FISETIN, A FLAVONOID, INDUCES CELL CYCLE ARREST AND APOPTOSIS IN HUMAN BREAST CANCER CELLS

by

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Abstract

Significant morbidity and mortality continues to be associated with breast cancer and its treatments. Fisetin, a phytochemical that is present in many fruits and vegetables, has demonstrated anticancer activity. My research explores fisetin as a possible novel therapeutic modality for breast cancer. Breast cancer cell lines (MDA-MB-468, MDA-MB-231, MCF-7, T47-D, SKBR-3; mitoxantrone-resistant (MITX) and paclitaxel-resistant (Tx400) cell lines) were exposed to fisetin and cell survival was assessed by MTT, crystal violet, acid phosphatase, and colony-forming assays. Normal cells (human mammary epithelial cells, fibroblasts, human umbilical vein endothelial cells) were used as negative controls. The mechanism of action of fisetin was explored using cell cycle analysis and assays for apoptosis/necrosis, including Annexin V-propidium iodide staining and LDH-release. Apoptosis induction pathways were studied using Western blotting, as well as caspase inhibitors and cell viability assays. Flow cytometry was used to assess mitochondrial membrane stability ($DiOC_6$ staining) and reactive oxygen species (ROS) production (dihydroethidium staining). Fisetin had a dose- and time-dependent cytotoxic effect on breast cancer cell lines (e.g., 100 μM fisetin decreased MDA-MB-468 cell number by 70% at 72h in both crystal violet and acid phosphatase assays). In contrast, the viability of normal cells was not substantially affected by concentrations of fisetin that killed breast cancer cells. Fisetin-treated breast cancer cells showed cell cycle arrest (MDA-MB-468 cells arrested at G_2/M phase; MDA-MB-231 cells arrested in S-phase) and death by apoptosis (e.g., MDA-MB-468 cells showed up to 50% apoptosis and 8% late apoptosis/necrosis by Annexin V-staining; cell cycle analysis and LDH-release assays supported these results). Fisetin-induced apoptosis was associated with mitochondrial membrane permeabilization, as well as activation of the caspase cascade since the pro-apoptotic effect of fisetin was reduced in the presence of a pan-caspase inhibitor. In addition, fisetin did not cause ROS production in MDA-MB-468 or 231 cells, ruling out a role for ROS in fisetin-mediated cytotoxicity. My findings suggest that fisetin may be useful in the treatment of breast cancer.

List of Abbreviations Used

Ab	Antibody
ABC	ATP-binding cassette
AJCC	American Joint Committee on Cancer
AIF	Apoptosis-inducing factor
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor 1
ATF6	Activating transcription factor 6
ATP	Adenosine Triphosphate
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
Bcl-X_L	B-cell lymphoma-extra large
Bim	Bcl-2-like protein 11
Boc-D-fmk	BOC-Asp(OMe)-FMK Caspase Inhibitor
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BCRP	Breast cancer resistance protein
C	Cytokinesis checkpoint
°C	Degrees Celsius
CAD	Caspase-Activated Deoxyribonuclease
$CaCl_2$	Calcium chloride
CAM	Complementary and alternative medicine
CAK	CDK activating enzymes
CARD	Caspase recruitment domain
Caspase	Cysteine aspartate-specific protease
CD95 (APO-1/Fas)	Cluster of Differentiation 95
CDK	Cyclin-dependent kinases
CDMEM	Complete Dulbecco's Modified Eagle's Medium
CKI	Cyclin-dependent kinase inhibitor
CMF	Cyclophosphamide, methotrexate, 5-FU
CO	Carbon Dioxide
Cu	Copper
DD	Death domain
ddH_20	Double distilled water
DFF	DNA fragmentation factor
DHE	Dihydroethidium
DHFR	Dihydrofolate reductase
DIABLO	Direct IAP binding protein with low PI
DiOC6	3,3'-dihexyloxacarbocyanine iodide
DISC	Death-inducing signaling complex
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DR	Death receptor pathway

dTMP	Deoxythimidine monophosphate
dUMP	Deoxyuridine monophosphate
DTT	Dithiothreitol
DVT	Deep vein thrombosis
EC_{50}	Half Maximal Effective Concentration
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic Reticulum
ER+	Estrogen receptor
FADD	Fas-associated death domain
rhFGF	Recombinant human Fibroblast Growth Factor
FBS	Fetal bovine serum
FCC	Fibrocystic changes
Fe	Iron
FGM-2	Fibroblast growth medium-2
FL2	Fluorescence label 2
FNA	Fine needle aspiration
5-FU	5-Fluorouracil
5-FdUMP	5-fluoro-deoxyuridine monophosphate
g	Gravity
G_0	Quiescent Phase
G_1	Gap Phase 1
G_2	Gap Phase 2
GSH	Glutathione Reduced
Gy	Gray
H_2O_2	Hydrogen peroxide
HEPES	5 mM N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid
OH	Hydroxyl radical
h	Hours
HER-2	Herceptin
HMEC	Human mammary epithelial cells
HUVEC	Human umbilical vein epithelial cells
HRP	Horseradish Peroxidase
HRT	Hormone replacement therapy
ICAD	CADs inhibitor
IgG	Immunoglobulin G
IL-1β	Interleukin 1β
INK4	Inhibitor of Cdk4
IRE1	Inositol-requiring 1 protein
kDa	Kilodalton
J	Joules
Kcl	Potassium chloride
kg	kilogram
Kip	Kinase Inhibitor Protein
LA/N	Late apoptosis/necrosis
LCIS	Lobular carcinoma in situ

LDH	Lactate dehydrogenase
LD50	Lethal Dose 50
LHRH	Lutenizing hormone releasing hormone
M	Mitotic Phase
MAb	Monoclonal antibody
MDM2	murine double minute2
MDR	Multiple drug resistance
mg	milligram
$MgSO_4$	Magnesium sulphate
min	minute
MITX	mitoxantrone
ml	milliliter
mm^2	milimeter squared
mM	millimolar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MRI	Magnetic resonance imaging
2N	Diploid
4N	Tetraploid
NAC	N-acetylcysteine
NaCl	Sodium chloride
NaF	Sodium fluoride
NaOH	Sodium hydroxide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
Na_3VO_4	Sodium orthovanadate
nm	nanometer
nM	nanomolar
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NS	Non-significant
OCP	Oral contraceptive pill
$O_2^{\cdot -}$	Superoxide anion
4-OH	4 hydroperoxy cyclophosphamide
OOR	Peroxy radical
p21	21 kDa Cyclin-dependent kinase inhibitor
p27	27 kDa Cyclin-dependent kinase inhibitor
p53	53 kDa tumour suppressor protein
PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate buffered saline
PERK	Double-strand RNA-activated protein-kinase like ER-kinase
PE	Pulmonary embolus
p-Gp	P glycoprotein
PFA	Paraformaldehyde

pH	Power of hydrogen (measure of acidity)
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PMSF	Phenylmethyl sulfonyl fluoride
PR	Progesterone receptor
pRb	Phosphorylated Retinoblastoma Protein
PS	Phosphotidylserine
PTEN	Phosphatase and tensin homolog
PTU	Propylenethiourea
R	Restriction Point
Rb	Retinoblastoma Protein
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
S	Synthesis Phase
SEM	Stand Error of the Mean
SERM	Selective estrogen receptor modulator
SD	Standard Deviation
SDS	Sodium dodecylsulphate
Smac	Second mitochondria-derived activator of caspases
SubG1	Sub Gap 1 phase
TMN	Tumor, metastasis, node staging
Tris-HCl	Tris-Hydrochloric acid
Triton-X 100	Octylphenolpoly(ethyleneglycolether)$_x$
TrypLE	Phenol red negative trypsin replacement
T-TBS	Tween Tris-buffered saline
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUPT-biotin nick end labeling
µg	Microgram
µL	Microlitre
µm	Micron
µM	Micromolar
UPR	Unfolded Protein Response
Z-VAD-FMK	Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone

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Chapter 1.0 Introduction

1.1 Cancer

Cancer is the loss of normal cellular control that leads to unregulated cell growth, and cells having the ability to spread beyond their point of origin (Gabriel 2007; NIH 2011). This uncontrolled cellular expansion kills patients by invading and destroying normal cell tissue and function (Evan and Vousden 2001). Current data suggest there are over one hundred different types of cancer (NIH 2011). In 2000, Hanahan and Weinberg described several "hallmarks" of this disease, which include the cells' ability to sustain an abnormal proliferative signal, to evade growth suppressors and to resist cell death. In addition, cancers possess unlimited replicative potential, are able to induce angiogenesis and activate tissue invasion and distant organ metastasis (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Since that original description, two additional hallmarks have been added: the ability to reprogram energy metabolism and the capacity to evade destruction by the immune system (Hanahan and Weinberg 2011). Understanding the biopathophysiology of these features is paramount in attempting to develop treatments for this disease.

1.2 Breast Cancer

1.2.1 Epidemiology and Risk Factors

Breast cancer is the most common type of cancer diagnosed in women (Parkin, Bray et al. 2001; CCS 2011). Breast cancer is the leading cause of cancer death in 20-59 year olds (Jemal, Siegal et al. 2007) and the second leading cause of cancer death in women overall (CCS 2011). In Canada, 1 in 9 women will be diagnosed with breast

cancer and 1 in 29 of these women, despite advances in diagnosis and treatment, will die from their disease (CCS 2011). While the incidence of breast cancer has increased steadily between 1978 and 1999, recent reports indicate that the incidence has stabilized or even decreased in the last decade (CCS 2007) and the mortality rate is the lowest documented since the 1950s (Gaudette, Gao et al. 1997). These trends are promising, however, there remains significant morbidity and mortality associated with both the disease and its treatments.

Multiple risk factors have been identified in large population-based studies and through studying special populations with increased incidence of breast cancer. Age is the risk factor with the highest correlation for the development of breast cancer (Townsend, Beauchamp et al. 2008). The risk for women under age 30 of developing breast cancer is 2%, but the incidence doubles every decade until menopause (McPherson, Steel et al. 2000; CCS 2011), with the majority of breast cancers occurring between the ages of 50 and 69 years of age (CCS 2011). Sex is another significant risk factor, in that the overwhelming majority of breast cancer occurs in females, with only 1% of all breast cancers diagnosed in males (Townsend, Beauchamp et al. 2008).

Geographic location seems to play a role in breast cancer etiology, as the incidence of breast cancer in Western countries is five times higher than in other countries, although this difference is diminishing (McPherson, Steel et al. 2000). Radiotherapy may also increase risk, as radiation treatment for other medical conditions such as lymphoma has been found to predispose to the development of breast cancer (Goss and Sierra 1998; De Bruin, Sparidans et al. 2009) This has not been found to translate into an increased risk in the contralateral breast when receiving radiation therapy

for breast cancer. However, these patients already have a significantly increased risk for the development of a second breast cancer, simply by having had a previous diagnosis of breast cancer (Boice, Harvey et al. 1992).

Hormone exposure, be it endogenous or exogenous, represents another risk factor. Lifetime exposure to estrogen and progesterone confers an increased risk for developing breast cancer, as do early menarche, nulliparty, late age at first conception (over 30 years) and delayed menopause (McPherson, Steel et al. 2000; Townsend, Beauchamp et al. 2008). Furthermore, there is a link between breast cancer and exogenous sources of estrogen and progesterone like hormone replacement therapy (HRT) (McPherson, Steel et al. 2000). It must be emphasized that no increased risk has been associated with the the use of oral contraceptive pills (OCP) or with estrogen-only HRT (McPherson, Steel et al. 2000).

The Gail model for stratification of breast cancer risk is used to facilitate clinical decision-making about the appropriate time to initiate screening and preventative measures across varying demographics; it was developed in 1989 and incorporates the risk factors outlined above (Gail, Brinton et al. 1989; Costantino, Gail et al. 1999; Gail and Costantino 2001). Genetic mutations, discussed below, are not currently incorporated in the Gail model; however, they can aid when it comes to decision-making, especially for whether or not genetic testing should be offered (Hampel, Sweet et al. 2004).

Breast cancer demonstrates sporadic and inheritable patterns of disease. For example, 10% to 20% of individuals diagnosed with breast cancer have a positive family history (McPherson, Steel et al. 2000; Box and Russell 2004). The correlation is strongest

when cancer occurs in a first degree relative diagnosed under the age of 40 and/or having bilateral disease (Townsend, Beauchamp et al. 2008).

Mutations in the Breast Cancer 1 and 2 (BRCA-1, -2 respectively) genes, p53 gene, PTEN and CHEK-2 genes confer a marked increase in risk for developing breast cancer (Turnbull and Rahman 2008). BRCA1 and BRCA2 are tumour suppressor genes that account for approximately 70% of known genetic mutations leading to breast cancer (Box and Russell 2004; Townsend, Beauchamp et al. 2008), with the lifetime risk associated with the BRCA1 gene mutation being as high as 50-85% in some studies (Struewing, Hartge et al. 1997; Box and Russell 2004). Not only do these mutations carry the risk for breast cancer, but they predispose to the development of other forms of fatal malignancy such as ovarian cancer, which in the case of BRCA1 mutations has a lifetime prevalence of 15-45% (Struewing, Hartge et al. 1997; Box and Russell 2004).

1.2.2 Diagnosis, Pathology and Staging

The diagnosis of breast cancer generally occurs when patients present to their physician complaining of symptoms such as a palpable lump or nipple retraction (Townsend, Beauchamp et al. 2008). Other times diagnosis is made following the identification of a lesion on routine radiological screening (Pruthi 2001; Townsend, Beauchamp et al. 2008). With increased public awareness and widespread availability of screening tools, one study demonstrated that 70.1% of women over the age of 40 in the United States undergo mammography every two years, making this a common method of detection (Swan, Breen et al. 2003; Esserman, Shieh et al. 2009). Although in Nova Scotia the target screening rate is great than 70%, in 2008-2009 the percentage achieved

was 57.6% (Health 2010). The imaging modalities used to evaluate a breast mass include mammography, ultrasound and magnetic resonance imaging (MRI) (Pruthi 2001).

Following identification of a breast mass, tissue examination is required for diagnosis. This can be accomplished by fine needle aspirate (FNA), core needle biopsy (with or without imaging localization) or excisional biopsy (Pruthi 2001; Townsend, Beauchamp et al. 2008). The information that can be obtained as well as the sensitivity and specificity vary by method. All three procedures allow identification of malignant cells and the determination of receptor status (i.e. estrogen, progesterone and HER-2), but only core and excisional biopsies provide information about invasiveness of the tumour (Townsend, Beauchamp et al. 2008). If the lesion is malignant, patients require further investigation to rule out concurrent bilateral disease and/or metastatic disease. This workup includes chest radiographs, bilateral mammography, complete blood count and liver panel. Other investigations should be pursued as symptoms evolve. For example, a bone scan should be performed to look for bone metastasis if the patient describes bony pain. Similarly, spinal cord imaging should be obtained in the setting of paralysis or paresthesias (Townsend, Beauchamp et al. 2008).

Ductal carcinoma is the most common form of breast cancer, representing 70-80%, of cases (Townsend, Beauchamp et al. 2008), followed by lobular carcinoma representing 10% of cases. The remainder consist of a variety of less common types of breast cancer (Townsend, Beauchamp et al. 2008). Of importance in this "remainder group" is the rare inflammatory carcinoma, as it carries with it the poorest prognosis for survival (AJCC 2010).

Following diagnosis, the cancer is staged. Staging can only be completed after imaging and surgical resection are complete (Townsend, Beauchamp et al. 2008). The American Joint Committee on Cancer (AJCC) provides the most widely used staging system for breast cancer today, what is referred to as the "TNM" (tumour, nodal status and metastasis) staging system. This system is based on tumour size, extension into other tissue, and whether or not the lesion is an inflammatory carcinoma. Nodal status is determined based on the number and location of histologically positive lymph nodes. The final category is distant metastases, which may be either microscopic circulating tumour cells or distant metastases (AJCC 2010). Staging using this method allows physicians to provide appropriate therapy and establish prognosis. It is also used in research and allows comparison among similar groups when monitoring for response to treatment.

1.2.3 Breast Cancer Treatment

As is the case for most maladies, treatment of breast cancer has been constantly evolving to keep up with vastly improved understanding of the disease at a cellular level. The two basic tenets of therapy are (i) local and (ii) systemic. Local (or regional) treatment involves disease control with surgery and radiation, whereas systemic therapy utilizes chemotherapy, hormone therapy and molecularly targeted therapy (Box and Russell 2004; CCS 2007; Townsend, Beauchamp et al. 2008). Treatments are available for the complications of breast cancer and the side effects of its treatment; however, these topics lie outside the scope of this paper. The following sections will give an overview of the approach to the treatment of breast cancer, followed by a discussion of the biological mechanisms of chemotherapy, hormone therapy and molecularly targeted therapy.

1.2.3.1 Surgery

Surgery is a critical intervention when breast cancer is localized. An example of tsurgical intervention is radical mastectomy described by Halstead in 1890, in which he removed the entire breast, skin, pectoralis muscles and all associated lymph nodes from the axillary vein to the costoclavicular ligament (Townsend, Beauchamp et al. 2008). This was the first intervention that demonstrated benefit in the survival of breast cancer, but also had significant morbidity (e.g., lymphoedema resulting from the extensive node dissection), and patients still died from metastatic disease at an unchanged rate (Townsend, Beauchamp et al. 2008). Attempts at even more radical operations with en bloc resections did not improve survival. More recent approaches have attempted to maximize survival benefit yet minimize complications. This has led to modified radical mastectomy and breast conserving surgery (Townsend, Beauchamp et al. 2008).

Studies have demonstrated that there is no survival advantage to modified radical mastectomy versus lumpectomy plus radiation, and although local control was better with the more invasive procedures, local recurrence post-lumpectomy can be managed with mastectomy plus delayed nodal dissection (Fisher, Anderson et al. 2002; Veronesi, Cascinelli et al. 2002; Clarke, Collins et al. 2005; Townsend, Beauchamp et al. 2008). Careful selection of patients undergoing breast-conserving therapy versus mastectomy is necessary to maximize survival benefit.

The extent of nodal dissection has also been reevaluated. Lymph nodes are an important indicator for prognosis (based on location and number affected) and therefore, “nodal status” aids in the development of a treatment plan. Positive nodal status requires

more aggressive systemic and local therapy (Townsend, Beauchamp et al. 2008). Given the significant morbidity associated with nodal dissection, preoperative information about node positivity and extent of disease can be acquired by "sentinel" lymph node biopsy in patients who do not have clinically positive nodes, locally advanced or inflammatory breast cancer (Lyman, Giuliano et al. 2005). A sentinel lymph node is the first node or nodes receiving lymphatic drainage from the tumour site and is the most likely location of metastasis, if present. The identification of these nodes is completed by preoperative or intraoperative lymphatic mapping (Camp, Feezor et al. 2005).

1.2.3.2 Radiation

X-rays and gamma (γ) -rays are part of the electromagnetic spectrum and are moving packets of energy called photons. Ionizing radiation is the term used for the process of photons displacing electrons and, in turn, inducing a positive charge (Tannock, Hill et al. 2005). This is accomplished in modern radiation therapy with the use of linear accelerators, $Cobalt^{60}$, $Cesium^{137}$ or charged particle accelerators (Tannock, Hill et al. 2005). Radiation is measured by Gray (Gy), which is the amount of energy (joules [J]) absorbed per unit of mass (kilogram [kg]); 1 Gy equals 1 J/kg (Tannock, Hill et al. 2005). The mechanism of action is either direct, via reactive free electrons on deoxyribonucleic acid (DNA), or indirect by interaction with water molecules as an intermediate free radical close to DNA. Ionizing radiation causes damage not only to DNA but also ribonucleic acid (RNA), protein synthesis, respiration and/or metabolism (Tannock, Hill et al. 2005). This damage can cause cell death.

Radiation therapy is used both for cure and for the palliative treatment of breast cancer. Radiation is necessary for local control when using wide local excision as opposed to mastectomy, in order to minimize local recurrence. The radiation protocols that are associated with wide local excision (lumpectomy) involve whole breast irradiation for a total dose between 4500 and 5000 cGy given as approximately 200cGy per fraction with an initial tumour bed "boost" of 1000 to 1200 cGy (Townsend, Beauchamp et al. 2008). Radiation is given over several weeks with the most common fraction of 200 cGy (Whelan, MacKenzie et al. 2002).

1.2.3.3 Systemic Therapy

Systemic therapy has become the major means of increasing survival in breast cancer patients. Prior to the use of adjuvant systemic therapy, the 10-year survival rate was 65% for node negative disease, 40% for 1 to 3 positive nodes and 15% for greater than 4 positive nodes (Box and Russell 2004). The primary cause of death was, and still is, metastatic disease (Townsend, Beauchamp et al. 2008). Systemic therapy includes chemotoxic medications (chemotherapy), hormone therapy and molecularly targeted therapy. In 2005, there were 45 cytotoxic drugs and biologically active agents (excluding hormones) licensed in North America for use in the treatment of all cancer (Tannock, Hill et al. 2005). The goal of systemic therapy is to prolong survival, control symptoms and/or improve the quality of life while minimizing treatment toxicity.

The categories of chemotherapeutic agents used in cancer care include the anthracyclines (eg. doxorubicin or epirubicin) and anthracendione (eg. mitoxantrone); antimetabolites (eg. methotrexate, 5-Fluorouracil (5-FU)); alkylating agents (eg. cyclophosphamide); taxanes (eg. docetaxel and paclitaxel); platinum-based drugs (eg.

cisplatin); topoisomerase-II inhibitors (eg. etoposide) (Stockler, Wilcken et al. 2000). The most widely used protocols for breast cancer treatment today employ anthracycline-based therapies such as FAC/CAF (5-FU; doxorubicin (also known as adriamycin); cyclophosphamide) or AC plus a taxane (doxorubicin; cyclophosphamide; docetaxel/paclitaxel) but others like CMF have been used in the past (cyclophosphamide; methotrexate; 5-FU) (Stockler, Wilcken et al. 2000; Box and Russell 2004). This is only a general overview of the current protocols as there is constant evaluation of combinations, timing and the addition of new agents as they become available.

Prior to the description of individual compounds, the issue of multidrug resistance (MDR) is important to review here. Resistance in cancer cells to toxic agents occurs by several mechanisms including decreased uptake, increased efflux of drugs or drug metabolites from the cell, reduced drug activation or increased drug inactivation, and increased repair of or increased tolerance to DNA damage (Tannock, Hill et al. 2005). Exposure to one agent can confer resistance to other compounds (Sparreboom, Danesi et al. 2003). Adenosine-triphosphate (ATP)-binding cassette (ABC) transporters move compounds from the cytoplasm out of the cell as an efflux pump or into intracellular organelles (Sparreboom, Danesi et al. 2003; Tannock, Hill et al. 2005). Although there are over 50 identified proteins in this family, the two of interest for this research are p-glycoprotein (p-Gp) and breast cancer resistant protein (BCRP) (Sparreboom, Danesi et al. 2003; Tannock, Hill et al. 2005). Both p-Gp (Bao, Haque et al. 2011) and BCRP (Doyle, Yang et al. 1998) have been identified in breast cancer cells.

Doxorubicin is an analog of the original anthyacycline, danorubicin, but has greater activity against solid tumours (Tannock, Hill et al. 2005). Doxorubicin exhibits its

cytotoxic effects through DNA intercalation (Momparler, Karon et al. 1976), inhibition of topoisomerase II function an enzyme that creates double stranded cuts in the DNA helix to decrease supercoiling and allow DNA manipulation (Tewey, Rowe et al. 1984; George, Ghate et al. 1992), formation of free radicals that have unpaired electrons (Xu, Tang et al. 2001) and effects on the cellular membrane (Siegfried, Kennedy et al. 1983; Tannock, Hill et al. 2005).

DNA topoisomerases are required for DNA replication, transcription, recombination and chromatin remodeling since they create single and double strand breaks in DNA (Champoux 2001). This interaction allows proteins to access DNA for these processes, as well as inhibiting supercoiling that would cause damage (Champoux 2001). The main side-effects of topoisomerase inhibitors are those commonly seen with most chemotherapeutic agents, and include myelosuppression, hair loss, nausea and vomiting, mucositis and local tissue necrosis after interstitial administration (Tannock, Hill et al. 2005). A specific concern regarding the toxicity of doxorubicin is the development of an irreversible cardiomyopathy (Xu, Tang et al. 2001). Some patients' disease is resistant to doxorubicin because of up-regulation of the intracellular free radical scavenging system, which limits the cytotoxic effect of free radicals. However, even without maximum free radical production, as it would occur in an hypoxic environment, doxorubicin can still exert its cytotoxic effects (Teicher 1994; Tannock, Hill et al. 2005).

Mitoxantrone is an anthracenedione and initiates cell death by mechanisms similar to those of the anthracyclines. It is a synthetic drug that intercalates DNA between two base pairs and/or by electrostatic interaction involving the phosphate groups. It may

also inhibit topoisomerase II (Alberts, Peng et al. 1985). The end result is inhibition of RNA and DNA synthesis (Alberts, Peng et al. 1985). Resistance to mitoxantrone may occur through altered topoisomerase II activities (Harker, Slade et al. 1991; Errington, Willmore et al. 1999), overexpression of drug efflux pump P-glycoprotein (p-Gp) (Consoli, Van et al. 1997), and/or overexpression of Breast Cancer Resistant Protein (BCRP) (Diah, Smitherman et al. 2001). This drug is also an option for palliative regimens, because of its acceptable toxicity profile compared to anthracycline (Alberts, Peng et al. 1985).

Alkylating agents were first discovered during World War I following soldiers' experiences with nitrogen mustard gas (Tannock, Hill et al. 2005). Cyclophosphamide is a synthesized alkylating agent, and at present is the most widely used and beneficial anticancer agent (Tannock, Hill et al. 2005; Emadi, Jones et al. 2009). Cyclophosphamide is an inactive prodrug that is hydroxylated by hepatic mixed function oxidase to form an intermediate, 4-hydroxycyclophosphamide (4-HO), which freely diffuses into cells and spontaneously decomposes to phosphoramide mustard and acrolein (Tannock, Hill et al. 2005; Emadi, Jones et al. 2009). Phosphoramide mustard creates the interstrand DNA crosslinks at the most common site of alkylation, the N-7 position (Tannock, Hill et al. 2005; Emadi, Jones et al. 2009). Cyclophosphamide is detoxified in the cell by aldehyde dehydrogenase (Tannock, Hill et al. 2005; Emadi, Jones et al. 2009). Toxicities associated with cyclophosphamide are myelosuppression, nausea, vomiting, hair loss, gonad damage, cardiac damage and potential carcinogenesis. One of the unique toxicities is caused by acrolein, which causes hemorrhagic cystitis of the bladder (Tannock, Hill et al. 2005; Emadi, Jones et al. 2009). Resistance to the alkylating agents may occur via

decreased entry into the cell, increased intracellular thiol concentrations that decrease damage to DNA, and increased amounts of detoxification enzymes such as aldehyde dehydrogenase (Tannock, Hill et al. 2005).

Antimetabolites represent a category of chemotherapeutic agents that interfere with normal cellular function, especially DNA synthesis (Townsend, Beauchamp et al. 2008). Methotrexate is an analog of folic acid (Tannock, Hill et al. 2005). The reduced form of folate is a coenzyme required for DNA synthesis (i.e., purine synthesis), as well as for the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP). Methotrexate is a potent inhibitor of dihydrofolatereductase (DHFR), which is required for regeneration of reduced folate from dihydrofolate, and leads to an intracellular decrease in the amount of reduced folate available (Bleyer 1978; Bertino, Gorlick et al. 1996; Tannock, Hill et al. 2005). There are various ways that cells can be resistant to the actions of methotrexate. For example, decreased intracellular accumulation due to either impaired transport into the cell or decreased ability to maintain drug concentrations in the cell. The inability to maintain an adequate drug concentration generally occurs in the setting of decreased polyglutamation, a process that decreases methotrexate efflux from the cell (Bertino, Gorlick et al. 1996; Tannock, Hill et al. 2005). Since methotrexate is an inhibitor of DHFR, an increase in quantity of DHFR or mutated DHFR will create resistance. An increase in catabolism of polyglutamatemethotrexate and its removal from the cell also contributes to decreased activity of methotrexate (Bertino, Gorlick et al. 1996).

5-FU is a pyrimidine analog, the mechanism of action of which is the impairment of DNA and RNA synthesis, although the exact mechanism remains unclear (Tannock,

Hill et al. 2005). The current understanding is that phosphorylation of 5-FU to 5-fluoro-deoxyurideine monophosphate (5-FdUMP) inhibits thymidylate synthetase, which in turn depletes dexoythymidine monophosphate (dTMP). The decrease in dTMP, which is a critical component of DNA replication, will exert its effect on cells in S-Phase at which time DNA is being synthesized. If, however, 5-FU is phosphorylated to create 5-UTP, there is an inhibition RNA (Grem 2000; Tannock, Hill et al. 2005). Toxicities associated with 5-FU include neutropenia, gastrointestinal toxicity (nausea and diarrhea), and palmoplantar erythrodysesthesia or, hand-foot syndrome (Cohen 1993; Grem 2000; Tannock, Hill et al. 2005). Resistance to 5-FU occurs by increased drug catabolism by dihydropyrimidine dehydrogenase, decreased drug activation by decreases in thymidine phosphorylase, uridine phosphorylase and orotate phosophoribosyl transferase and/or alteration in the target enzyme such as an increase in thymidylate synthetase (Mader, Muller et al. 1998; Longley and Johnston 2004).

The taxanes (paclitaxel and docetaxel) are agents that cause microtubule stabilization, mitotic arrest and cell death by binding to tubulin in the microtubule (Milross, Mason et al. 1996; Vaishampayan, Parchment et al. 1999). This binding causes a G_1/M arrest (Schiff, Fant et al. 1979). Paclitaxel is derived from the North American yew tree, also called the Western or Pacific yew tree, and docetaxel is a semisynthetic compound derived from the European yew (Vaishampayan, Parchment et al. 1999; Townsend, Beauchamp et al. 2008). Paclitaxel toxicities include neutropenia, peripheral neuropathy and cardiotoxicity; docetaxel causes myelosuppression and fluid retention syndrome (Vaishampayan, Parchment et al. 1999). Resistance to the taxanes occurs by efflux by p-glycoprotein (p-Gp), which decreases drug concentration in the cells (Thomas

and Coley 2003) and alterations of microtubule structure, which alters binding to tubulin (Dumontet and Sikic 1999).

Platinum agents are generally reserved for use after first line agents have failed in breast cancer patients. Platinum drugs exist in a 2^+ or 4^+ oxidative state, the binding sites of which interact with DNA and form adducts at the N7 position of guanine and adenine, which interfere with DNA transcription and replication (Fuertes, Castilla et al. 2003; Siddik 2003). The toxicities of cisplatin include nausea and vomiting, mild myelosupression, nephrotoxicity, neurotoxicity, and ototoxicity (Fuertes, Castilla et al. 2003; Tannock, Hill et al. 2005). Most tumours become resistant to cisplatin due to reduced adduct formation, decreased uptake into the cell, increased DNA repair, increased tolerance to DNA damage, and/or increased binding of cisplatin to thiol scavengers like reduced glutathione (Johnson, Ferry et al. 1998; Siddik 2003).

