cancer cells has been shown to induce cell death and here it has been shown that extract treatment does indeed result in an increase in intracellular ROS. However, it is not clear what the consequences of the increase are, as the relative increase observed in the DCF-DA assay is not necessarily large enough to exert an effect.

The results from this chapter highlight the potential for an aqueous extract to be useful in the treatment of breast cancer. However, the mechanisms governing its activity, have yet to be elucidated. The subsequent chapters focus on the characterisation of the aqueous extract because although it did not show the greatest efficacy for reducing MCF-7 cell viability our understanding of *Fagonia cretica* as a breast cancer therapeutic is based on its administration as a herbal tea.

Chapter 4: Extract induced cell cycle arrest and apoptosis

4.1: Rationale

Cancer cells exhibit heightened proliferation and consequently are susceptible to stresses that affect cell cycle progression such as DNA damage or nutrient deprivation. With respect to DNA damage, activation of the DNA damage response and subsequent DNA repair mechanisms confers cell survival. When cellular DNA damage is too great to repair, initiation of the apoptotic machinery ensures organism survival by removing potentially tumorigenic cells. This principle is exploitable for cancer therapy because an increased proliferation rate exposes DNA from the protection of histones during replication, making the cell susceptible to DNA damaging agents and subsequently to growth inhibition by cell cycle arrest and cell death by apoptosis. As well as this, many cancers exhibit mutations within the DNA repair system and consequently struggle to repair radio or chemotherapy induced DNA damage, ultimately leading to apoptotic cell death.

Cell cycle arrest and apoptosis are two major targets for cancer chemotherapy as they allow for reduced tumour growth (and disease progression) and the removal of mutant cells in a controlled, non-damaging manner. The previous chapter demonstrated that an aqueous extract could reduce breast cancer cell viability *in vitro.* The limitations of the MTT assay mean that it is not possible to determine the action that results in a reduction in MTT conversion to formazan, thus further investigations are required to elucidate the mechanism of apparent loss of viability. In this chapter, the effects of extract treatment on the cell cycle and induction of apoptosis are investigated in order to understand

how treatment reduces cell viability. The effects on important proteins associated with controlling cell cycle checkpoints and apoptosis are also investigated.

4.2: Introduction

Cancer cells exhibit many characteristics that distinguish them from healthy cells and in fact many of these unique molecular features provide potential targets for therapy [343,344]. One important difference of a cancer cell is an increased replicative capacity, which ultimately, is responsible for tumour formation. Loss of cell cycle inhibition and increased cycling rate is also a target for cancer treatment as during cell replication DNA is unwound and relatively exposed. This loss of histone protection allows for DNA damaging agents to exert an effect and cause damage to DNA bases or interfere with DNA replication [343]. For example, cisplatin, a platinum based DNA inter and/or intra-strand cross-linking agent, is widely used in cancer chemotherapy and is effective by inducing cell cycle arrest and apoptosis in cancer cells by stalling of replication forks [279]. This in turn activates DNA damage response signalling leading to cell cycle inhibition and protection from further DNA damage. As well as this, starvation or nutrient deprivation can induce a quiescent state by initiating cell cycle arrest [345].

The eukaryotic cell cycle is regulated by formation of phase-specific cyclin/CDK complexes that allow progression through cell cycle checkpoints and are discussed in detail in chapter 1.2 [145]. In response to DNA damage, activation of cell cycle regulatory proteins such as p53 and p21, control cell cycle progression by preventing activation or transcription of specific cyclins [146]. The tumour suppressor p53 can be activated by various direct post-translational

modifications such as ATM/ATR-dependent phosphorylation as a result of DNA damage or indirectly such as modification of its negative regulator MDM2. Upon activation, p53 is translocated to the nucleus where it is transcriptionally active. An important cell cycle regulatory protein, p21, is a p53 transcription target and is responsible for inhibiting cell cycle progression [170]. The CDK-inhibitor, p21, directly binds to and inhibits cyclin-CDK2/1 complexes and prevents progression through cell cycle checkpoints [171].

DNA damage-activated cell cycle arrest serves to allow DNA repair mechanisms to repair DNA lesions and protect against possible lethal genomic mutations [111]. Under certain conditions, such as excessive exposure to UV light, the level of DNA damage can exceed the repair capabilities and in order to protect the organism, the apoptotic machinery is activated [286]. The DNA repair pathways are often dysregulated in cancers, thus providing a target for cancer therapy through an increased susceptibility to DNA damage [343]. p53 also plays an important role in modulating apoptosis through binding to its transcriptional target BAX. The bax protein is a pro-apoptotic member of the Bcl-2 gene family that regulates apoptosis. It has been shown that p53 promotes apoptosis by decreasing expression of the anti-apoptotic Bcl-2 protein and increasing expression of bax [285]. It has also been demonstrated that cytoplasmic p53 can act like a BH3-only Bcl-2 protein and activate bax independent of transcription [294]. Under pro-apoptotic conditions this has been shown to promote apoptosis by inducing mitochondrial outer membrane permeabilisation [263].

It was shown previously in chapter 3 that treatment with an aqueous extract of *Fagonia cretica* can significantly reduce MTT reduction as a measure of viability in MCF-7 cells.

However, it is not yet known how this occurs. In this chapter the effects of extract treatment on cell cycle and apoptosis in MCF-7 cells are described. The effects of extract treatment on p53 expression and its transcriptional targets, p21 and bax, will also be investigated as they are influential in the activation of cell cycle arrest and apoptosis in response to cellular stress.

4.3: Methods

4.3.1: Cell cycle analysis of aqueous extract treated cells

The effect of aqueous extract treatment on MCF-7 cells was determined using flow cytometry as detailed in section 2.6.

4.3.2: Detection of apoptosis in aqueous extract treated cells

Apoptosis in treated MCF-7 cells was detected by flow cytometry as detailed in section 2.7.

4.3.3: Quantification of p53, p21 and Bax expression in aqueous extract treated cells

The effects of aqueous extract treatment on p53, p21 and Bax protein expression in MCF-7 cells was determined by SDS-PAGE and western blot as outlined in section 2.10. The protein (20 µg per lysate) was separated on a 16% SDS-PAGE gel before western blot procedure. Membranes were probed with rabbit anti-human p53 (1:1000), mouse anti-human p21 (1:100) or rabbit anti-human bax (1:1000) before addition of sheep anti-rabbit or anti-mouse HRP conjugated antibody (1:10000). Bands were visualised using ECL reagent exposed to photographic film for 5 minutes.

4.4: Results

4.4.1: Aqueous extract treatment indues G0/G1 growth arrest

The effect of aqueous extract treatment on MCF-7 cell cycle was determined in order to ascertain the mechanism by which extract treatment reduces MCF-7 cell viability. MCF-7 cells were treated with 2 mg/ml extract for up to 24 hours before cell cycle analysis was carried out by flow cytometry. Extract treatment induced significant growth arrest in G0/G1 phase of the cell cycle after 5 hours treatment (figure 4.4.1a); after 5 hours, extract treatment resulted in an increase from 44.6% to 62.5% of cells in G0/G1 population which increased further to 73.3% after 24 hours treatment. In parallel with an increasing G0/G1 population, extract treatment resulted in a significant decrease from 16.3% to 10.2% of cells in G2 after 7 hours which decreased further to 5.4% after 24 hours (figure 4.4.1b).

4.4.2: Aqueous extract treatment increases expression of p53 and p21

Cell cycle arrest in G0/G1 phase of the cell cycle is regulated by activation of the p53 transcription target p21. The CDK-inhibitor, p21, inhibits cell cycle progression via inhibition of CDK activity, resulting in hypo-phosphorylation of pRB, inactivation of E2F and blockade of progression through G1. Thus, the protein expression levels of p53 and p21 in response to aqueous extract treatment were determined by western blot. MCF-7 cells were treated with 2 mg/ml extract for up to 24 hours before lysate collection and protein expression analysis. Figure 4.4.2a shows that MCF-7 cells increase expression levels of p53 and p21 in a time dependent manner in response to extract treatment.

Densitometric analysis of protein expression revealed a 48.4% increase in p53 after 5 hours treatment and a 129.2% increase after 24 hours (figure 4.4.2b). The increase in p53 expression was paralleled by a 49.1% increase in p21 expression after 5 hours and an 82% increase after 24 hours (figure 4.4.2c).

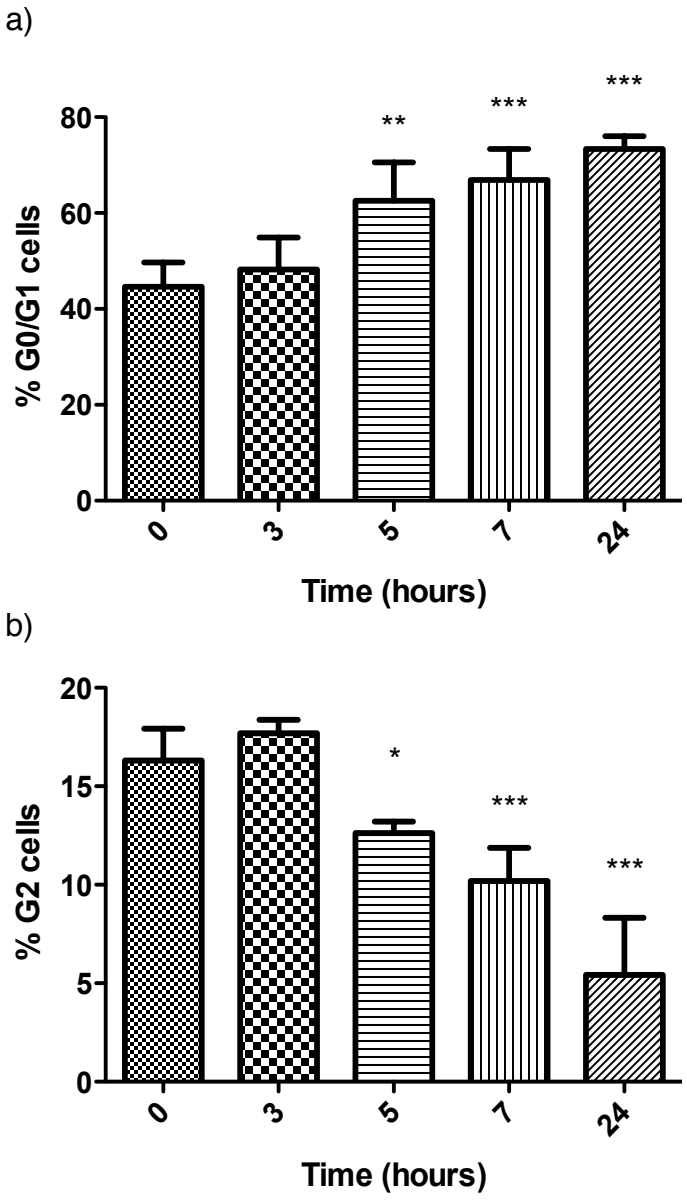


Figure 4.4.1: ***Extract treatment induces G0/G1 arrest in MCF-7 cells.*** MCF-7 cells were treated with 2mg/ml extract for up to 24 hours before cell cycle analysis by flow cytometry. Cell populations (a) G0/G1 and (b) G2 were determined based on fluorescence profiles. Data denoted * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control (*time=0 hours*) as analysed by one-way ANOVA with Dunnett's multiple comparison post test (mean ± standard deviation). Data is from three independent experiments performed in triplicate.

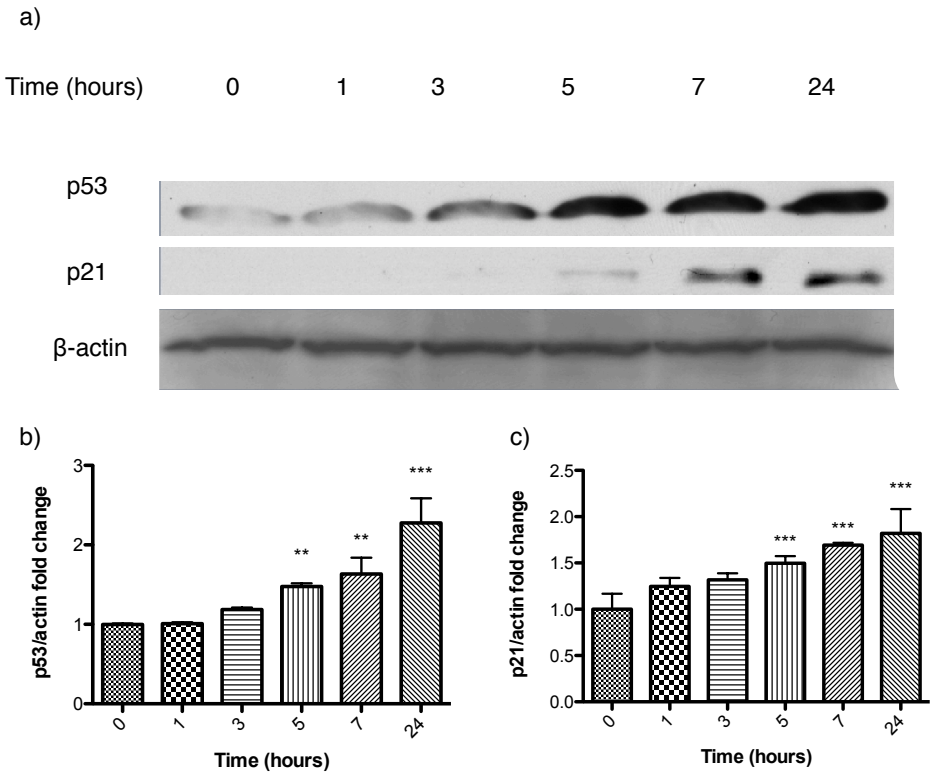


Figure 4.4.2: ***Extract treatment increases p53 and p21 expression in MCF-7 cells.*** MCF-7 cells were treated with 2mg/ml extract for up to 24 hours before expression of (a) p53 and p21 was determined by SDS-PAGE and western blot. β-actin was used as a loading control. Densitometry for (b) p53 and (c) p21 was calculated as a ratio to β-actin and expressed as fold change compared to control (*time=0 hours*). Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control analysed by one-way ANOVA with Dunnett's multiple comparison post test (mean ± standard deviation). Data is representative of three independent experiments.

4.4.3: Aqueous extract treatment induces apoptosis

Activation of apoptosis pathways in response to chemotherapy is an important outcome for effective treatment. The tumour suppressor, p53, plays an important role in apoptotic cell death via transcription of pro-apoptotic genes such as *BAX*. Apoptosis in treated MCF-7 cells was detected using flow cytometry after staining for the presence of phosphatidylserine on the cell surface using using the annexin V binding assay. Extract treatment induced significant levels of apoptosis in MCF-7 cells after 24 hours treatment (figure 4.4.3b), which increased further after 48 and 72 hours (figure 4.4.3c and d). After 24 hours treatment, the level of detected apoptosis had increased from 3.9% to 28.5% and further increased to 73.4% after 72 hours (figure 4.4.4b). In parallel, the level of live cells decreased from 93.2% to 60.2% after 24 hours and further decreased to 18.5% after 72 hours (figure 4.4.4a). The pro-apoptotic Bcl-2 family gene, *BAX*, is a transcriptional target for p53 and is up-regulated upon p53-mediated apoptotic signalling. The effects of extract treatment on bax protein expression were also investigated to determine whether or not induction of apoptosis was related to the increase in p53 expression seen in 4.4.2. Extract treatment of 2 mg/ml significantly increased Bax protein levels after 48 hours and sustained treatment (up to 72 hours) resulted in a further increase in bax protein expression (figure 4.4.5a). Bax expression was increased 19-fold in response to extract treatment after 48 hours and 22-fold after 72 hours (figure 4.4.5b).

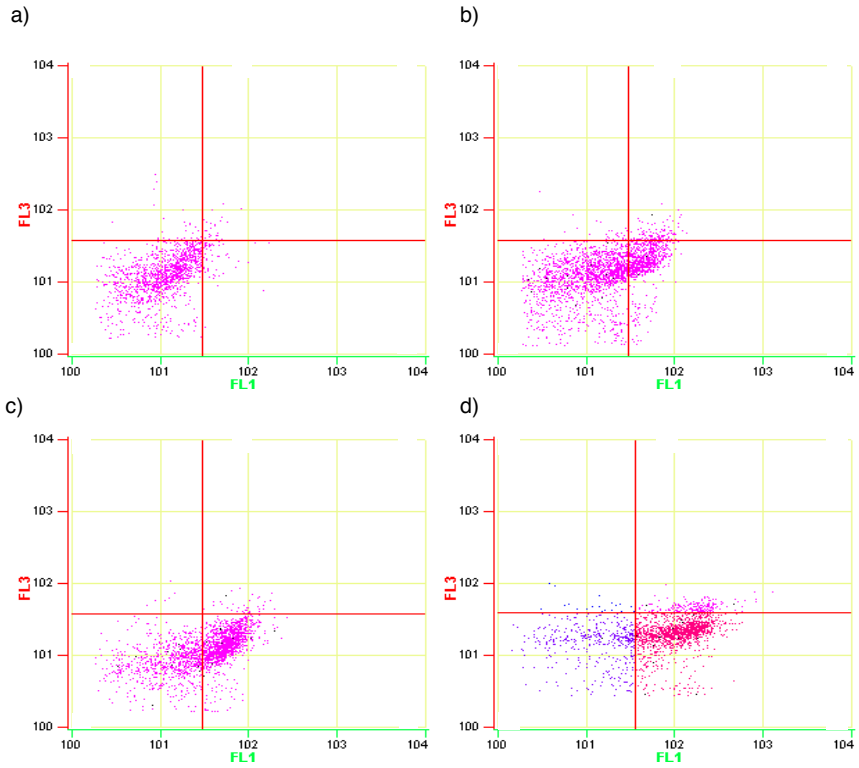


Figure 4.4.3. ***Extract treatment induces apoptosis in MCF-7 cells.*** MCF-7 cells were treated with 2mg/ml extract for either (a) 0, (b) 24, (c) 48 or (d) 72 hours prior to detection of apoptosis by flow cytometry. Histograms were generated according to log annexin V-FITC (FL1) and log propidium iodide (FL3) fluorescence. Data is representative of three independent experiments performed in triplicate.

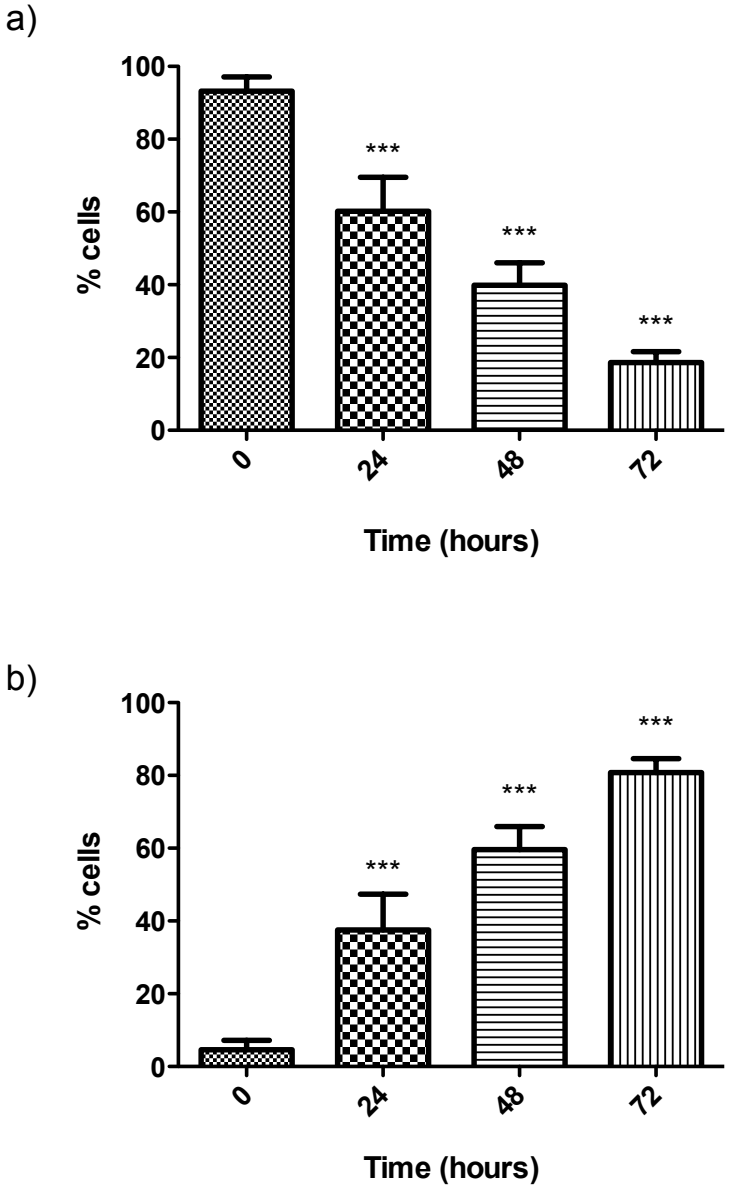


Figure 4.4.4. ***Extract treatment induces apoptosis in MCF-7 cells.*** MCF-7 cells treated with 2mg/ml extract for up to 72 hours were subjected to flow cytometric analysis for the detection of apoptosis. Cell populations of (a) live cells [% annexin V -ve/PI -ve] and (b) apoptotic cells [% annexin V +ve/PI -ve] were isolated based on fluorescent profiles. Data denoted *** ($p<0.001$) is significant compared to control (*time=0 hours*) analysed by one-way ANOVA with Dunnett's multiple comparison post test . Data is from three independent experiments performed in triplicate and presented as mean ± standard deviation.

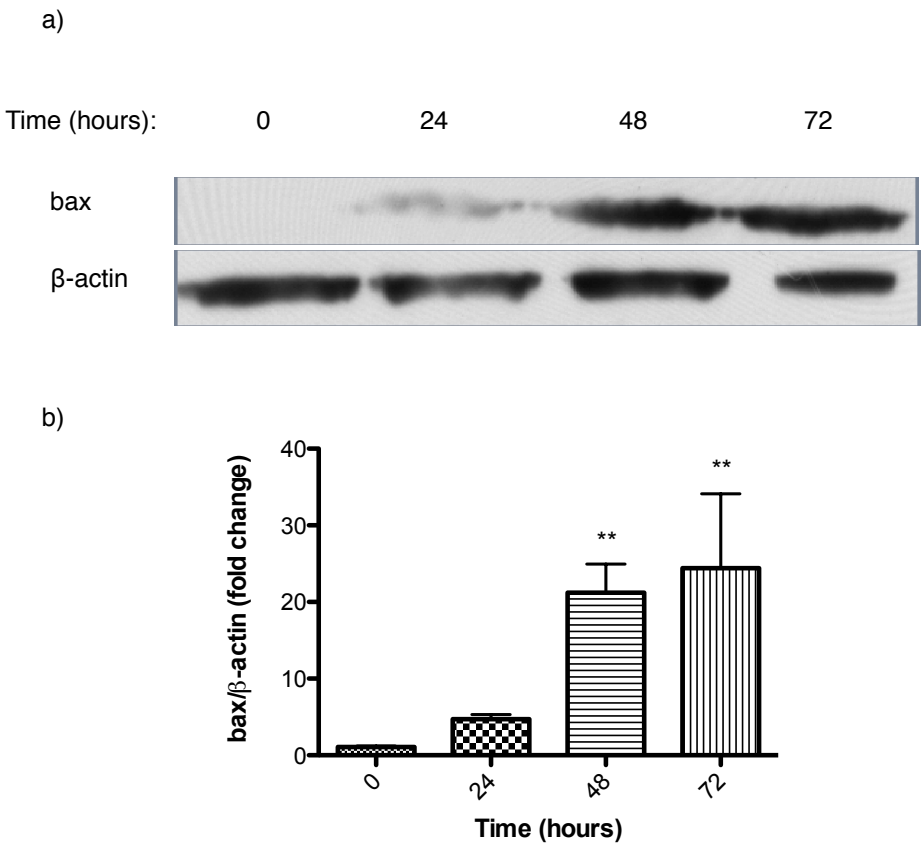


Figure 4.4.5. ***Extract treatment induces bax expression in MCF-7 cells.*** MCF-7 cells were treated with 2mg/ml extract for up to 72 hours before expression of (a) bax was determined by SDS-PAGE and western blot. β-actin was used as a loading control. Densitometry for (b) bax was calculated as a ratio to β-actin and expressed as fold change compared to control (*time=0 hours*). Data denoted ** ($p<0.01$) is significant compared to control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and is presented as mean ± standard deviation.

4.5 Discussion

Cell cycle checkpoints are crucial in regulating cell cycle progression and provide a defence against lethal genomic mutations. These restriction points provide decision points at different phases of the cell cycle that allow the cell to cease replication whilst the environment is optimised for successful cell division. For example, following genomic insult, DNA repair is initiated and cell cycle halted to prevent replication of possibly tumorigenic lesions [343]. Cancer cells are particularly sensitive to cell cycle modulation due to their hyper-proliferative phenotype and DNA damaging agents have been shown to induce growth arrest in a variety of breast cancer cell types [46]. Another important characteristic and drug target of many cancers is dysregulation of the DNA repair systems [343]. Thus, DNA damaging agents which activate cell cycle arrest can ultimately cause apoptotic cell death if the damage is irreparable.

This chapter has demonstrated that cell cycle arrest and apoptosis can be induced upon extract treatment of MCF-7 cells after both 5 and 24 hours. The results also show that cell cycle arrest and apoptosis may be associated with stabilisation of p53 and an increase in its transcriptional activity leading to activation of p21. Stabilisation of p53 can occur in response to stress-induced dissociation of p53 from its negative regulator MDM2. This has been shown to be regulated by kinase activity of ATM and ATR which, in response to DNA damage, can activate p53 directly through site-specific phosphorylation or indirectly by phosphorylation of MDM2 and destabilisation of the MDM2-p53 complex. Extract treatment induced time dependent growth arrest in G0/G1 followed by apoptosis after 24 hours, suggesting that after 24 hours treatment, a threshold is reached that

activates apoptosis. This effect has been shown with other plant extracts, for example the work of *Hu et al.* showed that an extract of *Ganoderma lucidum* induces G1 arrest at 12 hours followed by significant apoptosis at 36 hours in MCF-7 cells [346]. A delayed on-set of apoptosis suggests the possibility of the extract containing DNA damaging agents as,longer extract incubation times may increase the level of DNA damage and eventually lead to apoptosis when damage is beyond repair. Alternatively, prolonged activation of DNA repair mechanisms may invoke metabolic catastrophe, which could induce autophagy and apoptosis or autophagic cell death [312].

A central cell cycle regulator is p21, a CDK inhibitor that blocks cyclin kinase activity by inhibiting the formation of cyclin-CDK complexes. The protein, p21, is transcriptionally regulated by p53 which is rapidly stabilised under stress conditions by dissociation from its negative regulator MDM2 [101]. The results described in this chapter show that in response to aqueous extract treatment, expression levels of p53 and p21 are both increased in a time dependent manner. The expression levels of p53 were significantly increased after 5 hours treatment which coincides with the significant level of G1 arrest seen also after 5 hours. The level of p53 and G1 arrest also both increased towards 24 hours suggesting that the latter may be effected by the former. This effect was also seen in the expression levels of p21 in response to extract treatment, suggesting that in response to increased p53 levels, transcriptional activity of p53 may be driving an increase in p21 expression resulting in cell cycle arrest. The p53-p21 axis in cell cycle arrest is well documented and agrees with the current findings [347]. It was also evident, that in response to extract treatment, bax protein levels increased. Bax expression correlated with induction of apoptosis detected by flow cytometry and provides good evidence that extract treatment creates a pro-

apoptotic environment that may be regulated by induction of bax. As bax, like p21, is a transcriptional target of p53, the results provide further evidence that extract-induced p53 expression drives cell cycle arrest and apoptosis in MCF-7 cells.

These results demonstrate that loss of MCF-7 cell viability (chapter 3) is caused by extract induced G1 arrest and apoptosis, and that this effect may be a result of p53 transcriptional activation and/or protein stabilisation. It is well known that cell cycle arrest and apoptosis are major consequences of DNA damage, and the susceptibility of genomic material in cancer cells highlights the possibility that the extract may contain DNA damaging agent(s).

4.6: Conclusion

This chapter describes the potential mechanisms by which an aqueous extract of *Fagonia cretica* reduces MCF-7 cell viability. Flow cytometric and western blot methods were used to assess the effects of aqueous extract treatment on cell cycle and apoptosis with particular emphasis on p53 mediated effects. Cell viability in MCF-7 cells appears to be reduced as a result of cell cycle arrest and apoptosis. This could be controlled by transcriptional activation of p53 because levels of p53 transcriptional targets p21 and bax were increased alongside increases in p53 expression. This may be a result of extract induced stress, potentially, DNA damage. Increases in expression of p53 transcriptional targets p21 and bax, which are involved in cell cycle arrest and apoptosis respectively, demonstrate a potential role for p53 in extract induced cytotoxicity towards MCF-7 cells.

Further work to characterise the initiation steps leading to p53 stabilisation would provide a clearer insight into the

mechanism by which the aqueous extract exerts activity towards breast cancer cells.

Chapter 5: Aqueous extract-induced DNA damage and activation of the DNA damage response

5.1: Rationale

Cell cycle arrest and apoptosis can be induced by a variety of stimuli such as DNA damage and nutrient deprivation [146]. DNA damage results in activation of the DNA damage response which halts cell cycle progression in order to repair damaged DNA. In response to irreparable DNA damage the cell initiates apoptosis in order to remove itself from the population [51]. As cancer cells are hyper-proliferative they are highly susceptible to DNA damaging agents and thus can be targeted efficiently with treatments that modulate the DNA damage response or induce genomic lesions. Plant extracts contain a large variety of phenolic compounds that commonly can protect against oxidative DNA damage through their radical scavenging properties [348]. However, there is also evidence of natural products inducing DNA damage in tumour models. For example Doxorubicin was derived from daunorubicin, an antibiotic from Streptomyces peucetius that was discovered to have DNA damage-inducing activity towards murine tumours [349].

The investigations described in chapters 3 and 4 have shown that treatment of MCF-7 cells with an aqueous extract of *Fagonia cretica* leads to cell cycle arrest and cell death probably via p53-mediated mechanisms. In this chapter, the effect of extract treatment on activation of the DNA damage response is described. The involvement of p53 in the DNA damage response was also investigated to further understand how extract treatment initiates cell cycle arrest and apoptosis in MCF-7 cells.

5.2: Introduction

The DNA damage response (DDR) is a complex signal transduction network comprised of sensors, transducers and effectors. In response to DNA damage, DNA damage sensors recruit mediators to the lesion which in turn signal transducers and effectors leading to a wide range of cellular responses such as cell cycle inhibition, autophagy, chromatin remodelling, DNA repair and apoptosis [50]. This network is critical to maintaining genomic integrity and prevent tumorigenesis, but is also utilised as a chemotherapeutic target for DNA damaging agents.

DNA damage can occur through a myriad of sources including UV-light, oxidative stress and chemo/radiotherapy. DNA damage lesions include, methylation or oxidation of bases and single or double strand breaks, which all corrupt the structural integrity of DNA [39]. The most lethal lesion to occur is a double strand break and results in recruitment of sensor proteins that are charged with eliciting a cellular response [60]. Detection of genomic lesions activates members of the phosphoinositide kinase (PIK)-related protein family, including ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK) which relay the signal to transcription factors, such as p53, to elicit a cellular response [68]. Indeed, it has been shown that ATM activation is crucial for DNA damage-induced cell cycle arrest via its kinase activity on key transcription factors such as p53 [65]. In response to infra-red radiation, activation of ATM as measured by site specific phosphorylation at serine-1981 is maximal within minutes. This highlights the importance of ATM within the DDR [114].

In response to DNA damage, p53 becomes rapidly stabilised, in-part through site specific phosphorylation of serine-15 by the ATM kinase [82]. Post-translational modifications of p53 such as phosphorylation and acetylation play a role in transactivation of p53 and serine phosphorylation within the C-terminus can activate the DNA binding function of p53 [118]. Previous studies have demonstrated that DNA damaging agents can induce cell cycle arrest and apoptosis in cancer cells via activation of the DNA damage response, specifically through ATM/p53-mediated pathways [88,350]. Pharmacological inhibitors of ATM, such as caffeine, have been shown to sensitise tumour cells to genotoxic stress by reducing serine-15 phosphorylation of p53 and preventing DDR signalling [351]. However, caffeine-mediated suppression of ATM has also been reported to attenuate DNA damage induced cell cycle arrest and apoptosis [352].

It has been shown that loss of wild-type p53 accelerates tumour development in mice and that p53-dependent apoptosis is critical to regulating tumorigenesis [353]. It has been established that over 50% of tumours produce mutant, non-functional p53 and that mutations within the TP53 gene are the most frequent oncogenic mutation in human cancers [98]. The high frequency of TP53 mutations in tumours highlights the need for chemotherapeutic treatments that work independently of p53-mediated effects. In fact many p53-independent mechanisms that lead to cancer cell death have been described [290,292,294]. The anti-diabetic drug metformin has been shown to reduce tumour growth of $p53^{-/-}$ xenografts via alterations in cellular metabolic state [354]. Studies carried out by *Li et al.* have also shown that DNA damage-induced XPA translocation activates cell cycle checkpoints independent of cellular p53-status and is not regulated by ATM/ATR pathways [355].

The research described in previous chapters has shown that treatment with an aqueous extract of *Fagonia cretica* can significantly reduce MCF-7 cell viability by initiating cell cycle arrest and apoptosis with increased expression of p53, p21 and bax. The current study aims to identify a possible mechanism for extract induced-cytotoxicity in MCF-7 cells and to investigate upstream targets involved in initiating cell cycle arrest and apoptosis.

5.3: Methods

5.3.1: DNA damage detection by comet assay

The ability of aqueous extract treatment to induce strand breaks in MCF-7 cells was assessed using the comet assay as detailed in section 2.9.

5.3.2: Protein expression analysis by western blot

The effects of aqueous extract treatment on γ-H2AX, p53 and p21 protein expression in MCF-7 cells were determined by SDS-PAGE and western blot as outlined in section 2.10. A total of 20µg protein per lysate was separated on a 16% SDS-PAGE gel before western blot procedure. Membranes were probed with rabbit anti-human γ-H2AX (1:1000), rabbit anti-human p53 (1:1000) or mouse anti-human p21 (1:100) before addition of sheep anti-rabbit or anti-mouse HRP conjugated antibody (1:10000). Bands were visualised using ECL reagent exposed to photographic film for 5-20 minutes.

5.3.3: MTT - cell viability assay

The effect of aqueous extract treatment on MCF-7, MDA-MB-231 cell viability was determined using the MTT assay as detailed in section 2.5. Cells were pre-treated with 3 mM caffeine or 3 mM NAC for 60 minutes prior to aqueous extract treatment of up to 2 mg/ml for 24 hours prior to analysis of cell viability using the MTT assay.

5.3.4: siRNA interference

TP53 siRNA interference was used to knockdown p53 expression in MCF-7 cells as detailed in section 2.11.

5.3.5: Cell cycle analysis of aqueous extract treated cells

The effect of aqueous extract treatment on the cell cycle of MDA-MB-231 cells was determined using flow cytometry as detailed in section 2.6.

5.4: Results

5.4.1: Aqueous extract treatment induces non-oxidative DNA damage in MCF-7 cells

DNA damage initiates cell cycle arrest in order to permit repair of damaged DNA before replication. Many cancers demonstrate dysfunctional DNA repair activity, thus rendering them susceptible to sustained DNA damage resulting in cell death. The previous studies have shown that extract treatment induces cell cycle arrest and apoptosis in MCF-7 cells associated with activation of p53 and an associated increase in its transcriptional targets p21 and bax. DNA damage sensing via ATM/ATR is one mechanism by which p53 can become transcriptionally active. Therefore, the effects of aqueous extract treatment on the level of DNA damage in MCF-7 cells, was investigated. MCF-7 cells were treated with 1 or 2 mg/ml extract for either 3 or 24 hours, prior to analysis using the comet assay. DNA damage was measured as the mean percentage of tail DNA taken from at least 50 comets per test. The results in figure 5.4.1 show that extract treatment could induce significant dose dependent DNA damage in MCF-7 cells after 3 hours treatment (figure 5.4.1a) which was increased after 24 hours (figure 5.4.1b). After 3 hours treatment, the percentage of tail DNA increased by 73.2% in response to 1 mg/ml extract treatment and 157.3% in response to 2 mg/ml extract treatment, compared to untreated control (figure 5.4.1a). This increase was elevated further after 24 hours with a 226.6% and 304.3% increase in DNA damage compared to untreated control, after treatment with 1 mg/ml and 2 mg/ml extract respectively (figure 5.4.1b).

Oxidation by free radicals is one of the main sources of DNA damage. Oxidation of DNA commonly results in the

formation of 8-oxoguanine which can be measured as a marker of oxidative DNA damage [39]. Formamidopyrimidine DNA glycosylase (FPG) protein, a key enzyme in the base excision repair (BER) pathway, is involved in the recognition and removal of 8-oxoguanine. Therefore, it can be used in a modified comet assay to specifically detect oxidative DNA damage. Aqueous extract treatment may induce oxidative DNA damage in MCF-7 cells based on the data shown previously in chapter 3 that extract treatment increases levels of intracellular ROS. To investigate this hypothesis, MCF-7 cells were treated as before, and prepared for analysis of DNA damage by comet assay with the addition of FPG incubation prior to alkaline unwinding of DNA and electrophoresis. The results show that there is no significant increase in the percentage of tail DNA in the presence of FPG protein for any of the extract treatments at any time point compared to non-FPG treated cells (figure 5.4.1a and b). To verify the role of oxidative stress in extract-induced cytotoxicity, the effects of extract-induced cytotoxicity on MCF-7 cells was determined after pre-treatment with NAC. In chapter 3 pre-treatment of 3 mM NAC was shown to attenuate extract-induced ROS. However, this modulation had no effect on extract-induced cytotoxicity, suggesting that increases in ROS by extract treatment may not play a role in cytotoxicity (figure 5.4.2).

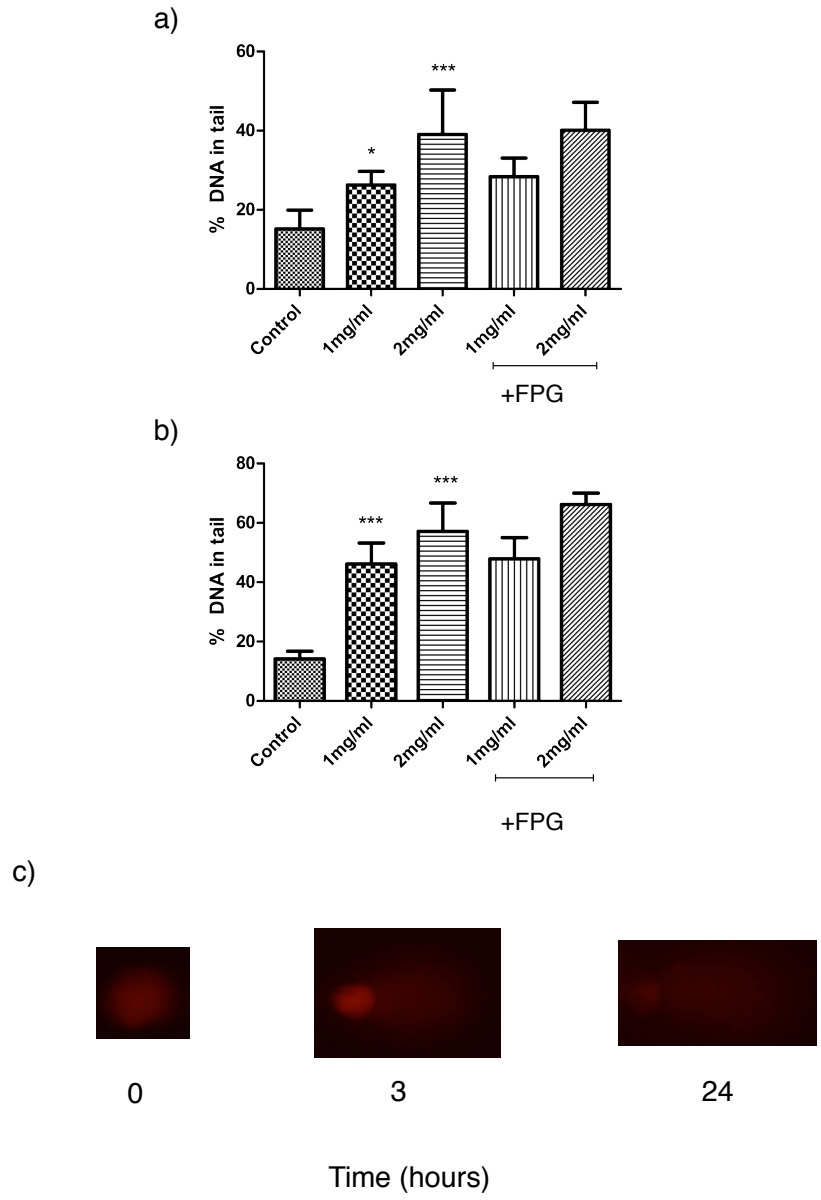


Figure 5.4.1: ***Extract treatment induces non-oxidative DNA damage in MCF-7 cells.*** MCF-7 cells were treated with up to 2mg/ml extract for up to 24 hours prior to detection of DNA damage using the comet assay with and without FPG protein. Comets were visualised after staining with propidium iodide (c), and DNA damage was determined as an average percentage of DNA in comet tails after (a) 3 or (b) 24 hours treatment. Data denoted * ($p<0.05$) and *** ($p<0.001$) are significant compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data are from three independent experiments performed in triplicate and presented as mean ± standard deviation.

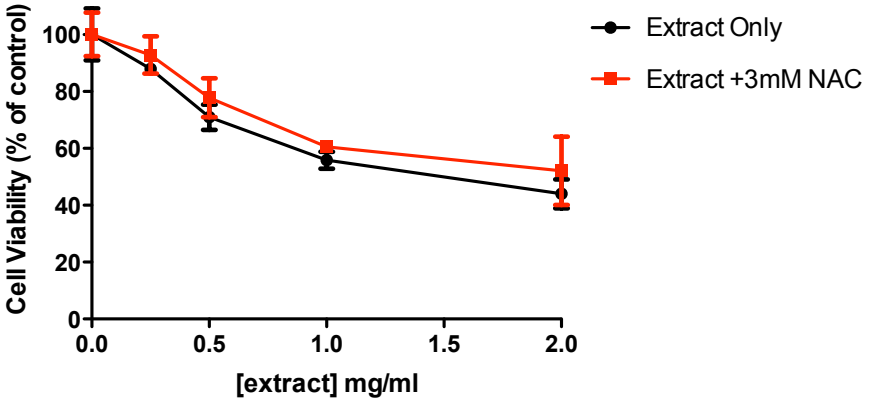


Figure 5.4.2. ***Pre-treatment with NAC does not attenuate extract-induced loss of MCF-7 cell viability.*** MCF-7 cells were pre-treated with 3mM N-acetyl-cysteine (NAC) for 60 minutes before treatment with up to 2mg/ml extract for 24 hours. Cell viability was determined by MTT assay. Data is from at least three independent experiments performed in triplicate and presented as mean ± standard deviation.