The use of other systemic therapies relies on specific tumour markers, which include the estrogen receptor (ER), the progesterone receptor (PR) and the HER-2 receptor. Hormone receptor status is the single most important predictor of patient response to hormone therapy; having both ER and PR positivity confers a 70% response rate to therapy, which drops to 30-40% in the setting of only one receptor being positive, and to less than 10% response when neither receptor is present (Muss 1992; Keen and Davidson 2003). The strategy for treatment of ER positive breast cancer consists of oophorectomy or luteinizing hormone-releasing hormone (LHRH) analogues in younger women, aromatase inhibitors in postmenopausal patients, anti-estrogens (e.g., fulvestrant), or selective ER modulators (SERMs) like tamoxifen and raloxifen in women in the intervening age groups (Keen and Davidson 2003). These therapies reduce tumour

burden, decrease the numbers of tumour cells in S-phase and induce apoptosis (Keen and Davidson 2003; Arpino, De Angelis et al. 2009). The major complications of ER modulators are endometrial cancer, venous thromboembolic disease (deep venous thrombosis (DVT) and pulmonary embolism (PE) (Smith 2005) . HER-2 is a receptor in the epidermal growth factor family that is overexpressed in 30% of breast cancers (Hudis 2007). Trastuzumab is a monoclonal antibody that targets the HER-2 receptor and prevents the activation of HER-2 intracellular tyrosine kinase, causing cell death and inhibiting angiogenesis (Hudis 2007). The toxicities associated with trastuzumab are myelosuppresion, nausea, vomiting and a hypersensitivity type reaction that occurs in 10% of patients, in response to the monoclonal antibody (Hudis 2007).

1.3 Cell Death

Cell death has been classically described in terms of apoptosis and necrosis. This apparently dichotomous description has come under scrutiny over the past two decades. New research has demonstrated that the two forms of cell death are more likely to represent different points along a continuum (Zeiss 2003). Apoptosis is the active or energy-dependent form of programmed cell death. This scripted or organized process does not induce an inflammatory response (Majno and Joris 1995; Fink and Cookson 2005). Necrosis is the passive or energy-independent form of cell death, which occurs in response to environmental conditions, is uncontrolled and pro-inflammatory (Fink and Cookson 2005). The word “necrosis”, however, is more of a pathological description and represents the final common pathway of all cell death (Schwartz and Bennett 1995). A shift in terminology to the word “oncosis” has emerged, but many variations of

programmed cell death have been identified (Majno and Joris 1995; Fink and Cookson 2005).

Apoptosis is critical in the living organism and provides the balance to mitosis and cytokinesis needed for the maintenance of healthy tissue. New cells are continuously developing, and in order to maintain homeostasis, approximately ten billion cells die per day in the adult human (Renehan, Booth et al. 2001; Nagata 2006; Widlak and Garrard 2009). Disregulation of cellular proliferation with suppression of apoptosis results in carcinogenesis (Evan and Vousden 2001). To understand cancer and possible therapeutics, it is necessary to first review the process of cell division and death. The following sections outline the main types of programmed cell death and the cell cycle in terms of currently accepted terminology, as well as an overview of the mechanisms of action.

1.3.1 Apoptosis

After witnessing cells undergoing death under microscopic examination the word "apoptosis" was proposed to describe the cells' dying process. "Apoptosis", literally means "falling off", as leaves fall off a tree (Kerr, Wyllie et al. 1972). Apoptosis is critical in embryonic development, it provides protection against invasive pathogens, functions in the elimination of autoimmune cells, plays a role in remodeling several adult organs such as postovulation follicular atresia or post-weaning mammary gland involution, and aids in the elimination of damaged cells, which might otherwise develop into a malignancy (Norbury and Hickson 2001; Elmore 2005).

The characteristics that define apoptosis are cell shrinkage, pyknosis (condensation of chromatin), plasma membrane blebbing, karyorrhexis (fragmentation of the nucleus), and the creation of apoptotic bodies (Kerr, Wyllie et al. 1972). The apoptotic bodies, which consist of cytoplasm with tightly bound organelles with or without nuclear fragments, develop during "budding" (Kerr, Wyllie et al. 1972; Hacker 2000). Apoptosis is a highly organized and complex process with multiple independent pathways that also exhibit cross-communication.

The initial studies of apoptosis were completed on *Caenorhabitis elegans*, a transparent nematode used extensively in research (Hope 1999). This organism has exactly 131 cells that undergo apoptosis and therefore, allowed detailed study of this process (Horvitz 1999). *Caenorhabitis elegans ced* were used to identify gene products required in the development of the nematode embryo (Hedgecock, Sulston et al. 1983), including CED-3, which is similar to interleukin-1β (IL-1β)-converting enzyme in mammalian species (Yuan, Shaham et al. 1993). The subsequent identification of a family of protein kinases (caspases) that are cysteine-dependent aspartate specific proteases was critical for the understanding of apoptosis. The nomenclature was changed with IL-1β converting enzyme being renamed caspase-1 and all subsequent members identified being named in sequence (Alnemri, Livingston et al. 1996).

Caspases have a common amino acid sequence but diverse physiological roles (Thornberry, Rano et al. 1997). The two recognized physiological caspase categories are (1) inflammatory (caspase-1,-4,-5,-13,-14); and (2) apoptotic (caspase-3,-6,-7,-8,-9,-10); the latter being the focus of this section (Creagh, Conroy et al. 2003). The apoptotic

caspase group is further subdivided into initiator caspases (caspase-8,-9,-10) and executioner caspases (caspase-3,-6,-7) (Los, Wesselborg et al. 1999).

Initiator caspases are activated by a proximity-induced dimerization without cleavage (Gu, Wu et al. 1995), which is accomplished by the common caspase prodomain binding to either the caspase recruitment domain (CARD) or death effector domain (DD) (Boatright and Salvesen 2003). A 'cascade-like' propagation then occurs to activate the executioner caspases to dismantle the cell. The executioner caspases are activated by cleavage of proform zymogens by proteolysis at the internal asparate residue forming a heterotetramer (Thornberry 1998; Boatright and Salvesen 2003). This form of activated caspase induces apoptosis by cleaving cellular proteins involved in all aspects of cell structure and function (Boatright and Salvesen 2003).

The dissolution of proteins in the caspase-dependent pathway is the underlying biochemical process that creates the morphological changes identifying apoptosis. This begins with the cleavage of DNA into approximately 180 base pairs by endonuclease (Wyllie 1980). Caspase-activated deoxyribonuclease (CAD), also known as DNA fragmentation factor (DFF), is activated by the cleavage of CADs inhibitor (ICAD) by caspases-3 and -7 (Enari, Sakahira et al. 1998; Widlak and Garrard 2009). Caspases proteolyse the scaffold proteins (e.g., lamins) of the nuclear envelope, leading to chromatin condensation, nuclear shrinkage and fragmentation (Rao, Perez et al. 1996). Another critical effect of the caspases on the nucleus is the inhibition of DNA repair proteins such as Poly [ADP-ribose] polymerase 1 (PARP-1). Since PARP-1 is a nuclear enzyme involved in DNA strand breakage, disregulation of this enzyme by caspases facilitates cellular dismantling (Song, Lees-Miller et al. 1996).

Cytoskeleton proteins are also cleaved leading to the loss of the cell's asymmetric shape (Hacker 2000). Phosphatidylserine (PS), which is usually localized to the inner leaflet of the cell membrane, is exposed on the outer leaflet during apoptosis (Fadok, Voelker et al. 1992; Verhoven, Schlegal et al. 1995). This phospholipid component is one of the signals for macrophages and T-lymphocytes to engulf dying cell and is therefore referred to as the "eat me" signal (Fadok, Voelker et al. 1992; Verhoven, Schlegal et al. 1995). Inflammation does not occur during this process due to the controlled reaction to ingestion of apoptotic bodies by phagocytes, which prevents apoptotic cells from releasing their intracellular contents (Haslett, Savill et al. 1994; Savill and Fadok 2000). These apoptotic bodies are created by cleavage of a member of p21-activated kinase family (Kothakota, Azuma et al. 1997; Rudel and Bokoch 1997; Wen, Fahrn et al. 1997). The cleaving of adhesion complexes by caspases cause the cell to detach from the basement membrane and neighboring cells (Wen, Fahrn et al. 1997).

Activation of the caspase cascade can be initiated via two main pathways, the extrinsic/death receptor pathway (DR) and the intrinsic/mitochondrial pathway. Although both pathways are distinct, they converge at the activation of executioner caspases and also cross-communicate. These pathways are constantly being reevaluated as knowledge increases. An upstream pathway that initiates mitochondrial caspase activation is the endoplasmic reticulum (ER) stress pathway. This will also be introduced briefly.

The DR-pathway involves transmembrane receptors that are members of the tumor necrosis factor receptor (TNFR) gene superfamily (Locksley, Killeen et al. 2001). TNFR share cysteine-rich extracellular domains with a "death domain" (DD) in the cytoplasm (Ashkenazi and Dixit 1998; Gupta 2001). Multiple receptors within this family

have been studied but the most completely studied is the Fas ligand/Fas receptor (FasL/FasR) or CD95L/CD95R (CD95 ligand/CD95 receptor) (Ashkenazi and Dixit 1998). Fas ligand binds to the cytoplasmic Fas receptor and recruits proteins with corresponding DD. These domains create an area for further initiation of cell death and, in the case of Fas, creates Fas-associated death domain (FADD) (Ashkenazi and Dixit 1998). FADD contains an effector domain termed the caspase recruitment domain (CARD) that can bind to procaspases with large prodomains such as procaspase -2, -8, -9 and -10, forming the death-inducing signaling complex (DISC). This complex activates procaspase-8 and/or caspase-10 into its active form and the cascade is propagated (Ashkenazi and Dixit 1998; Gupta 2001; Khan, Afaq et al. 2007).

The mitochondrial pathway is the other main signaling pathway that can initiate apoptosis. The mitochondria are the "engines" or energy producing organelles of cells, and consist of the inner membrane (which surrounds the matrix), the intermembrane space, and the outer membrane. The inner membrane contains ATP-synthase, electron transport chains and adenine nucleotide translocators (Gupta 2001). The inner membrane is also the location of some members of Bcl-2 family which are involved in the maintenance of the mitochondrial membrane potential (Gupta 2001). There is complex interaction with various Bcl-2 proteins, of which 25 genes have been identified to date, that have both antiapoptotic (like Bcl-2 and Bcl-xL) and proapoptotic (Bax, Bak, Bad, Bid and Bim) functions (Tannock, Hill et al. 2005).

The outer membrane of mitochondria, where voltage-dependent anion channels are located, becomes permeable via the action of the pores created by apoptotic signals. Opening of the permeability transition pore complex results in the loss of membrane

potential and the release of proapoptotic compounds (Kroemer, Zamzami et al. 1997; Gupta 2001; Cory and Adams 2002; Khan, Afaq et al. 2007). The proapoptotic compounds are located in the intermembrane space and are pro-caspase initiators of apoptosis like cytochrome-*c* and a second mitochondria-derived activator of caspases, also known as direct inhibitor of apoptosis-binding protein with low pI (SMAC/Diablo). Cytochrome-*c*, one of the major components of mitochondria induced apoptosis, binds and activates apoptotic protease activating factor 1 (APAF-1) and procaspase-9, forming the "apoptosome", which further propagates the caspase cascade (Chinnaiyan 1999). There are also caspase-independent compounds that cause apoptosis such as apoptosis-inducing factor (AIF) and endonuclease G (Gupta 2001; Wang 2001; Elmore 2005).

The mitochondrial pathway can be initiated by negative stimuli such as the absence of growth factors, hormones and cytokines, or positive stimuli such as radiation, toxins, or the creation of reactive oxygen and/or nitrogen species (Gupta 2001). In the literature, an emerging initiator of apoptosis by interactions with the mitochondria and through other mechanisms is endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). The endoplasmic reticulum is an organelle responsible for calcium storage/release, protein folding/secretion, and lipid biogenesis (Tsai and Weissman 2010; Shore, Rapa et al. 2011). UPR is a generally a protective mechanism, however, it can be severe and result in cell death, mainly through apoptosis. Apoptosis is accomplished by membrane proteins including inositol-requiring protein 1 (IRE-1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Tsai and Weissman 2010; Shore, Rapa et al. 2011) as well as the release of calcium from the ER and through members of

the BCL-2 family (BAK, BAX and Bid) (Ron and Walter 2007). Calcium can also activate cellular proteases and caspases (caspase-12) (Ron and Walter 2007).

Although the DR and mitochondrial pathways are described as distinct mechanisms there is considerable "cross talk" between them. For example, caspase-8 activation via the DR pathway causes the activation of Bid, a pro-apoptotic Bcl-2 family member. Generation of the truncated form of Bid causes the mitochondria to release the pro-apoptotic compounds described above (Gupta 2001; Esposti 2002).

1.3.2 Oncosis

Necrosis, the passive and accidental form of cell death has been described as the "counter" to apoptosis; however, this definition has come under scrutiny since understanding that the organized removal of cells by apoptosis can in fact lead to necrosis, under certain conditions (Fink and Cookson 2005). An example of this occurs during apoptosis in tissue culture. Since no phagocytes are present, apoptotic bodies are not removed and eventually lose membrane integrity and proceed to undergo secondary or "apoptotic" necrosis (Majno and Joris 1995; Fink and Cookson 2005). For this reason, alternate terms are used and a variety of alternate routes of cell death have been identified.

Oncosis has now been proposed as the counterpart to apoptosis in some literature (Majno and Joris 1995; Fink and Cookson 2005). The term is derived from the Greek "onkos" meaning swelling, and represents a cellular pathway for cell death characterized by cellular and organelle swelling, blebbing (albeit to a lesser degree than apoptosis), and increased cell membrane permeability (Majno and Joris 1995; Fink and Cookson 2005).

Oncosis is thought to be activated by the involvment of enzymes including p53, caspases and Bax but can be inhibited by other Bcl-2 family members (Trump, Berezesky et al. 1997).

Toxic agents can cause oncosis by decreasing the production of ATP or increasing the depletion of energy stores within the cell. Energy-dependent mechanisms are critical for normal function of the cell (Fink and Cookson 2005). An example of this energy depletion involves PARP-1, which was previously described as a substrate for the caspase-dependent apoptotic pathway of cell death. PARP-1 is a DNA repair enzyme and if damage is moderate, can repair the DNA damage and the cell can recover. However, during severe DNA damage resulting in cell death, caspase activation causes PARP-1 to become inactive (Herceg and Wang 2001). During this massive DNA damage, if PARP-1 is not cleaved and continues to use energy to attempt repair, it leads to its depletion that can cause oncotic cell death (Walisser and Thies 1999).

1.3.3 Oxidative Stress

Oxidative stress is defined as a disturbance in the prooxidant/antioxidant balance that can lead to cell damage (Pan, Ghai et al. 2008). The production of reactive oxygen species (ROS) can be through normal cellular metabolism in an aerobic environment but excess ROS production can damage the cell and/or cause cancer (Dreher and Junod 1996). Two examples of normal production of O_2^- occur during ATP electron transport reaction when leakage of a single electron can be transferred to O_2 or in the endoplasm reticulum when an electron can be transferred from NADPH by cytochrome p450 reductase to O_2 (Kamata and Hajime 1999). Cells have mechanisms to maintain the redox

cellular environment, including superoxide dismutase or intracellular antioxidants like reduced glutathione (GSH) (Kamata and Hajime 1999; Pan, Ghai et al. 2008) The types of ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$), and peroxy radical ($^{\cdot}OOR$) (Kamata and Hajime 1999; Pan, Ghai et al. 2008). Although H_2O_2 and O_2^- are reactive with other molecules, conversion to $^{\cdot}OH$ in the presence of iron (Fe) and copper (Cu) can cause severe damage to the cell (Kamata and Hajime 1999).

The oxidative stress system is important in cell survival/cell death and cellular signaling pathways (Nakamura, Nakamura et al. 1997). ROS signaling activates the same cellular pathways as growth factors such as protein tyrosine kinases, serine/threonine kinases, phospholipases, and calcium changes (Kamata and Hajime 1999). When ROS are produced by death stimuli, both apoptosis and necrosis can occur. Apoptosis is induced when there is moderate oxidative stress caused by production of ceramide (Verheij, Bose et al. 1996), stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Verheij, Bose et al. 1996), activation of p53 (Yin, Terauchi et al. 1998) and/or the induction of regulatory phosphatidylinositol 3-kinase (PI3-kinase) (Yin, Terauchi et al. 1998). Necrosis can be induced by high oxidative stress or even at lower concentration of ROS if caspase-dependent apoptosis is inhibited (Hampton and Orrenius 1997).

1.4 Proliferation and Cell Cycle

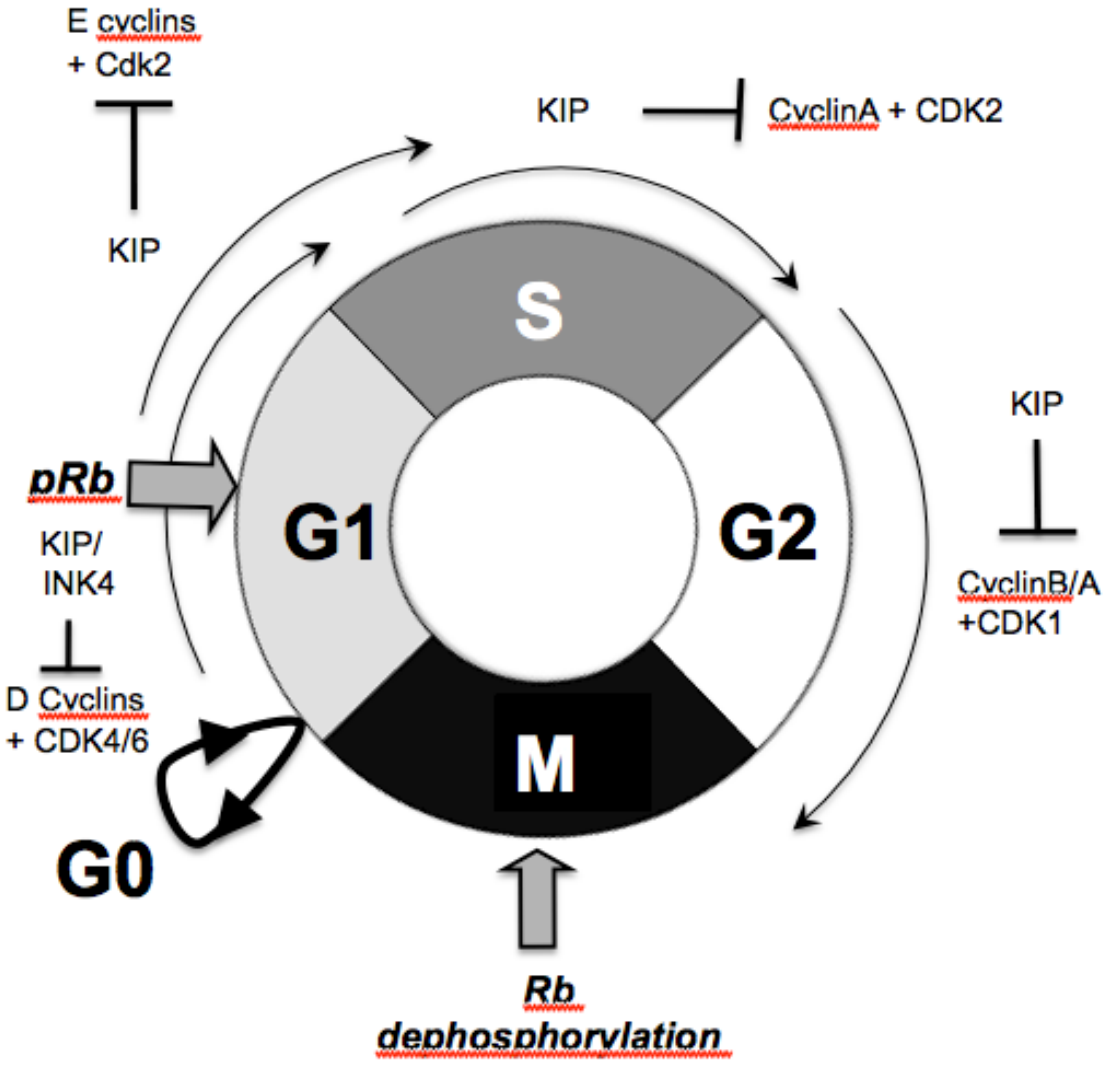
Cell proliferation (Figure 1) which constantly renews and maintains healthy tissues, and occurs via the cell cycle characterized by (i) DNA replication and (ii) division into two

daughter cells (Vermeulen, Van Bockstaele et al. 2003). The final stage of cell division is termed mitosis (M-phase), which is comprised of prophase, metaphase, anaphase and telophase. It is at this point that the two sets of DNA are relocated to the daughter cell (Blow and Tanaka 2005). Cytokinesis is the equal division of the other cellular components of the cell and, ultimately, the creation of two daughter cells at the end of mitosis (Glotzer 2005). Interphase, where the majority of the cell's life is spent, is the time between two M-phases. The cell functions normally or prepares for cell division with DNA replication and cell size increase (Vermeulen, Van Bockstaele et al. 2003; Blow and Tanaka 2005). Interphase is divided into S-phase (synthesis) and two G-phases (Gap). DNA replication occurs during S-phase (Vermeulen, Van Bockstaele et al. 2003). G_1 represents the time during which the cell is preparing for DNA replication or is in a resting state (G_0) and performing its function. G_2 falls between S-phase and M-phase (Vermeulen, Van Bockstaele et al. 2003). Cell division is a complex process with various controls to ensure proper replication. Dysregulation of cell division can cause mutations in DNA, resulting in cell death or tumour initiation (Tannock, Hill et al. 2005). It is estimated that mutations occur at 1 in 20 million per gene during cell division (Evan 1998). This represents a large number of cells in a person's lifetime and reinforces the concept that the body has naturally occurring potent defense mechanisms against cancer.

Cyclin-dependent kinases (CDK) are a family of serine/threonine protein kinases involved in many functions during the cell cycle, such as phosphorylation of retinoblastoma protein (Rb) (Morgan 1995; Evan and Vousden 2001). The activity of CDKs are controlled by phosphorylating enzymes referred to as CDK-activating enzymes (CAK), as well as cyclins and inhibitors (Kaldis 1998; Tannock, Hill et al. 2005).

Figure 1. Overview of the cell cycle. The cell cycle is divided into several phases. The majority of cells are in G_0/G_1, a resting phase (G_0) or normal functioning phase (G_1). When the cell is preparing to divide, several components are required to progress through G_1, S, G_2 and M-phases. This figure outlines the current understanding of this process (Vermeulen et al. 2003).

Figure 1.


E cyclins
+ Cdk2
KIP
KIP
CyclinA + CDK2
S
G1
G2
M
G0
pRb
KIP/
INK4
D Cyclins
+ CDK4/6
KIP
CyclinB/A
+CDK1
Rb
dephosphorylation

Expression of CDKs remains relatively stable throughout the cell cycle but cyclin levels increase and decrease during cell cycle progression (Pines 1995). This is true with the exception of the cyclins involved in G_1-phase of the cell cycle (e.g., Cyclin-D_1, D_2 and D_3), which have an increased expression induced by growth factors (Assoian and Zhu 1997; Vermeulen, Van Bockstaele et al. 2003). Cyclin-D binds to CDK4 and CDK6 to become active (Vermeulen, Van Bockstaele et al. 2003). The regulator for progression from G_1 to S-phase is the cyclin-E/CDK2 (Ohtsubo, Theodoras et al. 1995). Cyclin-A is needed in S-phase and is associated with CDK2 (Walker and Maller 1991; Vermeulen, Van Bockstaele et al. 2003). Cyclin-B regulates mitosis when bound with CDK1 (Arellano and Moreno 1997). Cyclin-H, with CDK7, phosphorylates CDK, similar to CAK (Vermeulen, Van Bockstaele et al. 2003).

Activation of the CDKs, which initiates the cell cycle, is regulated by activating proteins and growth signals, as well as inhibitors (Vermeulen, Van Bockstaele et al. 2003; Tannock, Hill et al. 2005). There are two categories of inhibitors, Inhibitor of CDK4 (INK4) and Kinase Inhibitory Protein (KIP) (Sherr and Roberts 1999). The INK4 includes p15 (Hannon and Beach 1994), p16 (Serrano, Hannon et al. 1993), p18 and p19 (Hirai, Roussel et al. 1995), which inhibit CDK4 and CDK6 by forming stable complexes and preventing cyclin-D from binding to progress through G_1 (Vermeulen, Van Bockstaele et al. 2003). Unlike the INK4 family of inhibitors, the KIP family of inhibitors p21, p27 and p57 bind to and inactivate the CDK-cyclin complexes (Harper and Elledge 1996; Denicourt and Dowdy 2004). Specifically, KIP inhibits CDK-cyclin complexes in G_1, as well as the cyclin-B/CDK1 in mitosis (Vermeulen, Van Bockstaele

et al. 2003). The p21 inhibitor, which is controlled by p53 tumour suppressor gene, can also halt cell proliferation by inhibiting DNA synthesis (Pan, Reardon et al. 1995).

Once the CDK-cyclin complex is activated, it phosphorylates targeted proteins required for the cell cycle. The classic example is the retinoblastoma tumour suppressor gene (pRb), which is a restriction point for transition from G_1 to S-phase of the cell cycle (Vermeulen, Van Bockstaele et al. 2003; Tannock, Hill et al. 2005). Once pRb has become phosphorylated it causes the release of transcription factors that produce products for S-phase such as cyclin-A, cyclin-E and Cdc25 (Kato, Matsushime et al. 1993). pRb maintains phosphorylation through the cell cycle by one of its products, cyclin-E, which combines with CDK2 to create its own negative feedback by phosphorylating its inhibitor p27 (Vermeulen, Van Bockstaele et al. 2003). Other than the transcription factors and products, CDKs also regulate cell structure required for cell division such as components of the cytoskeleton (Vermeulen, Van Bockstaele et al. 2003).

The process outlined above is complex, and clearly a mechanism that ensures that daughter cells are mutation-free and function normally is required for normal growth and development. This is accomplished by restriction points as well as checkpoints. The restriction point (R) falls between G_1 and S-phase; once a cell passes R, it is committed to complete the cell cycle (Hartwell and Weinert 1989; Vermeulen, Van Bockstaele et al. 2003; Tannock, Hill et al. 2005). The checkpoints identified to date include quiescent checkpoint, G_1/S checkpoint, replicative/S checkpoint, G_2 checkpoint, mitotic checkpoint, cytokinesis or C-checkpoint, and DNA damage checkpoint (Dash and El-Deiry 2004). DNA damage and checkpoints arrest the cell cycle. For example, tumour suppressor gene p53 is involved in multiple checkpoints, including G_1/S checkpoint

(Levine 1997). There are several genes that are stimulated by p53 to cause cell cycle arrest and cell death such as p21, Mdm2, Bax, Fas and those involved in the oxidative stress pathway (Ko and Prives 1996; Agarwal, Taylor et al. 1998; Vermeulen, Van Bockstaele et al. 2003).

The loss of this complex control can lead to mutations in proto-oncogenes and/or tumour suppressor genes, leading to uncontrolled cell proliferation. Mutations occur in genes encoding CDKs, cyclins, CDK-activating enzymes, CKI, CDK substrates and checkpoint proteins (Sherr 1996). An example of mutations leading to uncontrolled proliferation specific to some breast cancer is overexpression of cyclin-D_1 (Hall and Peters 1996) or cyclin-E (Vermeulen, Van Bockstaele et al. 2003). Two of the most common tumour suppressor genes mutated in cancer are Rb, which is associated with unrestricted cell cycle progression (Hall and Peters 1996) and p53, which removes some of the checkpoints from the cell cycle (Nataraj, Trent II et al. 1995; Vermeulen, Van Bockstaele et al. 2003). Mutations in these common tumour suppressor genes have been identified in some forms of breast cancer (Jiang, Jones et al. 2011).

1.5 Complimentary and Alternative Medicines

Complimentary and alternative medicine (CAM) is defined as any medical intervention that is not taught widely in medical schools or available in hospitals (Eisenberg, Kessler et al. 1993). CAMs are a broad category of interventions that vary between cultures. Examples include acupuncture, chiropractic, massage therapy, support groups, exercise, prayer, vitamins and herbal/natural products (Eisenberg, Kessler et al. 1993; Rockwell, Liu et al. 2005). Although herbal medicines are perceived by the general

public as being safe as they are “natural”, these compounds contain potent bioactive agents (Bent and Ko 2004). This is evidenced by the fact that 30% of all pharmaceutical agents (Winslow 1998) including 70% of all drugs used in cancer treatment (Newman, Gragg et al. 2002) are derived from or based on natural products.

CAMs are employed by patients for a wide variety of conditions including arthritis, chronic back pain, acquired immunodeficiency syndrome, gastrointestinal problems, and asthma to name a few (Eisenberg, Kessler et al. 1993; Bent and Ko 2004). The use of CAMs in the general population has been reported to be as high as 71% (Bent and Ko 2004). This comprises a multibillion dollar market is rapidly expanding (Cassileth 1999). Various studies in the literature, including systematic reviews and clinical trials, have estimated that 31-60% of cancer patients use CAMs (often they use more than one form) (Eisenberg, Kessler et al. 1993; Ernst and Cassileth 1998; Cassileth 1999; Sparber, Bauer et al. 2000). Specifically in the breast cancer population, 10.6% of patients reported using CAM at the time of diagnosis with an additional 28.1% CAM use after surgery (Burstein, Gelber et al. 1999).

Phytochemicals are components of herbal/natural medications. They have been explored for use in both prevention and treatment of cancer. Breast cancer is more prevalent in Western countries and this observation has lead to the identification of diet as a risk factor for development of the disease. It has been observed that people who immigrate to Western countries and adopt our diet have an increased risk for breast cancer (Lee and Gourley 1991), whereas those that maintain their traditional diet do not (Ziegler, Hoover et al. 1993). Phytochemicals are plant based compounds that do not provide nutrition but have been found to be anticarcinogenic and antimutagenic (Surh

2003). Phytochemicals can be divided into phenolics, carotenoids, alkaloids, organosulfer compounds and terpanoids (Pan, Ghai et al. 2008). The National Cancer Institute has identified 35 plant based foods with over 1,000 different phytochemicals that have shown cancer preventative properties (Surh 2003). Although there is significant research interest cancer prevention, my research will focus on the use of a flavonoid/phenolic compound, fisetin, in cancer treatment.

1.5.1 Flavonoids

Flavonoids are a category of phytochemicals that contain more than 8000 known individual polyphenolic compounds (Pietta 2000; Ren, Qiao et al. 2003). This is constantly expanding with an estimated 2 million compounds possibly included in this category (Havsteen 2002). The massive number of compounds in this group is due to subtle variation of ring structure, extent of hydroxylation and substitution of the hydroxyl group (Havsteen 2002). The compounds can be subdivided according to variations in the heterocyclic ring into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids (Hollman and Katan 1999; Ren, Qiao et al. 2003). It is estimated that the average person ingests 1 gram of flavonoids per day (Kuhnau 1976). This large group of compounds has been extensively investigated for potential therapeutic benefit in human disease and the following is a brief overview.

Flavonoids are synthesized from phenylalanine and contribute the taste, flavour, and colour of many fruits and vegetables (Havsteen 2002). This group of compounds are important for normal growth, development and natural defense in plants (Havsteen 2002; Treutter 2006). Flavonoids have been extensively studied for their use in a variety of

human disease processes including cholesterol reduction (Havsteen 2002) prevention of atherosclerosis, asthma and chronic inflammation (Hollman and Katan 1999; Havsteen 2002). Flavonoids have antimicrobial properties and have been shown to inhibit/kill bacteria, interfere with viral replication and prevent/treat protozoal infections (Havsteen 2002). Flavonoids also have analgesic (Beretz, Anton et al. 1978; Liang, Huang et al. 1999) and anxiolytic properties (Paladini, Marder et al. 1999). They appear prominently in the literature as scavengers of free radicals and an example of this occurs during γ-radiation, flavonoids quenching nitrogen free radicals (van Acker, Tromp et al. 1995) and oxygen free radicals (Nijveldt, van Nood et al. 2001). A significant proportion of the beneficial effects of flavonoids has been linked to their antioxidant properties (Pietta 2000).

Flavonoids have been investigated for their effect on cancer. They have demonstrated both antiproliferative and cytotoxic effects (Cushman and Nagarathnam 1991; So, Guthrie et al. 1997). Apoptosis is induced in a variety of cancer cell lines by numerous mechanisms. The induction of apoptosis by flavonoids has been demonstrated through inhibition of topoisomerase I/II, ROS production, regulation of heat shock proteins, modulation of signaling pathways, release of cytochrome-*c*, downregulation of Bcl-2 and Bcl-XL with promotion of Bax and Bak, activation of endonuclease, suppression of Mcl-1 protein and effect on NF-κB (Ren, Qiao et al. 2003). They can also have an effect on growth signaling pathways via their anti-aromatase activity (Jeong, Shin et al. 1999) and effect on the estrogen receptor (Han, Tachibana et al. 2001; Pouget, Lauthier et al. 2001).

The use of flavonoids can be complicated by their individual toxicity/adverse effect as well as their influence on the metabolism and absorption of drugs (Havsteen 2002). Flavonoid aglycone LD_{50} (lethal dose, 50%) is approximately 2 g/kg (Havsteen 2002), however, due to the pharmacodynamics it is extremely unlikely for humans to experience lethal toxicity with the exception of an acute allergic reaction (Havsteen 2002). Toxicities associated with flavonoids that have been identified in the literature include liver failure, contact dermatitis, hemolytic anemia, and estrogenic-related concerns like effects on sexual reproduction (Galati and O'Brien 2004). Flavonoids have been shown to interact with drugs or food components with resultant effects on the drug's therapeutic window. An example of this in the literature is the flavonoid naringenin derived from grapefruit juice, which suppresses the expression of p450 (CYP) 3A4 gene and can affect bioavailability by altered intestinal absorption of a variety of drugs (Fukuda, Ohta et al. 1997; Kane and Lipsky 2000).

1.5.2 Fisetin

Fisetin, 3,3',4',7-tetrahydroxyflavone (Figure 2), is a flavonol that has 4 phenolic groups but is rare due to the fact that it does not contain a 5-hydroxy substitution (Shia, Tsai et al. 2009). It is found in many plants including in the wood of *Rhus continues, Rhus rhodanthema* (Sando and Bartlett 1918), *Continus coggygriai* Scop (Valianou, Stathopoulou et al. 2009) and *Acacia catechu* Willd (Hathway and Seakins 1957). Fisetin is also found in various fruits and vegetables including strawberries (160 μg/g), apples (0.6 μg/g), grapes (3.9 μg/g), onions (4.8 μg/g), cucumber (0.1 μg/g), and persimmons (10.5 μg/g) (Arai, Watamabe et al. 2000). Although dietary intake varies considerably

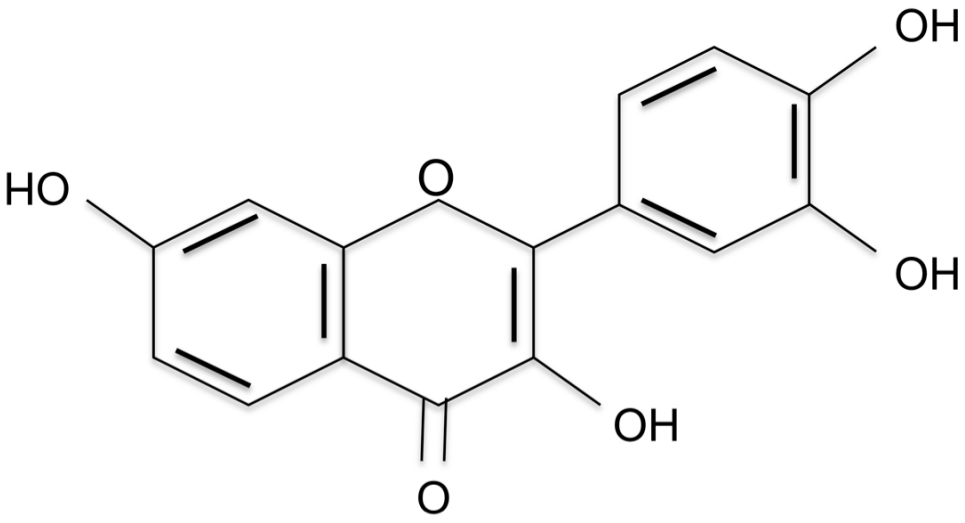
across cultures, one study demonstrated a daily intake of 0.39 mg of fisetin in Japan (Kimira, Arai et al. 1998).

Fisetin, like other flavonoids, has been studied extensively for use in a variety of human diseases and has various physiological effects including antioxidant, anti-inflammatory and anti-allergenic (Sagara, Vanhnasy et al. 2004; Sengupta, Banerjee et al. 2004). Specific examples of these effects include demonstrated cardioprotective properties through both its effect on cholesterol (Lian, Wang et al. 2008) and by induction of nitric oxide production in arterial endothelial cells resulting in vasorelaxation (Taubert, Berkels et al. 2002). Fisetin has also been shown to have neuroprotective effects (Sagara, Vanhnasy et al. 2004). Fisetin has the potential to exert various effects on different cells through the interactions with cell membranes or by uptake into the cytosol (de Sousa, Queiroz et al. 2007).

In relation to cancer, fisetin has been shown to be anti-angiogenic (Fotsis, Pepper et al. 1997; Sung, Pandey et al. 2007), anti-metastatic (Chien, Shen et al. 2009; Liao, Shih et al. 2009), cytotoxic, antiproliferative and to affect metabolism by inhibiting uptake of glucose (Park 1999),. Cytotoxicity and antiproliferative effects have been induced by exposure of fisetin to various cancer cell lines including bladder cancer (Li, Cheng et al. 2011), colon cancer (Kuntz, Wenzel et al. 1999; Lu, Chang et al. 2005; Lu, Jung et al. 2005; Lim do and Park 2009; Suh, Afaq et al. 2009; Yu, Yang et al. 2011), prostate cancer (Haddad, Venkateswaran et al. 2006; Haddad, Fleshner et al. 2010), pancreatic cancer (Murtaza, Adhami et al. 2009), hepatocellular cancer (Chen, Shen et al. 2002; Kim, Jeon et al. 2010), cervical cancer (Salmela, Pouwels et al. 2009), lung cancer

Figure 2. Molecular structure of fisetin. (Shia et al., 2009)

Figure 2.



(Sung, Pandey et al. 2007; Touil, Seguin et al. 2010), Burkitt's lymphoma (Sung, Pandey et al. 2007) and leukaemia (Lee, Shen et al. 2002; de Sousa, Queiroz et al. 2007). Although many studies have elucidated pathways of fisetin-induced cell death as well as fisetin's anti-proliferative effects, the mechanism of action is cell type specific.

There are few studies investigating fisetin's effect on breast cancer in the literature. In previous studies where breast cancer was included, only one cell line was used and the mechanism of action of the effects of fisetin were not included. Two studies investigated the effect of fisetin on MCF-7 breast cancer cell line proliferation at an EC_{50} (half of the maximal effective concentration) dose of 118.1+/-3.5 μM but did not demonstrate cytotoxicity (Kuntz, Wenzel et al. 1999). This finding was consistent with a study that was unable to determine the EC_{50} at concentration of fisetin up to 80 μM (Haddad, Venkateswaran et al. 2006). Yet another study did, however, reveal an EC_{50} of 10.6 μM (Fotsis, Pepper et al. 1997). Fisetin has been shown to inhibit of 17β-hydroxysteriod dehydrogenase type 1 (17β-HSD) at a 6 μM concentration. This enzyme converts estrone to estradiol, which is a potent growth factor in estrogen positive breast cancer. Although fisetin inhibited 17β-HSD, this did not translate into a significant antiproliferation in estrogen receptor positive, T-47D breast cancer cells (Brožič, Kocbek et al. 2009).

Other benefits identified for the treatment of cancer with fisetin includes anti-angiogenic as well as anti-metastatic effects. Angiogenesis is the generation of new blood vessels and in a normal adult organism it only occurs in very specific instances like wound healing and female reproduction (Fotsis, Pepper et al. 1997). Angiogenesis is a key step in the development of solid tumours, and as such, has gained prominence as a

possible therapeutic target (Weidner, Semple et al. 1991). Fisetin has demonstrated antiangiogenic activity *in vitro* (Fotsis, Pepper et al. 1997) and *in vivo* (Touil, Seguin et al. 2010). The anti-metastatic effects have been linked to fisetin's interaction and inhibition of matrix metalloproteinases as well as inhibiting signaling pathways involved in invasion and metastasis (Liao, Shih et al. 2009).

Although general toxicities of flavonoids were outlined above, a specific concern with fisetin relates to the possible increase in developing leukemia. Flavonoids have been linked to leukemia by their action as topoisomerase poisons and by inducing topoisomerase II-mediated DNA damage (Olaharski, Mondrala et al. 2005; Lopez-Lazaro, Willmore et al. 2010).

1.5 Research Rationale

CAMs have become more prominent in patient care and in particular the use of herbal compounds including phytochemicals. Although there is significant evidence of phytochemical use in the general population as well as in cancer patients, there are limited studies in the current literature pertaining to their specific effects and mechanisms of action. Fisetin, which has demonstrated beneficial effects on numerous cancer cell lines, has had limited investigations in breast cancer.

The review of the literature was the basis for my hypothesis that fisetin will decrease proliferation and induce cell death in breast cancer cells. The purpose of my research was to examine fisetin as a novel therapy against breast cancer by investigating its effect on multiple breast cancer cells lines, as well as its mechanisms of action. My research also examined the effects of fisetin on normal cells as well as exploring its effect

on zebrafish, a relatively novel *in vivo* model for the study of cancer, in preparation for translation research into an *in vivo* model.

Chapter 2.0 Materials and Methods

2.1 Cell Lines and Normal Cells

MDA-MB-468 human breast carcinoma cells (triple negative, mutated p53, pRb negative (Carlson, Dubay et al. 1996; Hirsh, Iliopoulos et al. 2009)) were provided by Dr. P. Lee (Dalhousie University, NS), MDA-MB-231 human breast carcinoma cells (triple negative, mutated p53 (Liu, Fan et al. 2009; Zhuang and Miskimins 2011)) were provided by Dr. S. Dover (Memorial University of Newfoundland, NL), SK-BR-3 human breast carcinoma cells (over-express HER-2 (Moulder, Yakes et al. 2001)) were provide by Dr. G. Dellaire (Dalhousie University, NS), MCF-7 (ER^+, PR^+, wild type p53, pRb positive (Carlson, Dubay et al. 1996; Rockwell, Liu et al. 2005)) and T47D (mutated p53, ER^+ (Zhuang and Miskimins 2011)) human breast carcinoma cells were provided by Dr. J. Blay (Dalhousie University, NS). The MCF-7 MITX breast cancer cells (mitoxantrone resistant), MCF-7 TX400 (paclitaxel resistant) breast cancer cells and the native MCF-7 breast carcinoma cells, from which the drug resistant lines were derived, were provided by Dr. K. Goralski (Dalhousie University, NS). MITX and TX400 cells were generated from the native MCF-7 breast carcinoma cells by exposure to progressive concentration increases to mitoxantrone (MITX) or paclitaxel (TX400). Human mammary epithelial cells (HMEC), normal adult human dermal fibroblasts and human umbilical vein epithelial cells (HUVEC) were purchased from Lonza Inc. (Walkersville, MD).

2.2 Culture Medium and Incubation Conditions

Breast cancer cells were maintained in a humidified 10% CO_2 incubator at 37°C and cultured in complete Dulbecco's Modified Eagle's Medium (cDMEM; Sigma-

Aldrich Canada Ltd., Oakville, ON), supplemented with 10% heat-inactivated (56˚C for 30 min) fetal bovine serum (FBS), 5 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 7.4 pH), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Corp., Burlington, ON). The MITX breast cancer cells and the TX400 breast cancer cells were cultured in cDMEM with the addition of mitoxantrone (100 μM) and paclitaxel (400 ug/ml), respectively. HMECs, human dermal fibroblasts, and HUVECs were maintained in a humidified 5% CO_2 incubator at 37°C. HMECs were cultured in mammary epithelium basal medium (MEBM) containing supplements provided by the supplier (Lonza Inc., Walkersville, MD) that included recombinant human insulin, recombinant human epidermal growth factor, hydrocortisone, gentamicin sulphate, amphotericin, and bovine pituitary extract. Fibroblasts were grown in fibroblast growth medium-2 (FGM-2) supplemented with insulin, recombinant human fibroblast growth factor (rhFGF), GA-100, and FBS provided by the supplier (Lonza Inc., Walkersville, MD). HUVECs were cultured in RPMI 5% heat-inactivated FBS, 5 mM HEPES buffer, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Lonza Inc., Walkersville, MD).

2.3 Reagents

Fisetin (3,3',4',7-Tetrahydroxyflavone, 5-dexoyquercetin, Naural Brown-1; Sigma-Aldrich Canada Ltd.,Oakville, ON) was dissolved in dimethyl sulfoxide (DMSO) to 100 mM stock and stored at -20°C. Reduced L-glutathione (GSH), N-acetylcysteine (NAC), phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), paclitaxel, 5-fluorouracil (5-FU), cis-platinum (II)

diamminedichloride (cisplatin), 4-amino-10-methylfolic acid hydrate (methotrexate), doxorubicin hydrochloride (doxorubicin), docetaxel, phosphatase substrate, and crystal violet were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). An active metabolite of cyclophosphamide, 4-hydroperoxy cyclophosphamide (4-OH), was purchased from Toronto Research Chemicals Inc. (North York, ON). Pan-caspase inhibitors BOC-D-FMK (caspase inhibitor VI) and Z-VAD-FMK (caspase inhibitor III) were purchased from EMD Biosciences (San Diego, CA). Z-VAD-fmk and BOC-D-FMK were dissolved in DMSO to 100 mM stock solutions and were stored at -20°C. Cell Trace™ Oregon Green® 488 carboxylic acid diacetate, and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Molecular Probes (Eugene, OR). Trypsin and TrypLE (phenol red negative trypsin replacement) were purchased from Invitrogen (Burlington, ON).

2.4 Cell Quantity Assays

2.4.1 Crystal Violet Assay

The crystal violet colorimetric assay was based on the method described by Saotome et al. (1989) to determine the quantity of cells exposed to a range of experimental treatments at various time points (24, 48 and 72 h) (Saotome, Morita et al. 1989). Crystal violet binds to all proteins within the cell, as well as DNA. Increased binding of crystal violet, which leads to a darker purple colour, indicates an increase in proteins and correlates with an increase in cell number. Cells were harvested and plated in quadruplicate at a concentration of 5,000 cells per well on a 96-well flat-bottomed plate. The cells were allowed to adhere for 24 h prior to treatment. Fisetin, vehicle or

medium alone treatments were then applied for specific time periods. After the desired time point was reached, the supernatants were carefully removed with a multi-channel pipette (Biohit, Helsinki, Finland) and cells were washed by addition of 0.2 ml of room temperature PBS to each well. The PBS was removed and 50 μl of 0.4% crystal violet (dissolved in methanol) was added for 10 min. Following a 10-min exposure, the plate was washed twice in one liter of double distilled water (ddH_2O). The plate was inverted and allowed to dry on a paper towel. After drying, the crystal violet stain was dissolved in 0.1 ml DMSO. A EL×800 UV Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT), provided by Dr. J. Marshall (Dalhousie University, NS), was used to read absorbance at 570 nm. Optical densities of the treatment groups were compared to the medium control using the equation (1 - (experimental reading/ medium control reading)) × 100 to calculate the percent cell quantity change for each treatment group.

2.4.2 Acid Phosphatase Assay

The acid phosphatase assay, based on the method outlined by Yang et al (1996), uses the cytosolic portion of acid phosphatase activity to determine the quantity of cells exposed to a range of experimental treatments at time points (24, 48 and 72 h). The cytosolic phosphatases in viable cells hydrolyze the substrate p-nitrophenyl phosphatase, causing a colorimetric change in the presence of a strong base, 1N sodium hydroxide (NaOH). The colorimetric change associated with viable cells is yellow and a darker yellow colour indicates an increase in the quantity of cells. Cells were harvested and plated in quadruplicate at a concentration of 5,000 cells per well on a 96-well flat-bottomed plate. The cells were allowed to adhere for 24 h prior to treatment. Fisetin,

vehicle or medium alone treatments were then applied for specific time periods. After the desired time point was reached, the supernatants were carefully removed using multi-channel pipette (Bioit, Helsinki, Finland) and cells were washed with 0.2 ml of room temperature PBS for each well. The PBS was removed and 0.1 ml of fresh PBS was added to each well. A 0.1 ml volume of the acid phosphatase buffer (0.2 M sodium acetate, pH 5.5; 0.1% Triton X-100; 4 mg/ml phosphatase substrate) was then added to the wells and incubated for 90 min. After this incubation period, 10 µl of 1 N NaOH was added to each well to stop the chemical reaction. A EL×800 UV Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT), provided by Dr. J. Marshall (Dalhousie University, NS), was used to read absorbance at 405 nm. Optical densities of the treatment groups were compared to the medium control using the equation (1 - (experimental reading/ medium control reading)) × 100 to calculated the cell quantity percent change across treatment groups.

2.4.3 MTT Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used determine the quantity of cells exposed to a range of experimental treatments at various time points (24, 48 and 72 h). MTT is a yellow tetrazolium salt, which is converted to a purple formazan crystal by mitochondrial succinate dehydrogenase. The increased conversion of the MTT to formazan, which leads to a darker purple colour, indicates an increase in cell number (Mosmann 1983). Cells were harvested and plated in quadruplicate at a concentration of 5,000 cells per well on a 96-well flat-bottomed plate. The cells were allowed to adhere for 24 h prior to treatment.

Fisetin, vehicle or medium alone treatments were then applied for the desired time periods. After the time point was reached, 20 μl of MTT was added to each well and incubated for 2 h at 37°C in 10% CO_2. At the completion of the incubation, the cells were centrifuged at 1400 × *g* for 5 min, the supernatant was discarded, and 0.1 ml DMSO was added to each well to solubilize the formazan crystals. The plate shaker was used until all crystals were solubilized in the DMSO. An EL×800 UV Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) provided by Dr. J. Marshall (Dalhousie University, NS), was used to read absorbance at 490 nm. Optical densities of the treatment groups were compared to the medium control using the equation (1 - (experimental reading/ medium control reading)) × 100 to calculate the percent cell quantity change for each treatment group.

An additional control mechanism was to visually inspect the cells prior to the initiation of the quantity assays. This was to insure the results were consistent with direct observation of the approximate numbers of cells.

2.5 Oregon Green 488® Proliferation Assay

The Oregon Green 488® cell proliferation assay was used to assess the effect of fisetin on proliferation of MDA-MB-468 and MDA-MB-231 cell lines. Oregon Green 488® is a fluorescent dye that couples irreversibly by amine group reactions with proteins in the cell and on the cell membrane (Wallace, Tario et al. 2008). When cells undergo mitosis, the dye will be equally distributed in both daughter cells. During each division, the dye per cell will be decreased by half and the exponential rate of fluorescence decrease allows assessment of cell proliferation. The protocol was based on information

provided by Invitrogen (Burlington, ON) and excitation/emission maxima are ~495/525 nm. The breast cancer cell lines were harvested from tissue culture flasks using trypsin, centrifuged at 500 × *g*, washed with 4 ml of room temperature PBS, centrifuged at 500 × *g*, and then resuspended in 4 ml of PBS containing 1 µl of Oregon Green 488®. The tubes containing cells were covered in tin foil and placed on the rocker for 10 min at room temperature. After the incubation period, 5 ml of HI-FBS was added to the Oregon Green 488® labeled cells, which were then centrifuged at 500 × *g*, followed by the addition of 1 ml of medium to cells. The cells were counted and plated at 50,000 cells per well on a 6-well plate. The cells were incubated for 24 h to allow adherence. After this time, the non-proliferative control was harvested using TrypLE, fixed in 4% paraformaldehyde (PFA) and stored at 4°C until final analysis with flow cytometry. Fisetin, vehicle or medium alone treatments were then applied to the remaining wells for 72 h. After the incubation period, the cells were harvested using TrypLE and analysed using parameter FL1 flow cytometer (FACSCalibur BD Biosciences, Mississauga, ON) the change in fluorescence was measured was in comparison to the non-proliferative control. The median channel fluorescence difference was calculated using $MCF_{control} = 2^n$ x $MCF_{experiment}$.

2.6 Clonogenic Assay

A modified clonogenic assay was used to assess the colony-forming capacity of cells treated with fisetin (Puck and Marcus 1956). This assay was originally used to assess the effects of radiation on cells but has been expanded to include cytotoxic agents and environmental conditions (Pomp, Wike et al. 1996). MDA-MB-468 and MDA-MB-231 breast cancer cells were harvested with trypsin, centrifuged at 500 × *g*, and 700,000

cells were plated in 75 mm^2 tissue culture flasks. The cells were allowed to adhere for 24 h. Fisetin, vehicle and medium alone treatments were then applied for 24 h. The cells were harvested, centrifuged at 500 × *g*, resuspended in medium and plated from 125 to 4000 cells per well on a flat-bottomed 6-well plate. The cells were incubated for 13 days and the medium was changed every 2 days. At the end of the 13-day period, the medium was removed and the wells washed with 2 ml of room temperature PBS. The PBS was then removed and 0.5 ml of crystal violet (0.4% in methanol) was added to the well for a 10 min. The crystal violet was then removed with a pipette and the plates were thoroughly washed with ddH_2O. The plates were allowed to dry and stained colonies were counted. The colonies per 1000 cells plated were calculated.

2.7 Cell Cycle Analysis

Cell cycle analysis was used to assess the effect of fisetin on the progression of cells to division. MDA-MB-468 and MDA-MB-231 breast cancer cell lines that were 50-60% confluent were incubated with serum-free DMEM for 12 h. Serum starvation of the cells was to synchronize the cell cycle (G_1) across the entire population. The cells were then harvested using trypsin and plated at 50,000 cells per well in a 6-well flat-bottomed plate. The cells were allowed to adhere for 24 h and treated with fisetin, vehicle or medium alone treatments were applied for 48 h. Following the incubation period, the cells were harvested, centrifuged at 500 × *g*, and washed with 5 ml of ice cold PBS. The cells were resuspended in 0.5 ml of ice cold PBS and ice cold ethanol (70%) was slowly added while vortexing the cells. The ethanol fixed cells were stored at -20°C for a minimum of 24 h. After storage, the cells were washed with 5 ml of PBS, centrifuged at 500 × *g*, and resuspended in PBS with 0.02 mg/ml propidium iodide (PI), 0.1% v/v Triton

X-100, and 0.2 mg/ml DNase-free RNase A. PI binds to nucleic acids of DNA (Riccardi and Nicoletti 2006). The cells were incubated for 30 min at room temperature in the dark. Flow cytometric analysis was completed with the FL2 parameter using a FACSCalibur instrument (BD Biosciences, Mississauga, ON). Events were limited to 20-40 per sec to minimize G_0/G_1 doublets. The data were analyzed using ModFitLT V2.0 software (Becton Dickson, CA) provided by Dr. T. Lee (Dalhousie University, NS) to determine percentage of cells in each phase of the cell cycle. The subG1/0 population was also quantified and represents cells that are undergoing apoptosis (Riccardi and Nicoletti 2006).

2.8 Annexin-V-FLUOS/PI Assay

The annexin-V-FLUOS/PI assay was used to determine whether fisetin induced cell death by apoptosis and/or necrosis. Phosphotidylserine, an anionic phospholipid, is normally located on the inner leaflet of healthy cells. During apoptosis, which is characterized by disruption of the cell membrane symmetry, phosphotidylserine is exposed on the outer leaflet. Annexin-V-FLUOS is a calcium-dependent phospholipid-binding protein, with high affinity of phosphatidylserine, which identifies the disruption of the cell membrane during apoptosis (Meers and Mealy 1993). PI is a DNA-intercalating agent that enters the cells when the cell membrane becomes permeable, as occurs during necrosis. Although PI staining suggests necrosis, it is not definitive in the environment of tissue culture. *In vivo*, cells that undergo apoptosis signal via phosphatidylserine exposure (Fadok, Voelker et al. 1992; Verhoven, Schlegal et al. 1995) for phagocytes to engulf the apoptotic bodies or cellular debris. If this does not occur, the

membrane undergoes further damage, which allows PI into the cell. During analysis, the presence of annexin-V-FLUOS and PI staining suggests a cell undergoing late apoptosis or necrosis. MDA-MB-468 and MDA-MB-231 breast cancer cells were harvested and plated at a concentration of 50,000 cells per well in a 6-well plate. The cells were allowed to adhere for 24 h. Fisetin, vehicle or medium alone treatments were applied for specific time periods (24, 48 and 72 h). After incubation, cells were harvested with TrypLE and centrifuged at 500 × *g*. The cells were labeled with Annexin-V-FLUOS prepared according to the manufacturer's protocol (Roche Diagnostics, Laval, QC; (Rode, Eisel et al. 2009)) and PI (1 µg/ml) in staining buffer (10 mM HEPES, 10 mM NaCl, and 5 mM $CaCl_2$) for 15 min at room temperature. Flow cytometric analysis was performed using a FACSCalibur instrument (BD Bioscience, Mississauga, ON).

2.9 Lactate Dehydrogenase (LDH) Release Assay

Loss of cell membrane integrity allows LDH to exit the cell, which is an indication of cell damage that is consistent with necrosis. The protocol used was a modification of the original protocol (Korzeniewski and Callewaert 1983). MDA-MB-468 and MDA-MB-231 breast cancer cells were harvested and centrifuged at 500 × *g*. The cells were plated at a concentration of 5000 cells per well in a 96-well flat-bottomed plate. After cells were allowed to adhere for 24 h. Fisetin, vehicle or medium alone treatments were then applied for specific time periods. The supernatants from each well were transferred to a new 96-well flat bottomed plate. LDH release was measured from the supernatants using the CytoTox 96© nonradioactive cytotoxicity assay (Promega Corp, Madison, WI). Maximal cell lysis control was achieved by 3 freeze/thaw cycles

and mechanical manipulation with a pipette tip. A EL×800 UV Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) provided by Dr. J. Marshall (Dalhousie University, NS), was used to read absorbance at 490 nm. The percent LDH release was calculated using the equation [(E/S)/(M/S)] x 100, where E equals experimental, S equals spontaneous/medium control and M equals maximal LDH release.

2.10 Mitochondrial Membrane Potential Assay

The $DiOC_6$ fluorescence-based assay was used to assess the change in mitochondrial membrane potential as a surrogate of a change in mitochondrial membrane integrity (Pringle, Preston et al. 1989). $DiOC_6$ accumulates with a high affinity in mitochondria that have a normal transmembrane potential, creating a higher mean channel fluorescence. If the mitochondrial membrane looses its charge, pores are created and the $DiOC_6$ dye is released. This movement of fluorescence is represented as a left shift on the x-axis when cells are analyzed by flow cytometry. MDA-MB-468 and MDA-MB-231 breast cancer cells were harvested, plated at 50,000 cells per well on 6-well flat-bottomed plate and allowed to adhere for 24 h. The cells were treated with fisetin, vehicle or medium alone for 72 h. After this time period, the cells were harvested, centrifuged at 500 × *g*, washed with PBS and incubated with 40 nM $DiOC_6$ for 15 min at room temperature. This concentration of $DiOC_6$ preferentially accumulates in the mitochondria versus the other membrane bound organelles (Koning, Lum et al. 1993). The cells were analyzed using parameter FL1 by flow cytometry using a FACSCalibur instrument (BD Biosciences, Mississauga, ON).

2.11 Dihydroethidium (DHE) Reactive Oxygen Species Assay

Dihydroethidium (DHE; Invitrogen Corp., Burlington, ON) is a non-polar, non-fluorescent compound that diffuses into the cell and, in the presence of superoxide anions, is converted to ethidium (Fink, Laude et al. 2004). Since ethidium is a fluorescent compound, an increase in reactive oxygen species generation shifts the curve to the right on the x-axis when cells are analysed using flow cytometry. MDA-MB-468 breast cancer cells were harvested and plated at 50,000 cells per well on a 6-well plate. The cells were allowed to adhere for 24 h. Fisetin, vehicle or medium alone treatments were applied for 48 h. After this time period was completed, the cells were harvested, washed and resuspended in 0.5 ml of DMEM in the presence of 20 μl of 0.01 μM DHE. The cells were incubated for 15 min and analyzed on parameter FL2 with a FACSCalibur instrument (BD Biosciences, Mississauga, ON).

2.12 Crystal Violet Based Antioxidant Pretreatment

MDA-MB-468 breast cancer cells were harvested, centrifuged and plated at 5000 cells per well in a 96-well flat-bottomed plate. After allowing the cells to adhere for 24 h, a 1 h pretreatment with antioxidant (5mM GSH or 10 mM NAC) was completed and then fisetin, vehicle or medium alone was applied. The dose of the antioxidants was used since it is in the normal physiological range (1 to 10 mM) and required for rapid refolding of proteins (Hwang, Sinskey et al. 1992). After the time period was completed, the cells were washed, treated with crystal violet and analyzed as described above.

2.13 Annexin-V- FLUOS /PI Based Caspase Inhibitor Assay

MDA-MB-468 breast cancer cells were harvested and plated at 50,000 cell per well on a 6-well plate. The cells were allowed to adhere for 24 h. Pan-caspase inhibitors Z-VAD-FMK or BOC-D-FMK at 50 μM concentration were applied to the cells for 30 min prior to the fisetin, vehicle or medium alone treatments. The plates were incubated for 72 h. After incubation, cells were harvested with TrypLE and centrifuged at 500 × *g*. The cells were labeled with Annexin-V-FLUOS diluted according to the manufacturer's protocol (Roche Diagnostics, Laval, QC) and PI (1 μg/ml) in staining buffer (10 mM HEPES, 10 mM NaCl, and 5 mM $CaCl_2$) for 15 min at room temperature. Flow cytometric analysis was performed using a FACSCalibur instrument (BD Bioscience, Mississauga, ON).