The presence of DNA damage in MCF-7 cells was also confirmed by the presence of the double strand break marker γ-H2AX. Figure 5.4.3 shows that in expression of γ-H2AX is increased in a time dependent manner after treatment with 2 mg/ml extract. Expression of γ-H2AX was significant after 3 hours treatment (figure 5.4.3a). In response to aqueous extract treatment, γ-H2AX expression was increased 26.9% after 3 hours, with sustained treatment increasing γ-H2AX expression by 75.0% after 24 hours (figure 5.4.3b).

5.4.2: Extract induced p53 expression is ATM/ATR dependent

The DNA damage response is controlled by activation of sensors such as ATM and ATR. In response to single (ATR) or double (ATM) strand breaks, these proteins exerts kinase activity towards p53 resulting in phosphorylation, rapid stabilisation and transcriptional activation of p53. ATM/ATR

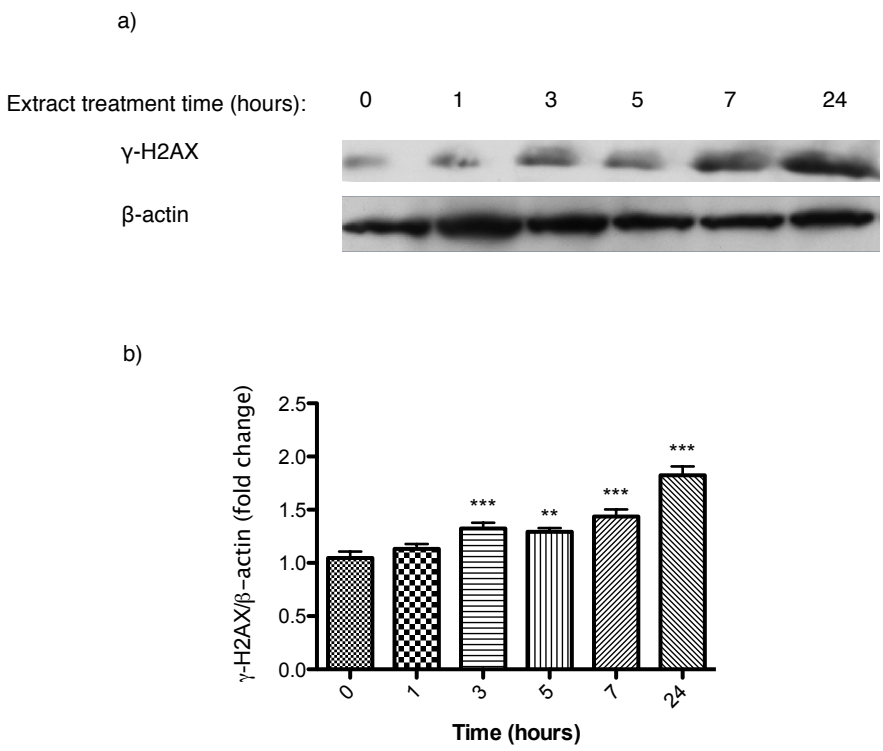


Figure 5.4.3. Aqueous *extract treatment time increases γ-H2AX expression in MCF-7 cells.* MCF-7 cells were treated with 2mg/ml extract for up to 24 hours before expression of (a) γ-H2AX was determined by SDS-PAGE and western blot. β-actin was used as a loading control. Densitometry for (b) γ-H2AX was calculated as a ratio to β-actin and expressed as fold change compared to control (*time=0 hours).* Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

activity can be modulated pharmacologically by caffeine, a protein kinase inhibitor with activity towards ATM and ATR [351]. Both ATM and ATR exert site specific kinase activity towards serine-15 of p53 in response to DNA damage and play a critical role in DDR signalling [83]. Here, caffeine has been used to inhibit ATM/ATR in MCF-7 cells to investigate the effects of kinase inhibition, on extract induced p53 and

p21 expression. The effects of ATM/ATR inhibition and extract treatment on cell viability was also determined. MCF-7 cells were pre-treated with 3 mM caffeine or vehicle control for 60 minutes prior to 2 mg/ml extract treatment for up to 24 hours. Cell lysates were collected and expression of p53 and p21 was analysed by western blot. Pretreatment with 3mM caffeine significantly reduced extract-induced p53 and p21 expression after 5 hours and 24 hours treatment (figure 5.4.4a). Extract treatment alone induced a 112.0% increase in p53 expression after 5 hours and a 118.2% increase after 24 hours (figure 5.4.4b). Pretreatment with caffeine prior to extract treatment induced a 17.3% increase after 5 hours and a 30.9% increase after 24 hours compared to caffeine treated controls (figure 5.4.4b). This effect was also seen with p21 expression. Extract treatment alone increased p21 protein expression 47.2% after 5 hours and 82.1% after 24 hours. Cells pretreated with caffeine prior to extract treatment demonstrated an insignificant change in p21 expression after 5 or 24 hours compared to control (figure 5.4.4c).

The effect of ATM/ATR inhibition on extract induced cell death was determined by measuring cell viability using the MTT assay. MCF-7 cells were treated for 60 minutes with 3 mM caffeine or vehicle control prior to extract treatment for 24 hours. Inhibition of ATM/ATR resulted in a significant reduction in extract induced loss of cell viability at concentrations above 0.5 mg/ml. Extract treatment alone (2 mg/ml) reduced MCF-7 cell viability by 39.1%, while pre-treatment with caffeine followed by 2 mg/ml extract treatment resulted in a 21.7% reduction in cell viability (figure 5.4.5).

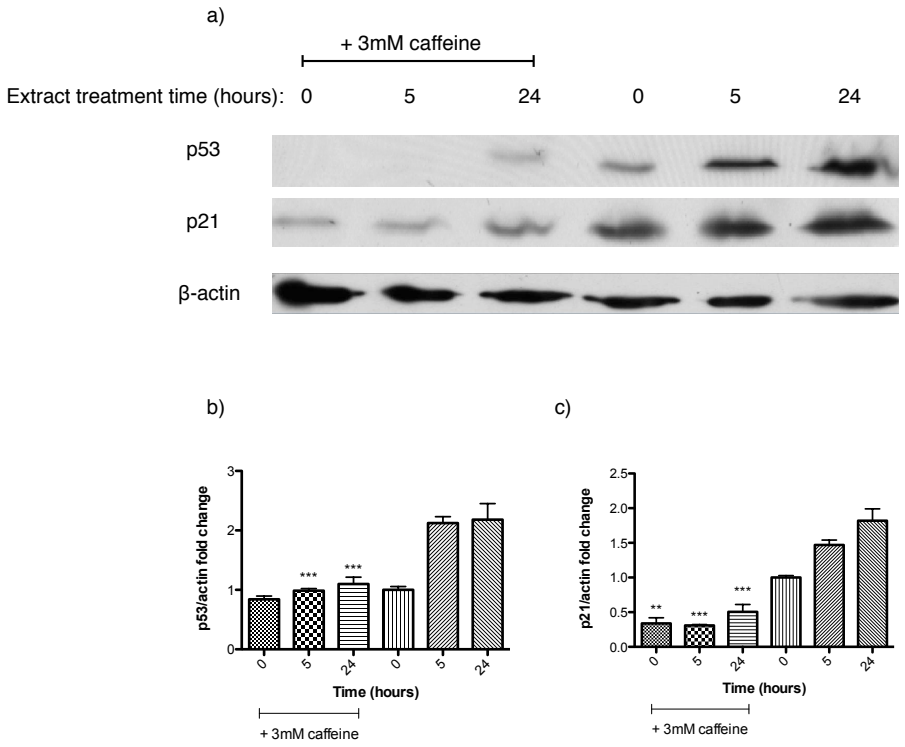


Figure 5.4.4. Aqueous *extract induced p53 and p21 expression is dependent on ATM/ATR activity.* MCF-7 cells pretreated with and without 3mM caffeine for 60 minutes were treated with 2mg/ml extract for up to 24 hours before expression of (a) p53 and p21 protein expression by SDS-PAGE and western blot. β-actin was used as a loading control. Densitometry for (b) p53 and (c) p21 was calculated as a ratio of β-actin and expressed as fold change compared to control (*time=0 hours*). Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to non-caffeine treatment analysed by one-way ANOVA with Bonferroni's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

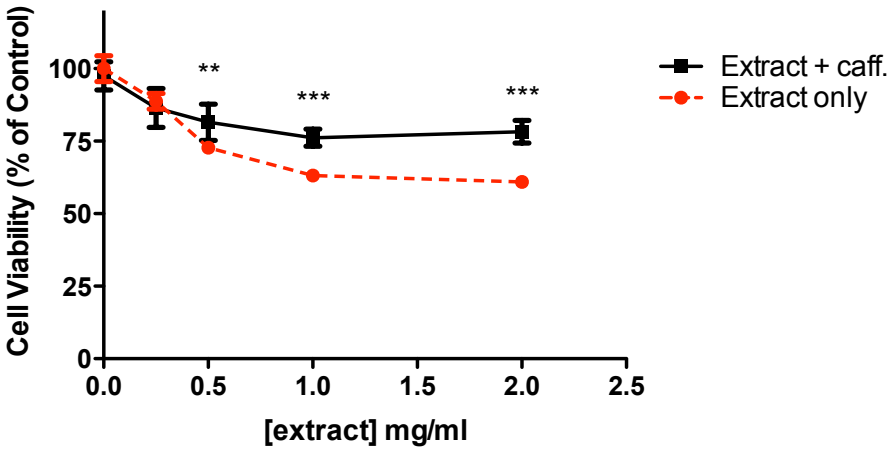


Figure 5.4.5. ***Inhibition of ATM/ATR partly inhibits extract induced MCF-7 cell death.*** MCF-7 cells pretreated with 3mM caffeine or vehicle control for 60 minutes were treated with up to 2mg/ml extract for 24 hours prior to MTT assay. Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to extract only as analysed by two-way ANOVA with Bonferroni's multiple comparison post test. Data is from at least three independent experiments performed in triplicate.

5.4.3: Extract cytotoxicity is not fully dependent on p53

Although caffeine is known to be an effective inhibitor of ATM and ATR it also has other pharmacological functions that could potentially affect p53 and p21 expression not related to inhibition of the DNA damage response. In order to determine whether extract induced-cytotoxicity is completely dependent on a p53 mediated response in MCF-7 cells, *TP53* was transiently knocked down by transfection with silencer siRNA. Cells treated with and without *TP53* siRNA were subjected to 2 mg/ml aqueous extract treatment for up to 24 hours prior to analysis of cell viability by MTT assay and quantification of p53 protein levels by western blot. As shown previously, extract treatment significantly increased p53 expression up to 24 hours. Transfection of *TP53* siRNA significantly reduced the extract mediated increase in p53

expression at 5 or 24 hours (figure 5.4.6a). Extract treatment for 5 and 24 hours induced a 29.8% and 72.3% increase in p53 expression, respectively (figure 5.4.6b). Knockdown of p53 translation with siRNA prior to extract treatment resulted in an insignificant increase in p53 expression after 5 or 24 hours treatment with extract. Basal p53 expression was also significantly reduced by 12.3% in siRNA transfected cells. After successful *TP53* knockdown, the effect of aqueous extract treatment on MCF-7 cell viability was assessed by MTT assay. Transfection of *TP53* siRNA significantly attenuated extract treatment-induced loss of cell viability in MCF-7 cells but a significant decrease in cell viability was still apparent after 24 hours compared to untreated cells. Extract treatment of 2 mg/ml reduced MCF-7 cell viability by 47.5% after 24 hours and had an IC_{25} of 0.5 mg/ml. In comparison, in siRNA transfected cells, viability was reduced 31.7% under the same conditions and had an IC_{25} of 1.6 mg/ml (figure 5.4.7a). It was shown in chapter 3 that an aqueous extract of *Fagonia cretica* could reduce cell viability in MDA-MB-231 breast cancer cells, albeit, not as effectively as against MCF-7 cells. In fact, the loss of cell viability in extract treated MDA-MB-231 cell, is similar to the loss of cell viability of extract treated MCF-7 cells post *TP53* siRNA interference (figure 5.4.7c).

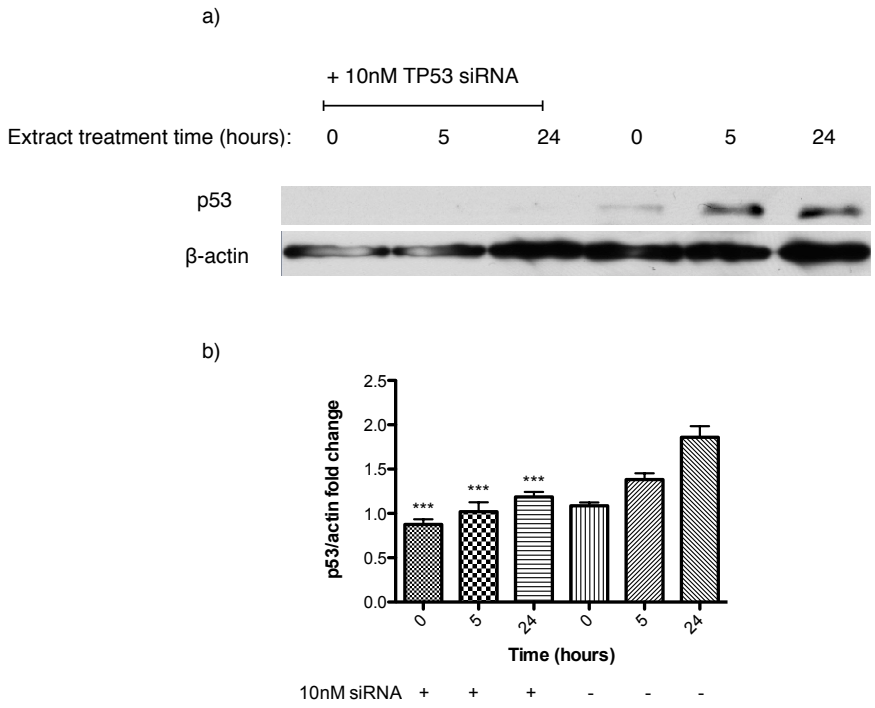


Figure 5.4.6. ***Aqueous extract induced p53 expression is blocked with transfection of TP53 siRNA.*** MCF-7 cells were transfected with and without 10nM TP53 siRNA for 24 hours prior to 2mg/ml extract treatment for up to 24 hours. Cell lysates were collected and p53 protein expression was assessed by western blot. β-actin was used as a loading control. Data is expressed as a fold change in p53 density normalised to β-actin. Data denoted *** ($p<0.001$) are significant compared to non-siRNA treated counterpart. Data was analysed by one-way ANOVA with Bonferroni's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

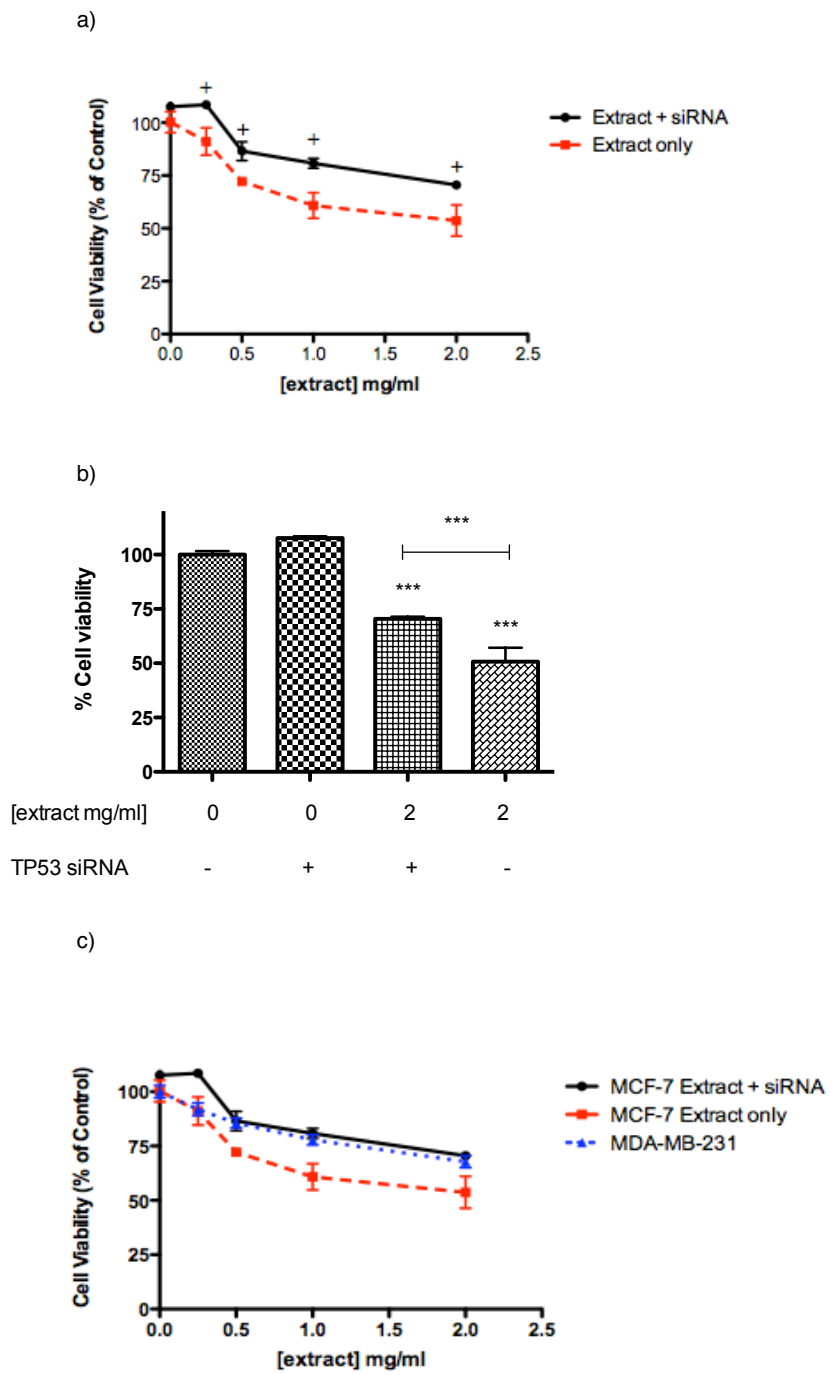


Figure 5.4.7. ***Cell viability following aqueous extract treatment is improved in p53-deficient cells.*** (a, b) MCF-7 cells were transfected with and without TP53 siRNA for 24 hours before extract treatment up to 2mg/ml for 24 hours. Cell viability was determined by MTT assay. (c) MCF-7 cells transfected with and without TP53 siRNA and MDA-MD-231 cells were treated with up to 2mg/ml extract for 24 hours prior to MTT assay. Data denoted *** ($p<0.001$) are significant compared to untreated control or as indicated, analysed by one-way ANOVA with Bonferroni's multiple comparison post test. Data denoted + ($p<0.001$) are significant compared to 'no siRNA' as analysed by two-way ANOVA with Bonferroni's

5.4.4: Aqueous extract treatment induces G0/G1 arrest in MDA-MB-231 cells

In order to assess the impact of p53 deficiency on aqueous extract activity, against breast cancer cells, the effects of aqueous extract treatment on cell cycle status of MDA-MB-231 cells, was investigated. Extract treatment induced significant cell cycle arrest in G0/G1 phase after 24 hours treatment. Treatment with 2 mg/ml aqueous extract for 24 hours increased the percentage of cell in G0/G1 phase from 20.3% to 64.0%, which coincided with a decrease from 30.4% to 5.0%% in the G2 cell population (figure 5.4.8 a and b). In contrast, the results from chapter 4 show that in extract treated MCF-7 cells, significant G0/G1 arrest is present as early as 5 hours treatment.

5.4.5: Aqueous extract treatment increase Y-H2AX expression in MDA-MB-231 cells

The results in this chapter show that p53-deficient breast cancer cells are less susceptible to aqueous extract-dependent loss of viability then those with functional p53. However, cell cycle arrest and loss of cell viability, is still apparent. The extract induced p53 response in MCF-7 cells appears to be initiated by activation of the DNA damage response, therefore, induction of γ-H2AX in MDA-MB-231 cells was assessed as a marker of DNA damage in p53 deficient cells. Western blot analysis of aqueous extract treated MDA-MB-231 cells revealed a time-dependent increase in γ-H2AX expression (figure 5.4.9a). Expression of γ-H2AX was significantly increased by 93.3% after 1 hour treatment and 317.3% after 24 hours (figure 5.4.9b).

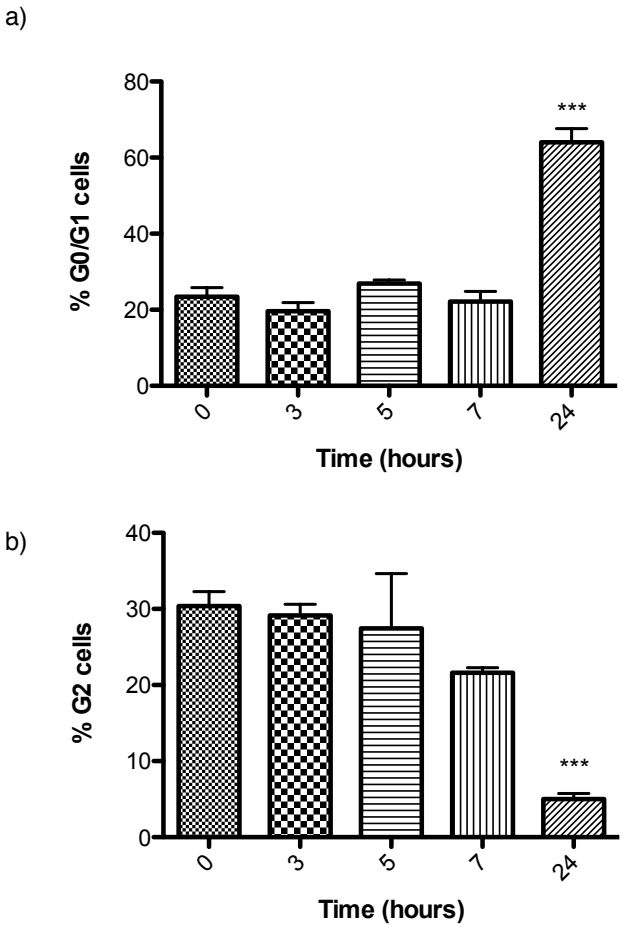


Figure 5.4.8. ***Extract treatment induces G0/G1 arrest in MDA-MB-231 cells.*** MDA-MB-231 cells were treated with 2mg/l extract for up to 24 hours before cell cycle analysis by flow cytometry. Populations (a) G0/G1 and (b) G2 were determined according to cyclin A-FITC (FL3) and propidium iodide (FL1) fluorescence. Data denoted *** ($p<0.001$) are significant compared to control *(time = 0 hours)* analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments performed in triplicate and presented as mean ± standard deviation.