2.14 Western Blotting

2.14.1 Preparation of Total Cell Lysates

MDA-MB-468 breast cancer cells were harvested, centrifuged and a total of 700,000 cells were placed in 75 mm^2 culture flasks. The cells were allowed to adhere for 24 h. Fisetin, vehicle or medium alone treatments were then applied for a range of time points (12, 24 and 48 h). After the desired time period, cells were harvested, centrifuged at 500 × *g*, resuspended in 1 ml of ice cold PBS and transferred to a 1.5 ml Eppendorf tube. The cells were centrifuged at 1000 × *g* for 5 min at 4°C. The supernatants were removed and the pellet was resuspended in 60 μl RIPA lysis buffer solution (0.1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% sodium dodecylsulphate (SDS); 20 mM

Tris-HCl; 150 mM sodium chloride (NaCl); 1mM ethylenediaminetetraacetic acid (EDTA), 1mM ethylene glycol tetraacetic acid (EGTA) pH 7.5 with 5 μg/ml pepstatin, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1mM phenylmethyl sulfonyl fluoride (PMSF); 100 μM sodium orthovanadate (Na_3VO_4), 1mM dithiothreitol (DTT), 10 mM sodium fluoride (NaF) and 10 μM phenylarsine oxide (PAO)). The cells were incubated for 30 min on ice and then centrifuged at 4°C at 14,000 × *g* for 10 min. The supernatant, which contained cellular protein, was transferred to a 1.5 ml Eppendorf tube and stored at -80°C.

2.14.2 Digitonin Lysis Preparation of Cytosolic Protein

Digitonin, a gentle detergent, in a salt-containing buffer was used to permeabilize the plasma membrane without damaging the mitochondrial membrane or other organelles in order to isolate cytosolic cytochrome-*c* (Adam, Marr et al. 1990; Gottlieb and Granville 2002). MDA-MB-468 breast cancer cells were prepared in a similar fashion to the preparation of total cell lysates; however, digitonin lysis buffer (190 μg/ml digitonin; 1 mM NaH_2P0_4; 8 mM Na_2HPO_4; 75 mM NaCl; 250 mM sucrose; 100 μM Na_3VO_4; 1 mM DTT; 10 mM NaF; 10 μM phenylarsine oxide with 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 10 μg/ml aprotinin) was used use instead of RIPA lysis buffer. The cells were lysed for 15 min with 40 μl digitonin lysis buffer, centrifuged at 10,000 × *g* for 10 min at 4°C and the supernatants collected.

2.14.3 Western Blot Analysis

After the protein content across treatment groups was standardized by Bradford assay (Kruger 2002) (Bio-Rad Laboratories Ltd., Mississauga, ON), equal amounts of protein (10-20 μg) were loaded into wells of a SDS-polyacrylamide gel. The consistency of the gel (7.5, 12, or 15% polyacrylamide) was selected on the basis of the size of the protein of interest. Proteins were resolved for 1 h at 200 volts. The gel was removed from the cassette and the proteins were transferred to a nitrocellulose membrane using the iBlot transfer system according to the manufacturer's protocol (Invitrogen, Burlington, ON). The membrane was blocked in 5% mass per volume of skim milk powder, which was mixed in Tris-buffered saline (200 mM Tris, 1.5 M NaCl (pH 7.6)) containing 0.05% Tween-20 (T-TBS). After completion of the blocking period, the membrane was washed extensively for 1 h with T-TBS (changed every 5 min) and incubated with the selected primary antibody (Ab) overnight at 4°C. Blots were washed again with T-TBS for 60 min and incubated with the appropriate HRP-conjugated secondary antibody in 5% w/v skim milk powder in T-TBS for 1 h. Protein bands were detected after using chemiluminescence western blotting detecting reagent (Bio-Rad Laboratories Inc., Hercules, CA). The blots were reprobed using goat anti-actin Ab for 1 h, washed and HRP-conjugated bovine anti-goat IgG Ab was applied. The detection of the bands was performed as above. Actin quantification was completed to control for possible differences in protein loading. Protein expression was determined relative to β-actin expression using densitometry and the Scionimage program (Scionimage.com).

Primary Abs used included antihuman PARP-1 monoclonal Ab, (mAb) antihuman actin polyclonal Ab, as well as horseradish perodidase (HRP)-conjugated bovine antigoat

IgG, antimouse IgG-HRP secondary Ab (Biotechnology Inc., Santa Cruz, CA). Other primary antibodies used were mouse antihuman cytochrome-*c* mAb was from BD Pharmingen (Mississauga, ON), antirabbit ABCB1 mAb (Rockland Inc., Gilbertsville, PA) and antirabbit ABCG2 mAb (Cell Signaling Technology, Daners, MA). All Ab were prepared as a dilution of 1 in 1000 except for anticytochrome-*c*, which was at 1 in 250.

2.15 Crystal Violet Assay with Chemotherapeutic Agents and Fisetin Combination

MDA-MB-468 and MDA-MB-231 breast cancer cells were harvested, centrifuged and plated at 5000 cells per well in a 96-well flat-bottomed plate as previously described. The cells were allowed to adhere for 24 h. The cells were pretreated with the fisetin treatment, vehicle or medium alone for 1 h prior to treatment with standard chemotherapeutic agents (methotrexate, cisplatin, 5-FU, docetaxel, doxorubicin, or 4-OH). The cells were incubated for 72 h and then crystal violet was applied as described above. An EL×800 UV Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT), provided by Dr. J. Marshall (Dalhousie University, NS), was used to read absorbance at 570 nm. Optical densities of the treatment groups were compared to the medium control using the equation (1 - (experimental reading/ medium control reading)) × 100 to calculate the percent cell quantity change for each treatment group.

2.16 Crystal Violet Assay with Radiation and Fisetin Combination

MDA-MB-468 and MDA-MB-231 breast cancer cells were harvested, centrifuged at 500 × *g*, and plated at 3000 cell per well in a 4-well flat-bottomed plate. The cells were allowed to adhere for 24 h. The cells were then pretreated with fisetin, vehicle or medium

alone for 1 h and then exposed to h 0-4 Gy γ-radiation using the GC3000 ^{137}Cs source (MDS Nordion, Ottawa, ON). The cells were incubated for 72 h, the medium was removed and room temperature PBS was used to wash the cells. Crystal violet (0.4% in methanol) 0.2 ml was added to each well for 10 min. The crystal violet was removed and washed with ddH_2O. The plates were dried on paper towel and 0.5 ml of DMSO was added to each well. Once the stained cells were dissolved, 100 µl of liquid was transferred to a well in a 96-well flat-bottomed plate for each treatment group (quadruplicate). A EL×800 UV Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT), provided by Dr. J. Marshall (Dalhousie University, NS), was used to read absorbance at 570 nm. Optical densities of the treatment groups were compared to the medium control using the equation (1 - (experimental reading/ medium control reading)) × 100 to calculate the percent cell quantity change for each treatment group.

2.17 *In Vivo* Toxicity Screen

Zebrafish (*Danio rerio)* were provided by Dr. J. Berman (Dalhousie University, NS). The fish were maintained, bred and developmentally staged according to Westerfield's protocol (Westerfield 1995). The use of zebrafish in this experiment was approved by the Dalhousie University Animal Care Committee. Zebrafish were maintained in 28.5°C water with a salinity of 1100-1300 us and a pH between 6 and 8. Fish were exposed to light for 14 h. Zebrafish embryos were collected and incubated at 28.5°C in egg water (5mM NaCL; 0.17 mM KCL; 0.4 mM $CaCl_2$; 0.16 mM $MgSO_4$). Embryos were treated with pronase (10mg/ml) (Roche, Indianapolis, IN) in propylenethiourea (PTU) water (0.003% 1-phenyl-2-thiourea; Sigma-Aldrich Canada

Ltd., Oakville, ON) to remove their chorions. The embryos were incubated to 72 h post fertilization and plated at one embryo per well in a 96-well flat-bottomed plate. Fisetin, vehicle or egg water alone treatments were applied to the embryos (total volume was 300 µl per well). The embryos were incubated at 37°C and assessed for viability every 24 h over a total of 72 h. The viable embryos were counted and dead embryos were removed. The treatment groups were compared for survivability.

2.18 Statistical Analysis

Statistical analysis was conducted using the Instat statistics program (GraphPad Software Inc., San Diego, CA). Statistical comparisons were performed using Student's t-test or one-way analysis of variance (ANOVA) and the Tukey–Kramer multiple comparisons test; $p < 0.05$ was considered statistically significant.

Chapter 3.0 Results

3.1 Fisetin reduced the quantity of breast cancer cells.

The effect of fisetin on breast cancer cells was studied using a panel of breast cancer cell lines and several different assays. The different breast cancer cell lines were characterized mainly on the basis of hormone receptor type (estrogen or progesterone), presence or absence of the HER-2/neu receptor, and whether or not they expressed wild-type or mutated p53. Human mammary epithelial cells (HMEC), human dermal fibroblasts and human umbilical vein epithelial cells (HUVEC) were used to investigate the effect of fisetin on normal cell growth. Various assays were used as markers of cell quantity including the crystal violet assay to assess the amount of protein present (Saotome, Morita et al. 1989), the MTT assay to measure mitochondrial succinate reductase activity (Mosmann 1983), and the acid phosphatase assay to determine cytosolic phosphatase activity (Yang, Sinai et al. 1996). A reduction in optical density in these assays suggests a decrease cell quantity. All cell lines were investigated using the crystal violet assay and at least one other assay, either the acid phosphatase assay or MTT assay, was used for breast cancer cell lines.

Breast cancer cell lines demonstrated variable responses to fisetin exposure. MDA-MB-468 (Oliveras-Ferraros, Vazquez-Martin et al. 2008; Hirsh, Iliopoulos et al. 2009) and MDA-MB-231 (Liu, Fan et al. 2009) triple negative breast cancer cell lines (ER, PR and HER-2 negative) had different patterns of response (Figure 3). MDA-MB-468 cells showed a significant decrease in protein concentration when exposed to fisetin for 24 hours at two different concentrations: 26% at 50 μM and 42% at 100 μM. These results were consistent with those obtained when mitochondrial succinate dehydrogenase

activity was measured at 24 hours, which demonstrated a 26% and 38% decrease in formazan crystal formation at fisetin concentrations of 50 μM and 100 μM respectively. The dose- and time-dependent responses for both breast cancer cell lines were significant at 48 hours and 72 hours, although MDA-MB-231 cells demonstrated some resistance at the lower concentrations of fisetin (25 and 50 μM) for these time points. MDA-MB-231 cells demonstrated maximal decrease at fisetin concentrations of 100 μM at 72 hours, with an 81% decrease of cytosolic phosphatase activity, 50% decrease in protein levels and 34% decrease in mitochondrial succinate reductase activity. Since these cell lines demonstrated a range of dose- and time-related responses to fisetin as well as both being triple negative phenotype, they were the focus of the remainder of the research.

SK-BR-3 (Figure 4A, 4B) and MCF-7 (Figure 4A, 4B, 4C) breast cancer cells demonstrated dose- and time-dependent responses to fisetin. At 24 hours of exposure, SK-BR-3 breast cancer cells demonstrated a 30% decrease in protein and acid phosphatase activity at a fisetin concentration of 50 μM. At a 100 μM fisetin concentration, SK-BR-3 cells showed a 38% decrease in protein quantity and an associated 35% decreased in acid phosphatase activity. MCF-7 cells, at 48 hours, demonstrated a significant decrease in cell quantity in all three assays (Figure 4). The above findings are in contrast to those observed in T47-D breast cancer cells (Figure 4A, 4C), which, demonstrated resistance to fisetin at all time points and fisetin concentrations; however, significant decrements in protein quantity were seen using the crystal violet assay, with a 27% decrease in protein quantity at 72 hours exposure to 100 μM fisetin concentrations. Of note, MTT is converted by fisetin to formazan crystal in the

absence of viable cells (Figure 5), which leads to an underestimation of the decrease in cell quantity for this assay.

The effect of fisetin on normal cells was examined using the crystal violet assay (Figure 6). HMEC and fibroblasts were resistant to those concentrations of fisetin that were effective at causing breast cancer cell number decrease. HMEC only demonstrated a significant decrease of cell quantity at 72 hours, i.e., 23% and 25% reduction for fisetin concentrations of 50 and 100 μM, respectively (Figure 6A). Fibroblasts demonstrated no significant decrease in cell number for any treatment (Figure 6B). In contrast HUVEC demonstrated a significant decrease in cell quantity at all concentrations of fisetin over all time points (Figure 6C).

3.2 Fisetin decreases the ability of breast cancer cells to proliferate

Fisetin was investigated for its effect on the ability of breast cancer cells to proliferate and form colonies. An Oregon Green 488® cell proliferation assay, which uses a fluorescent dye that enters the cell or binds irreversibly to the cell membrane, was used to measure the effect on cell proliferation (Wallace, Tario et al. 2008). At each cell division, the total fluorescence for each daughter cell decreases by half, which can be detected by flow cytometry. Each treatment group was stained and incubated for 72 hours, at which point its fluorescence was assessed and compared to the maximum fluorescence achieved by harvesting a non-proliferating control culture at the time of staining. A shift to the left on the x-axis represents cell proliferation (Figure 7). Untreated MDA-MB-468 breast cancer cells underwent a median of 3.2 cycles of cell division but only 2.4, 2.2 and 1.7 cycles of division at fisetin concentrations of 25 μM, 50

μM and 100 μM, respectively. These results were statistically significant at all three concentrations of fisetin. MDA-MB-231 breast cancer cells demonstrated similar results, with a median of 3.1 cycles of division for untreated cells but only 2.5, 2.1 and 1.2 divisions for 25 μM, 50 μM and 100 μM concentrations of fisetin, respectively. Statistical significance was reached for at 50 μM and 100 μM concentrations of fisetin. These data were a compilation of three individual experiments and calculated using the equation: $MCF_{control} = 2^n \times MCF_{experiment}$.

A second assay, the clonogenic assay, was used to assess the ability of breast cancer cells to form colonies; this assay exposed cells of varying concentrations of fisetin (25 μM, 50 μM and 100 μM), vehicle or medium alone for 24 hours. The cells were then harvested, counted and plated at different dilutions to facilitate counting of individual colonies after 13 days of incubation. By definition, cells that maintained their proliferative capacity were those that generated a colony count of 50 cells or more (Tannock, Hill et al. 2005). Colonies per 1000 cells plated were calculated.

MDA-MB-468 breast cancer cells demonstrated a statistically significant decrease in colony forming ability at fisetin concentrations of 25 to 100 μM (Figure 8C). The average colonies per 1000 cells plated decreased from 62 when incubated with vehicle control to 1 when exposed to 100 μM of fisetin. MDA-MB-231 breast cancer cells also showed negative effects on proliferation when exposed to fisetin with statistically significant decreases from an average of 272 colonies per 1000 cells for vehicle control to 86 and 6 colonies per 1000 cells at 50 μM fisetin and at 100 μM fisetin, respectively (Figure 8C). A visual representation of this assay is shown in Figures 5.

The ability of the two breast cancer cell lines to create colonies varied. MDA-MB-231 breast cancer cells generated more colonies in the presence of medium alone, creating 313 colonies per 1000 cells plated, as compared to MDA-MB-468 breast cancer cells that created only 62 colonies per 1000 cells plated. This suggests that MDA-MB-231 breast cancer cells proliferate at an increased rate and may therefore, be more aggressive.

3.3 Fisetin induces cell cycle arrest

In order to investigate the effect of fisetin on cycle progression, cell cycle analysis was performed using permeabilized cells and a fluorescent compound (PI) that stained DNA. This allowed DNA quantity to be measured per cell using flow cytometry. A normal cell's complement of DNA is 2N (G_0/G_1), while cells in mitosis (G_2/M) have a 4N complement of DNA; any quantity of DNA between 2N and 4N thus represents the stage of synthesizing DNA (S) (Tannock, Hill et al. 2005). The cells were serum starved for 12 hours to synchronize the cell population, since cells denied serum before the restriction point to S-phase enter a G_0, while cells starved after the restriction are already committed to mitosis (Pardee 1974). MDA-MB-468 breast cancer cells treated with 50 μM fisetin showed a significant increase in percentage of cells in G_2/M phase (38%) compared to the vehicle control (23%). There was also a significant increase in the number of cells undergoing apoptosis (subG_1), with 21% of cells in the fisetin-treated group as compared to 2% of cells in the vehicle control group. This increase in these two phases corresponds to a significant decrease in cells in the G_1 (Figure 9A). MDA-MB-231 breast cancer cells demonstrated a different pattern of cell cycle arrest with a

significant increase in S-phase for both 50 μM and 100 μM fisetin concentrations; the was no significant increase in apoptosis (Figure 9B).

3.4 Fisetin is cytotoxic to breast cancer cells

Since fisetin exhibited anti-proliferative effects and the possibility of cytotoxicity was established in the apoptotic population (subG1) of MDA-MB-468 breast cancer cells shown in cell cycle analysis (Figure 9A), I next investigated the cytotoxic effect of fisetin using Annexin-V-Fluos/PI and LDH assays.

The Annexin-V-Fluos/PI assay uses combined staining with a fluorescent phospholipid-binding protein (Annexin-V-Fluos), which binds to phosphotidylserine (PS) that is expressed on the outer leaflet of the cell membranes during apoptosis (Meers and Mealy 1993), and PI, which is permeable to the disrupted cellular membrane of necrotic cells or apoptotic cells in the final stage of cell death. A representative plot graph for Annexin-V is demonstrated in Figures 10A and 10B. This assay demonstrates a dose- and time-dependent response induction of apoptosis in fisetin treated MDA-MB-468 (Figure 10C) and MDA-MB-231 breast cancer cell lines (Figure 10D).

MDA-MB-468 breast cancer cells demonstrated significant cytotoxicity in response to fisetin, with a predominance of apoptosis occurring at all time points. The total cytotoxicity of the cell population treated with 100 μM fisetin at 24 hours was 46%, with apoptosis being the dominant component (38%). At 72 hours the total cytotoxicity was 61% and apoptosis and late apoptosis/necrosis occurred in 38% and 22% of the cell population, respectively (Figure 10C). Statistical significance was also reached at 50 μM fisetin. However, MDA-MB-231 cells demonstrated a different pattern of cytotoxicity

(Figure 10D). Significant total cytotoxicity was not achieved until after 48 and 72 hours exposure to fisetin, and only at 100 μM fisetin (20% and 32%, respectively). Both apoptosis and late apoptosis/necrosis were significant in these treatment groups. This demonstrates a relative resistance of MDA-MB-231 cells to fisetin, consistent with previous observations of the effect of fisetin on cell quantity and proliferation assays.

Lactate dehydrogenase (LDH) release was used to further assess the loss of membrane permeability as a component of cell death; loss of LDH from cells indicates necrosis (Korzeniewski and Callewaert 1983). Neither, MDA-MB-468 (Figure 11A) nor MDA-MB-231 breast cancer cells (Figure 11B) demonstrated LDH release when compared to the vehicle treated cells. This finding was consistent across all treatment groups and time points. Although there was a component of late apoptosis/necrosis observed in the Annexin-V-Fluos/PI assay for MDA-MB-468 cells at 72 hours, and for MDA-MB-231 cells at 48 and 72 hours at 100 μM fisetin, the LDH assay did not suggest a major role for necrosis at these time points in fisetin induced cytotoxicity.

3.5 Fisetin destabilizes the mitochondria and releases cytochrome-*c*

Mitochondria are critical for the intrinsic pathway of apoptosis (Gupta 2001). $DiOC_6$ is a fluorescent dye compound that enters the cell and binds to membrane-bound organelles, and at a 40 nM concentration preferentially binds to mitochondria (Koning, Lum et al. 1993). A loss of mitochondrial membrane potential leads to a corresponding loss of fluorescence that can be measured using flow cytometry. A left shift on the x-axis represents a loss of mitochondrial membrane potential and the release of proapoptotic compounds such as cytochrome-*c*. MDA-MB-468 (Figure 12A) and MDA-MB-231

(Figure 12B) breast cancer cell lines after 72 hours exposure to 100 μM fisetin demonstrate this leftward shift.

Mitochondrial destabilization and release of proapoptotic compounds was confirmed by Western blotting for cytochrome-*c*, is major component of mitochondria-induced apoptosis. Cytochrome-*c* binds to and activates apoptotic protease activating factor 1 (APAF-1) and procaspase-9, forming the "apoptosome" which propagates the caspase cascade of apoptosis (Chinnaiyan 1999). A pilot study was performed using cells treated for 12, 24 and 48 hours for treatment with 50 μM or 100 μM fisetin to identify the optimal time point of maximal cytochrome-*c* release. Three individual experiments were then completed using the 24 hour time point and significance was identified for both concentration of fisetin using densitometry (Figure 12C).

3.6 Fisetin induces caspase-dependent apoptosis

Previous experiments showed that fisetin has cytotoxic effects on MDA-MB-468 breast cancer cells, predominantly by apoptosis. An investigation was therefore performed using the Annexin-V-Flous/PI assay and pretreatment of two different pan-caspase inhibitors, Z-VAD-FMK and BOC-D-FMK. Caspases are divided into initiator (Caspase-8, -9, -10) and executioner (caspase-3, -6, -7) caspases (Los, Wesselborg et al. 1999). The executioner caspases are the common components of both the external and internal pathways of apoptosis (Los, Wesselborg et al. 1999).

MDA-MB-468 breast cancer cells were treated for 1 hour with one of the two pan-caspase inhibitors at a concentration of 50 μM prior to treatment with fisetin (50 or 100 μM). Figure 13A shows representative data obtained from one experiment (n=3), and

demonstrates a decrease in total cytotoxicity as well as a decrease in the number of cells undergoing apoptosis. There was a significant decrease in total cytotoxicity between 100 μM fisetin alone (80%) and pretreatment with Z-VAD-FMK (42%) or BOC-D-FMK (32%). The decrease in total cytotoxicity that occurred due to the decreased number of cells undergoing apoptosis with 100 μM fisetin alone (60%) versus Z-VAD-FMK and BOC-D-FMK pretreatment with 100 μM (16% and 19%, respectively). There was no significant change in the proportion of cells undergoing late apoptosis/necrosis when fisetin concentrations of 100 μM were compared to the group pretreated with pan-caspase inhibitors (Figure 13B).

After the fisetin intragroup comparison for each treatment (i.e. 100 μM fisetin alone versus 100 μM with each pan-caspase pretreatment), the data was analyzed to assess if there was a significant difference between the pan-caspase pretreatment groups with fisetin versus the pan-caspase inhibitors alone. A significant increase in total cytotoxicity of 42% was maintained for both pan-caspase pretreatments with 100 μM fisetin when compared to the pan-caspase treatment alone (Z-VAD-FMK 13%; BOC-D-FMK 18%). This corresponded with a significant increase in late apoptosis/necrosis with pan-caspase pretreatment with fisetin 100 μM (Z-VAD-FMK and fisetin 26%; BOC-D-FMK and fisetin 23%) versus pan-caspase control (Z-VAD-FMK 8%; BOC-D-FMK 10%) (Figure 13B).

PARP, a substrate of executioner caspases, was then investigated in order to identify downstream effects the fisetin induced caspase cascade (Herceg and Wang 2001). MDA-MB-468 cells were treated with 50 μM of fisetin, vehicle or medium alone for 24 hours, then protein was collected and a Western blot was performed. Figure 14

shows a representative blot for PARP (112 kDa) and its cleavage products (87 kDa). Densitometry was performed and showed a significant decrease in intact PARP and an increase in the cleavage product (n=3), which is consistent with caspase activation.

3.7 ROS was not involved in the cytotoxicity of fisetin

Moderate ROS production in cells can lead to apoptosis through several signaling pathways (Yin, Terauchi et al. 1998) while severe oxidative stress induced by ROS production will cause the cell to undergo necrosis (Hampton and Orrenius 1997). I therefore, investigated of ROS's role in fisetin induced cell death.

Superoxide anion (O_2^-) is a common form of ROS; production of this anion was investigated using dihydroethidium (DHE) to stain breast cancer cells. DHE is a non-polar, non-fluorescent compound that incorporates into the cell and is converted to ethidium, a fluorescent compound, in the presence of O_2^- (Fink, Laude et al. 2004). Figure 15 illustrates the data obtained from one representative experiment (n=3). While the shift of the x-axis to the right suggests increased fluorescence and O_2^- production, there was, however, an equivalent shift in the absence of DHE. Thus no conclusions can be made because the flow cytometry results are confounded by the fluorescence caused by the presence of fisetin alone.

Since DHE only measures O_2^- production, a general assay was performed to evaluate the effect of fisetin on breast cancer cell numbers in the presence of fisetin with and without antioxidants. Using the crystal violet assay, cells were pretreated with 10 μM n-acetylcysteine (NAC) or glutathione (GSH), and then exposed to cytotoxic concentrations of fisetin. In the presence of these antioxidants, there was no decrease in

cell quantity caused by fisetin. In fact at lower concentrations of fisetin there is an increase in cytotoxicity in the presence of antioxidants (Figure 16). Although the production of ROS by fisetin cannot be excluded, it is unlikely since there was no protective effect of NAC and GSH.

3.8 MDR breast cancer cells are also relatively resistant to fisetin

Resistance of cancer cells to potentially curative chemotherapeutic agents is a concern for the treatment of cancer. Two drug-resistant breast cancer cells lines were developed by incubating MCF-7 breast cancer cells with increasing concentrations of mitoxantrone (MCF-7 MITX) and paclitaxel (MCF-7 TX400) in a step-wise fashion (Kars, Iseri et al. 2006). Both MCF-7 MITX and MCF-7 TX400 cells were resistant to fisetin (50, 100 or 200 μM) in comparison to the native MCF-7 breast cancer cell line. This effect was demonstrated using the crystal violet and acid phosphatase assays. Native MCF-7 cells were significantly decreased in number at 24 hours following treatment with fisetin 100 μM (crystal violet 44%; acid phosphatase 38%) and 200 μM (crystal violet 53%; acid phosphatase 50%). However, MCF-7 MITX and MCF-7 TX400 cells required higher concentrations of fisetin and/or longer exposure (Figure 17). For example, the acid phosphatase assay performed at 72 hours on cells treated with 200 μM fisetin, showed an 80% decrease in MCF-7 cell number while MCF-7 MITX and MCF-7 TX400 showed a 66% and 36% decrease in cell number, respectively.

BCRP, an ATP-binding cassette (ABC) transport protein that mediates the efflux of several chemotherapeutic agents from the cell (Tannock, Hill et al. 2005), was confirmed by Western blotting to be present in MCF-7 MITX cells and not in native

MCF-7 or MCF-7 TX400 cells (Figure 18A). MCF-7 TX400 cells were also evaluated for resistance to paclitaxel and shown to be refractory to paclitaxel in comparison to the native MCF-7 breast cancer cells (Figure 18B).

3.9 Increased cytotoxic effect with the combination of fisetin and radiation

The general population, in particular, those individuals undergoing cancer therapy or having survived cancer, often ingest "natural" or homeopathic products in the hopes of improving their outcome (Sparber, Bauer et al. 2000). It is therefore important to explore possible interactions between natural products and treatments used in the gold standard of care.

Radiation is used after wide local excision of breast tumours and in advanced cancer post-mastectomy (Box and Russell 2004). Fisetin (25 or 50 μM) was used in combination with radiation treatment of breast cancer cell cultures in clinically relevant fractionated doses (0, 1, 2 or 4 Gy) since on average 2 Gy fractions are employed in current practice (Whelan, MacKenzie et al. 2002). MDA-MB-468 (Figure 19A) and MDA-MB-231 (Figure 19B) breast cancer cells were pretreated with the indicated concentrations of fisetin and then exposed to various doses of radiation. The preliminary data shows increased cytotoxicity after fisetin pretreatment followed by irradiation for both cell lines when compared to radiation monotherapy. Intra-group comparisons of the cytotoxic effect of fisetin concentrations with various doses of radiation demonstrated that MDA-MB-468 breast cancer cells treated with 50 μM fisetin and 4 Gy ionizing radiation demonstrated a 53% decrease in cell number versus only a 43% decrease after treatment with 50 μM fisetin alone (non significant). MDA-MB-231 breast cancer cells

showed a significant decrease in cell quantity when treated with 25 μM and 50 μM fisetin in combination with various doses of radiation. A 40% decrease was demonstrated with 50 μM fisetin and 4 Gy ionizing radiation while there was only a 23% decrease with 50 μM fisetin alone. This is preliminary data only, and complete analysis will require intra-group comparisons of cultures exposed to a single concentration of fisetin and escalating doses of radiation (i.e., the slope of the curve) and the use of assays performed for longer time periods.

3.9 Increased cytotoxic activity by fisetin in combination with chemotherapeutic agents

Systemic therapy for breast cancer involves using a number of chemotherapeutic agents and various protocols of administration (Stockler, Wilcken et al. 2000). Since flavonoids have demonstrated drug interactions in the literature (Galati and O'Brien 2004) and a preliminary *in vitro* comparison of the cytotoxic effects of fisetin alone or combined with various chemotherapeutic drugs was performed using MDA-MB-468 and MDA-MB-231 breast cancer cells. Fisetin (25 μM or 50 μM) ws used in combination with cisplatin (Figure 20), 4-hydroxycyclophosphamide (Figure 21), doxorubicin (Figure 22), 5-FU (Figure 23), docetaxel (Figure 24), or methotrexate (Figure 25) and all changes in cell number were determined by crystal violet assay. This preliminary data demonstrates that fisetin, used in combination with all of the chemotherapeutic agents used in these experiments decreased cell quantity, with the exception of methotrexate. Methotrexate did not show increased killing in combination with fisetin.

3.10 Fisetin does not affect zebrafish embryo viability

Fisetin –induced toxicities have been reported in the literature, including the possibility of inducing neonatal leukemia (Olaharski, Mondrala et al. 2005) by its action as a topoisomerase II inhibitor (Lopez-Lazaro, Willmore et al. 2010). Some other generalized toxicities of flavonoids identified in the literature include liver failure, contact dermatitis, hemolytic anemia, and estrogenic-related concerns (Galati and O'Brien 2004). A preliminary study of the effect of fisetin on zebrafish embryos was therefore, completed. Zebrafish were exposed to a range of fisetin concentrations (0 to 200 μM), vehicle or egg water alone at 72 hours post fertilization and monitored every 24 hours for impact on viability. The final assessment at 72 hours demonstrates that there was no significant effect on embryo viability at any fisetin concentrations (Figure 26).

Figure 3. Fisetin decreases MDA-MB-468 and MDA- MB-231 breast cancer cell number in culture. MDA-MB-468 and MDA-MB-231 cells were treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone for specific time periods (24, 48 or 72 h). (A) Crystal violet (0.4% in methanol) was applied to the cells to stain all protein. Cells were then dissolved in DMSO. The graph represents the mean of 6 individual experiments +/- SEM. (B) Phosphatase assay measured the quantity of cytosolic phosphatase from viable cells. Phosphatase buffer was combined with cells to create an acidic pH, then the cell membrane was lysed to release phosphatase and substrate was provided for the chemical reaction one and a half hours prior to analysis. The graph represents the mean of 3 individual experiments +/-SEM. (C) MTT (455 μg/ml) was added to the cells to measure mitochondrial succinate dehydrogenase. After 2 h medium was removed and formazan crystals were dissolved in DMSO. The graph represents the mean of 5 individual experiments +/- SEM. Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 3.

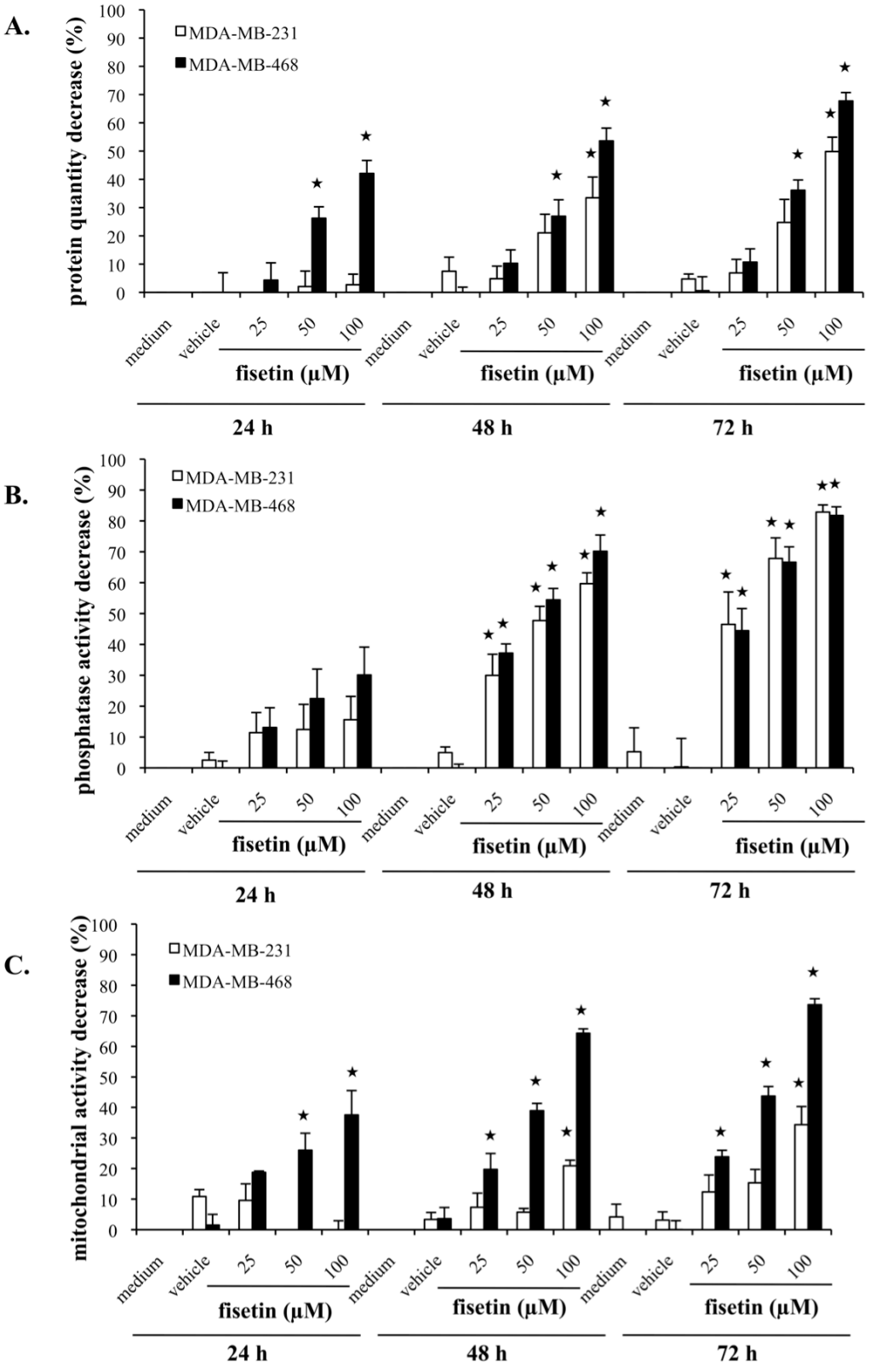

A.
protein quantity decrease (%)
MDA-MB-231
MDA-MB-468
medium
vehicle
25
50
100
fisetin (μM)
24 h
48 h
72 h
B.
phosphatase activity decrease (%)
C.
mitochondrial activity decrease (%)

Figure 4. Fisetin decreases T-47D, SK-BR-3 and MCF-7 breast cancer cell number in culture. T-47D, SK-BR-3 and MCF-7 cells were treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone for specific time periods (24, 48 or 72 h). (A) Crystal violet (0.4% in methanol) was applied to the cells to stain all protein. Cells were then dissolved in DMSO. The graph represents the mean of multiple individual experiments +/- SEM (T-47D, 4 experiments; SK-BR-3, 3 experiments; MCF-7, 6 experiments). (B) Phosphatase assay measured the quantity of cytosolic phosphatase from viable cells. Phosphatase buffer was combined with cells to create an acidic pH, then the cell membrane was lysed to release phosphatase and substrate was provided for the chemical reaction one and a half hours prior to analysis. The graph represents the mean of 3 individual experiments +/-SEM for MCF-7 and SK-BR-3. (C) MTT (455 μg/ml) was added to the cells to measure mitochondrial succinate dehydrogenase. After 2 h medium was removed and formazan crystals were dissolved in DMSO. The graph represents the mean of multiple individual experiments +/- SEM (T-47D, 3 experiments; MCF-7, 6 experiments). Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 4.

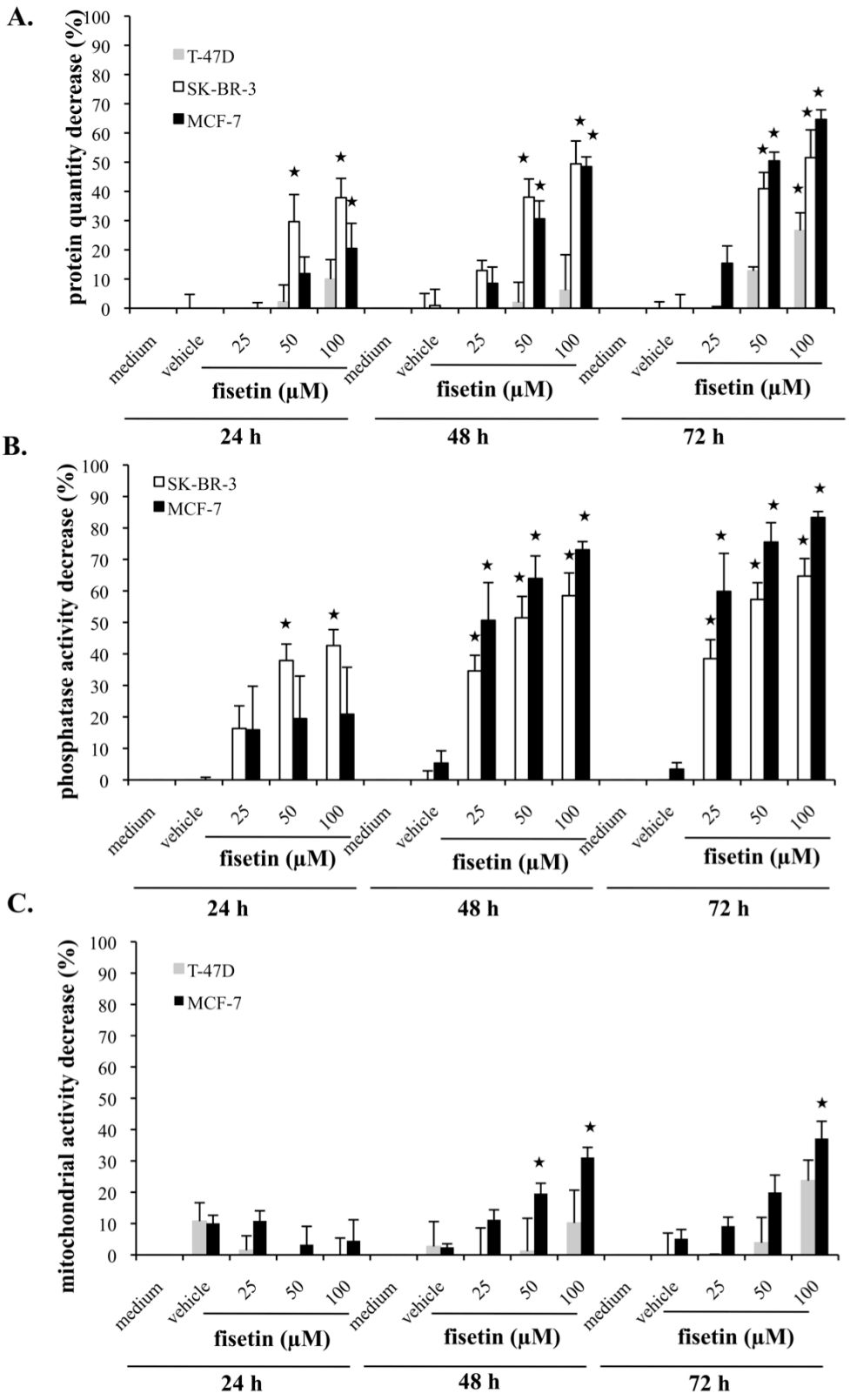

A.
protein quantity decrease (%)
T-47D
SK-BR-3
MCF-7
medium
vehicle
25
50
100
fisetin (μM)
24 h
48 h
72 h
B.
phosphatase activity decrease (%)
SK-BR-3
MCF-7
C.
mitochondrial activity decrease (%)
T-47D
MCF-7

Figure 5. Fisetin converts MTT to formazan crystals in the absence of cells. Medium, vehicle or 100 μM fisetin was added to MTT (455 μg/ml) in wells of a microtitre plate and left for 2 h in the incubator. Absorbance was then measured using the microplate reader at 470 nM. The graph represents the mean of three individual experiments +/- SEM. Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 5.

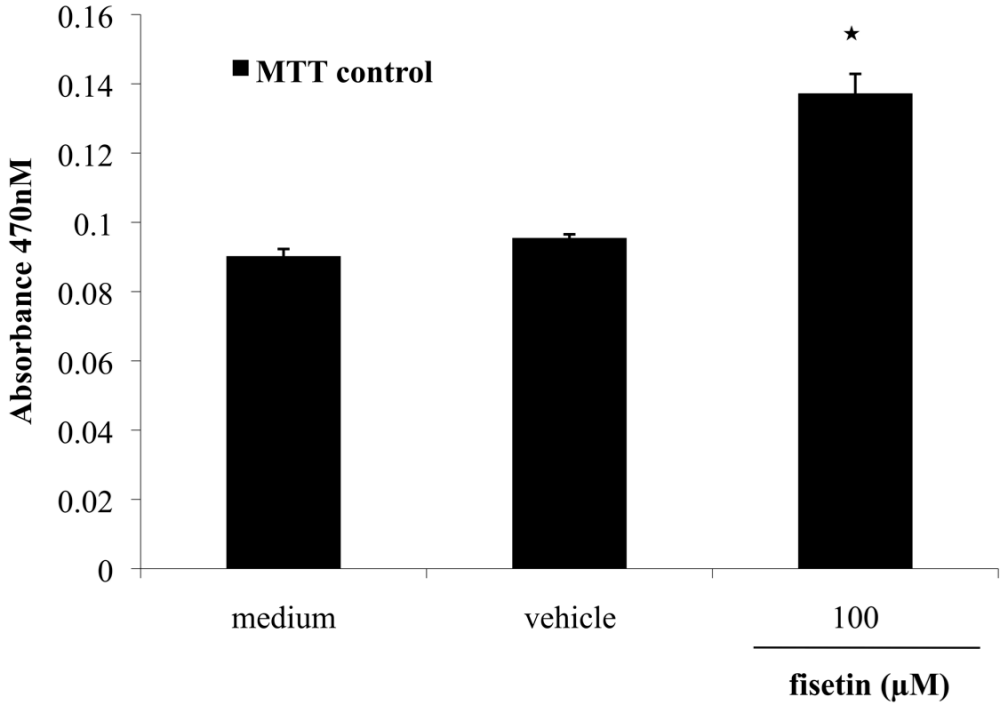

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance 470nM
MTT control
★
medium
vehicle
100
fisetin (μM)

Figure 6. HMECs and fibroblasts, but not HUVECs are resistent to fisetin. HMECs, fibroblasts and HUVECs cells were treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone were applied for specific time periods (24, 48 or 72 h). Crystal violet (0.4% in methanol) was applied to the cells, staining all protein, and then dissolved in DMSO. The graphs represent the mean of multiple individual experiments +/- SEM. (A) HMECs, 4 experiments; (B) Fibroblasts, 3 experiments; (C) HUVECs, 4 experiments. Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 6.

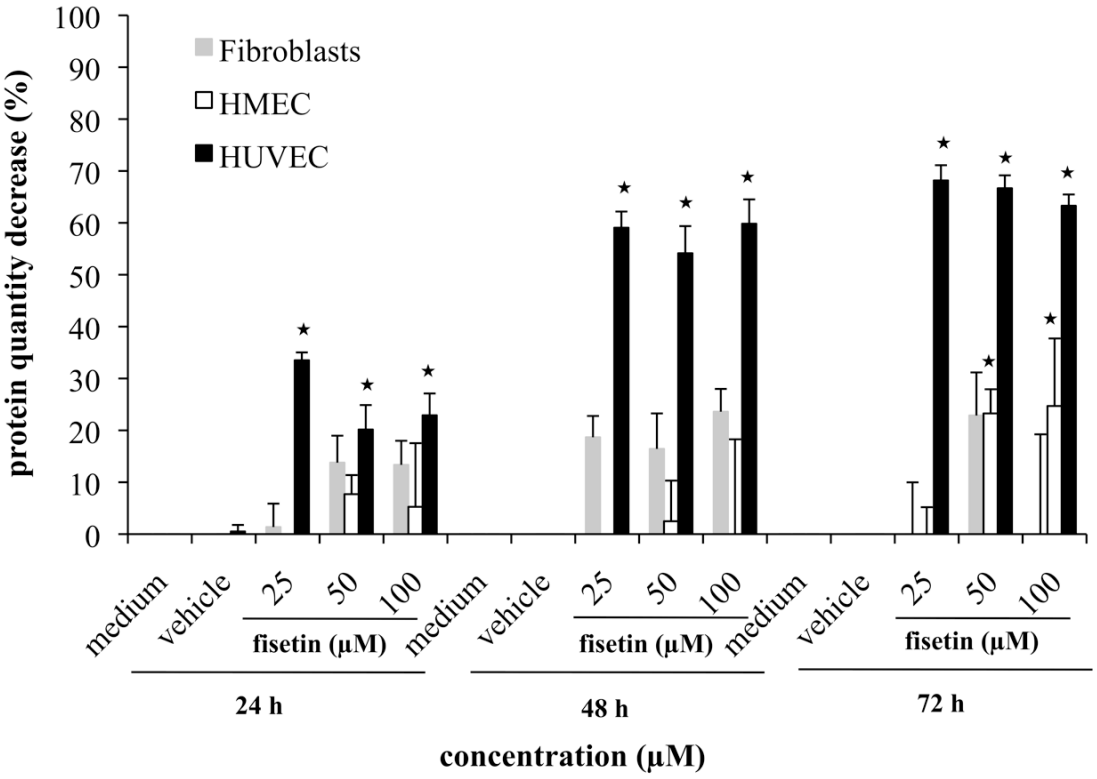
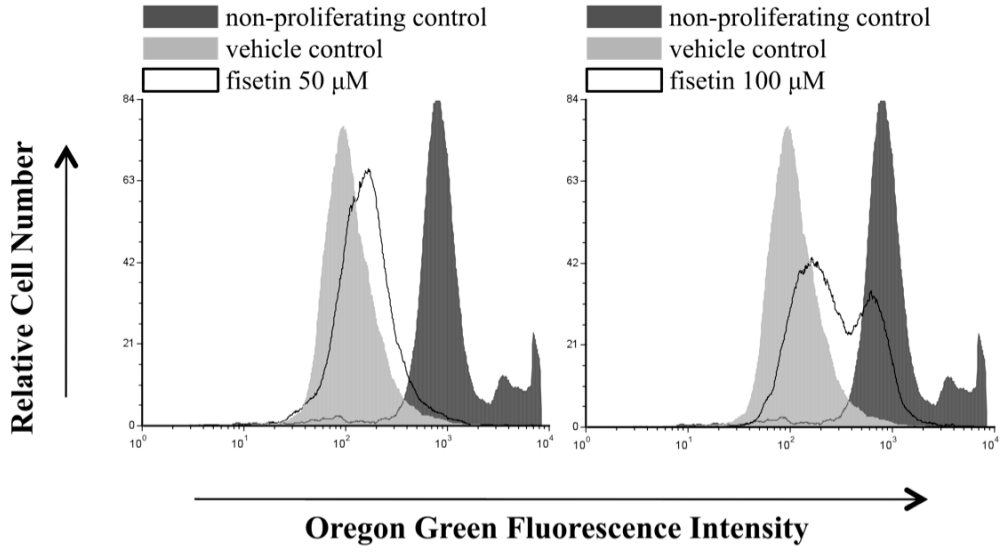

protein quantity decrease (%)
100
90
80
70
60
50
40
30
20
10
0
Fibroblasts
HMEC
HUVEC
medium
vehicle
25
50
100
fisetin (μM)
24 h
48 h
72 h
concentration (μM)

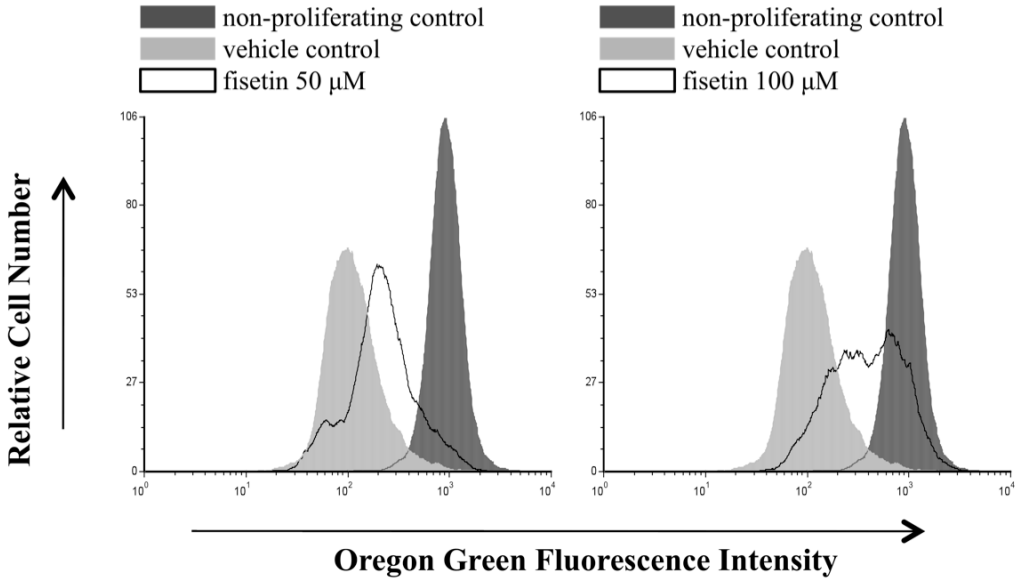
Figure 7. Fisetin inhibits MDA-MB-468 and MDA-MB-231 breast cancer cell proliferation. MDA-MB-468 and MDA-MB-231 breast cancer cells were plated for 24 h and treated with concentrations of fisetin (25, 50 and 100 μM), vehicle or medium alone for 72 h. A non-proliferative control was harvested at the time of treatment for standard comparison (no proliferation) and stored in 1% paraformaldehyde. (A) MDA-MB-468 and (B) MDA-MB-231 are representative of the three independent experiments. (C) The graph represents the mean of 3 individual experiments +/- SEM. Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 7.

A. MDA-MB-468



B. MDA-MB-231



C.

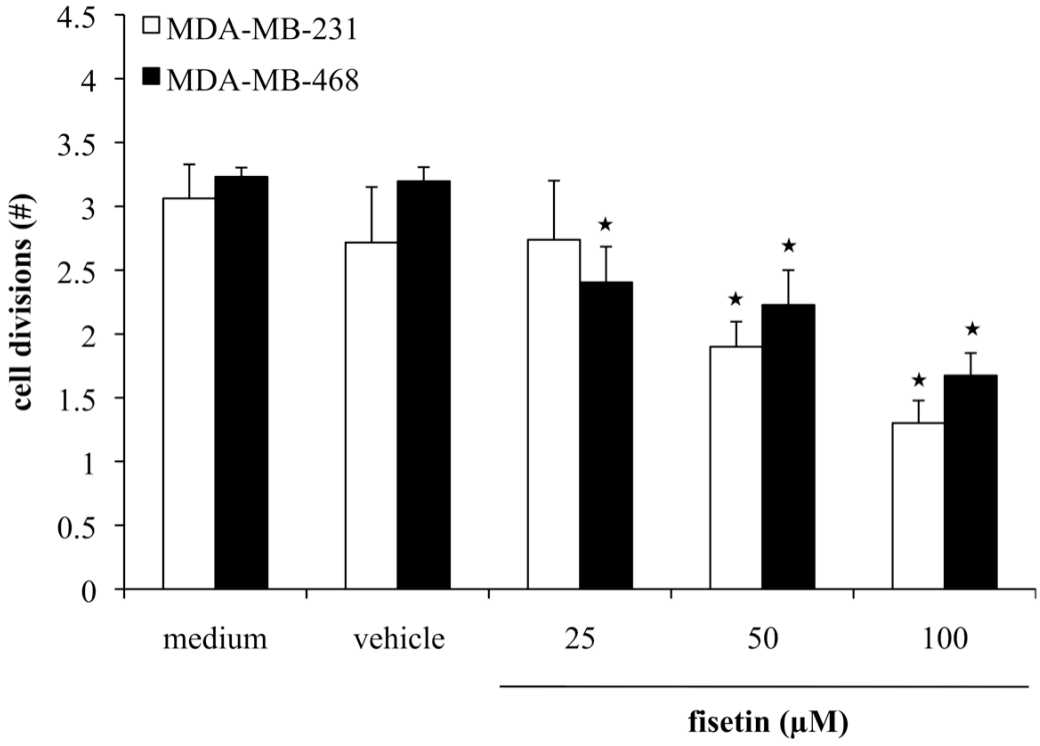
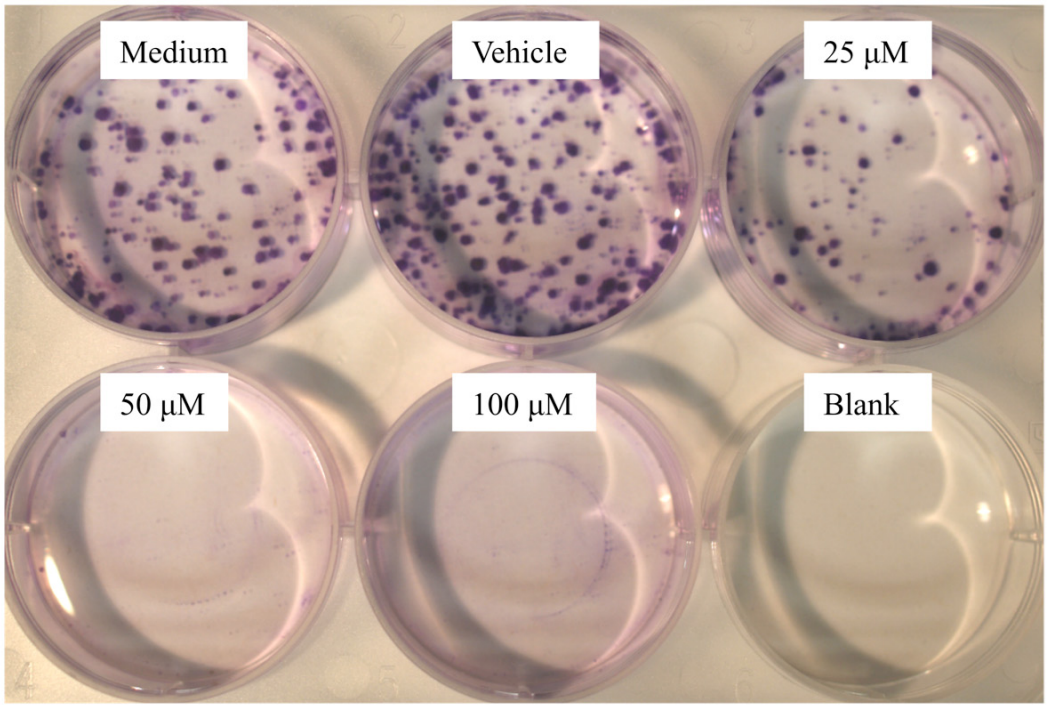

MDA-MB-231
MDA-MB-468
cell divisions (#)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
medium
vehicle
25
50
100
fisetin (μM)

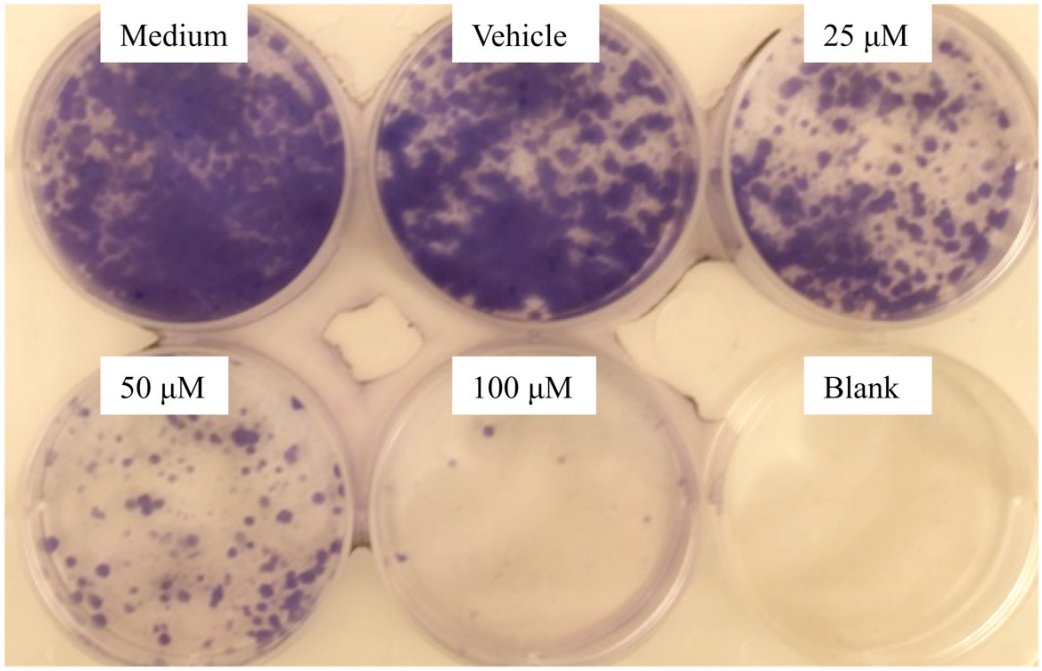
Figure 8. Fisetin decreases the quantity of colonies formed by MDA-MB-468 and MDA-MB-231 breast cancer cells. MDA-MB-468 and MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone were applied for 24 h. The cells were harvested, counted and plated in various dilutions in medium to allow for identification of individual colonies. Crystal violet (0.4% in methanol) was applied to the cells after 13 days and colonies counted. The pictures are a visual representation of the clongenic assay, (A) MDA-MB-468 cells were plated at 2000 cells/well and (B) MDA-M-231 cells were plated at 1000 cells/well as a representation of the assay. (C) The graph represents The graph represents the mean of 3 individual experiments+/- SD. Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 8.

A. MDA-MB-468



B. MDA-MB-231



C.

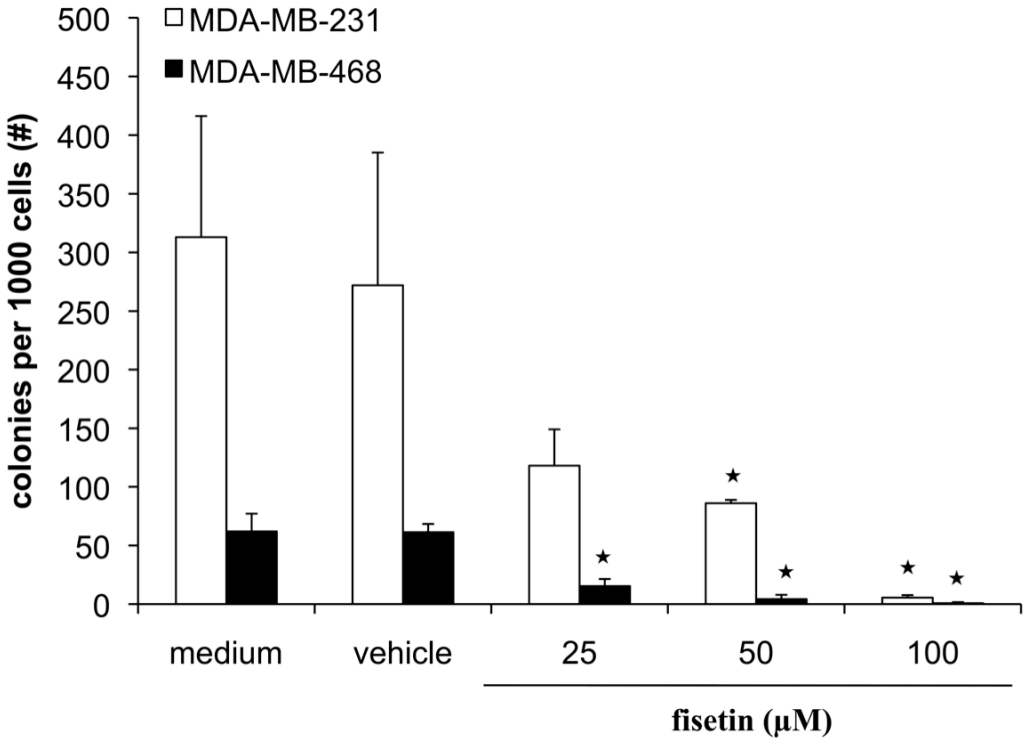
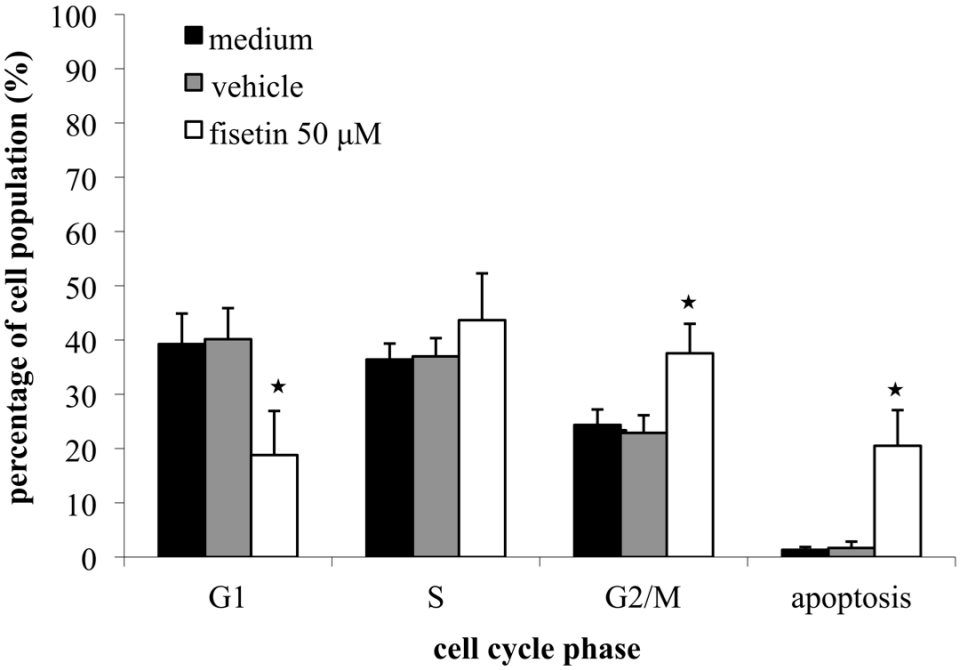

MDA-MB-231
MDA-MB-468
colonies per 1000 cells (#)
500
450
400
350
300
250
200
150
100
50
0
medium
vehicle
25
50
100
fisetin (μM)

Figure 9. Fisetin induces a G_2/M phase arrest as well as apoptosis (subG_1) in MDA-MB-468 breast cancer cells and a S-phase arrest in MDA-MB-231 breast cancer cells. (A) MDA-MB-468 breast cancer cells were treated with 50 μM concentration of fisetin, vehicle, or medium alone and (B) MDA-MB-231 breast cancer cells were treated with 50 μM and 100 μM concentrations of fisetin, vehicle or medium alone for 48 h. The cells were harvested, stained with PI, and analyzed by flow cytometry to quantify the percentage of cells in each phase of the cell cycle. The graph represents the mean of 6 individual experiments +/- SD. Statistical significance in comparison to the vehicle control was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p < 0.05$.