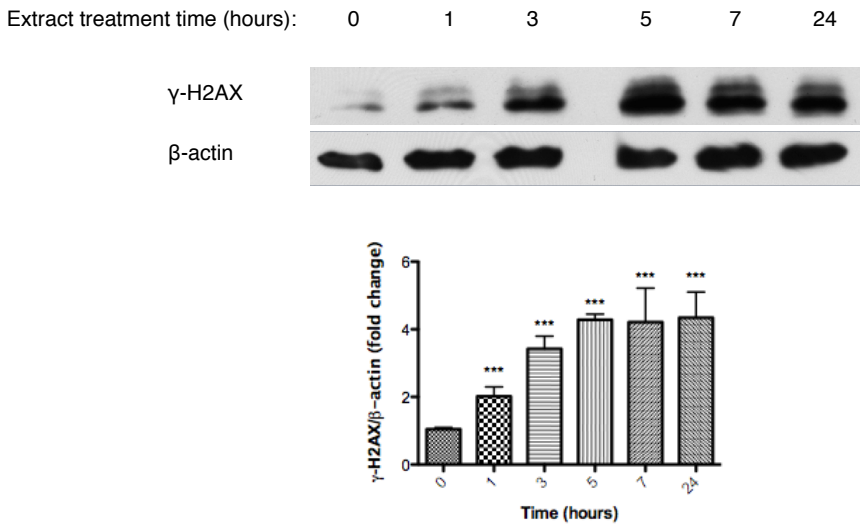


Figure 5.4.9. ***Aqueous extract treatment increases γ-H2AX expression in MDA-MB-231 cells.*** MDA-MB-231 cells were treated with 2mg/ml extract for up to 24 hours before expression of (a) γ-H2AX was determined by SDS-PAGE and western blot. β-actin was used as a loading control. Densitometry for (b) γ-H2AX was calculated as a ratio to β-actin and expressed as fold change compared to control (*time=0 hours).* Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

5.5: Discussion

Induction of DNA damage is an effective mechanism by which chemotherapeutic drugs selectively target cancer cells for growth arrest or death because they frequently have impaired DNA repair responses and are rapidly cycling. Activation of the DNA damage response initiates signalling pathways that result in cell cycle arrest, DNA repair and cell death. Central to the downstream control of the DDR is the transcription factor p53, which regulates transcriptional targets involved in DNA repair, cell cycle arrest and cell

death, in response to DNA damage. The importance of p53 in the DDR has been shown in experiments involving over-expression of functional p53 in mice, which show enhanced cellular response to DNA damage and significantly protected mice from cancer [356]. The data described in previous chapters has demonstrated that aqueous extract treatment of MCF-7 cells induces cell cycle arrest and apoptosis which may be related to increased expression of p53 and its transcriptional targets p21 and bax. In order to further understand the mechanisms by which these events occur, the ability of extract treatment to induce DNA damage and the DDR was investigated. The present study has shown that extract treatment of MCF-7 cells can induce significant levels of non-oxidative DNA damage resulting in ATM/ATR-dependent activation of p53. It was also shown that p53 is not essential for the bioactivity in the extract and that *TP53* knockdown MCF-7 cells and mutant-p53 MDA-MB-231 cells are susceptible to extract treatment.

DNA damage, measured by the comet assay was significant after 3 hours treatment and further increased after 24 hours in MCF-7 cells suggesting a sustained response to extract treatment. This agrees with the findings in chapter 3 as cell viability was decreased with longer incubation periods. The standard comet assay analyses all types of strand breaks and modifications to the assay using FPG protein can specifically detect the presence of oxidative DNA damage. Incubation with FPG protein prior to DNA damage analysis showed no significant increase in tail DNA. This indicates that the DNA damage is most likely not oxidative as FPG recognises and removes the most prevalent oxidative DNA lesions including 8-oxo-guanine, 8-oxo-adenine, fapy-guanine, methyl-fapy-guanine and fapy-adenine [357]. In chapter 3 it was shown that extract treatment can significantly increase intracellular ROS levels in MCF-7 cell

which may lead to oxidative DNA damage. However, it is not known what oxidative load would be necessary to induce DNA damage in this model or how localised the ROS production is to specific cellular compartments. The nucleus maintains a strong reducing potential which may resist cytoplasmic redox flux [358]. Therefore it is plausible that extract induced ROS production may not be sufficient in the nucleus to cause oxidative nuclear DNA damage. ROS are involved in a variety of cellular processes and excessive production can mediate other effects than DNA damage [335]. Hydrogen peroxide is produced as a signalling molecule that exerts oxidative power on cysteine residues of effector proteins in response to activation of cell surface receptors [359]. ROS production also accompanies signalling events leading to critical roles determining cell fate such as activation of MAP kinase pathways [360].

It was previously shown in chapter 4 that p53 and p21 expression are increased as a result of extract treatment. In order to determine if this was dependent on ATM/ATR-mediated stabilisation of p53, the effects of the ATM/ATR inhibitor caffeine on extract induced p53 and p21 expression was assessed. Inhibition of ATM/ATR prevented extract induced up-regulation of p53 and p21 expression suggesting that activation of p53 is dependent on ATM/ATR and most likely induced by single or double DNA strand breaks. The role of ATM/ATR in the DNA damage response is well characterised [59,88]. In response to DNA damage, both ATM and ATR can directly interact with p53 and induce site specific phosphorylation at serine-15, resulting in p53 transactivation [117]. The kinase activity of ATM could also increase p53 stabilisation through interaction with other kinases. For example ATM may play a role in activating c-Abl kinase activity on p53 and induce phosphorylation at serine-20. This post-translational modification can stabilise

p53 and reduce its interaction with MDM2 [361]. This suggests a potential role for ATM/ATR in increasing p53 expression in response to extract treatment and indeed results from this study show that inhibition of ATM/ATR with caffeine can prevent extract-mediated effects on p53 and its transcriptional target p21. Although caffeine is reported to inhibit ATM/ATR activity it has also been shown to influence other cellular responses. For example, caffeine may interfere with drug transport and DNA binding capability, by forming inhibitory complexes with poly-aromatic drugs [362].

An investigation by *Pabla et al.* demonstrated that the DNA damaging agent, cisplatin, induces ATR activation and its co-localisation with λ-H2AX at sites of DNA damage resulting in activation of p53 [363]. The current study shows evidence of increased γ-H2AX expression in response to extract treatment which is a common marker of DNA double strand breaks. Gamma-H2AX is generated by phosphorylation of serine-139 in H2AX by members of the PIK-related family including ATM and ATR. It has been shown that γ-H2AX is required for activation p53/p21 axis and p21 induced cell cycle arrest in response to replication stalling during DNA synthesis [364]. These findings suggest that extract treatment may be either inducing double strand breaks in DNA of MCF-7 cells resulting in recruitment of ATM/ATR to sites of DNA damage and site specific phosphorylation of H2AX or allowing DNA breaks to accumulate due to inhibition of DNA repair processes. It is important to note that H2AX activation can also occur independently of double strand breaks [365]. As well as this, the comet assay measures all types of DNA damage, so it is not possible to fully determine double strand breaks as the DNA damage induced by extract treatment.

It has been shown that caffeine increases radio-sensitivity in treated cells by inhibition of ATM activity and abrogation of the G2/M DNA damage checkpoint [362]. Potentiation of DNA damaging agents by caffeine is specific to the G2/M checkpoint and it has been shown that loss of G1-arrest does not lead to increased radiosensitivity [366]. In agreement with these findings, extract treatment in caffeine treated cells does not decrease cell survival beyond extract treatment alone. Taken together with previous results demonstrating extract induced G1-arrest and activation of DNA damage response it is expected that caffeine would not potentiate the effects of extract treatment. In fact, cell survival was improved with caffeine treatment, suggesting that an independent mechanism other then p53 associated G1-arrest and apoptosis may be working in parallel to reduce cell viability. This is expected as a crude aqueous plant extract would probably contain multiple bioactive compounds which may elicit an anti-cancer response either individually or in synergy with other compounds. This finding also highlights that extract treatment could be effective against tumours carrying mutant p53. This agrees with the data reported in chapter 3 that extract treatment could also reduce MDA-MB-231 cell viability after 24 hours (figure 3.4.3).

Although p53 is important in suppressing oncogenesis, and loss of function in tumours is responsible for disease progression and drug resistance, p53-independent mechanisms which can inhibit cellular growth in response to stress, are also important for homeostatic control [98]. The present study has demonstrated that although p53 expression is increased in response to ATM/ATR activation, it is not crucial to extract activity, as shown by siRNA inhibition of p53 expression. Knockdown of p53 expression attenuated some of the extract induced cytotoxicity suggesting that p53-mediated mechanisms only play a part in the overall

cytotoxic effect. This p53-independent effect was also shown in MDA-MB-231 cells, which carry a non-functional mutant p53, confirming that extract induced toxicity is not fully reliant on expression of p53. Evidence for p53-independent cell death can be found in the literature, for example, the antimicrobial agent sanguinarine has been shown to induce DNA damage and cell death independent of cellular p53 status in human colon cancer cells. Work by *Liu et al.* has also identified that the isoquinoline alkaloid, berberine, induces growth arrest and apoptosis in cancer cells, with arrest in G1 dependent on p53-status and G2/M arrest occurring independent of p53 [367,368]. Furthermore, E2F-1, a cell cycle regulator downstream of pRB, can induce apoptosis in p53 dependent and independent mechanisms [155]. There is also evidence that DNA damage activates JNK pathways to induce p53-independent cell death through up-regulation of the pro-apoptotic protein, bid [369].

In light of the results demonstrating that p53 deficient cells are less susceptible to extract, the effects of extract treatment on cell cycle status of MDA-MD-231 cells was investigated. G0/G1 arrest was induced in MDA-MB-231 cells in response to extract treatment which was also reported in MCF-7 cells. However, cell cycle arrest was delayed in MDA-MB-231 cells and did not present significantly until after 24 hours treatment. In comparison, G0/G1 arrest was significantly induced in MCF-7 cells after 5 hours treatment, suggesting that p53 is partly responsible for extract induced cell cycle arrest. This finding indicates that in the absence of p53, extract induced-stress, does not initiate a cellular response as rapidly as in the presence of functional p53. This provides further evidence for the role of p53-independent cell cycle arrest and cell death in extract treated-breast cancer cells. As extract treatment was shown to induce γ-H2AX expression in MCF-7 cells, the effect of

treatment on γ-H2AX expression in MDA-MB-231 cells, was also investigated. The results revealed that MDA-MB-231 cells also show increased γ-H2AX expression indicative of accumulating double strand breaks. This, together with earlier data obtained using MCF-7 TP53 siRNA treated cells, suggests that DNA damage could be the initiating step in extract-induced cytotoxicity and that downstream signalling only partly involves p53.

It is known that stress signals activate FOXO transcription factors which play a major role in regulating cell survival and cell death. For example, in Taxol (paclitaxel) treated breast cancer cells, increases in FOXO3a activity up-regulated pop-apoptotic bim and induced apoptosis [222]. The FOXO transcription factors have been implicated in a wide variety of physiological responses including glucose metabolism, oxidative stress, cell cycle regulation and apoptosis and are involved pathologically in many disease states including cancer [180]. DNA repair is an energy consuming process as recruitment and activation of the repair protein PARP requires NAD+ to generate ADP-ribose monomers. This reliance on NAD+ as a substrate results in a depletion in available ATP and subsequently prolonged PARP activation can lead to metabolic crisis and activation of FOXO. It has also been shown that FOXO proteins are involved in activation of DNA repair mechanisms through direct interaction with the DNA damage response gene *GADD45a* [209]. It has been reported that CDK2 dependent phosphorylation on FOXO1 allows normal cell cycle progression and that in response to DNA damage, cytoplasmic sequestering of FOXO1 is abrogated, which allows for FOXO-mediated cell cycle repression [175]. A study by *Tsai et al.* revealed that FOXO3a also has functional interaction with ATM. In particular, FOXO3a phosphorylates ATM in response to DNA damage and propagates nuclear

activity of downstream effectors [370]. The results from this investigation taken with evidence from the literature suggest a possible role for FOXO proteins in mediating extract induced cytotoxicity in both p53 proficient and deficient cells.

5.6: Conclusion

The work described in this chapter aimed to understand the initiation step in inducing growth arrest and apoptosis in extract treated MCF-7 cells. It is well documented that DNA damage is an important inducer of cell cycle inhibition and cell death, thus levels of extract-induced DNA damage were assessed using the comet assay. Evidence of strand breaks in MCF-7 cells was apparent after extract treatment. The role of the DNA damage sensors ATM/ATR and their influence on p53 expression was also investigated. Extract-induced DNA damage appeared to induce ATM/ATR-dependent p53 expression, which could be attenuated with caffeine. DNA damage was determined to be double stranded as there was increased γ-H2AX expression in response to extract treatment. This provides evidence implicating ATM as the mediator of p53 expression as ATM primarily responds to this type of DNA damage. It was also shown that DNA damage-induced p53 expression is responsible for increased p21 expression as both could be attenuated with caffeine treatment. This suggests that DNA damage is an initiating step in cell cycle arrest of extract treated MCF-7 cells. Inhibition of DNA damage-induced p53 expression with caffeine improved cell survival in response to extract treatment, suggesting that the aqueous extract is activating multiple functional pathways leading to cell death and that its activity is not fully dependent on p53. This was confirmed with *TP53* siRNA knockdown in MCF-7 cells and comparable with treatment effects on MDA-MB-231 cells. This result highlights the potential for extract treatment to be

effective towards cancer cells independent of their p53-status, thus, increasing the range of efficacy for chemotherapy. It was also shown that extract induced-cell cycle arrest is delayed but not abrogated in p53-deficient cells. This provides further evidence for the role of p53 in extract induced MCF-7 cytotoxicity and the activation of p53-independent mechanisms contributing to extract activity (figure 5.4.10).

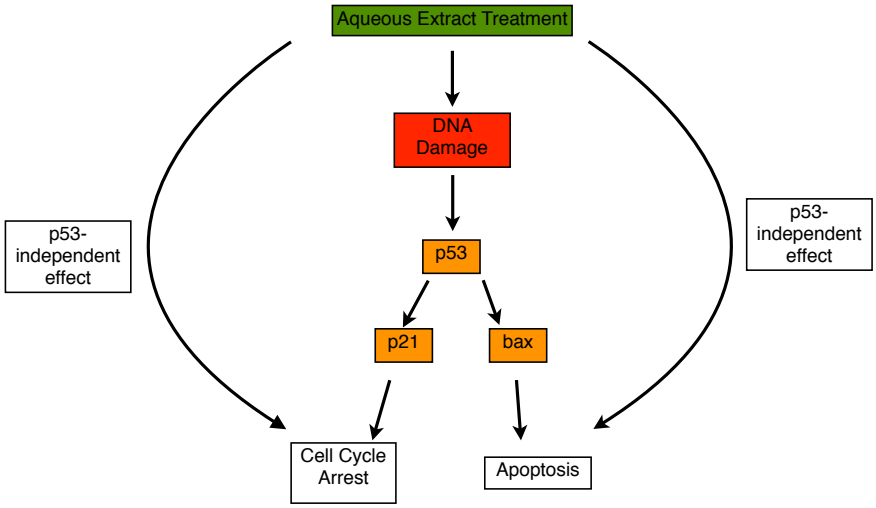


Figure 5.4.10. ***The role of p53 in aqueous extract-induced cell cycle arrest and apoptosis in MCF-7 cells.*** Treatment of MCF-7 cells with an aqueous extract of *Fagonia cretica* results in DNA damage and activation of the DNA damage response involving activation of p53 and its downstream transcriptional targets p21 and bax. Inhibition of the p53 response does not fully attenuate extract induced cytotoxicity in MCF-7 cells suggesting a role for p53-independent effects.

Chapter 6: Extract-induced p53-independent cytotoxicity

6.1: Rationale

The tumour suppressor protein, p53, is an important mediator of cell cycle arrest and apoptosis following exposure to DNA damaging agents. In fact, over 50% of human cancers contain a functional mutation within the TP53 gene, which contributes to their tumorigenic phenotype. Although this highlights the importance of p53 as a powerful guardian against tumorigenesis, there are also p53-independent mechanisms of tumour suppression, by which DNA repair and apoptotic machinery can be activated following DNA damage. It is becoming increasingly clear that utilising p53-independent driven apoptosis can be effective therapeutically for the treatment of cancer. For example, the FOXO transcription factors control cell fate in response to a variety of stresses, including DNA damage. Recent research has been aimed at understanding how FOXO signalling pathways can be used as a target for chemotherapy.

The investigation described in chapter 5 demonstrated that a p53-mediated DNA damage response was not vital to *Fagonia cretica* extract-induced cytotoxicity in human breast cancer cells. In this chapter, the p53-independent effects of aqueous extract treatment governing cell death in breast cancer cells will be investigated focused on the extract-induced effects on FOXO3a due to its importance in regulating cell cycle arrest and apoptosis in response to DNA damage. The effects of aqueous extract treatment on SIRT1, a negative regulator of FOXO3, have also been investigated to understand any protein-protein interaction that may contribute to extract-mediated cytotoxicity.

6.2: Introduction

Functional mutations within the *TP53* gene are a major contributor to tumorigenesis [98]. Loss of functional p53 also has an impact on chemotherapeutic drug efficacy and can drive drug resistance in certain tumours [371]. This is primarily due to loss of tumour suppressor functions that not only regulate cell cycle progression but also promote apoptosis in response to chemotherapy. However, p53 loss of function does not always confer tumour resistance to DNA damaging agents, as parallel p53-independent signalling pathways can also induce cell cycle arrest and apoptosis in response to DNA damage [286,312]. Functional p53 mutations are present in over 50% of tumours, thus, highlighting the importance for therapies that induce growth arrest or cell death in cancers independent of their p53-status [104].

The Forkhead box class O (FOXO) proteins are a group of transcription factors involved in cellular differentiation, cell proliferation, metabolic stress and apoptosis [175]. It has been shown that loss of FOXO function in cancer cells may be responsible for an impairment in promotion of cell cycle arrest and cell death in response to DNA damage [180]. The FOXO proteins are negatively regulated by the PI3K/AKT signalling pathway in the presence of survival signals and growth factors [198]. Under these conditions, phosphorylation of conserved serine/threonine residues increases cytoplasmic shuttling of FOXO transcription factors, thus reducing their nuclear activity [175]. The FOXO protein, FOXO3a, has been shown to control cell cycle progression and pro-apoptotic signalling through transactivation of its transcriptional targets such as p27 and bim [222]. Inhibition of FOXO3a has also been implicated in promoting tumorigenesis in multiple models where nuclear

translocation was restricted by promoting phosphorylation of FOXO3a [182,183]. It has also been shown that activation of FOXO3a in breast cancer models can repress epithelial-mesenchymal transition (EMT) as well as induce cell cycle arrest and apoptosis [222,223]. Furthermore, it has been shown that FOXO3a cytoplasmic retention is associated with poor prognosis in breast cancer patients due to repression of FOXO3a transcriptional activity, including up-regulation or cell cycle and pro-apoptotic genes [224].

It is unclear how FOXO transcription factors respond to DNA damage but ectopic over-expression of FOXO3a is not only able to reduce cellular proliferation but also potentiates growth arrest induced by DNA damage [372]. Moreover, induction of DNA damage by ionising radiation, activates FOXO3a and increases its nuclear translocation [221]. The resulting activation of bim and Fas ligand is associated with induction of apoptosis, and is observed independently of p53, highlighting a potential FOXO3a mediated response to DNA damage.

Similar to the regulation of p53, the transcription factor FOXO3a is subjected to multiple phosphorylation and acetylation modifications, which alter its functionality [196]. For example, analysis of the nematode worm homolog to FOXO, DAF-16, revealed four consensus sites for Akt-dependent phosphorylation [373]. Signalling in the presence of growth factors leads to Akt-mediated phosphorylation of FOXO3a, subsequent nuclear export and cytoplasmic retention via interaction with 14-3-3 proteins [198]. The protein kinase CK1 is known to target FOXO proteins that have primed or phosphorylated serine/threonine residues and the activation of CK1 kinase activity occurs in response to DNA damage [374].

Sirtuin 1 (SIRT1) is a NAD-dependent histone deacetylase which is involved in control of epigenetic gene silencing via histone modifications. SIRT1 also exhibits deacetylase activity against non-histone targets including the transcription factors p53 and FOXO3a [205]. Deacetylation of FOXO3a by SIRT1 has been shown to repress FOXO3a transcriptional activity, specifically by inhibiting binding to the promoter region of the cell cycle and pro-apoptotic target genes, p27 and bim [203]. As part of a negative feedback loop, FOXO3a is self-repressed by increasing SIRT1 transcription [175]. Cell survival was initially associated with SIRT1 activity due to deacetylation of p53. However, in certain cancers, over-expression of SIRT correlates with tumorigenesis and resistance to chemotherapy [375]. The tumour suppressor, deleted in breast cancer-1 (DBC-1), interacts with SIRT1, which in turn represses DBC-1 activity in vitro and in vivo [376]. DBC-1 is commonly mutated in carcinoma of the bladder and its down-regulation potentiates the anti-apoptotic effects of SIRT1 in response to genotoxic stress, thus, implicating SIRT1 in tumorigenesis [377].

It has been shown that acetylation of p53 is crucial to destabilise the MDM2-p53 repressor complex and activate the p53-mediated stress response [125]. However, increased acetylation of p53 as a result of pharmacological inhibition of SIRT1, does not potentiate DNA damage-induced cell death [378]. The natural product, resveratrol is a naturally occurring polyphenol with extensive pharmacological characteristics including activation of SIRT1 activity [379]. It has also been reported that resveratrol inhibits phosphorylation of FOXO proteins, thus, potentiating nuclear translocation and FOXO transcriptional activity [380].

Given the significance of the FOXO3a pathway as a mediator of growth arrest and apoptosis, in this chapter the effects of

extract treatment on FOXO3a expression, will be investigated. Furthermore, the extract-induced effects on SIRT1 and any role for SIRT1 in its toxicity will also be investigated, as SIRT1 plays an important role in not only regulating FOXO3a activity but also functioning as tumour promoter.

6.3: Methods

6.3.1: Protein expression analysis by western blot

The effects of aqueous extract treatment on SIRT1 protein expression in MCF-7 cells and FOXO3a protein expression in MCF-7 and MDA-MB-231 cells was determined by SDS-PAGE and western blot as outlined in section 2.10. A total of 20µg protein per lysate was separated on a 10% SDS-PAGE gel before western blot procedure. Membranes were probed with rabbit anti-human FOXO3a (1:1000), rabbit anti-human SIRT1 (1:1000) before addition of sheep anti-rabbit HRP conjugated antibody (1:10000). Bands were visualised using ECL reagent exposed to photographic film for 5-20 minutes.

6.3.2: MTT - cell viability assay

The effect of aqueous extract treatment following resveratrol pre-treatment or FOXO3a siRNA interference on MCF-7 and MDA-MB-231 cell viability was determined using the MTT assay as detailed in section 2.5. Cells were pre-treated with a non-toxic dose (50µM) of resveratrol (figure 6.3.1) for 60 minutes before co-incubation with extract for up to 2mg/ml over 24 hours.

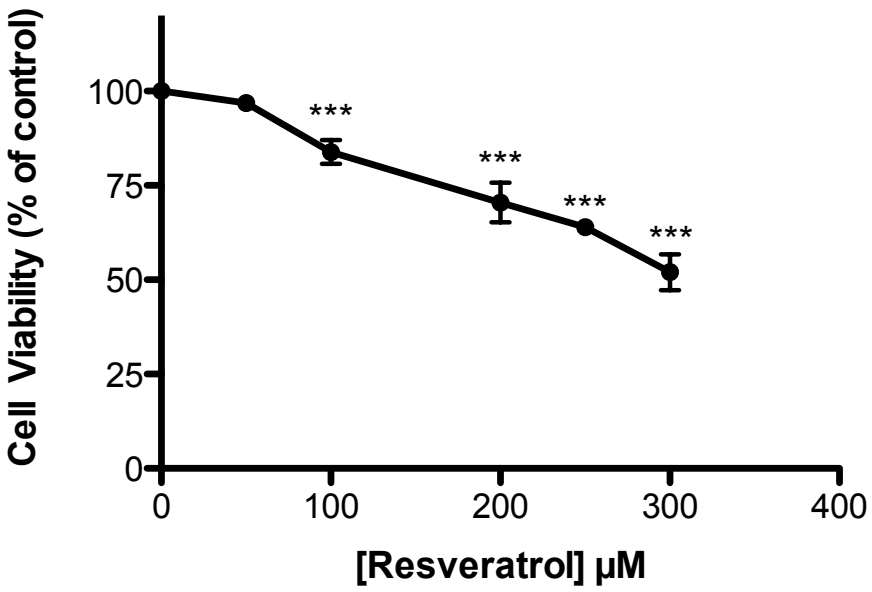


Figure 6.3.1. ***Effect of resveratrol treatment on MCF-7 cells.*** MCF-7 cells were treated with up to 300µM resveratrol for 24 hours prior to analysis of cell viability by MTT assay. Data denoted *** ($p<0.001$) is significant compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is from three independent experiments performed in triplicate and presented as mean ± standard deviation.

6.3.3: SIRT1 activity assay

The effect of aqueous extract treatment on SIRT1 activity in MCF-7 cells was determined using the SIRT1 activity kit (Sigma) as detailed in section 2.12.

6.3.4: siRNA interference

FOXO3 siRNA interference was used to knockdown FOXO3a expression in MCF-7 and MDA-MB-231 cells as detailed in section 2.11.

6.3.5: ATP assay

The effect of aqueous extract treatment on ATP levels in MCF-7 cells was assessed using the ATP assay kit (Promega) as detailed in section 2.13

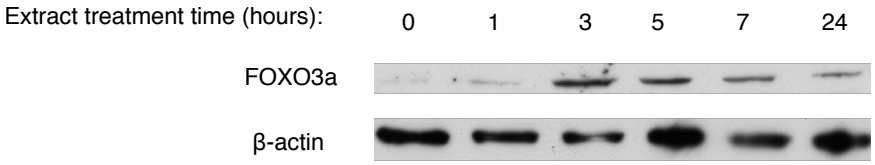
6.4: Results

6.4.1: Aqueous extract treatment increases FOXO3a expression in breast cancer cells.

The role of FOXO proteins as transcription factors controlling cell fate in response to stress is well documented. The results in chapter 5 demonstrate that aqueous extract treatment is cytotoxic towards breast cancer cells in a p53-dependent and independent manner. Therefore, it was hypothesised that FOXO proteins may play a role in extract-induced cell death. In particular, emphasis was placed on FOXO3a because of its role in inducing cell cycle arrest and apoptosis via induction of its target genes p27 and bim. MCF-7 and MDA-MB-231 cells were treated for up to 24 hours with 2 mg/ml aqueous extract prior to protein expression analysis by SDS-PAGE and western blot. The results in figure 6.4.1a and 6.4.2b show that in response to extract treatment both MCF-7 and MDA-MB-231 cells significantly increase FOXO3a protein expression within 3 hours. Protein expression of FOXO3a in MCF-7 cells was increased 8.4-fold after 3 hours treatment compared to control (time = 0 hours). Protein expression tapered off towards 24 hours treatment but remained significantly elevated by 3.8-fold compared to control levels (figure 6.4.1b). In extract-treated MDA-MB-231 cells, FOXO3a expression was increased 1.8-fold after 3 hours and peaked

at 5 hours treatment with a fold increase of 2.1 (figure 6.4.2b).

a)



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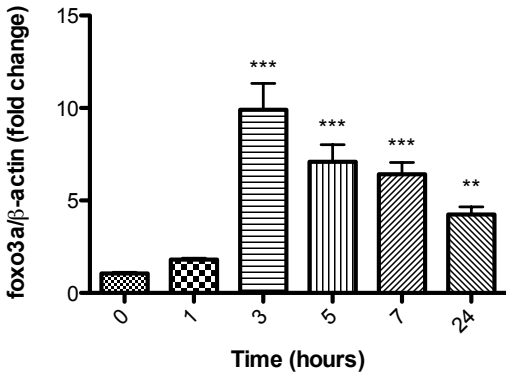
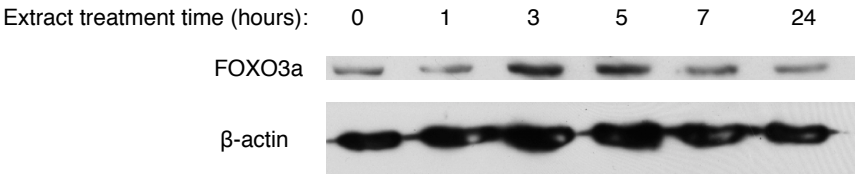


Figure 6.4.1. ***Aqueous extract treatment increases FOXO3a expression in MCF-7 cells.*** MCF-7 cells were treated with 2mg/ml aqueous extract for up to 24 hours before expression of (a) FOXO3a was determined by SDS-PAGE and western blot. β-actin was used as a loading control. Densitometry for (b) FOXO3a was calculated as a ratio to β-actin and expressed as fold change compared to control (*time=0 hours).* Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

a)



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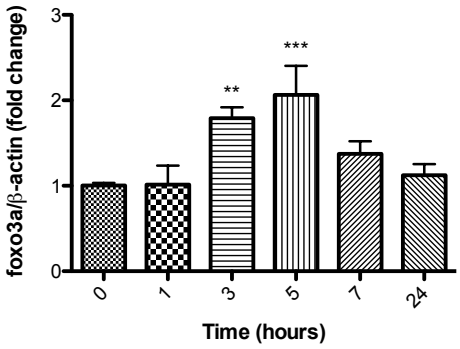


Figure 6.4.2. ***Aqueous* extract *treatment increases FOXO3a expression in MDA-MB-231 cells.*** MDA-MB-231 cells were treated with 2 mg/ml aqueous extract for up to 24 hours before expression of (a) FOXO3a was determined by SDS-PAGE and western blot. β-actin was used as a loading control. Densitometry for (b) FOXO3a was calculated as a ratio to β-actin and expressed as fold change compared to control (*time=0 hours).* Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

6.4.2: Expression of FOXO3a is required for aqueous extract-induced cytotoxicity.

In order to determine if an aqueous extract-induced increase in FOXO3a expression is required for loss of cell viability in breast cancer cells, MCF-7 and MDA-MB-231 cells were transiently transfected with FOXO3 siRNA, prior to extract treatment. Knockdown of FOXO3a by siRNA transfection

attenuated an extract-induced (2 mg/ml) increase in FOXO3a protein expression at 24 hours with complete abrogation of 5 hour response (figure 6.4.3a). In the presence of 5 nM siRNA, extract-treatment for 24 hours induced a significant 23.3% increase in FOXO3a expression, compared to siRNA-transfected controls (time = 0 hours). However, after 24 hours extract-treatment in siRNA treated cells, the level of FOXO3a expression was not significantly greater than basal levels in non-siRNA treated MCF-7 cells, demonstrating complete abrogation of a later FOXO3a response to extract treatment. In comparison, treatment with 2 mg/ml extract alone induced a 67.0% increase in FOXO3a expression after 5 hours and a 47.0% increase after 24 hours (figure 6.4.3b). Transfection of MCF-7 cells with FOXO3 siRNA also significantly reduced basal levels of FOXO3a expression by 32.1% which was consistent with the siRNA validation performed in section 2.11 (figure 2.11.5). A similar response was obtained in siRNA transfected MDA-MB-231 cells which also demonstrated a reduction in extract induced FOXO3a expression (figure 6.4.4). To determine wether or not expression of FOXO3a was required for extract induced cytotoxicity, both cell lines were transfected with siRNA prior to extract treatment, and assessment of cell viability by MTT assay. Knockdown of extract-induced FOXO3a expression reduced the loss of cell viability in MCF-7 cells and completely abrogated cytotoxicity in MDA-MB-231 cells (figure 6.4.5 a and b).

6.4.3: Extract treatment reduces ATP levels in MCF-7 cells

FOXO3a can be activated in response to metabolic stress brought on by energy depletion. Therefore, the effect of extract treatment on intracellular ATP levels, was assessed in MCF-7 cells. Extract treatment significantly reduced ATP

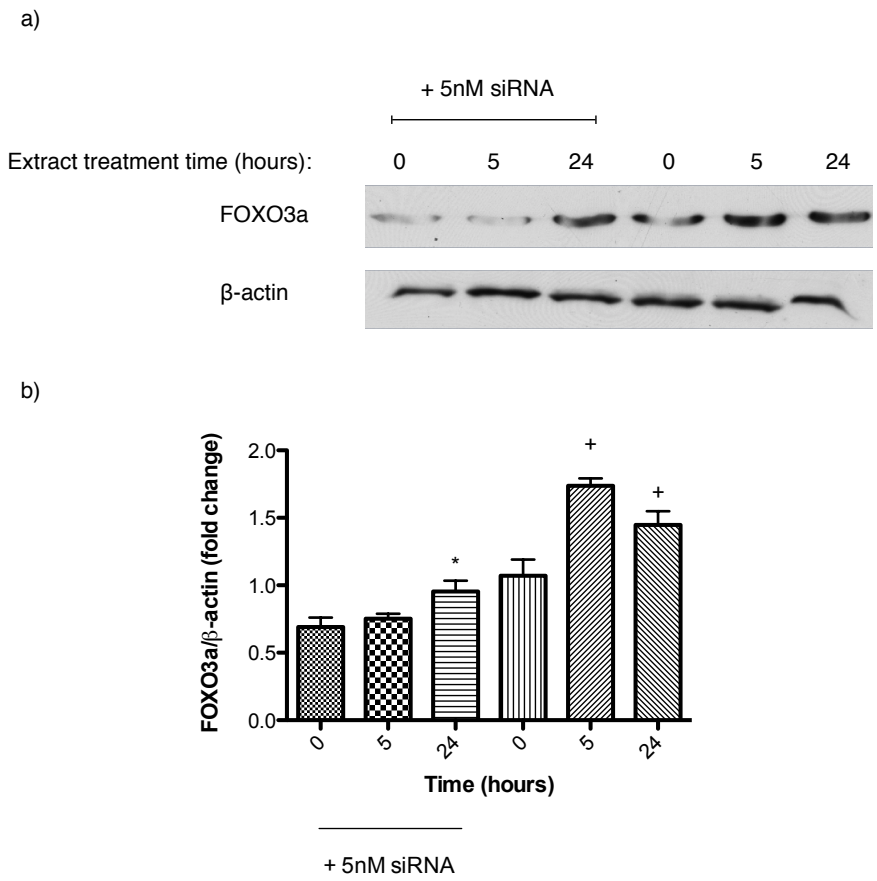


Figure 6.4.3. ***Aqueous extract-induced FOXO3a expression in MCF-7 cells is blocked with transfection of FOXO3 siRNA.*** MCF-7 cells were transfected with and without 5nM FOXO3 siRNA for 24 hours prior to 2 mg/ml aqueous extract treatment for up to 24 hours. (a) Cell lysates were collected and FOXO3a protein expression was assessed by western blot. β-actin was used as a loading control. (b) Data is expressed as a fold change in FOXO3a density normalised to β-actin. Data denoted * ($p<0.05$) is significant compared to siRNA treated control (time = 0 hours). Data denoted + ($p<0.001$) is significant compared to untreated control (time = 0 hours). All data was analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

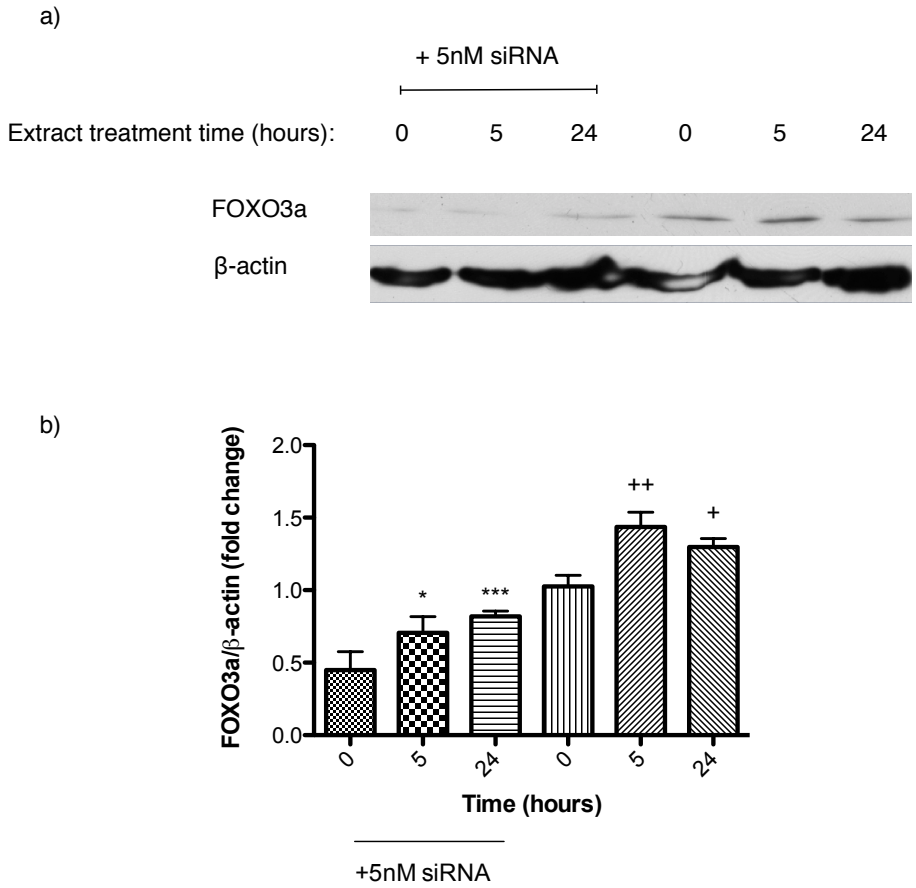
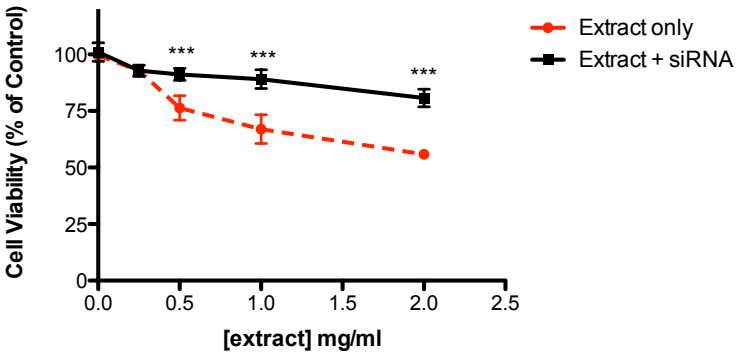


Figure 6.4.4. Aqueous *extract induced FOXO3a expression in MDA-MB-231 cells is blocked with transfection of FOXO3 siRNA.* MDA-MB-231 cells were transfected with and without 5nM FOXO3 siRNA for 24 hours prior to 2 mg/ml aqueous extract treatment for up to 24 hours. (a) Cell lysates were collected and FOXO3a protein expression was assessed by western blot. β-actin was used as a loading control. (b) Data is expressed as a fold change in FOXO3a density normalised to β-actin. Data denoted * ($p<0.05$) and *** ($p<0.01$) and *** ($p<0.001$) are significant compared to siRNA-transfected control (time = 0 hours). Data denoted + ($p<0.01$) and ++ ($p<0.001$) are significant compared to untreated control (time = 0 hours). All data was analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

a)



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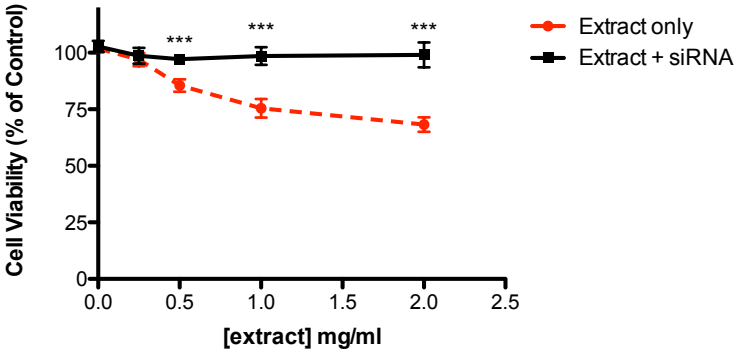


Figure 6.4.5. ***Aqueous extract-induced FOXO3a expression is required at least in part for cytotoxicity against human breast cancer cells.*** (a) MCF-7 and (b) MDA-MB-231 cells were transfected with and without 5nM FOXO3a sirNA for 24 hours prior to up to 2mg/ml aqueous extract treatment for 24 hours. Cell viability was determined by MTT assay. Data denoted *** ($p<0.001$) are significant compared to extract only analysed by two-way ANOVA with Dunnett's multiple comparison post test. Data is from three independent experiments performed in triplicate and presented as mean ± standard deviation.

levels after 3 hours treatment with 2 mg/ml extract. Furthermore, ATP levels were still depleted after 24 hours suggesting that the cells could not recover from the ATP depletion (figure 6.4.6).

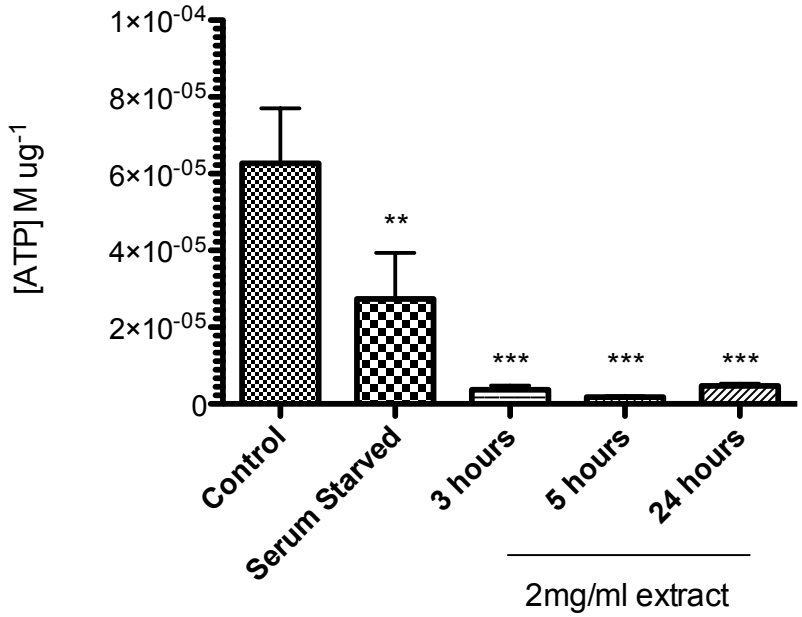


Figure 6.4.6. ***Aqueous 2xtract treatment reduces intracellular ATP levels in MCF-7 cells.*** MCF-7 cells were treated for up to 24 hours with 2mg/ml aqueous extract or serum starved for 24 hours prior to ATP extraction and analysis of ATP content using a ATP assay kit. ATP concentration was normalised to protein content determined by BCA assay and expressed as moles of ATP per µg of protein. Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is from two independent experiments performed in duplicate and presented as mean ± standard deviation.