Figure 9.

A MDA-MB-468.



B. MDA-MB-231

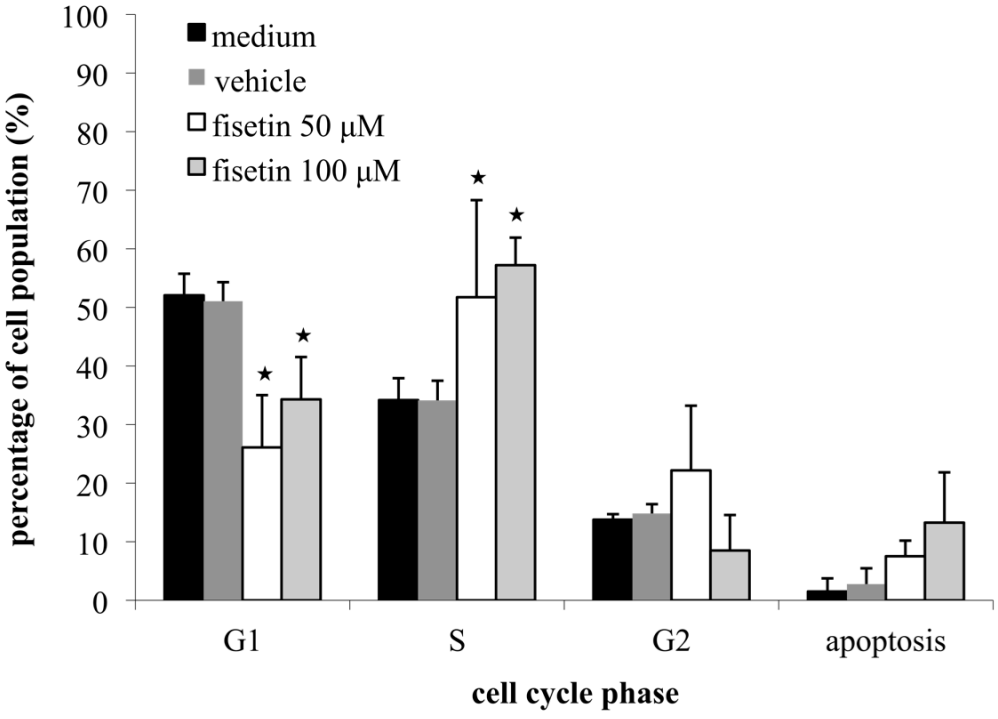
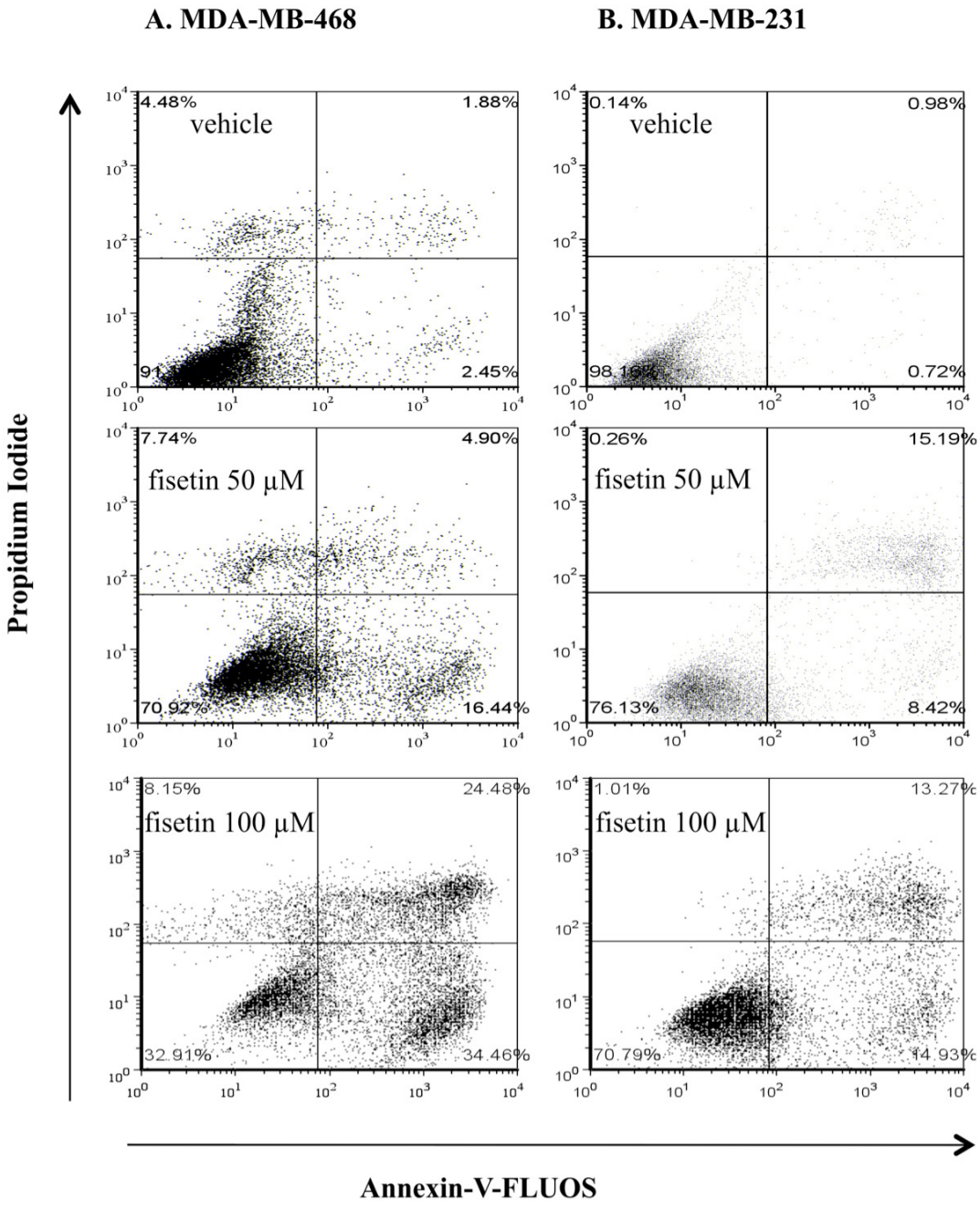
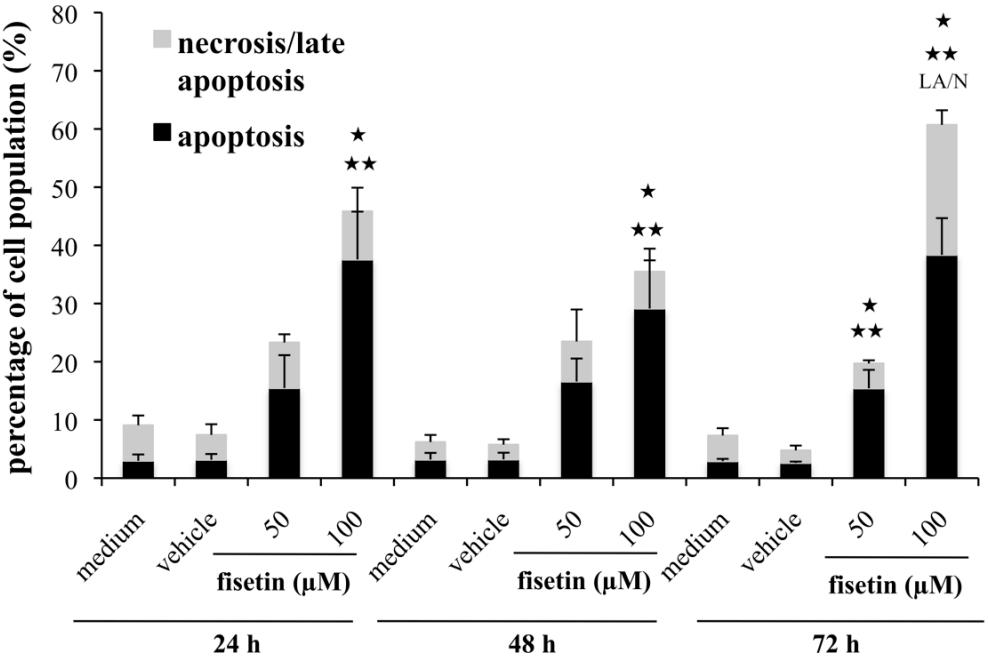


Figure 10. Fisetin induces cell death by apoptosis and late apoptosis/necrosis in MDA-MB-468 and MDA- MB-231 breast cancer cells. MDA-MB-468 and MDA-MB-231 cells were treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone for 24, 48 or 72 h. Cells were harvested with trypLE, washed with PBS, and labeled with annexin-V-FLUOS and propidium iodide. Cells stained with only annexin-V represent apoptosis and cells stained with both annexin-V and propidium iodide represent late apoptosis/necrosis. The analysis was performed by flow cytometry. (A) MDA-MB-468 and (B) MDA-MB-231 are representative graphs (n=3) after 72 h of treatment. (C) MDA-MB-468 and (D) MDA-MB-231 data from 3 individual experiments was averaged and expressed as total cytotoxicity with a division of percent of cells in apoptosis and late apoptosis/necrosis +/- SD. Statistical significance in comparison to the vehicle control was determined by the Tukey-Kramer multiple comparisons test for total cytotoxicity, apoptotosis and late apoptosis/necrosis; ⋆ denotes $p< 0.05$ for total cytotoxicity; ⋆⋆ denotes $p< 0.05$ for apoptosis; LA/N denotes $p< 0.05$ for late apoptosis/necrosis.

Figure 10.


A. MDA-MB-468
B. MDA-MB-231
vehicle
4.48%
1.88%
91
2.45%
0.14%
0.98%
98.16%
0.72%
fisetin 50 µM
7.74%
4.90%
70.92%
16.44%
0.26%
15.19%
76.13%
8.42%
fisetin 100 µM
8.15%
24.48%
32.91%
34.46%
1.01%
13.27%
70.79%
14.93%
Propidium Iodide
Annexin-V-FLUOS

C. MDA-MB-468



D. MDA-MB-231

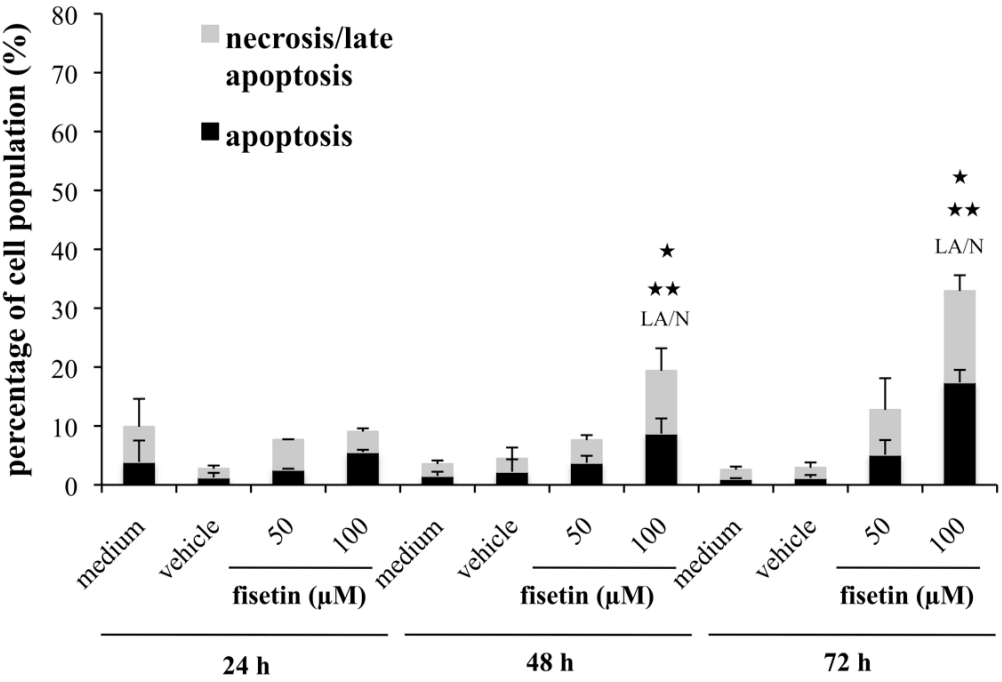
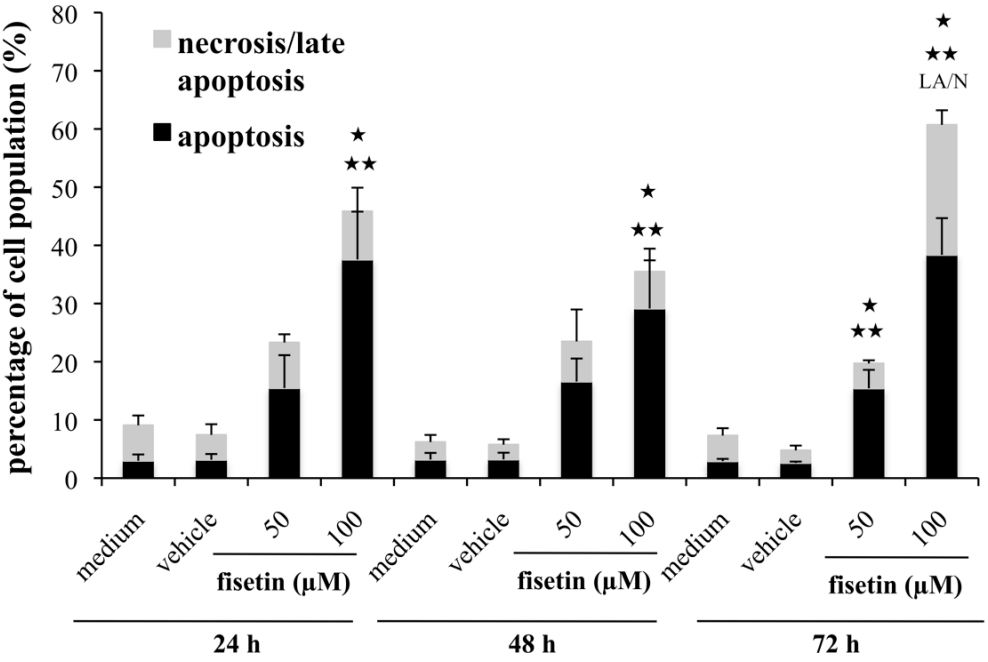


Figure 11. Fisetin does not induce LDH release by MDA-MB-468 and MDA-MB-231 breast cancer cells. MDA-MB-468 and MDA-MB-231 cells were treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone for 24, 48 or 72 h. LDH release was calculated using the equation [(T/S)/(M/S)] x 100, where T equals treatment induced LDH release, S equals spontaneous LDH release (medium control), and M equals maximal LDH release. Maximal lysis was achieved using repeated freeze/thaw cycles as well as mechanical manipulation. (A) MDA-MB-468 and (B) MDA-MB-231 graphs represent the mean of 3 independent studies +/- SEM. Statistical significance in comparison to the vehicle control was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

C. MDA-MB-468



D. MDA-MB-231

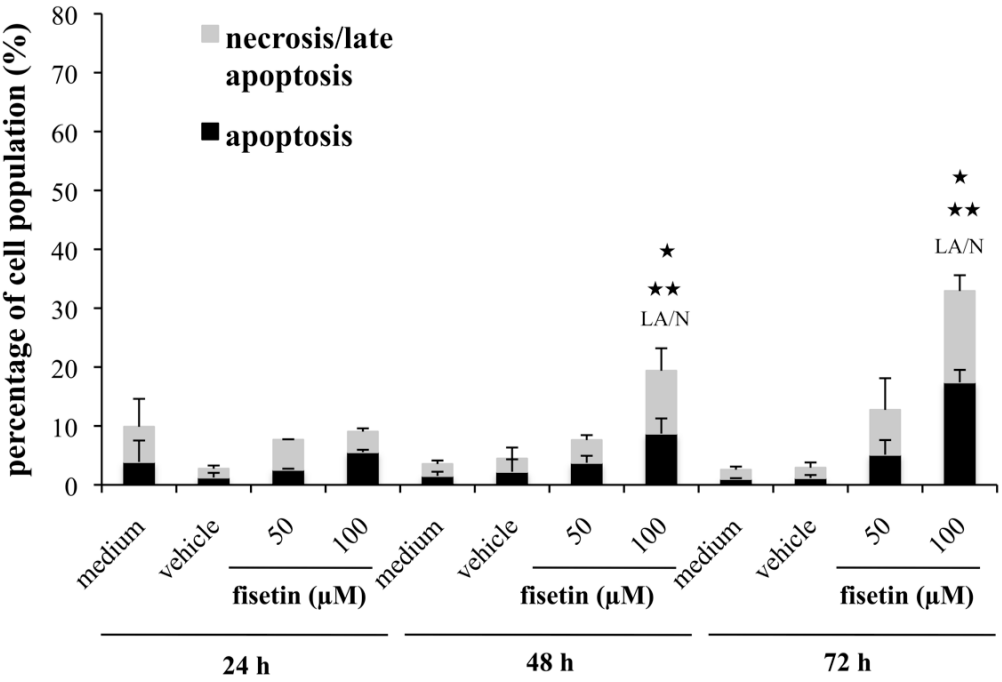
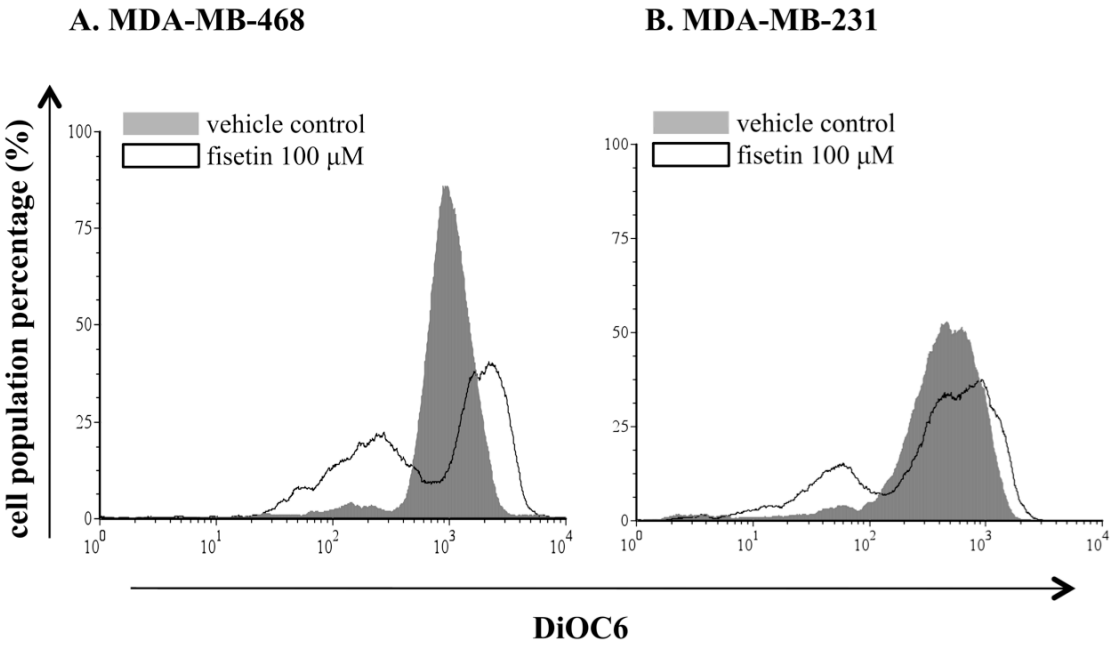


Figure 12. Fisetin induces loss of mitochondrial membrane potential in MDA-MB-468 and MDA-MB-231breast cancer cells with corresponding cytochrome *c* release in MDA-MB-468 breast cancer cells. MDA-MB-468 and MDA-MB-231 cells were treated with concentrations of fisetin (25, 50 or100 μM), vehicle or medium alone for 72 h. Cells were harvested, stained with $DiOC_6$, and analyzed by flow cytometry. (A) Data from MDA-MB-468 and (B) MDA-MB-231 breast cancer cells are shown as representative graphs (n=3) after 72 h of treatment with vehicle or fisetin concentration of 100 μM. The loss of mitochondrial membrane potential is represented by the leftward shift on the x-axis showing a reduction of $DiOC_6$ staining. (C) MDA-MB-468 breast cancer cells were treated with treated with concentrations of fisetin (25 or50 μM), vehicle or medium alone for 24 h. The cells were harvested and cellular protein was collected. Western blotting was performed for cytochrome *c*. Equal loading was confirmed using actin expression as a control. The graph represents densitometry analysis of 3 individual experiments +/-SEM. Statistical significance in comparison to the vehicle control was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

Figure 12.


A. MDA-MB-468
B. MDA-MB-231
vehicle control
fisetin 100 μM
cell population percentage (%)
DiOC6

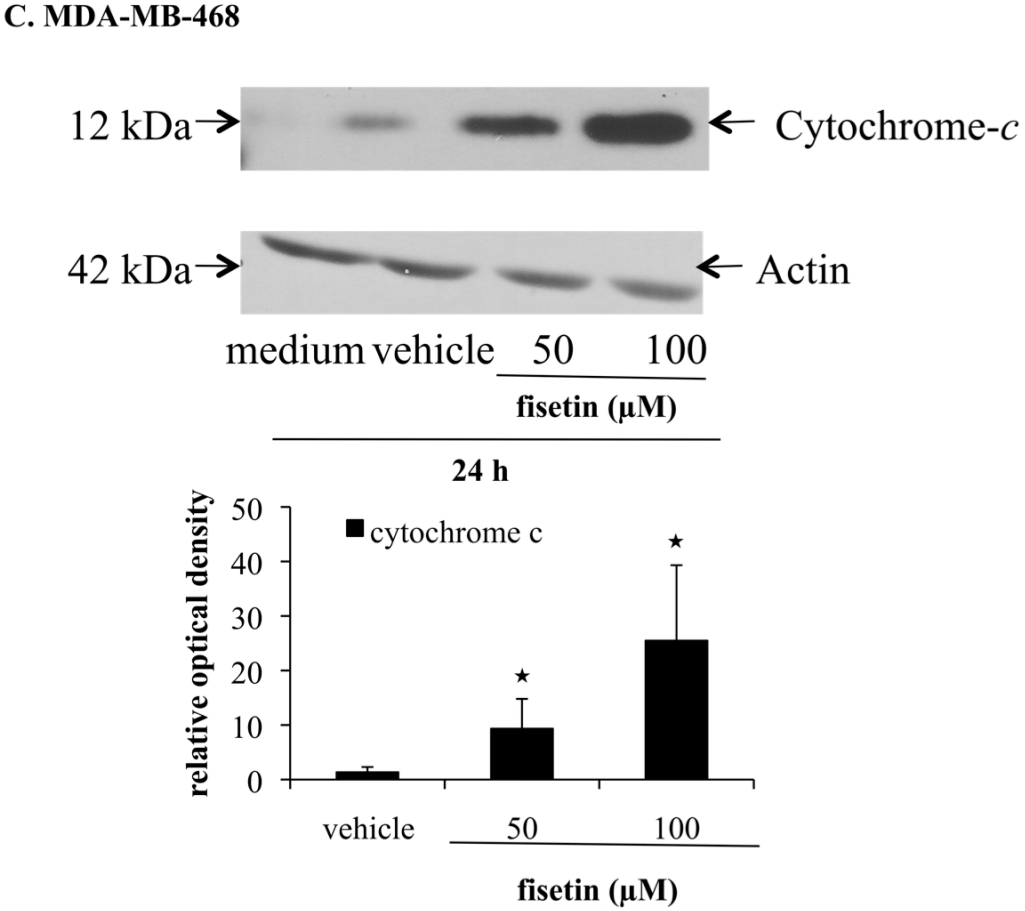
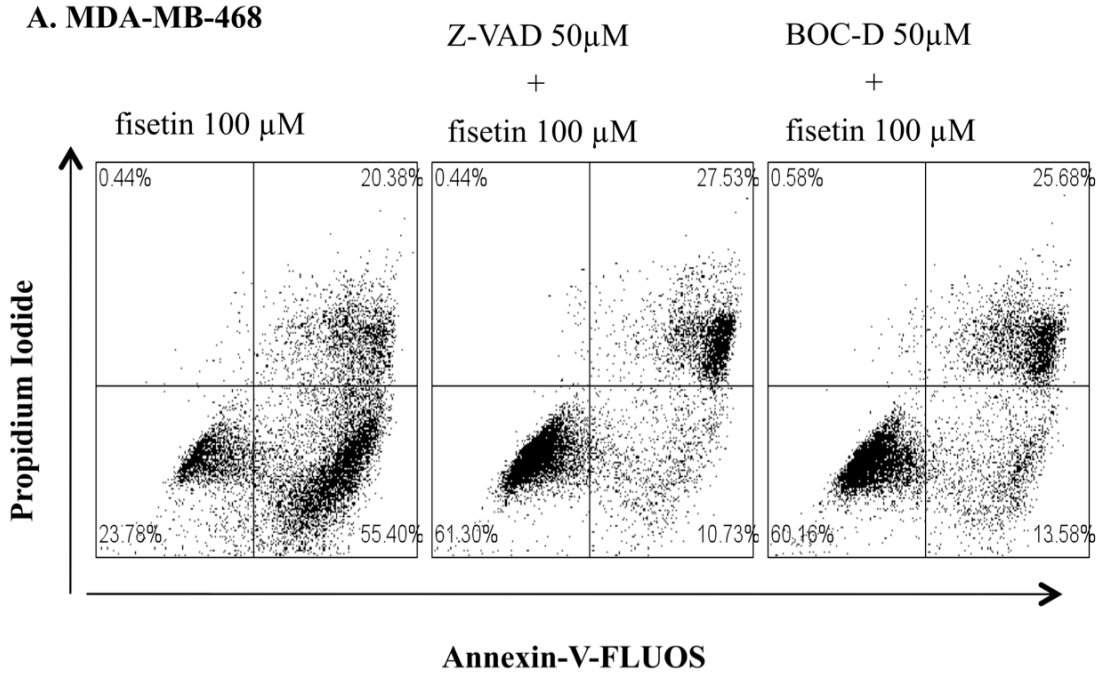

C. MDA-MB-468
12 kDa
Cytochrome-c
42 kDa
Actin
medium vehicle 50 100
fisetin (μM)
24 h
relative optical density
cytochrome c
vehicle 50 100
fisetin (μM)

Figure 13. Fisetin induces caspase-dependent apoptosis as well as necrosis in MDA-MB-468 breast cancer cells. MDA-MB-468 were pretreated with 50 μM concentration of either Z-VAD-FMK and BOC-D-FMK (pancaspase inhibitors), medium or vehicle for 1 h prior to treatment with fisetin (50 and 100 μM), vehicle or medium alone for 72 h. Cells were harvested with trypLE, washed with PBS, and labeled with annexin-V-FLUOS and propidium iodide. The analysis was performed by flow cytometry. (A) Representative data from a single experiment and (B) average data from 3 individual experiments expressed as total cytotoxicity with a division of cells in apoptosis and late apoptosis/necrosis ± SD. Statistical significance was determined by the Tukey-Kramer multiple comparisons test for total cytotoxicity, apoptosis and late apoptosis/necrosis; ⋆ denotes $p< 0.05$ for total cytotoxicity; ⋆⋆ denotes $p< 0.05$ for apoptosis. Statistical significance was also determined for Z-VAD-FMK and BOC-D-FMK pretreatment compared to fisetin or vehicle alone T denotes $p< 0.05$ for total cytotoxicity; LA/N denotes $p< 0.05$ for late apoptosis/necrosis; NS denotes non significant.

Figure 13.