6.4.4: Extract treatment decreases SIRT1 expression and activity in MCF-7 cells

SIRT1 is a negative regulator of FOXO3a activity via its deacetylase activity. It is also known to be over-expressed in some cancers and can inactivate proteins involved in tumour suppression. Therefore, the effects of extract treatment on SIRT1 were evaluated in MCF-7 cells. Extract treatment of 2 mg/ml reduced levels of SIRT1 protein expression in MCF-7 cells in a time-dependent manner over 24 hours (figure 6.4.7a). A significant reduction of 14.2% was seen by western blot after 1 hour treatment and by 24 hours, SIRT1 protein expression had decreased by 87.3%, compared to control (time = 0 hours) (figure 6.4.7b). The effects of extract treatment on SIRT1 activity in MCF-7 cells were determined, as a change in expressed protein does not necessarily correlate with any change in SIRT1 activity. In response to extract treatment (2 mg/ml) for 24 hours, relative SIRT1 activity was reduced by 27.4%, which could be attenuated by pre-treatment with 50 μM resveratrol (figure 6.4.8). This demonstrates that SIRT1 activity as well as protein expression is modulated by extract treatment in MCF-7 cells. SIRT1 inhibition can improve chemotherapeutic efficacy by increasing acetylation status of transcription factors involved in cell death. In fact, acetylation of p53 is crucial for its transcription independent and dependent functions, including induction of cell cycle arrest following DNA damage. Therefore, in order to test whether extract-induced SIRT1 inhibition plays a role in cytotoxicity, MCF-7 cells were pre-treated with the SIRT1 activator resveratrol for 60 minutes prior to 2 mg/ml extract treatment for 24 hours. Analysis of cell viability using the MTT assay showed that pre-treatment with resveratrol could not attenuate extract-induced cytotoxicity in MCF-7 cells (figure 6.4.9) even though it was

shown that 50μM resveratrol pre-treatment can attenuate loss of SIRT1 expression (figure 6.4.8 and 6.4.10).

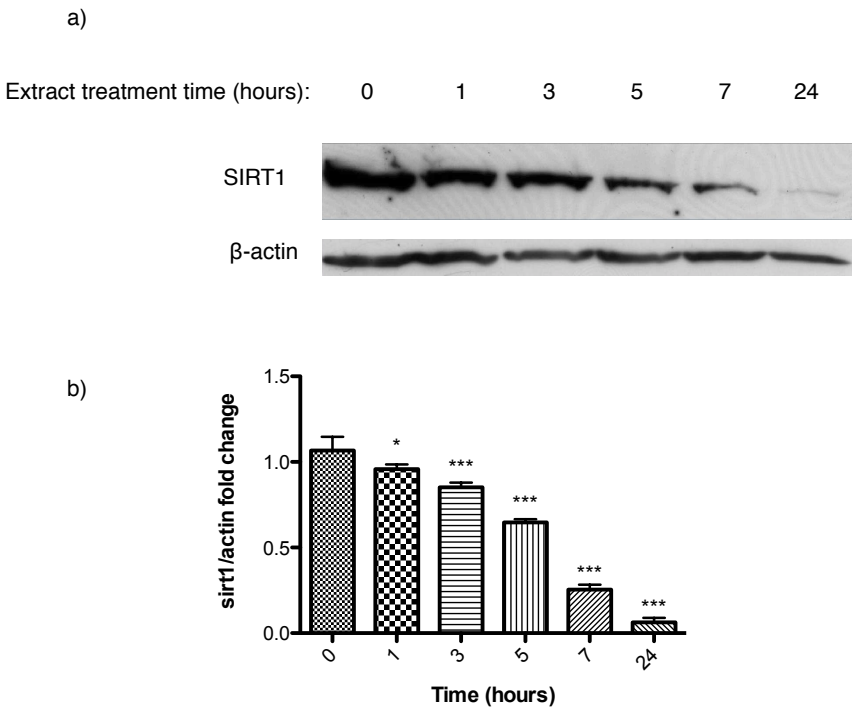


Figure 6.4.7. ***Aqueous extract treatment reduces SIRT1 protein expression in MCF-7 cells.*** (a) MCF-7 cells were treated with 2mg/ml aqueous extract for up to 24 hours before lysates were subjected to SDS-PAGE and western blot. β-actin was used as a loading control. (b) Protein expression was expressed as a fold change in SIRT1 density relative to β-actin density. Data denoted * ($p<0.05$) and *** ($p<0.001$) are significant compared to control (*time = 0 hours*) analysed by one-way ANOVA with Dunnett's multiple comparison post test Data is representative of three independent experiments and presented as mean ± standard deviation.

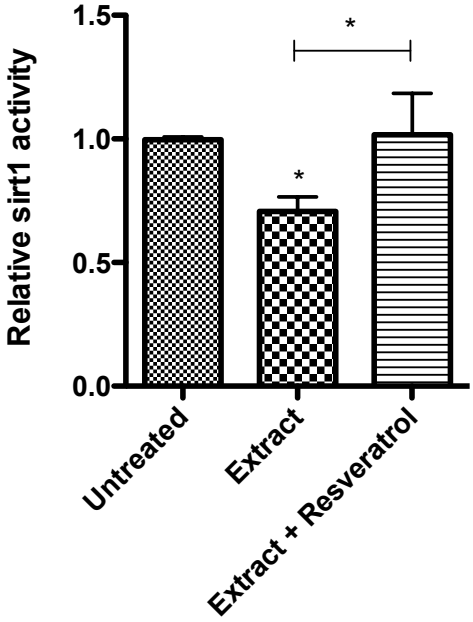


Figure 6.4.8. ***Aqueous extract treatment reduces SIRT1 activity in MCF-7 cells.*** MCF-7 cells were treated with and without 50µM resveratrol for 60 minutes prior to 2mg/ml aqueous extract treatment for 24 hours. Nuclear lysates were obtained and SIRT1 activity was determined based on relative deacetylase activity. Data denoted * ($p<0.05$) is significant compared to untreated control or as indicated analysed by one-way ANOVA with Bonferroni's multiple comparison post test. Data is from three independent experiments performed in duplicate and presented as mean ± standard deviation.

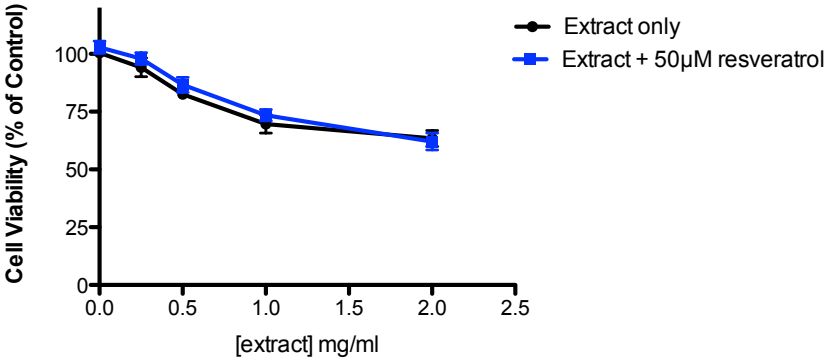


Figure 6.4.9. ***Resveratrol has no effect on aqueous extract-induced cytotoxicity in MCF-7 cells.*** MCF-7 cells were treated with and without 50µM resveratrol for 60 minutes prior to treatment of up to 2mg/ml aqueous extract for 24 hours. Cell viability was determined by MTT assay. Data is from three independent experiments performed in triplicate and presented as mean ± standard deviation.

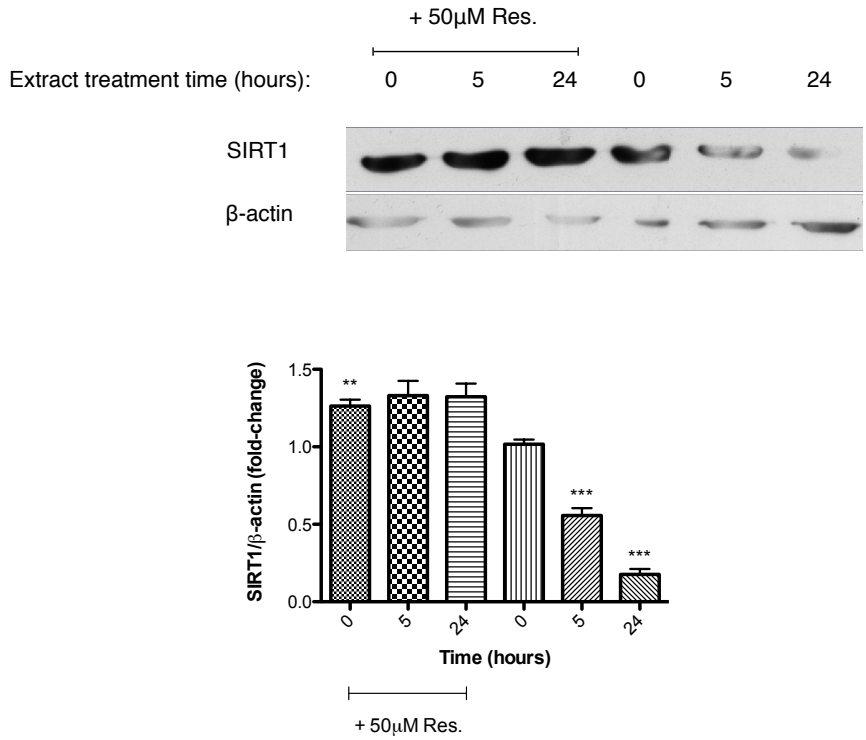


Figure 6.4.10. ***Aqueous extract induced decrease in SIRT1 expression is attenuated with resveratrol.*** MCF-7 cells were pre-treated with and without 50μM resveratrol (Res.) for 24 hours prior to 2mg/ml aqueous extract treatment for up to 24 hours. (a) Cell lysates were collected and SIRT1 protein expression was assessed by western blot. β-actin was used as a loading control. (b) Data is expressed as a fold change in SIRT1 density normalised to β-actin. Data denoted ** ($p<0.01$) and *** ($p<0.001$) are significant compared to control (time = 0 hours, no Res.). Data was analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is representative of three independent experiments and presented as mean ± standard deviation.

6.5: Discussion

Tumours carrying mutations within p53 are associated with aggressive cancer phenotypes and predict poor response to therapy due to the loss of cell death pathways in the face of genotoxic agents [98,111]. However, certain anti-cancer

drugs such as Paclitaxel, can effectively kill cancer cells harbouring a mutant-p53 phenotype [222]. It has also been shown previously that DNA damage can induce apoptosis in cancer cells irrespective of p53-status [286,369]. In chapter 5, it was shown that the cytotoxic activity of an aqueous extract of *Fagonia cretica* is not fully dependent on p53 functionality. In the current chapter it has been demonstrated that in response to extract treatment, the expression of SIRT1 and FOXO3a are decreased/increased, respectively. Furthermore, inhibition of FOXO3a with siRNA abrogated loss of cell viability in p53-deficient cells whereas inhibition of SIRT1 activity did not appear to be critical to toxicity. This provides evidence for FOXO3a-dependent cell death in response to extract treatment in human breast cancer cells.

The transcription factor FOXO3a regulates cellular responses to a variety of stressors including energy depletion, oxidative stress and genomic insult. Herein, it was shown that FOXO3a expression is increased in MCF-7 and MDA-MB-231 cells in response to extract treatment. It was described previously in chapter 4 that DNA damage-induced activation of p53 drives a cytotoxic effect in MCF-7 cells. Therefore, it can by postulated that FOXO3a activation may occur as a consequence of DNA damage, particularly as elevation of FOXO3a expression coincides with detectable DNA damage levels. Recent work by *Tsai et al.* elucidated a functional interaction of FOXO3a and ATM in response to DNA damage which suggests that ATM is directly regulated by FOXO3a [370]. It is also apparent that DNA damage induced by ionising radiation results in cell cycle arrest and apoptosis independent of p53-status [221]. FOXO3a transcriptional activity was increased by ionising radiation resulting in expression target genes including bim and Fas-L. In this chapter, abrogation of extract-induced FOXO3a expression did not fully alleviate cell death in MCF-7 cells

carrying functional p53. However, in the absence of functional p53, MDA-MB-231 cells were saved from extract-induced cytotoxicity by abrogation of FOXO3a expression. This suggests that in the presence of functional p53 in MCF-7 cells, an active DNA damage response pathway can mediate cell death. This agrees with the findings by *Stan et al.* which show that Withaferin A, a derivative from a medicinal plant, was able to decrease cell viability in MCF-7 and MDA-MB-231 cells in a FOXO3a-dependent manner that relied on induction of pro-apoptotic bim [381].

FOXO proteins are key regulators of cellular responses to metabolic stress including activation in response to energy depletion. In *C. elegans* it has been shown that dietary restriction activates AMPK which exerts changes in metabolic function in a FOXO-dependent manner [216]. Furthermore, in mammalian cells, activated AMPK directly phosphorylates FOXO3a at 6 specific regulatory sites, allowing nuclear retention and transcription of energy metabolism genes [215]. In response to double strand breaks, ATM coordinates multiple cellular responses following phosphorylation from DNA repair enzymes, which are recruited to sites of DNA damage. Subsequently, ATM activates a variety of downstream signalling molecules to elicit a response. For example, etoposide-induced DNA damage results in ATM-mediated phosphorylation of AMPK leading to mitochondrial biogenesis [382]. This provides evidence demonstrating that the DDR can challenge the cell metabolically and can induce survival responses which in part are mediated by FOXO proteins. In this chapter, increased FOXO3a expression correlated with time-points associated with DNA damage detected by comet assay and by the presence of γ-H2AX. Therefore, it is plausible that FOXO3a-mediated cell cycle arrest or apoptosis in response to extract treatment may be a direct result of DNA damage or as a consequence of

metabolic stress. It was shown in this chapter that intracellular ATP levels are depleted in MCF-7 cells in response to extract treatment. This could provide an explanation for the increased expression of FOXO3a, particularly as levels of ATP are depleted at time points coinciding with elevated FOXO3a expression.

As previously discussed, the transcription factors p53 and FOXO3a are both subjected to post-translational modifications, which are required for activity. Members of the sirtuin class of proteins are enzymes that possess deacetylase activity against histones and non-histone proteins. Sirtuin1 (SIRT1) is a class III histone-deacetylase (HDAC) that catalyses the deacetylation of acetyl-lysine residues in a NAD+ dependent manner. As well as suppressing gene expression via histone modifications, SIRT1 has catalytic activity towards p53 and FOXO3a, and inhibition of SIRT1 activity increases acetylation of both proteins [202,205]. It has been shown that over-expression of SIRT1 in *C. elegans* extends life-span dependent on the activity of the FOXO homolog DAF-16. Deacetylation of FOXO3a creates a shift in transcriptional program from cell death to cell survival that leads to increases in organismal lifespan [378]. However, it is clear that a cell which is switched to a survival program may become tumorigenic, suggesting that the role of SIRT1 in pathology is complex. It has been reported that SIRT1 represses the pro-apoptotic function of FOXO3a related to an inhibition in bim transcription [203]. On the other hand, SIRT1 over-expression has been shown to increase FOXO3-transcription of the DNA repair gene GADD45, suggesting that deacetylation of FOXO3a could contribute to tumorigenesis by inducing cell survival following DNA damage [383]. In light of these findings, the effect of extract treatment on SIRT1 was assessed in MCF-7 cells, in order to understand

any relationship between SIRT1 and treatment outcome. The results demonstrated that SIRT1 protein expression is decreased in extract-treated MCF-7 cells in a time dependent manner which was associated with a decrease in SIRT1 enzyme activity. However, because the assay used crude nuclear extracts as a purified source of SIRT1, it is difficult to ascertain if SIRT1 catalytic activity is inhibited or if the effect is due solely to reduced SIRT1 protein levels. It is known that p53 and FOXO3 regulate SIRT1 expression as part of a feedback loop that enables a self-regulated negative control [378]. Therefore, reduced SIRT1 expression in response to extract treatment, may help to sustain the tumour suppressor function of p53 and FOXO3a. This hypothesis was tested by using resveratrol to sustain SIRT1 protein expression during extract treatment of MCF-7 cells. It was shown that resveratrol treatment inhibited the extract-induced reduction in SIRT1 protein expression. However, maintaining SIRT1 levels did not block extract-induced loss of cell viability, suggesting that the reduction in SIRT1 does not affect cytotoxic activity of the extract.

6.6: Conclusion

The work described in this chapter identifies possible p53-independent mechanisms that may contribute to cell cycle arrest and death in extract-treated human breast cancer cells. The FOXO proteins, in particular FOXO3, are important in maintaining cellular homeostasis in response to nutrient deprivation, metabolic stress and oxidative stress. FOXO3 has also been implicated in DNA repair pathways. In the presence of DNA damage, FOXO3 is normally phosphorylated and retained in the nucleus where it is responsible for switching on apoptotic and DNA repair genes. Extract treatment was shown to increase FOXO3a expression and inhibition with siRNA fully abrogated extract-

induced cytotoxicity in mutant-p53 MDA-MB-231 cells. This demonstrates that in the absence of p53, extract-induced cell death, is dependent on FOXO3a. Interestingly, induction of FOXO3a expression following extract treatment coincides with detectable DNA damage, suggesting that FOXO3a may be directly increased by DNA damage. Extract treatment also reduced levels of SIRT1, a known negative regulator of FOXO3a and p53, in MCF-7 cells. However, inhibition of SIRT1 depletion by extract using resveratrol failed to attenuate extract-induced cytotoxicity, suggesting that SIRT1 may not play a role in extract activity towards breast cancer cells.

Chapter 7: Bioassay-guided fractionation of Fagonia cretica

7.1: Rationale

Many successful drugs have been derived from natural products. The rise of synthetic chemistry meant that drug discovery from natural products took a back seat to high-throughput analysis of compound libraries based on known molecular targets [384]. Recently, the pharmaceutical industry has moved back to the isolation of therapeutic compounds from natural products, due to the lack of leads from high-throughput chemistry and the successes experienced in the past with natural products [385]. One popular method of isolating compounds from natural products is known as bio-assay guided fractionation.

The aims of the work described in this chapter were to develop a method for the extraction and isolation of the compound(s) responsible for cytotoxicity in human breast cancer cells. A method involving sold-phase extraction (SPE) and high-performance liquid-chromatography (HPLC) was developed to isolate therapeutic compounds. The MTT cell viability assay was used to determine active fractions and indicated what fractions should be taken forward for further analysis. Identification and characterisation of compounds within active fractions was partially tested using electro-spray ionization mass-spectrometry (ESI-MS).

7.2: Introduction

The development of novel drugs from natural products has played a major role in past and present advancements of clinical science. Before the arrival of synthetic chemistry and high-throughput library screening, more than 80% of therapeutic compounds were of natural product origin [316]. Since 1994, almost half of all approved drugs have derived or modified structures isolated from natural products [384]. In fact, modern drug discovery is reverting to a 'back-to basics' approach due to the success seen with drug development from natural products in the past [315]. Anti-cancer drug development in particular has experienced success from natural product chemistry. For example, the widely used chemotherapeutic compounds paclitaxel, vinblastine and etoposide were all derived from plants [385].

Natural product drug discovery represents a field of research encompassing a wide range of disciplines that fall under the umbrella term pharmacognosy. It begins with identification of a potential therapeutic source, such as a plant or herb that is used in a traditional herbal remedy and preparation of extracts for screening [314]. Methodologies such as bioassay-guided fractionation are used to isolate compounds of interest from complex mixtures [333]. Bio-assay guided fractionation involves a mixture of separation and analytical techniques where fractionated material is periodically tested for activity in a bioassay and for purity by analytical methods. Initially a crude extract is produced with confirmed bioactivity such as demonstrating anti-cancer properties. This extract is then fractionated using HPLC, gel filtration or other chromatographic methods before testing isolated fractions in the same bioassay. Active fractions can then tested for purity by LC/MS. In the case of an impure fraction, further purification techniques can be used before re-testing in the

bioassay and rechecking for purity. Pure active compounds can be characterised structurally using analytical techniques such as mass-spectrometry and NMR (figure. 7.2.1) [316].

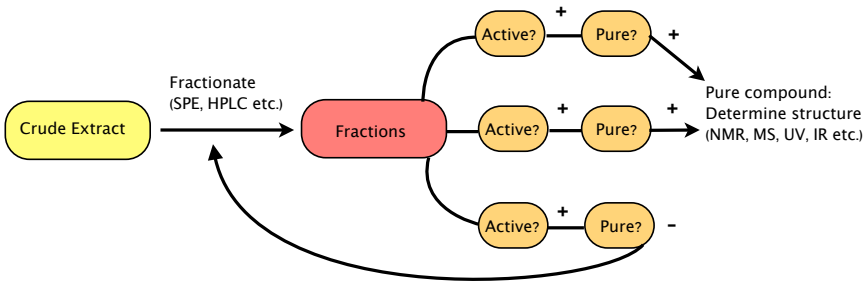


Figure 7.2.1. ***Bio-assay guided fractionation work flow.*** Adapted from *Koehn et al. 2005, the evolving role of natural products in drug discovery.* Bio-assay guided fractionation is a method for isolating and characterising bio-active natural products. Raw material is first processed and fractionated by chromatographic methods before fractions are analysed for bio-activity. Active fractions are tested for purity by liquid chromatography (LC) or mass spectrometry (MS) before structural characterisation using analytical techniques.