A. MDA-MB-468
Z-VAD 50μM
+
fisetin 100 μM
BOC-D 50μM
+
fisetin 100 μM
fisetin 100 μM
0.44%
20.38%
23.78%
55.40%
0.44%
27.53%
61.30%
10.73%
0.58%
25.68%
60.16%
13.58%
Propidium Iodide
Annexin-V-FLUOS

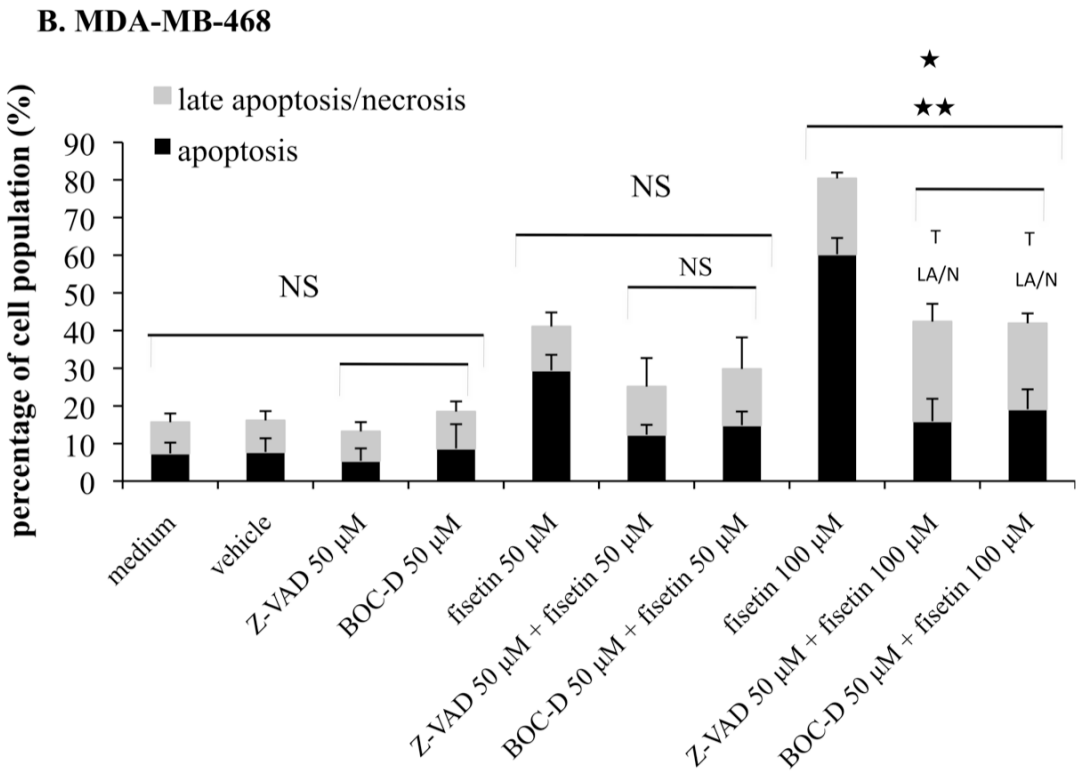
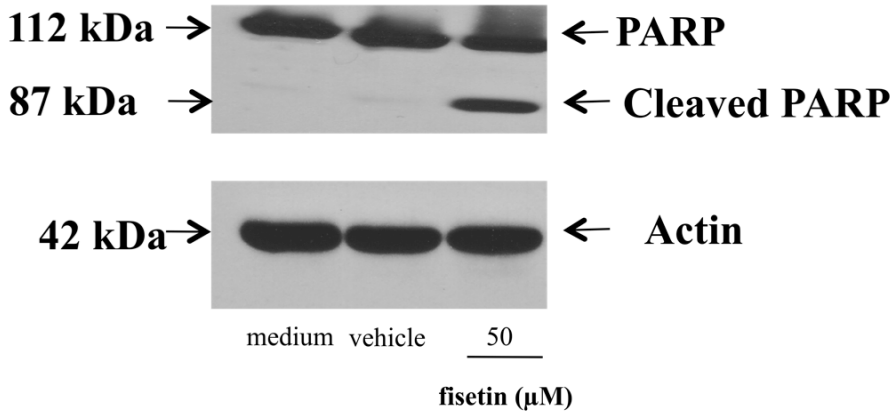
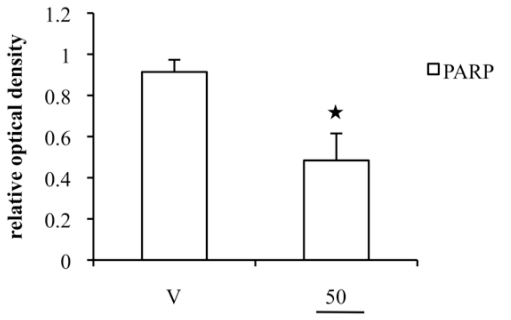

B. MDA-MB-468
late apoptosis/necrosis
apoptosis
percentage of cell population (%)
90
80
70
60
50
40
30
20
10
0
NS
NS
NS
★
★★
T
LA/N
T
LA/N
medium
vehicle
Z-VAD 50 μM
BOC-D 50 μM
fisetin 50 μM
Z-VAD 50 μM + fisetin 50 μM
BOC-D 50 μM + fisetin 50 μM
fisetin 100 μM
Z-VAD 50 μM + fisetin 100 μM
BOC-D 50 μM + fisetin 100 μM

Figure 14. Fisetin induces PARP-1 cleavage in MDA-MB-468 breast cancer cells. MDA-MB-468 were treated with 50 μM of fisetin, vehicle or medium alone for 24 h. Cells were harvested, cellular protein collected and Western blotting completed to detect PARP and PARP cleavage product. Data from as a representative Western blot is shown. Actin was used to ensure standardization to loading quantity. The graphs represent densitometry analysis with the mean of 3 individual experiments +/- SEM. Statistical significance in comparison to the vehicle control and fisetin treatment was determined by the Student's t-test; ⋆ denotes $p< 0.05$.

Figure 14.


112 kDa
87 kDa
PARP
Cleaved PARP
42 kDa
Actin
medium
vehicle
50
fisetin (μM)


relative optical density
1.2
1
0.8
0.6
0.4
0.2
0
PARP
V
50
fisetin (μM)

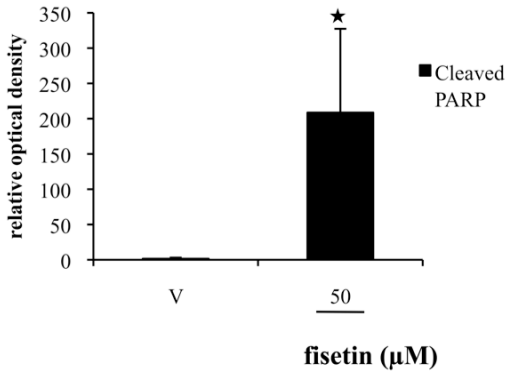

relative optical density
350
300
250
200
150
100
50
0
Cleaved PARP
V
50
fisetin (μM)

Figure 15. Fisetin did not induce ROS production. MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone for 48 h. Cells were harvested, stained with dihydroethidium (DHE), and analysed by flow cytometry. A shift of the curve on the x-axis to the right represents superoxide production but fisetin alone also shifts the curve from a representative experiment (n=3) is shown.

Figure 15.

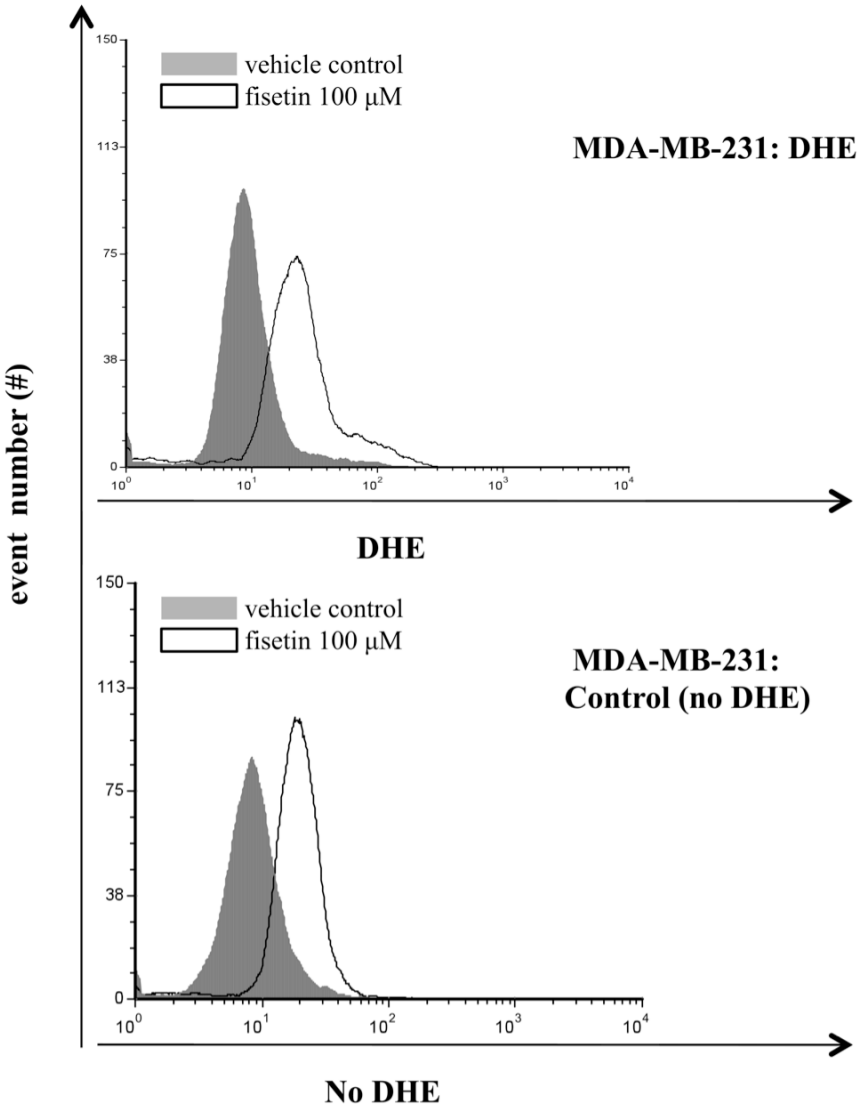

event number (#)
150
113
75
38
0
vehicle control
fisetin 100 µM
MDA-MB-231: DHE
10^0
10^1
10^2
10^3
10^4
DHE
150
113
75
38
0
vehicle control
fisetin 100 µM
MDA-MB-231:
Control (no DHE)
10^0
10^1
10^2
10^3
10^4
No DHE

Figure 16. Fisetin decreases the quantity of MDA-MB-468 breast cancer cells in the presence of antioxidants. MDA-MB-468 breast cancer cells were pretreated with medium alone or with 10 μM concentration of GSH or NAC for 1 h. The cells were then treated with concentrations of fisetin (25, 50 or 100 μM), vehicle or medium alone were applied for 72 h. Crystal violet (0.4% in methanol) was applied to the cells which were then dissolved in DMSO and absorbance measure. The graph is the mean of 3 individual experiments +/- SEM.

Figure 16.

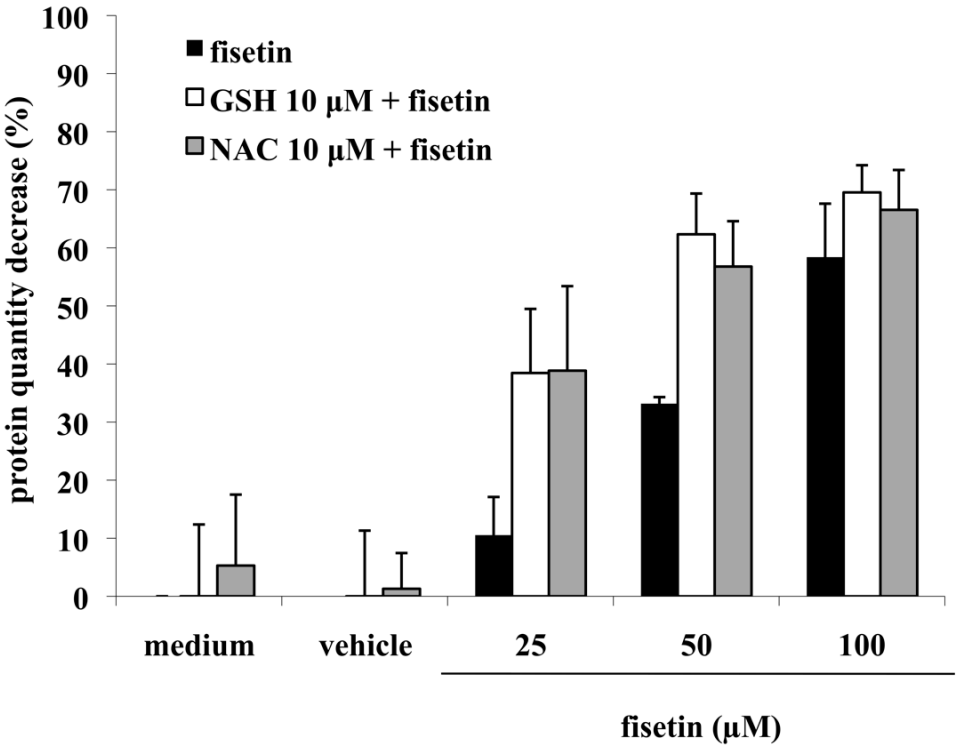

100
90
80
70
60
50
40
30
20
10
0
protein quantity decrease (%)
fisetin
GSH 10 µM + fisetin
NAC 10 µM + fisetin
medium
vehicle
25
50
100
fisetin (µM)

Figure 17. MCF-7 TX400 and MCF-7 MITX breast cancer cells are resistant to fisetin. MCF-7 TX400 (pacletaxel-resistant), MCF-7 MITX (mitoxantrone-resistant) and MCF-7 (native) breast cancer cells were treated with concentrations of fisetin (50, 100 or 200 μM), vehicle or medium alone for 24, 48 and 72 h. (A) Crystal violet (0.4% in methanol) was applied to the cells to stain all protein. Cell were then dissolved in DMSO. The graph represents the mean of 6 individual experiments +/- SEM. (B) Phosphatase assay measured the quantity of cytosolic phosphatase from viable cells The graph represents the mean of 3 individual experiments +/-SEM. Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 17.

A.

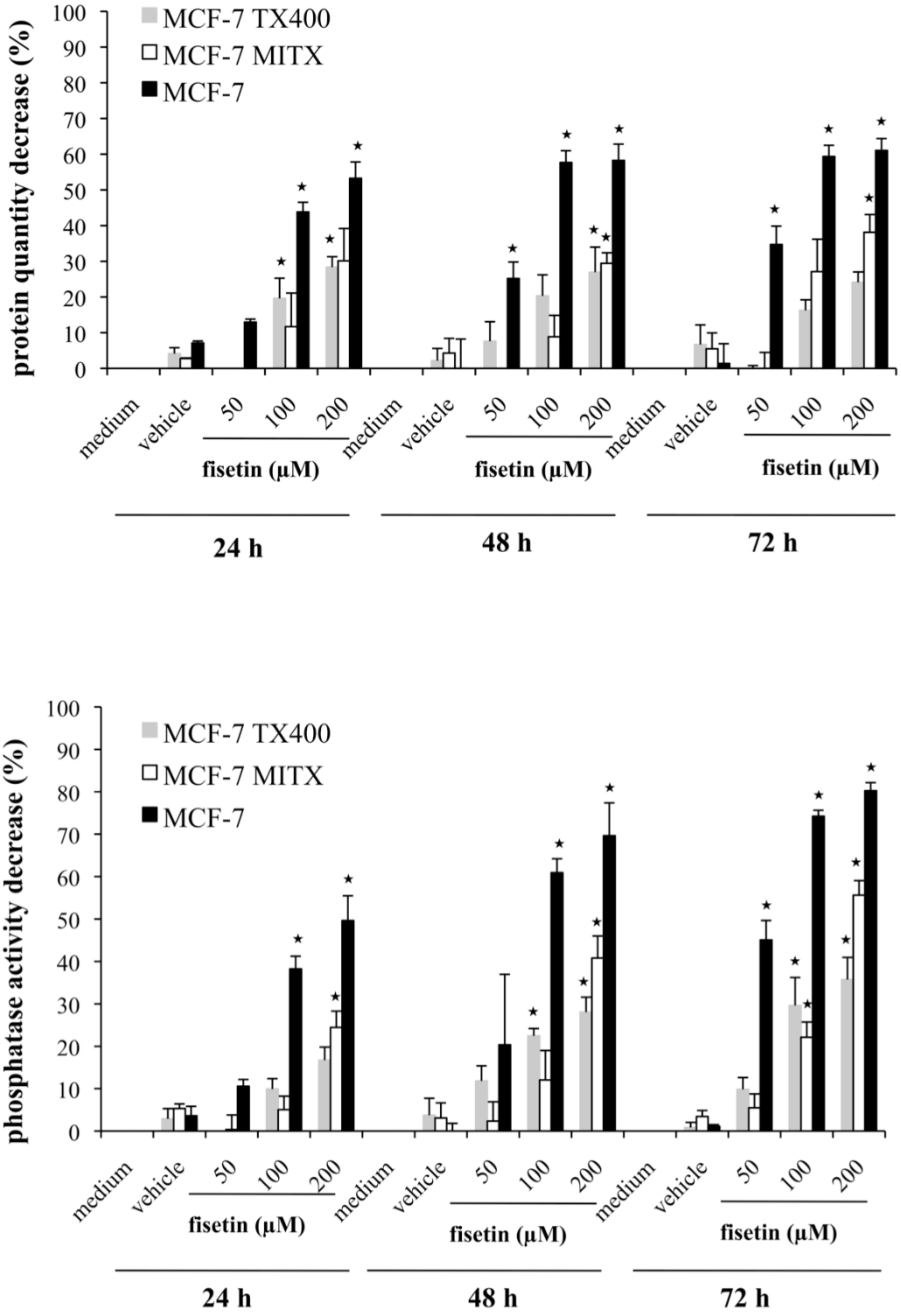
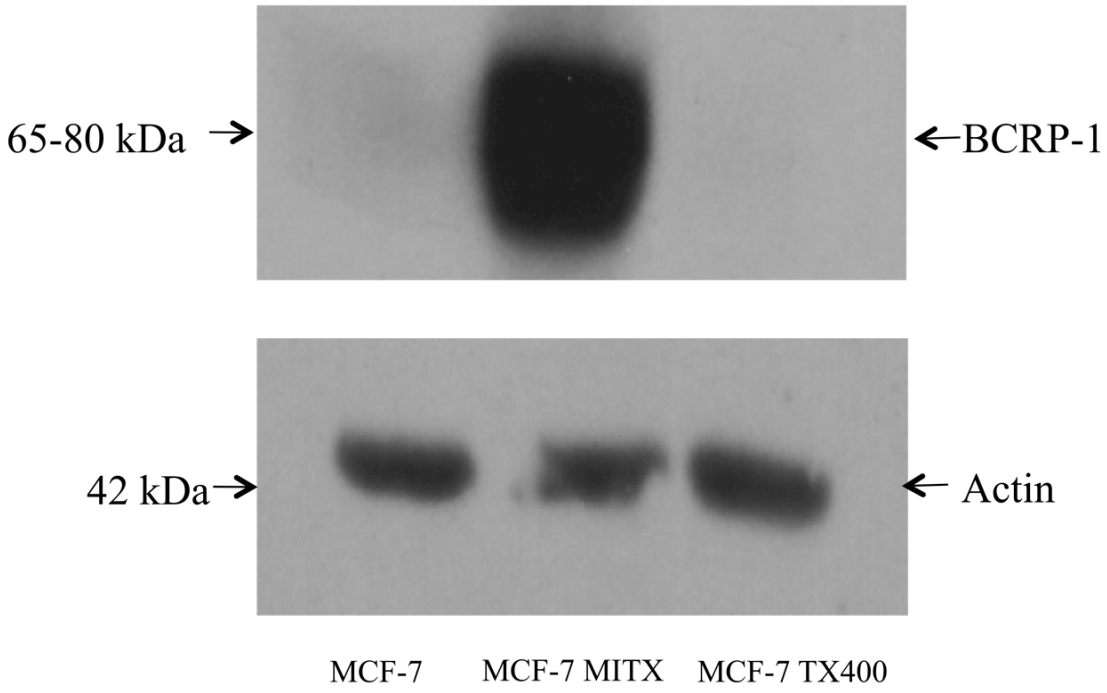

MCF-7 TX400
MCF-7 MITX
MCF-7
protein quantity decrease (%)
phosphatase activity decrease (%)
medium
vehicle
50
100
200
fisetin (μM)
24 h
48 h
72 h

Figure 18. MCF-7 MITX breast cancer cells over-expresses Breast Cancer Resistance Protein–1 (BCRP-1) while MCF-7 TX400; MCF-7 MITX and MCF-7 have the same resistance to mitoxantrone; MCF-7 TX400 are resistant to paclitaxel compared to MCF-7. (A) MCF-7, MCF-7 TX400 and MCF-7 MITX cells were harvested, cellular protein collected and Western blotting completed to detect BRCP-1. (B) MCF-7 or MCF-7 TX400 breast cancer cells were cultured in the absence or presence of the indicated concentrations of paclitaxel then phosphatase activity was measured. The graph represents the mean of 3 individual experiments +/- SEM. Statistical significance relative to the vehicle control was determined by Tukey-Kramer multiple comparison test; ⋆ denotes $p < 0.05$.

Figure 18.

A. Breast Cancer Resistant Protein -1



B. MCF-7 TX400

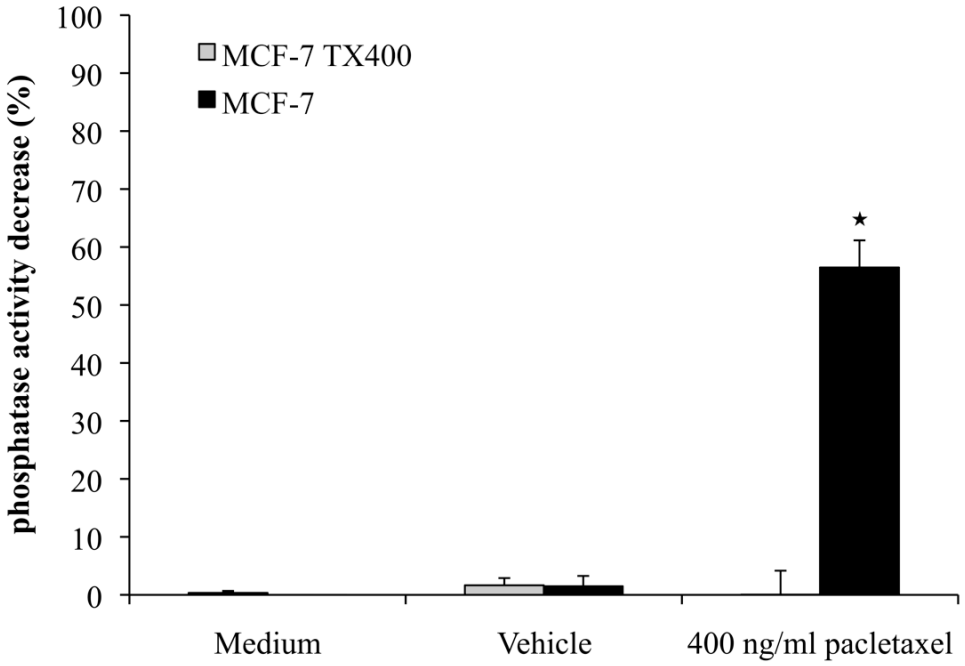
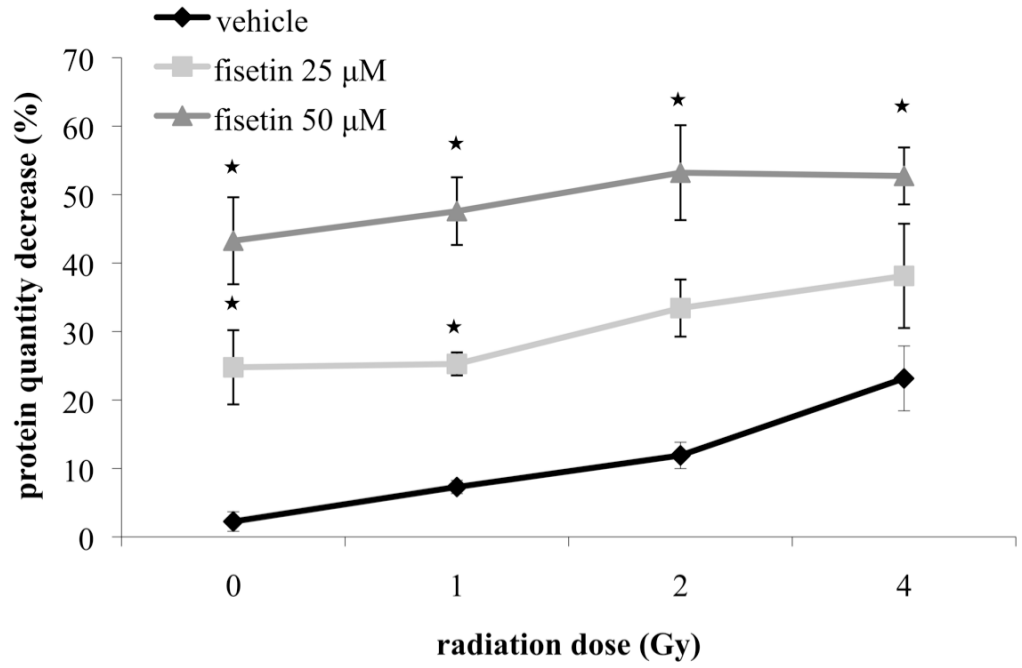


Figure 19. Increased cytotoxic effect by fisetin in combination with radiation. (A) MDA-MB-468 and (B) MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25 or 50 μM), medium alone or DMSO control for 1 hr prior to exposure to various doses of gamma radiation (0, 1, 2 or 4 Gy). The cells were then incubated for 72 h, washed and crystal violet was applied to stain protein. Cells were then dissolved in DMSO> The graphs represent the mean of 3 individual experiments +/- SEM. Statistical significance in comparison to the radiation dose control was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$. An intragroup comparison was also performed within fisetin concentrations to 0 Gy; ⋆⋆ denotes $p< 0.05$.

Figure 19.



B. MDA-MB-231

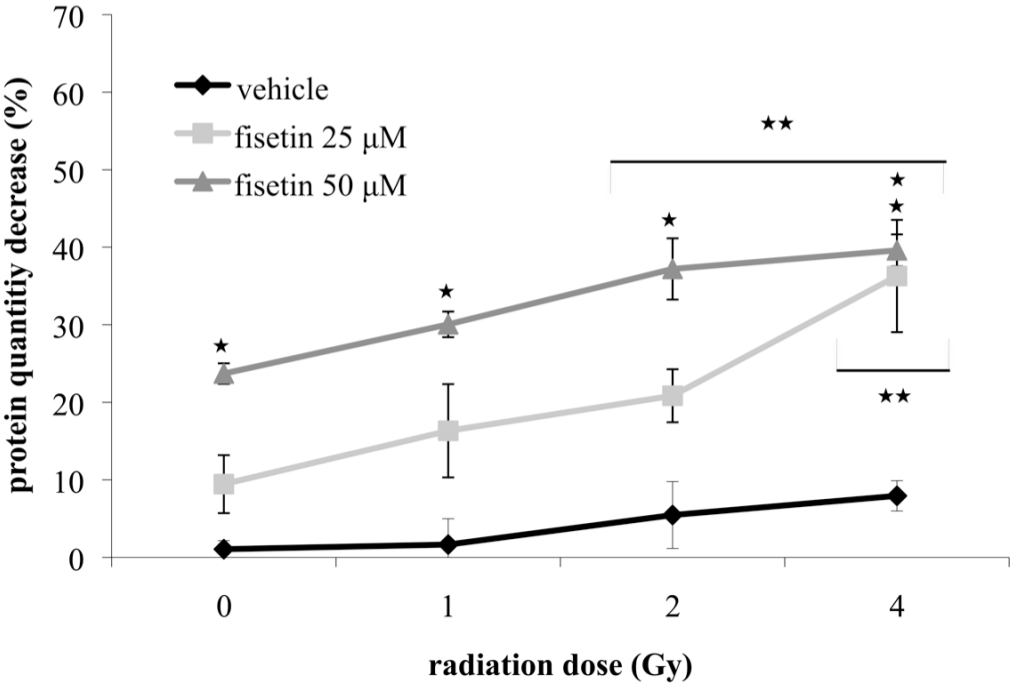
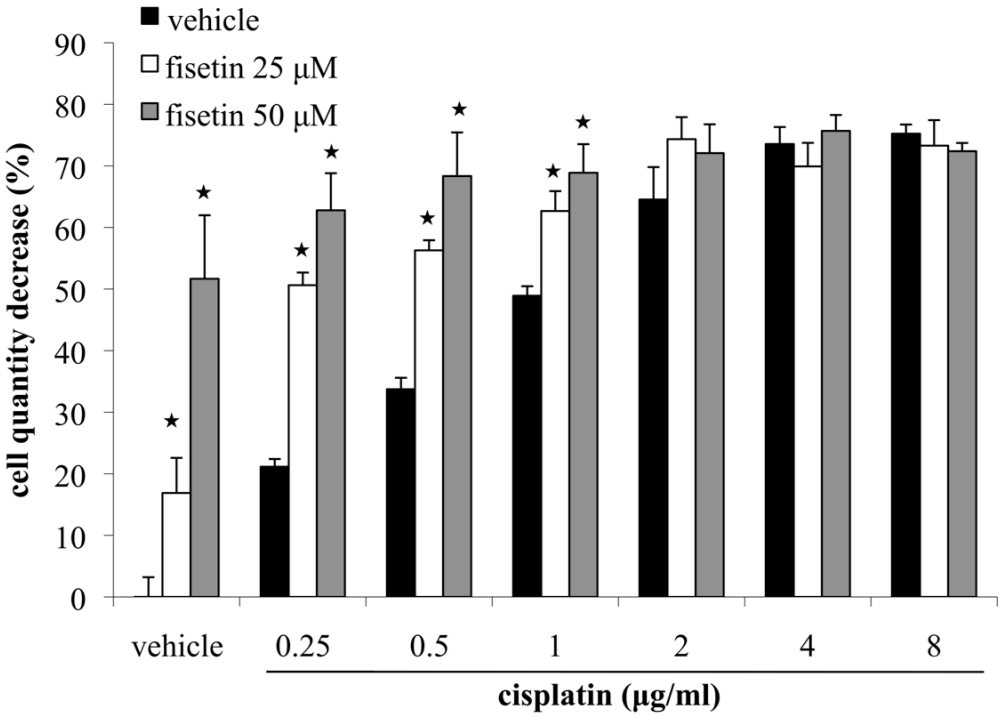


Figure 20. Increased cytotoxic effect by fisetin in combination with cisplatin. (A) MDA-MB-468and and (B) MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25 and 50 μM), vehicle or medium alone for 1 h prior to exposure to range of cisplatin concentrations (0 to 8 μg/ml). The cells were incubated for 72 h, washed, crystal violet was applied and the cells were solubilised in DMSO.The graphs represent the mean of 3 individual experiments +/- SEM. Statistical significance in comparison to the cisplatin monotherapy was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

Figure 20.