7.3: Methods

7.3.1: Solid-phase extraction

Solid-phase extraction (SPE) was carried out as outlined in section 2.14.2.

7.3.2: High-performance liquid chromatography

HPLC separation of extract was carried out as outlined in section 2.14.2

7.3.3: Mass spectrometry

Mass spectral (MS) analysis of HPLC fractions was carried out as outlined in section 2.14.2

7.3.4: MTT assay

Bioactivity of extract fractions generated by SPE and HPLC was determined using the MTT cell viability assay outlined in 2.5.2. Fractions were dried under vacuum and resuspended in 1ml of culture media. This was to ensure that the final test concentration of the fraction was approximately equal to the concentration that would be in 2mg/ml of whole extract (ignoring losses during the fractionation process). Elution and flow-through generated by SPE were tested neat at 1:2 and 1:4 for 24 hours. Fractions generated by HPLC were tested neat only for 24 hours.

7.4: Results

7.4.1: SPE and HPLC separation of an aqueous *Fagonia cretica* extract

Solid-phase extraction is a separation method used to isolate compounds from a mixture based on their specific chemical properties. In this investigation, a C18 matrix was used to separate compounds from an aqueous extract of *Fagonia cretica* based on their polarity. SPE was used as an initial work-up prior to higher resolution separation by HPLC. A total of 20 mg of plant material extracted into water as previously described in chapter 2 was resuspended in 0.5% v/v acetic acid (aq), separated by gravity flow and bound material was eluted with 100% methanol. Both the flow-through and elution were dried under vacuum, resuspended

in culture media and bioactivity determined by MTT assay. Treatment of MCF-7 cells for 24 hours with SPE flow-through induced no significant reduction in cell viability. In comparison, the SPE elution significantly reduced MCF-7 cell viability (20.5%) when applied as neat eluate but not at any other tested dilutions (figure 7.4.1).

The SPE elution (100% methanol) was subjected to further purification by HPLC using a C18 column. The elution was first dried under vacuum and resuspended in 500 µl of 0.5% v/v acetic acid (aq.) for injection into the HPLC system. Separation of compounds by HPLC generated the chromatogram in figure 7.4.2. Compounds detectable by UV absorbance at 254 nm were eluted in all mobile phases. Fractions were collected at 1 ml intervals and pooled according to the retention time to generate 6 fractions (table 7.4.1). Each fraction was dried under vacuum and resuspended in culture media prior to bioactivity testing using the MTT assay. Treatment of MCF-7 cells for 24 hours with any of the fractions, except for fraction 5, failed to significantly reduce cell viability (figure 7.4.3). Fraction 5 reduced MCF-7 cell viability by 40.1%, compared to untreated control, after 24 hours treatment.

7.4.2: Mass-spectral analysis of bioactive HPLC-purified fraction

The bioactive fraction was checked for total ion current (TIC) by electrospray ionisation (ESI)-MS. The bioactive fraction was eluted from the HPLC using the method described in section 2.14 in eight 1 ml fractions and each fraction was subjected to mass-spectral analysis in negative ion mode (figure 7.4.4). The major parent ion masses detected in each of the eight samples is outlined in table 7.4.2. Analysis of the mass-spectra revealed parent ions $[M-H]^-$ 367.2 and 403.2 to

be highly abundant in all eight of the fractions and $[M-H]^-$ 727.3 and 375.2 to be present in fraction 1 and 8 respectively. The high relative abundance of 367.2 and 403.3 suggests that they would both be good candidates to take forward for further bio-assay and structural analysis.

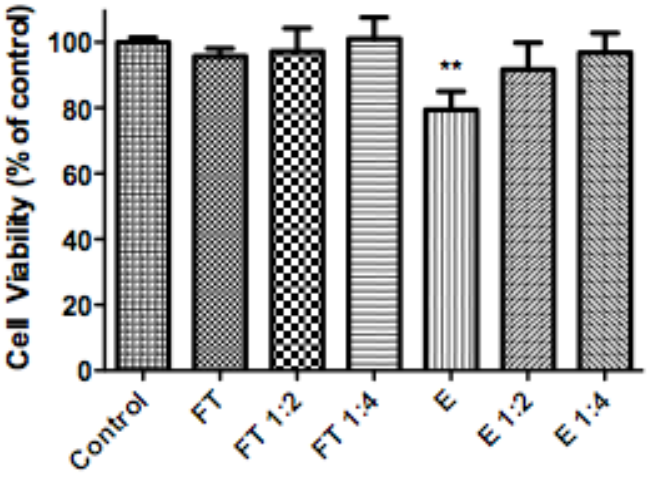


Figure 7.4.1. ***SPE elution reduces MCF-7 cell viability.*** Aqueous extract was separated by SPE on a C18 matrix by gravity flow. Compounds were eluted in 100% methanol. MCF-7 cells were treated with flow-through (FT) and elution (E) at indicated dilutions for 24 hours prior to assessment of cell viability by MTT assay. Data denoted ** ($p<0.01$) are significant compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is from three independent experiments and presented as mean ± standard deviation.

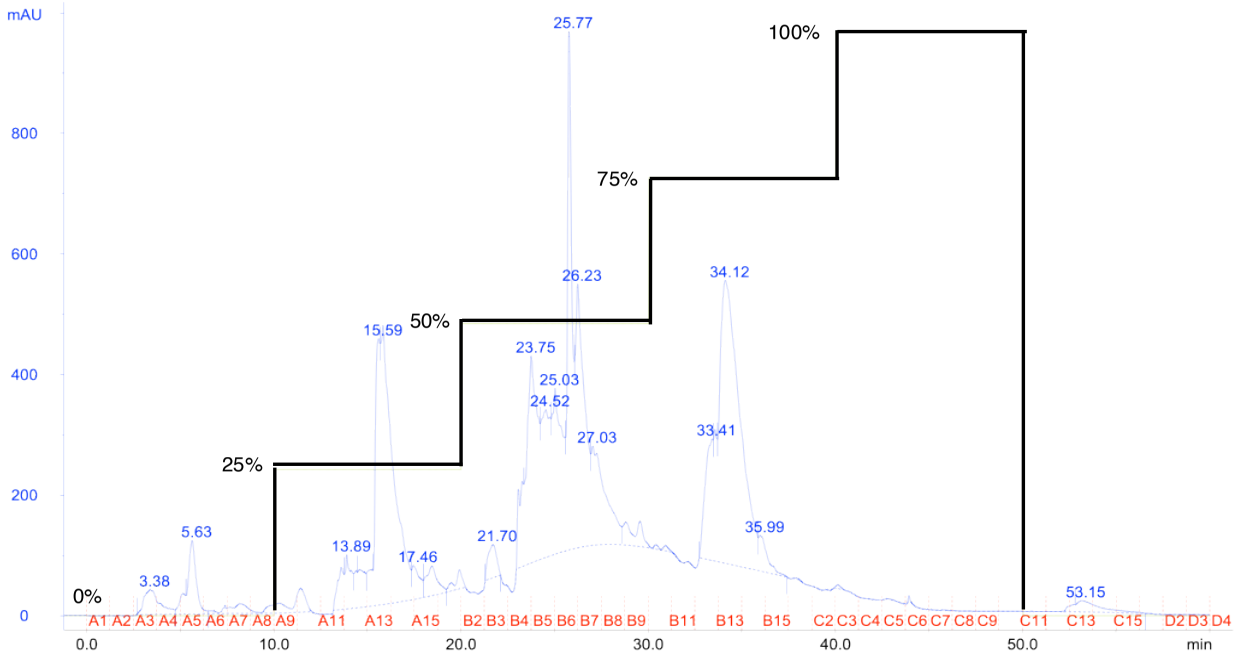


Figure 7.4.2. ***HPLC separation of an aqueous extract of Fagonia cretica.*** Following aqueous extract purification by SPE, the eluate was subjected to HPLC on a C18 column. This figure is typical of chromatograms observed using detection at 280nm. X axis - retention time, Y-axis - relative absorbance units (UV detection: 254nm). Black lines represent percentage of methanol in mobile phase.

Fraction	Retention Time (mins
1	0-8
2	8-16
3	16-24
4	24-32
5	32-40
6	40-48

Table 7.4.1. ***Retention times for pooled HPLC fractions.*** SPE-purified aqueous extract of Fagonia cretica was subjected to HPLC on a C18 column. Fractions were collected at 1ml intervals and pooled according to retention time.

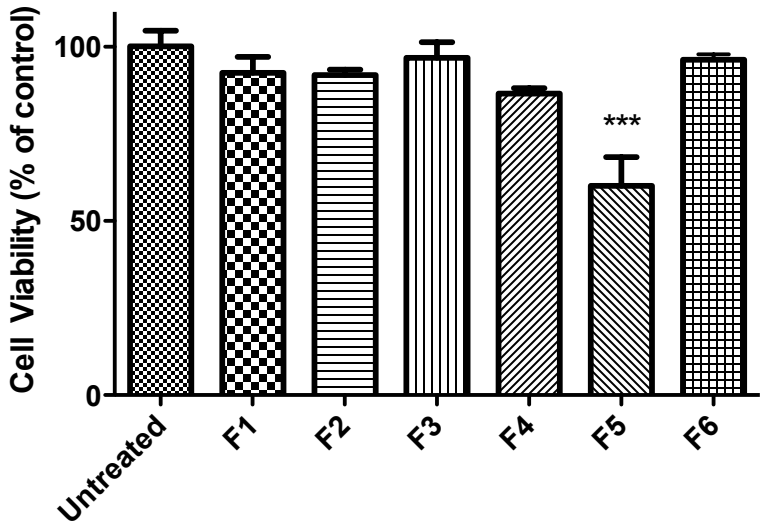
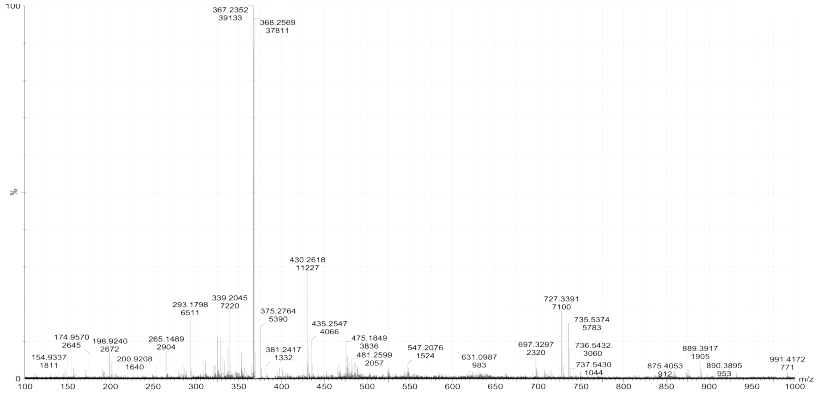


Figure 7.4.3. ***Effects of HPLC fractions on MCF-7 cell viability.*** SPE purified aqueous extract was subjected to separation by HPLC on a C18 column. MCF-7 cells were treated with isolated fractions (F1-F6) for 24 hours prior to analysis of cell viability by MTT assay. Data denoted *** ($p<0.001$) is significant compared to untreated control analysed by one-way ANOVA with Dunnett's multiple comparison post test. Data is from three independent experiments performed in triplicate and presented as mean ± standard deviation.

a)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6511
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375.2764
5390
435.2547
4066
727.3391
7100
735.5374
5783
174.9570
2645
198.9240
2672
265.1489
2904
475.1849
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381.2417
1332
547.2076
1524
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3060
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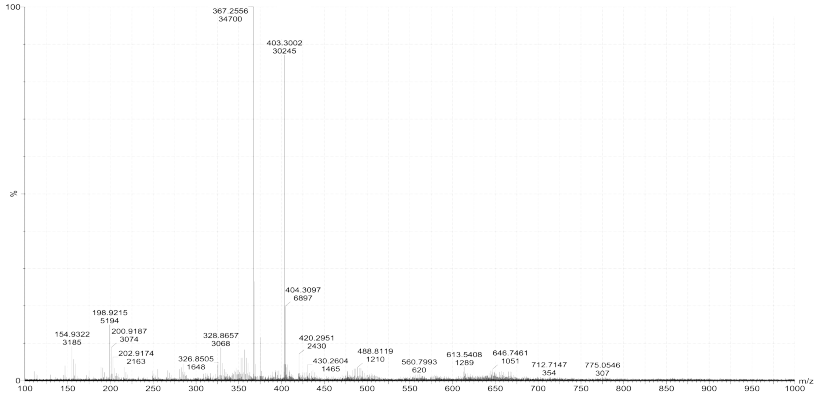
m/z

b)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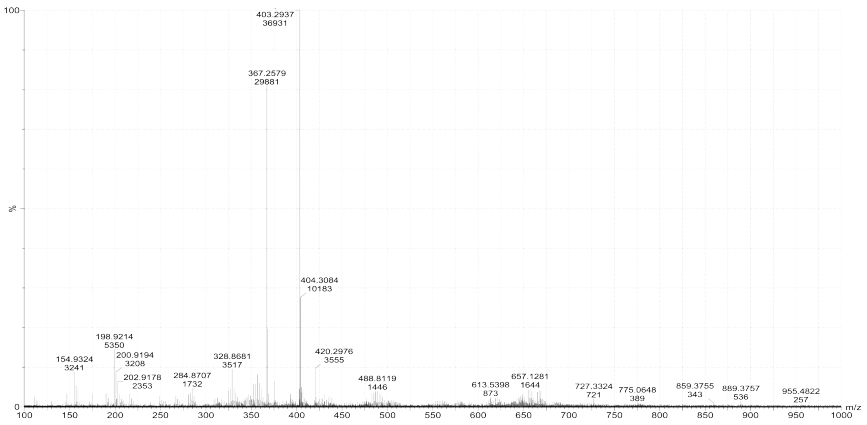
c)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3517
284.8707
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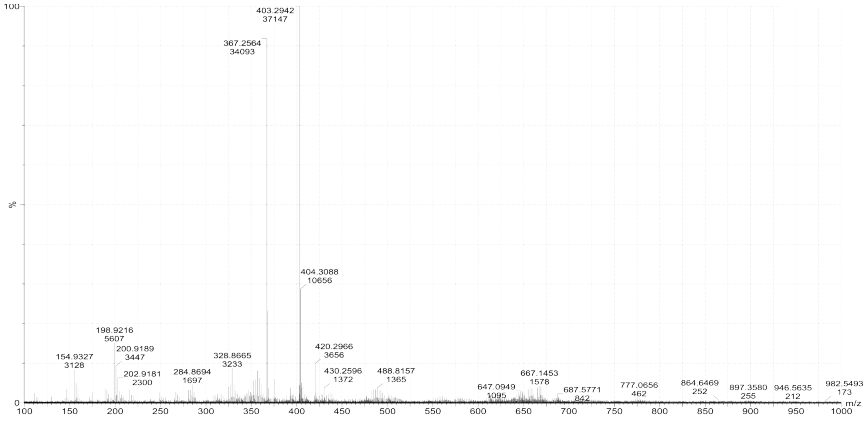
m/z

d)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202.9181
2300
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1365
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1095
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862
777.0656
462
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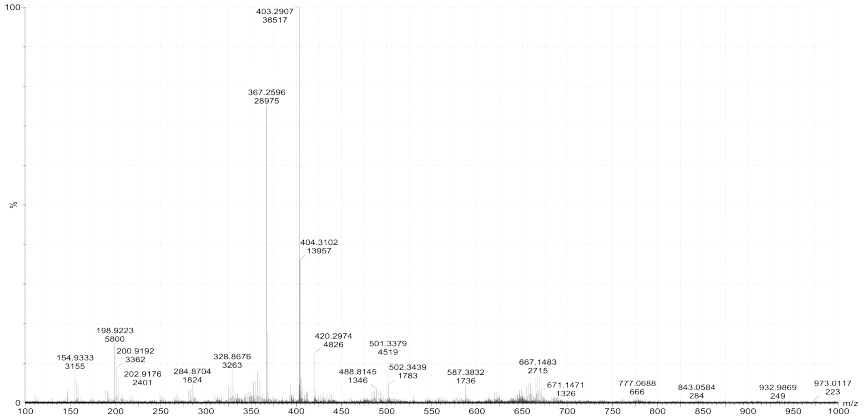
m/z

e)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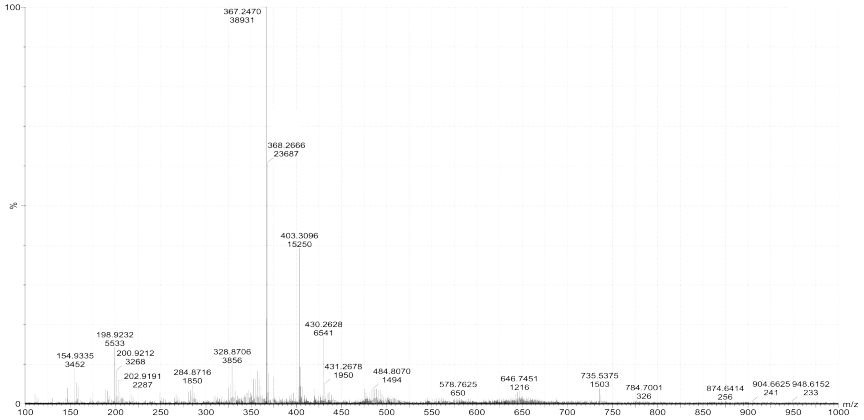
m/z

f)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202.9191
2287
284.8716
1850
328.8706
3856
431.2678
1950
484.8070
1494
578.7625
650
646.7451
1216
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1503
784.7001
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233
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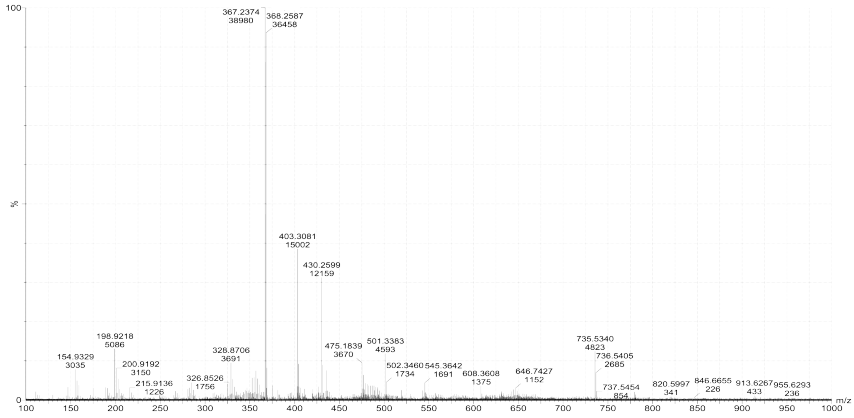
m/z

g)



h)

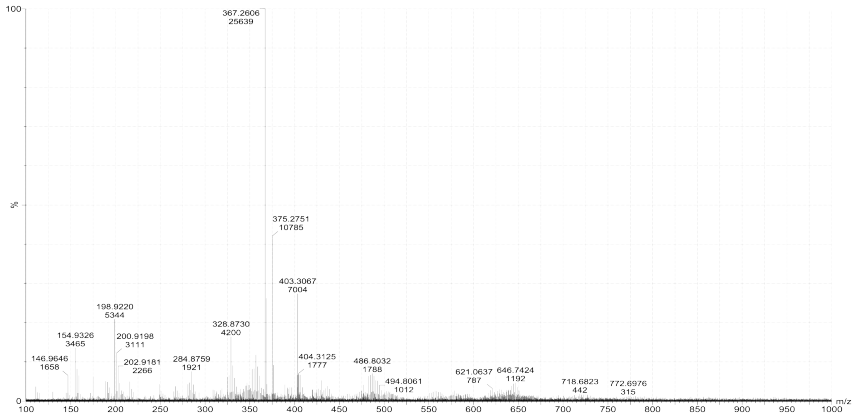


Figure 7.4.5. ***Mass spectral analysis of bioactive fraction from an aqueous extract of Fagonia cretica.*** An aquoues extract was subjected to SPE and HPLC purification to generate a bioactive fraction. The bioactive fraction was isolated as 8 x 1ml fractions(F1 (a), F2 (b), F3 (c), F4 (d), F5 (e), F6 (f), F7 (g), F8 (g)) and each fraction subjected to ESI-MS. Mass spectra were generated in negative ion mode.

Fraction	$[M-H]^-$
1	367.2, 727.3
2	367.2, 403.3
3	367.2, 403.2
4	367.2, 403.2
5	367.2, 403.2
6	367.2, 403.3, 430.2
7	367.2, 403.3, 430.2
8	367.2, 375.2, 403.3

Table 7.4.2. Charge to mass ratio of *parent ions* $[M-H]^-$ *detected by ESI-MS in bioactive fractions of Fagonia cretica.* An aquoues extract was subjected to SPE and HPLC purification to generate a bioactive fraction. The bioactive fraction was isolated as 8 x 1ml fractions and each fraction subjected to ESI-MS. Mass spectra were generated in negative ion mode.

7.5: Discussion

Bio-assay guided fractionation is a popular method for the extraction and purification of medicinal compounds from natural products. Recently, the use of natural products to source new therapeutic compounds has had a resurgence, primarily due to the clinical use of taxol, etoposide and artemisinin [316]. In this chapter, a bio-assay guided fractionation method was developed to isolate the active compound(s) in an aqueous extract of *Fagonia cretica*. A systematic approach involving purification of the extract on SPE and HPLC columns, punctuated with bioactivity testing by the MTT assay, revealed 4 potentially therapeutic compounds. These compounds with masses 368.2, 376.2,

404.3 and 728.3 were detected by ESI-MS analysis of the bioactive fraction.

Although, it is possible that just one of these compounds is responsible for the cytotoxic activity against human breast cancer cells, it cannot be disregarded that bioactivity may depend on synergistic action of multiple compounds. In fact, as previously shown in chapter 6, extract-treatment modulates SIRT1 which does not appear to contribute to cytotoxicity. Furthermore, in chapter 3 it was shown that extract-induced increases in intracellular ROS do not contribute to cytotoxicity. This demonstrates that ROS and SIRT1 modulation may be caused by a separate compounds to those which are responsible for cell death. In response to extract treatment, MCF-7 cells were subjected to DNA damage, which mediated a p53-dependent response. In MDA-MB-231 cells harbouring a non-functional form of p53, evidence of DNA damage was still apparent, and cell death was induced independently of p53. It was speculated that DNA damage may have increased FOXO3a expression leading to cell death in MDA-MB-231 cells. This would indicate that one DNA damaging agent present in the extract may be responsible for cytotoxicity in human breast cancer cell lines. However, no direct evidence has been presented that links DNA damage to both p53 and FOXO3a expression. Therefore, it is still plausible that a separate compound may be exerting an effect on FOXO3a, independent of DNA damage. It has recently been shown that troglitazone, a potent inhibitor of PPAR gamma and PI3K/AKT signalling, and aspirin, a NSAID, synergistically inhibit the growth of colon cancer cells [386]. This may in part be caused by FOXO3 activation as the authors report increased p27 expression, apoptosis and decreased PI3K/AKT signalling in response to synergistic treatment. Interestingly, is has recently been reported that a formulation of two DNA

damaging agents, doxorubicin and mitomycin C, was able to synergistically induce cell death in multi-drug resistance breast cancer cells [387].

7.6: Conclusion

In this chapter a novel method for the extraction and isolation of compounds from an aqueous extract of *Fagonia cretica* was created. The method involved bio-assay guided fractionation using a reduction in MCF-7 cell viability as a guide to nominating potential hits. It was reported that the chemotherapeutic activity of the aqueous extract can potentially be attributed to 4 compounds detected by mass-spectrometry. However, it is not known if the compounds act synergistically, or if one compound is solely responsible for extract-induced cytotoxicity.

Chapter 8: Discussion and Conclusion

8.1: Discussion

The investigation presented here aimed to (1) validate an aqueous extract of *Fagonia cretica* as a potential treatment for breast cancer based on ethno-pharmacological information, (2) characterise the mechanism responsible for any chemotherapeutic activity and (3) isolate and characterise the active compound(s) from the extract. Herein, it has been shown that an aqueous extract does demonstrate cytotoxic properties against human breast cancer cells (MCF-7 and MDA-MB-231 cells), with a markedly reduced effect against normal mammary epithelial cells (HMEpC). Furthermore, it has been shown in MCF-7 cells that extract-treatment was associated with p53-mediated cell cycle arrest and apoptosis, as a result of double strand breaks and activation of the DNA damage response (DDR). In the absence of extract-induced p53 induction or in extract-treated MDA-MB-231 cells, loss of cell viability was attenuated, but not completely abrogated. Therefore, a separate, p53-independent mechanism was sought that could drive cell death in both cell lines. Here, it has been shown that extract-induced FOXO3a expression was required for loss of cell viability in both MCF-7 and MDA-MB-231 cells. In addition to these findings, it was shown that in MCF-7 cells, expression and activity of the deacetylase SIRT1 was reduced in response to extract treatment. However, attenuation of extract-induced SIRT1 repression, did not affect cell viability in MCF-7 cells. The final section of this investigation dealt with the isolation and characterisation of the active compound(s) within the aqueous extract. In this report a novel, reproducible, bio-assay guided fractionation method was developed. Using chromatographic techniques coupled with a MTT cell viability assay, a final fraction was

produced, which revealed several compounds that may be responsible for the extracts chemotherapeutic activity. In addition to this, mass-spectral analysis determined the molecular weights of the most abundant compounds to be 368.2, 376.2, 404.3 and 728.3.

Chemotherapeutic compounds isolated from natural products are responsible for many of the clinical drugs in use today [384,385]. Compounds such as Paclitaxel are sourced directly from nature, but alternatively, many drugs are based on chemical structures derived from natural products. For example, the topoisomerase inhibitor, etoposide, is a semi-synthetic derivative of the natural product, epipodophyllotoxin [334]. Upon discovery of novel chemotherapeutic agents, it is important to understand the mechanisms by which they are active towards cells, in order to predict clinical efficacy and prepare for potential side-effects.

Modulation of the DNA damage response has been highlighted as a target for cancer therapy. DNA repair pathways exist to protect the cells from genotoxic stress that may induce tumorigenic mutations. Dysregulation in this system produces a paradox where loss of repair networks can drive tumorigenesis but also makes neoplastic tissue more susceptible to DNA damaging agents [343]. In particular, it has been shown that inhibition of the DNA repair enzyme, DNA topoisomerase II, increases the frequency of DNA double strand breaks, cell cycle arrest and apoptosis in cancer cells [388]. As well as inhibition of DNA repair pathways, direct induction of DNA damage from alkylating agents and platinum-based chemotherapy, can also target cancer cells through the DNA repair pathways [350]. For example, cyclophosphamide and etoposide, induce DNA damage-mediated activation of cell cycle arrest and apoptosis in cancer cells [50,343]. Although the DDR is

evolutionarily conserved as a barrier to cellular age-related pathology, targeting the mechanisms by which it ensures organismal survival such as DNA repair, cell cycle arrest and apoptosis can be used to treat cancers. Even in the presence of intact DNA repair pathways, sustained DNA damage eventually leads to cell death, as a means of removing potentially harmful cells from the population [284].

The work reported in this thesis has demonstrated that extract-induced DNA damage activates the DDR and mediates a p53/p21/Bax induced cell death in MCF-7 cells. Presence of the DNA damage marker, γ-H2AX, in extract-treated cells is indicative of double strand breaks (DSBs). It has been shown elsewhere that phosphorylated H2AX is essential to the recognition of DSBs [365]. It was also shown that extract-treatment most likely activated the DDR in an ATM/ATR dependent manner. The DNA-damage checkpoint kinases ATM and ATR are both activated in response to double strand breaks and both activate p53 by phosphorylation of serine-15 residue [83]. Inhibition of extract-induced ATM/ATR-mediated p53 expression using caffeine, resulted in a reduction in extract toxicity towards MCF-7 cells, suggesting that the p53-mediated DNA damage response is party involved in extract activity. Activation of this signalling pathway to induce cell death in cancer lines has been demonstrated by *Rong et al.* with the natural product gambogic acid. The authors show that gambogic acid treatment induces ATM/ATR-dependent p53 phosphorylation, via DNA damage, which results in p21-mediated cell cycle arrest [389].

The fact that extract treatment is still able to reduce MCF-7 cell viability in the absence of a p53-driven DNA damage response suggests that other mechanisms are involved in extract activity. This was confirmed by showing that extract treatment could reduce cell viability in MDA-MB-231 breast

cancer cells that harbour a non-functional p53 mutant, albeit, the effect was reduced compared to MCF-7 cells. Interestingly, the level of cytotoxicity demonstrated against MDA-MB-231 cells, was similar to the effect shown against MCF-7 cells which were transiently transfected with TP53 siRNA. This provides further evidence that the p53-mediated DDR is only part-responsible for the overall extract activity. However, this does not disregard DNA damage as the initiating step in extract-induced cell death in human breast cancer cells, as p53-independent DDR mechanisms exist that could drive similar effects [146]. DNA damage has been shown to activate the JNK pathway, resulting in expression of pro-apoptotic *hid* in Drosophila with mutant p53 [369]. As well as this, members of the Forkhead 'O' (FOXO) family of transcription factors have been shown to regulate cell survival and proliferation, in response to various stress signals [177]. It has been reported that FOXO3a directly interacts and phosphorylates ATM to activate the DDR in response to double strand breaks [370]. It has also been shown that DNA damage can induce FOXO3a-dependent DNA repair via transcription of Gadd45 [209]. Other transcriptional targets of FOXO3a including pro-apoptotic Bim and Fas-ligand and the CDk-inhibitors p21 and p27 are all inducible by DNA damage [182,221,372]. It has recently been reported that activation of FOXO3a reduces p53-transcriptional activity but increases cytoplasmic localisation and transcription-independent apoptosis [298]. Therefore a mechanism has been proposed by which FOXO3a can drive p53-dependent cell death in cells carrying a transcriptionally-inactive form of p53.

In this investigation it was shown that extract-treatment of MCF-7 and MDA-MB-231 cells increased FOXO3a protein expression. Furthermore, it was demonstrated that in the absence of p53, increased expression of FOXO3a was

required for extract-induced cytotoxicity. However, it is not clear if FOXO3a expression is being increased as a result of DNA damage, or as a result of an alternative stress response. Regulation of FOXO3a is controlled by the PI3K/Akt signalling pathway which negatively regulates FOXO3a transcriptional activity by promoting its nuclear exclusion [175]. In the presence of cytokine signalling, Akt is activated and phosphorylates FOXO3a at conserved thymidine and serine residues, which promotes binding to the adapter 14-3-3 proteins and subsequent nuclear export [374]. The chemotherapeutic agent, paclitaxel, has been shown to induce apoptosis in breast cancer cells in a FOXO3a-mediated mechanism. In fact, it was demonstrated that decreased Akt signalling in conjunction with increased JNK signalling, was responsible for nuclear translocation of FOXO3a following paclitaxel treatment [223].

Activation of FOXO3a can occur as a response to energy depletion and the AMP-activated protein kinase (AMPK)-FOXO3a axis has been implicated as a mediator of the cellular response to metabolic stress [211]. AMPK can detect fluctuations in the AMP/ATP ratio and is activated during energy depletion. It has been shown that AMPK phosphorylates FOXO3a and promotes transcription of stress resistance genes including energy metabolism genes such as aldehyde dehydrogenase and anti-oxidant genes such as thioredoxin [215,216]. Emerging research has highlighted AMPK signalling as a potential tumour suppressor by regulating metabolic homeostasis [246]. Furthermore, epidemiological studies have demonstrated a lower cancer incidence rate in diabetic patients treated with metformin, a powerful AMPK agonist [390]. It has also been shown recently, that AMPK is directly regulated by the tumour suppressor LKB1, which phosphorylates AMPK in response to ATP depletion [241]. Loss of LKB1 function has

been shown to induce cancer phenotypes in mouse models and is associated with an increased risk of developing cancer [214,243].

Cancer cells often exhibit a metabolic switch from oxidative phosphorylation to glycolysis as a major source of ATP. This phenomenon known as the Warburg effect results in 50% of cellular ATP being produced from glycolysis as opposed to only 10% in normal cells [213,227]. Interestingly, even under normal culture conditions this metabolic phenotype is sustained, suggesting a permanent alteration in the transcriptional program involved in metabolism [230]. It has recently been proposed that changes in cellular energy metabolism may be an effective target for cancer chemotherapy [229]. Indeed, a mechanism sustaining aerobic glycolysis in cancer cells was described, which involved activation of the transcription factor HIF-1 and may be reliant on over-activation of Akt signalling [231,237]. It was shown recently that inhibition of p38-MAPK can inhibit colorectal carcinogenesis by inducing a switch in transcriptional switch from HIF-1 to FOXO-dependent gene expression [218]. In the authors investigation they report that upon p38 blockade an AMPK/FOXO3a-mediated autophagic phenotype is induced as a survival response to impaired metabolism with sustained inhibition leading to cell cycle arrest and cell death. Interestingly, in the study presented here it was shown that extract-induced cell cycle arrest is delayed in the absence of functional p53. Potentially this could be a result of the cells activating an autophagic survival response before succumbing to cell cycle arrest and cell death via FOXO3a-dependent mechanisms. This may be a result of modulation of the metabolic program through the AMPK/FOXO3a axis.

Activation of the DNA damage response by ionising radiation can induce AMPK signalling, in tumour cells, independent of LKB1 [391]. It is also apparent that etoposide treatment can increase AMPK, independent of p53 status, suggesting that DNA damage induced AMPK activation may facilitate cancer cell death [392]. As well as this, extensive activation of the DNA repair enzyme, PARP-1, can induce cell death via cytosolic NAD+ depletion [393]. It is therefore possible that significant DNA damage may lead to metabolic stress. This in turn could be detected by AMPK and drive FOXO3a-dependent autophagy and cell death. Indeed, it has been shown that hyper-activation of PARP-1 can cause ATP depletion, eventually leading to irreversible energy failure and cell death [28]. As well as this, FOXO3a is known to regulate autophagy-related genes including LC3, ATG12 and BNIP3 [178].

In summary, the work presented in this investigation implicates FOXO3a as an important therapeutic target for *Fagonia cretica* treatment. In the presence of functional p53, other pathways connected to cell cycle arrest and death, are activated. However, these pathways are activated independently of FOXO3a expression, showing that they are not required for each other's activity. The broad spectrum of effects elicited by FOXO3a means that anti-cancer activity may not be the only beneficial effect of drinking a herbal tea containing *Fagonia cretica.* This may explain in part why the plant is considered useful to treat such a diverse range of illnesses and diseases in Pakistan. Alternatively, the fact that an extract would contain many diverse compounds, could provide a plethora of possible bioactive molecules effective against different cellular targets. As *in vitro* tests show, chemotherapeutic potential is independent of p53-status and presence of hormone receptors, revealing that *Fagonia cretica* may be effective against a diverse range of breast

cancer phenotypes. In particular, for treatment of the aggressive triple-negative phenotype, modelled in this investigation by the MDA-MD-231 cell line.

8.2: Conclusion

Fagonia cretica is a herbaceous plant which is commonly used in Pakistan as a treatment for a variety of ailments. For patients suffering from breast cancer, an aqueous extract in the form of a tea, is administered with the aim of inhibiting tumour growth and extending life-span. This investigation demonstrates that an aqueous extract of *Fagonia cretica* is cytotoxic towards human breast cancer cells and for the first time provides a mechanism of action. Extract treatment was able to induce a time and dose-dependent decrease in cell viability of two phenotypically different breast cancer cell lines while demonstrating a markedly decreased effect on normal mammary epithelial cells. Cell cycle arrest and apoptosis was induced in both MCF-7 and MDA-MB-231 cells. Loss of cell viability is associated with induction of DNA double strand breaks which was detected in both cell lines. In MCF-7 cells, stabilisation of p53 by ATM/ATR may have increased its transcriptional activity and induced up-regulation of the CDK inhibitor, p21, and pro-apoptotic Bax. Inhibition of extract-induced p53 expression using *TP53* siRNA reduced the cytotoxic effect against MCF-7 cells suggesting that p53 is important but not vital for extract-induced cytotoxicity. Extract treatment was also able to increase FOXO3a expression in MCF-7 and MDA-MB-231 cells. Inhibition of extract-induced FOXO3a expression using *FOXO3* siRNA reduced cytotoxicity against MCF-7 cells and completely abrogated cytotoxicity against MDA-MD-231 cells. This suggests that activation of FOXO3a in the absence of functional p53 plays a vital role in extract-induced cytotoxicity. Extract treated MCF-7 cells demonstrated a

depletion in ATP that coincided with increased FOXO3a expression and may provide an explanation for extract-induced FOXO3a expression. Figure 8.2.1 provides an overview of the proposed mechanisms governing the chemotherapeutic effects of an aqueous extract of *Fagonia cretica* against human breast cancer cells *in vitro.*

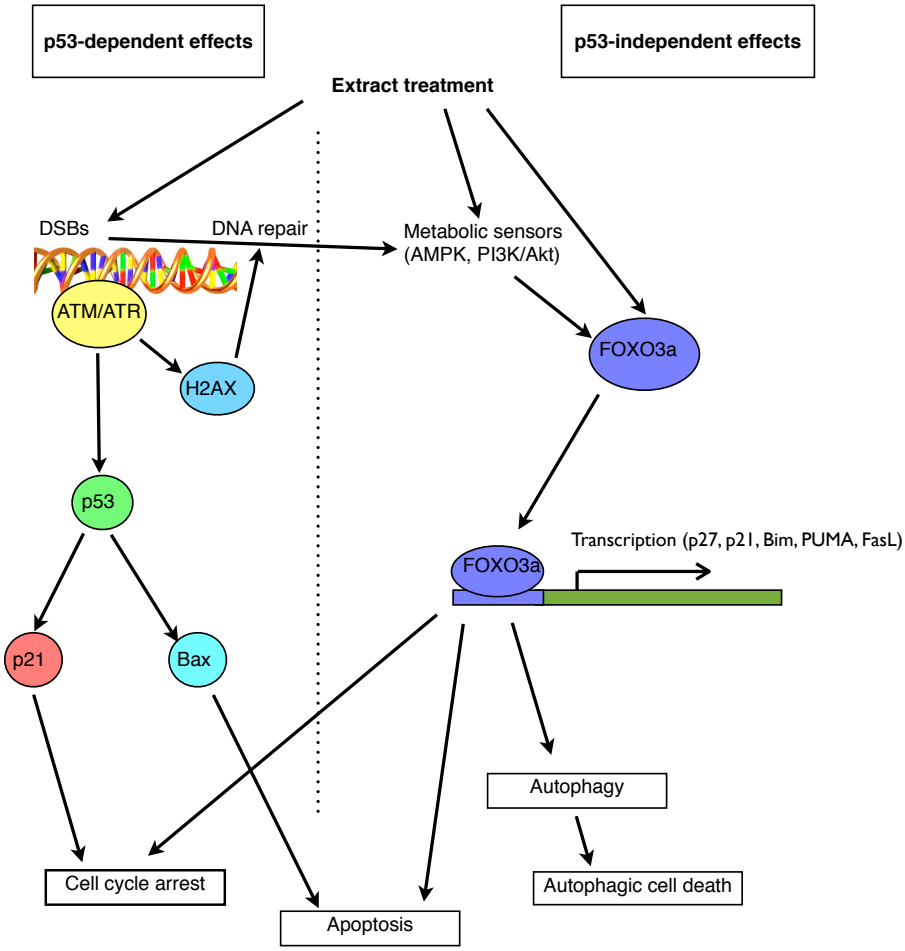


Figure 8.2.1. ***Proposed mechanisms governing the chemotherapeutic effects of Fagonia cretica.*** Extract treatment of human breast cancer cells induces double strand breaks (DSBs) to DNA associated with activation of ATM/ATR and the DNA repair protein γ-H2AX. This initiates downstream signalling of the DNA damage response involving p53, p21 and bax, which ultimately leads to cell cycle arrest and apoptosis. In the presence and absence of functional p53, extract treatment increases FOXO3a expression, which leads to cell death. This may be due to activation of metabolic sensors as a direct treatment effect or through over-activation of DNA repair mechanisms. Metabolic dysregulation could drive FOXO3a-dependent autophagy with sustained stress leading to autophagic cell death.

Chapter 9: Future Work

In this thesis the chemotherapeutic potential of an aqueous extract of *Fagonia cretica* has been well characterised. However, several key questions remain unanswered and warrant further investigation. Herein, it was shown that extract-induced FOXO3a expression is vital for activity against breast cancer cells, yet it is not clear how treatment causes this effect. It was postulated that metabolic dysregulation may be responsible and it was shown that extract-treatment reduces intracellular ATP levels. However, low experimental repeats (n=2) and issues regarding luminometer sensitivity, make the data from this experiment questionable. Therefore, a key future experiment would be to repeat this assay on a dedicated luminometer with higher sensitivity, in order to validate the results. Furthermore, it would be useful to repeat this experiment on MDA-MB-231 cells as so far it has only been carried out on MCF-7 cells. Investigating the role of AMPK and the PI3K/Akt signalling pathway would also provide further insight into the role of metabolism in extract-induced cytotoxicity. It is known that autophagic cell death mediated by FOXO3a can occur in response to prolonged metabolic stress. Investigating downstream FOXO3a targets that regulate autophagy in response to stress could provide a more detailed insight into the mechanism driving extract-induced cell death, particularly how it occurs in the absence of functional p53.

In this investigation extract toxicity profiles were developed against MCF-7 and MDA-MB-231 cells. For both these cell lines it would be useful to look at the effects of treatment combined with low-dose chemotherapy to see if it can potentiate activity. It would also be useful to look at colony forming assays to determine if clonality of viable post-treated cells is restricted. Another interesting experiment would be

to see what effects reducing treatment time or withdrawing treatment has on cell growth and viability.

It was demonstrated that extract treatment is cytotoxic towards two phenotypically different breast cancer cell lines. An interesting line of enquiry to pursue would be to screen the extract against a diverse range of cancer cell lines to determine the specificity of the treatment. It would also be useful to obtain data against human gut epithelial cells as they are at the front line of compound exposure following ingestion. Following this, the extract needs to be tested *in vivo* and on primary breast epithelial cells from a diverse range of tumours.

Human metabolism is a major influence on the therapeutic influence of natural products from dietary sources. Before an agent can enter the bloodstream and reach a therapeutic target it is first subjected to metabolic processes by the digestive system including foreign metabolism by the gut microflora and by gut epithelial cells responsible for nutrient uptake. This process can result in the modification of natural products through metabolic pathways that could effect their therapeutic potential once at their target site. This is an aspect that needs to be taken into consideration to fully understand the therapeutic potential of *Fagonia cretica.*

The key question currently surrounding this project is 'what is the active compound(s)?'. Although it was shown here that a reproducible fraction containing four major compounds can be produced by SPE and HPLC purification so far no compounds have been identified. This is due to the fact that yields after purification are too small to analyse by NMR. Further work is required to scale up the methods developed in this thesis to produce larger yields of a bioactive fraction that can be used to identify compound structures.

Identification of the key components should provide more information regarding the therapeutic targets associated with reduced breast cancer cell survival and enable effective progression of future research.

Chapter 10: References

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