A. MDA-MB-468



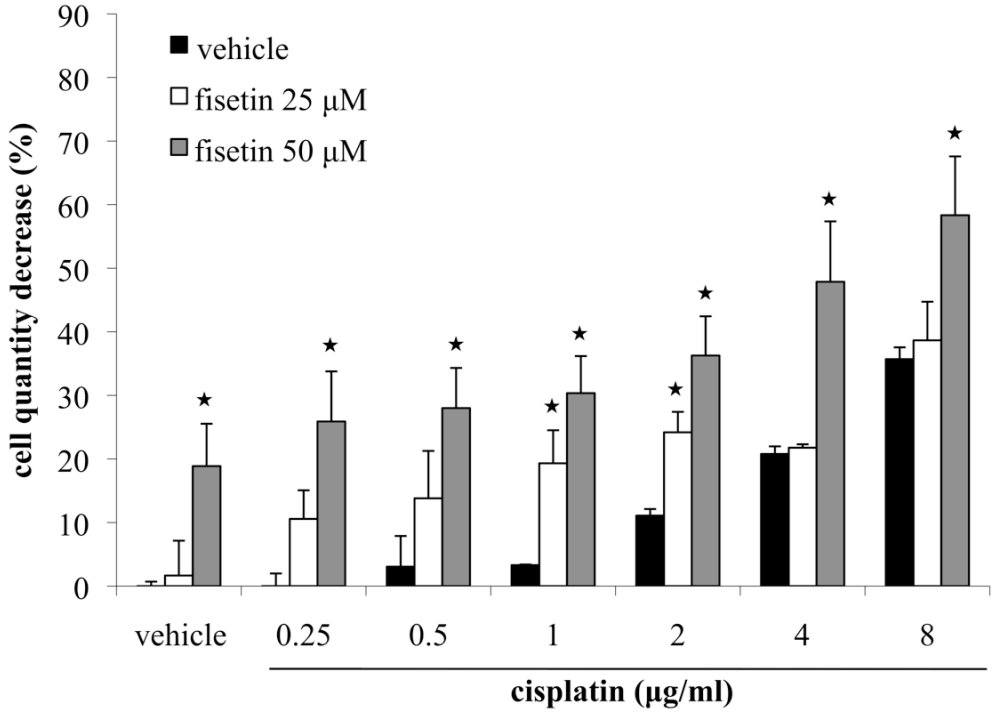
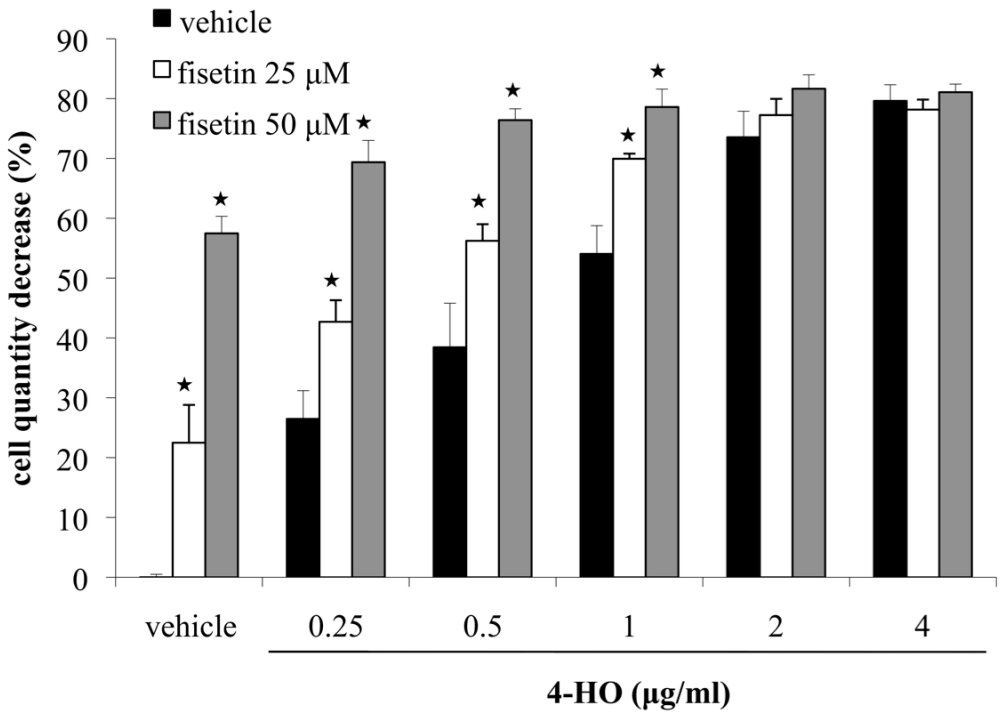


Figure 21. Increased cytotoxic activity by fisetin in combination with an active metabolite of cyclophosphamide, 4-hydroxycyclophosphamide (4-HO). (A) MDA-MB-468 and (B) MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25 and 50 μM), vehicle or medium alone for 1 h prior to exposure to a range of 4-HO concentrations (0 to 4 μg/ml), the active metabolite of cyclophosphamide. The cells were incubated for 72 h, washed, crystal violet was applied and the cells were solubilised in DMSO.The graphs represent the mean of 3 individual experiments +/- SEM. Statistical significance in comparison to 4-HO monotherapy was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

Figure 21.

A. MDA-MB-468



B. MDA-MB-231

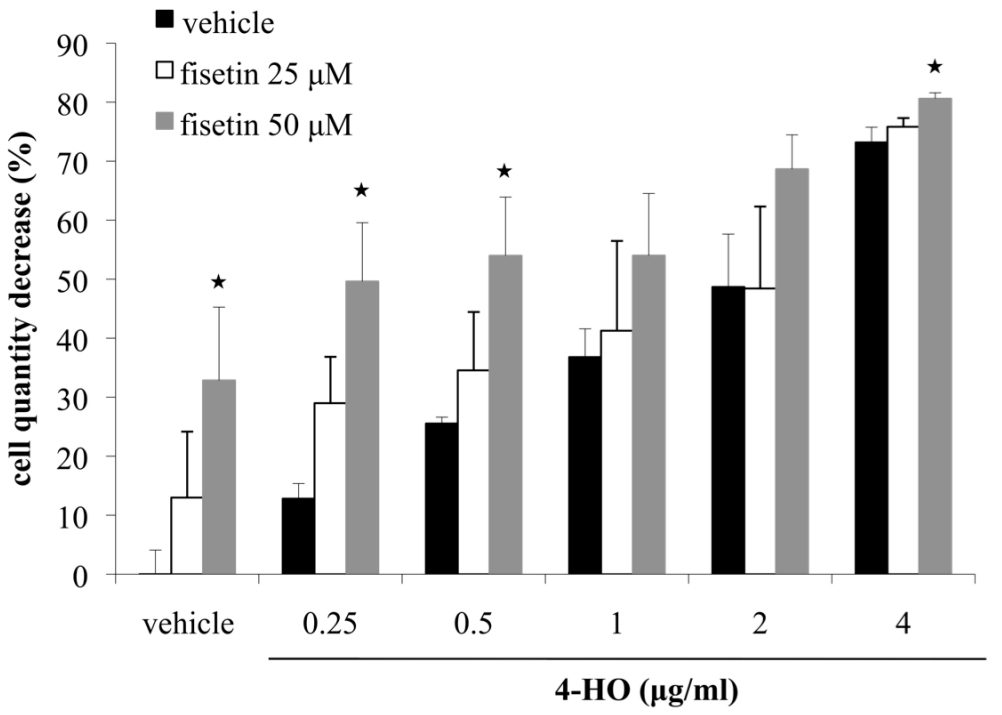
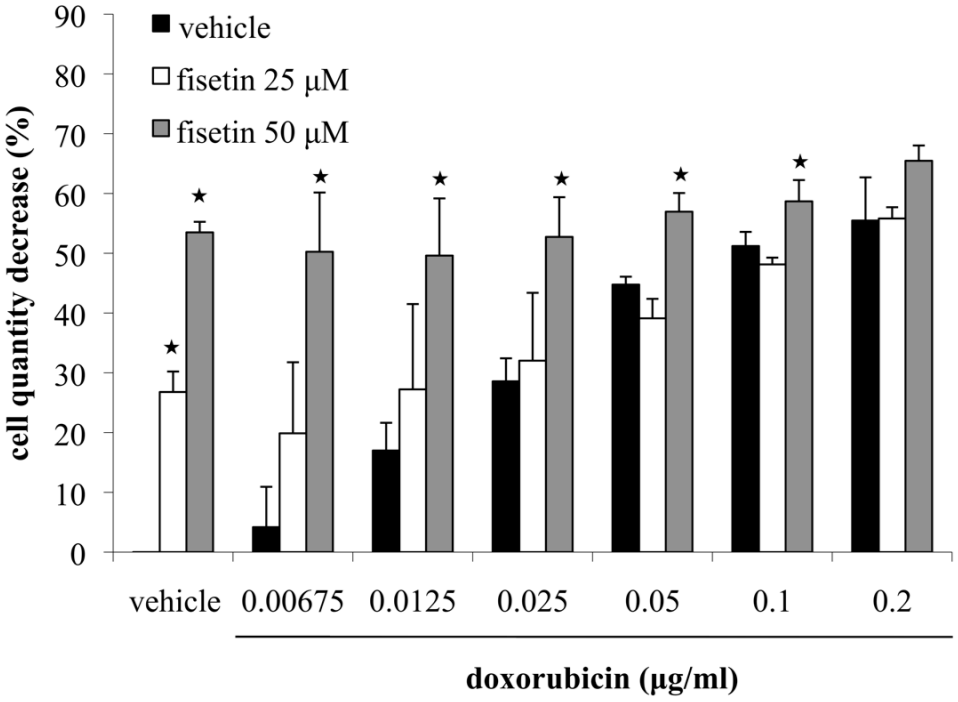


Figure 22. Increased cytotoxicity by fisetin in combination with doxorubicin. (A) MDA-MB-468 and (B) MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25 or 50 μM), vehicle or medium alone for 1 h prior to exposure to a range of doxorubicin concentrations (vehicle (0) to 0.2 μg/ml). The cells were incubated for 72 h, washed, crystal violet was applied and the cells were solubilized in DMSO. The graphs represent the mean of 3 individual experiments +/- SEM. Statistical significance in comparison to doxorubicin monotherapy was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

Figure 22.

A. MDA-MB-468



B. MDA-MB-231

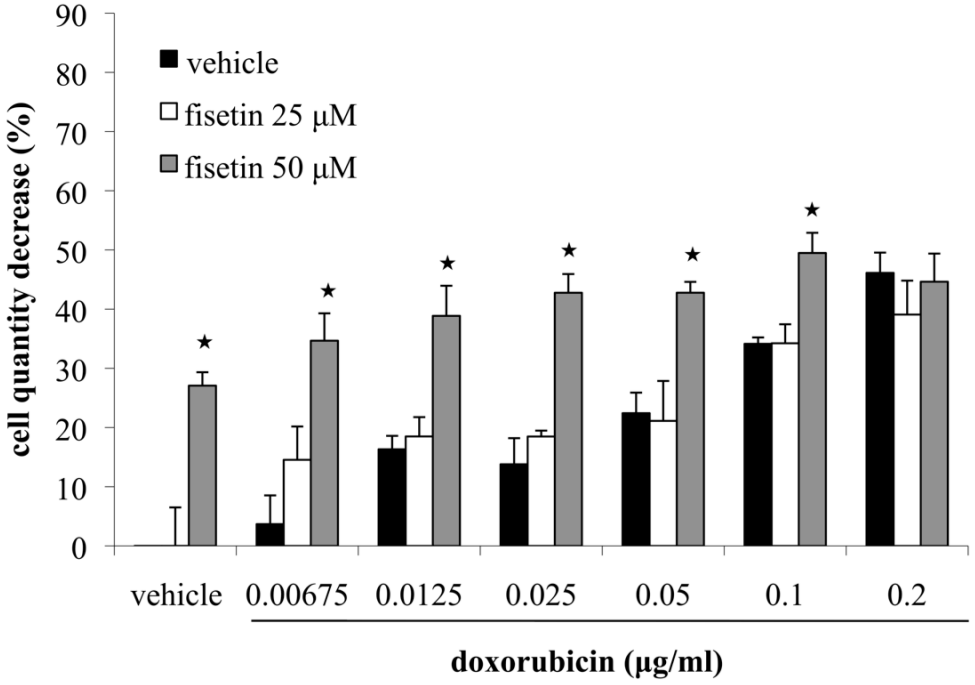


Figure 23. Increased cytotoxic effect of fisetin in combination with 5-FU. (A) MDA-MB-468 and (B) MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25 or 50 μM), vehicle or medium alone for 1 h prior to exposure to a range of 5-FU concentrations (vehicle (0) to 200 μM). The cells were incubated for 72 h, washed, crystal violet was applied and the cells solubilised in DMSO. The graphs represent the mean of 3 individual experiments +/- SEM. Statistical significance in comparison to 5-FU monotherapy was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

Figure 23.

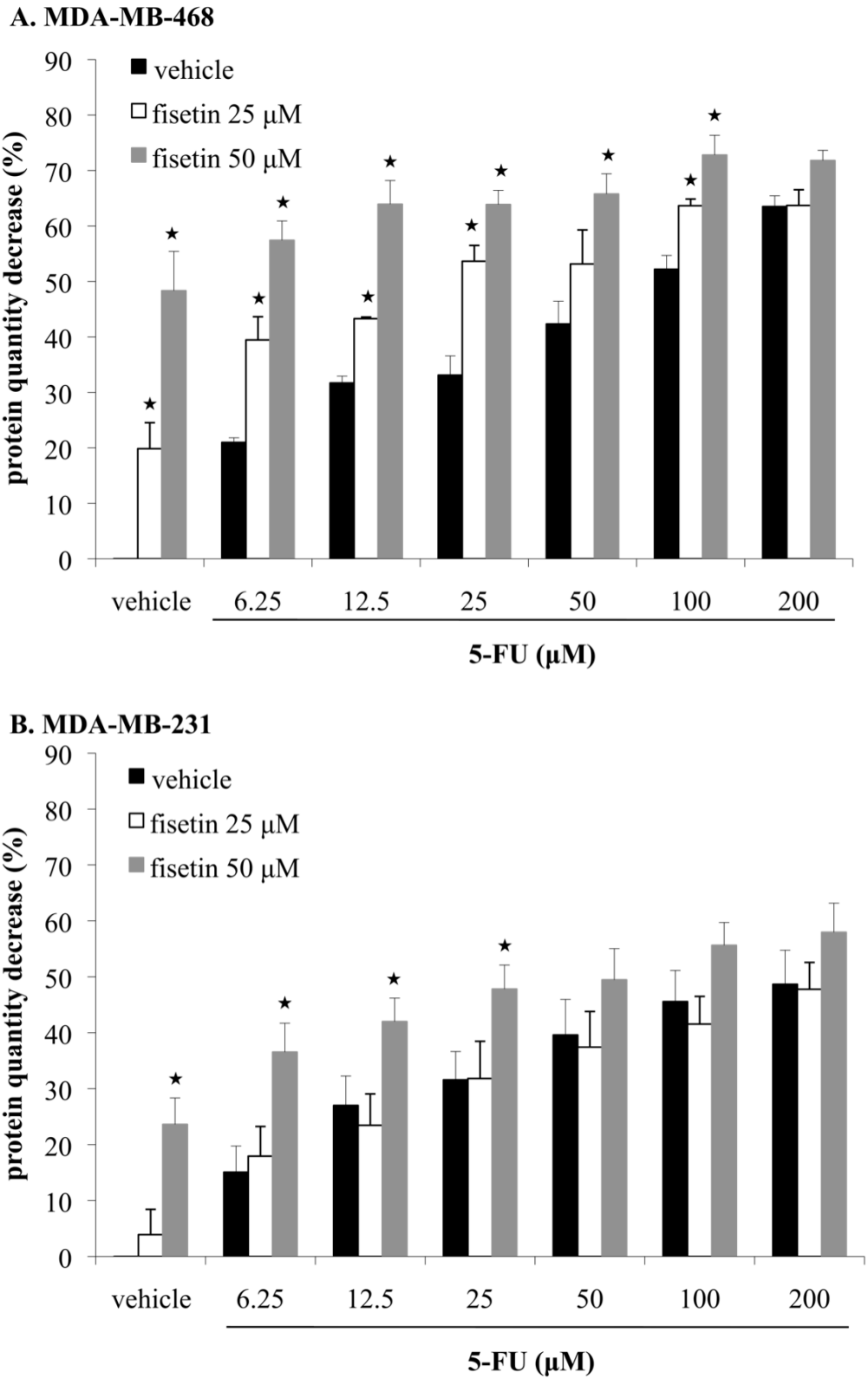
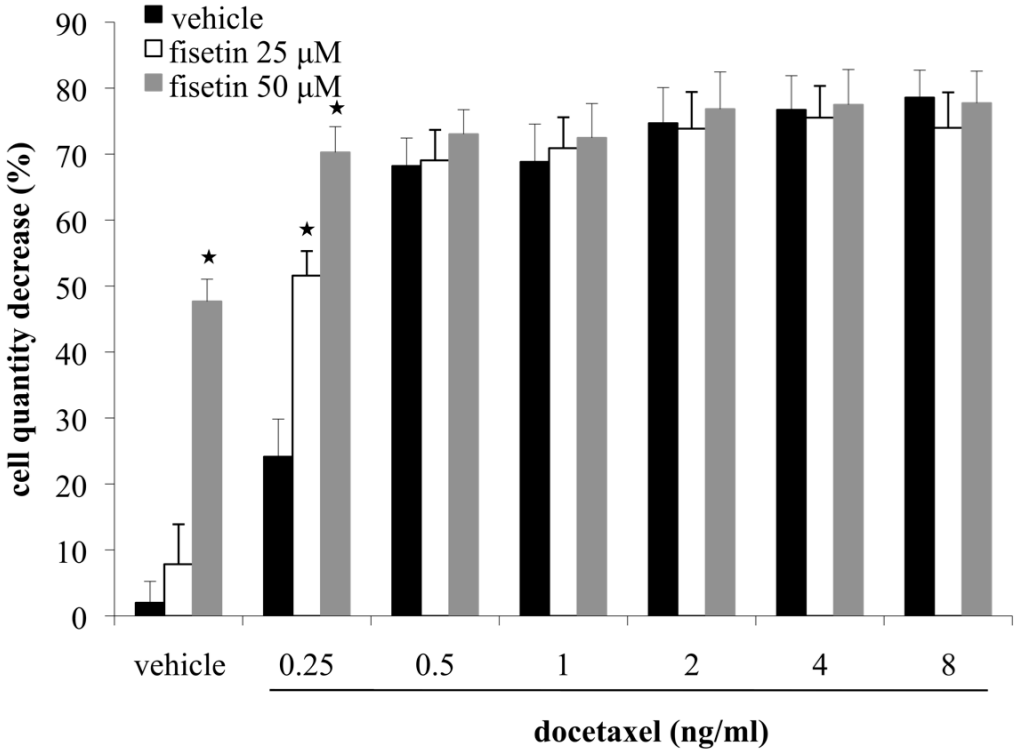

A. MDA-MB-468
vehicle
fisetin 25 μM
fisetin 50 μM
protein quantity decrease (%)
0
10
20
30
40
50
60
70
80
90
vehicle
6.25
12.5
25
50
100
200
5-FU (μM)
B. MDA-MB-231
vehicle
fisetin 25 μM
fisetin 50 μM
protein quantity decrease (%)
0
10
20
30
40
50
60
70
80
90
vehicle
6.25
12.5
25
50
100
200
5-FU (μM)

Figure 24. Increased cytotoxic effect of fisetin in combination with docetaxel. (A) MDA-MB-468 and (B) MDA-MB-231 breast cancer cells were treated with concentrations of fisetin (25 or 50 μM), vehicle or medium alone for 1 h prior to exposure to a range of docetaxel concentrations (vehicle (0) to 8 ng/ml). The cells were incubated for 72 h, washed, crystal violet was applied and the cells solubilised in DMSO. The graphs represent the mean of 3 individual experiments +/- SEM. Statistical significance in comparison to docetaxel monotherapy was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

Figure 24.

A. MDA-MB-468



B. MDA-MB-231

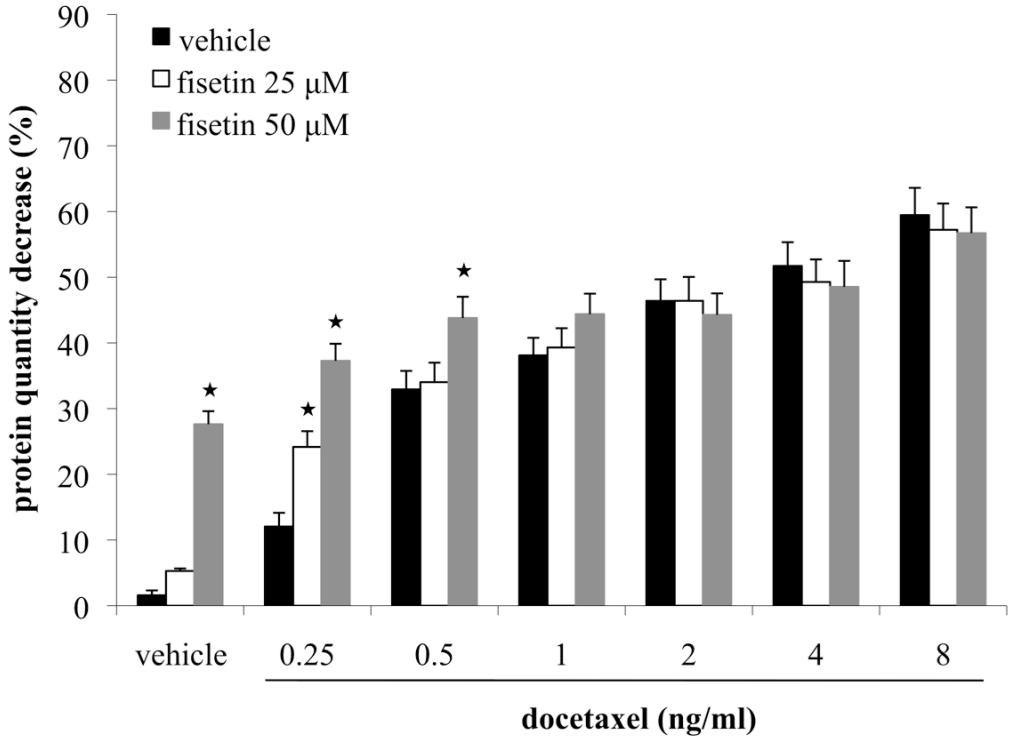
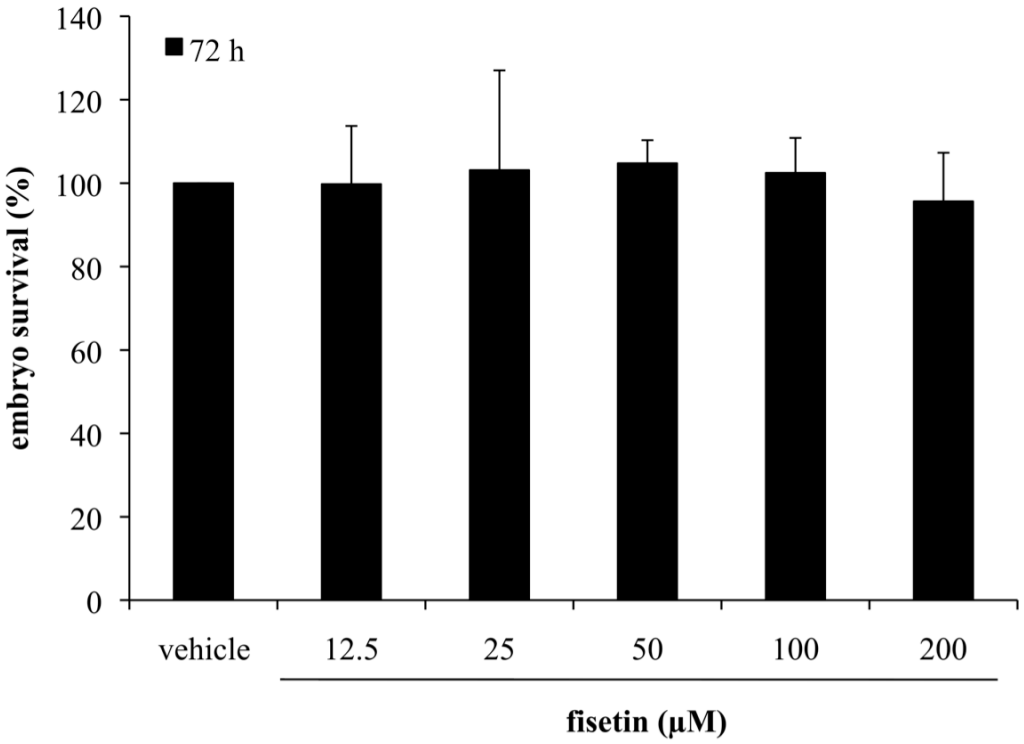


Figure 25. Fisetin does not affect Zebrafish embryo viability. Zebrafish were used to investigate the effect of various fisetin concentrations (12.5, 25, 50, 100 or 200 μM) on viability. The embryos were treated in a 96-well plate with one embryo per well at 72 h post fertilization. The embryos were treated for a 72 h time period and assessed for viability. The graphs represent the mean of 3 individual experiments +/- SD at 72h. Statistical significance in comparison to vehicle was determined by the Tukey-Kramer multiple comparisons test; ⋆ denotes $p< 0.05$.

Figure 25.


140
120
100
80
60
40
20
0
72 h
embryo survival (%)
vehicle
12.5
25
50
100
200
fisetin (µM)

Chapter 4.0 Discussion

4.1 Fisetin reduces breast cancer cell number in a cell specific manner

Fisetin has demonstrated a cytotoxic effect against numerous cancer cell lines, including bladder, hepatocellular, prostate, cervical, colon, pancreatic, and lung cancers, in addition to some lymphomas and leukemias (Kuntz, Wenzel et al. 1999; Chen, Shen et al. 2002; Lee, Shen et al. 2002; Lu, Chang et al. 2005; Lu, Jung et al. 2005; Haddad, Venkateswaran et al. 2006; de Sousa, Queiroz et al. 2007; Sung, Pandey et al. 2007; Lim do and Park 2009; Murtaza, Adhami et al. 2009; Salmela, Pouwels et al. 2009; Suh, Afaq et al. 2009; Haddad, Fleshner et al. 2010; Kim, Jeon et al. 2010; Touil, Seguin et al. 2010; Li, Cheng et al. 2011; Yu, Yang et al. 2011). To date, studies evaluating the effects of fisetin on breast cancer have primarily involved cell quantity assays, and were limited to one cell line in an attempt to identify EC_{50} (Kuntz, Wenzel et al. 1999; Haddad, Venkateswaran et al. 2006). The initial experiments of my study were designed to expose a panel of breast cancer cells (MDA-MB-468, MDA-MB-231, MCF-7, T-47D and SKBR-3 breast cancer cell lines) to fisetin in order to investigate fisetin's effects on a range breast cancers with different mutations. Descriptive characteristics of these cellular phenotypes in the literature include: MDA-MB-468 triple negative breast cancer cells (Oliveras-Ferraros, Vazquez-Martin et al. 2008; Hirsh, Iliopoulos et al. 2009) , which have mutated p53 and are pRb negative (Carlson, Dubay et al. 1996); MDA-MB-231 breast cancer cells are triple negative (Liu, Fan et al. 2009) and have mutated p53 (Zhuang and Miskimins 2011) ; MCF-7 breast cancer cells that are ER^+ (positive) and PR^+ (positive) (So, Guthrie et al. 1997; Rockwell, Liu et al. 2005), express wild type p53 (Carlson, Dubay et al. 1996; Zhuang and Miskimins 2011), are pRB positive (Carlson,

Dubay et al. 1996) and do not express caspase-3 (Zhuang and Miskimins 2011); T-47D breast cancer cells have mutated p53 and are ER^+ (Zhuang and Miskimins 2011); SK-BR-3 breast cancer cells over-express HER-2 (Moulder, Yakes et al. 2001). Note that there are several phenotypic disparities described in the literature that can be observed also in tissue culture.

The first series of experiments in my research was performed to assess the effect of fisetin exposure on breast cancer cell number. The crystal violet assay was used to measure the amount of protein present (Saotome, Morita et al. 1989); the MTT assay to measure mitochondrial succinate reductase activity (Mosmann 1983); and the acid phosphatase assay to determine cytosolic phosphatase activity (Yang, Sinai et al. 1996). The observed decrease in cell quantity was cell line specific with MDA-MB-468 breast cancer cells being most sensitive to fisetin, exhibiting a maximum reduction of 82% (Figure 3B) in contrast to the more resistant T-47D breast cancer cells, which only demonstrated a 27% reduction in number in response to fisetin (Figure 4A). The results for MCF-7 breast cancer cells were somewhat consistent with the EC_{50} value of 80-118 μM reported in the literature (Kuntz, Wenzel et al. 1999; Haddad, Venkateswaran et al. 2006).

All breast cancer cell lines were investigated using at least two of the aforementioned assays. Each assay was susceptible to different potentially confounding variables that were controlled for during each study. Note that control data not shown for acid phosphatase and crystal violet assays. In the MTT assay, fisetin caused tetrazolium salt to be reduced to formazen crystals despite the absence of cells (Figure 5). A possible explanation for this observation is that fisetin has the ability to donate an electron

(Markovic, Mentus et al. 2009) and thereby, cause direct reduction of tetrazolium salt. A similar effect has been previously described for other phytochemicals such as kaempferal and resveratrol (Bruggisser, von Daeniken et al. 2002). Although this is a potential confounder, any transition of tetrazolium salt due to fisetin would lead to an overestimation the amount of viable cells present and therefore, underestimate fisetin's cytotoxic effect.

The fisetin-treated cells were all viewed under the microscope to identify any discrepancies between assay outcomes and direct observed cell numbers; this provided a further internal control. Another assay to use in future experiments to evaluate a fisetin-induced change in cell quantity is to count cells under the microscope using the trypan blue exclusion assay (Bardon, Vignon et al. 1987).

The potential benefit of any novel treatment for breast cancer must always be weighed against its effect on normal cellular proliferation, as negative effects on normal cells can also translate into toxicity associated with the compound being studied. These effects on normal cells must also be investigated in preparation for translation into an *in vivo* model. It is known that various cancer cells rely on specific tumor-stromal interactions; for example, the [normal cellular growth] fibroblast-tumour symbiosis, in which fibroblasts enhance tumour initiation and progression (Bhowmick, Neilson et al. 2004). Another example is the necessity for proximal angiogenesis for growth and progression of solid tumours (Hanahan and Weinberg 2011). As such, toxic drug effects intended for cancer cells also often affect "normal" cells in order to interfere with this growth relationship.

The normal cell lines used for this research were HMEC, normal adult human dermal fibroblasts and HUVEC. The effect of fisetin on these cells was studied using the crystal violet assay, which was the most consistent assay, in my research, for cellular quantification assessment. The effects of fisetin on cell growth were, again, noted to be cell-type specific (Figure 6), fibroblasts did not demonstrate a significant decrease in response to fisetin treatment but HMEC did decrease in number at 100 μM fisetin. Although this may raise concern for a potentially negative effect of fisetin on the body's healthy normal cells, statistically significant cytotoxicity was reached in all breast cancer cell lines at lower doses and earlier time points than in normal cells (Figure 3; Figure 4). The only exception was in the T-47D breast cancer cell line. Further clarification of whether the effect of fisetin is cytostatic or cytotoxic on normal cells is necessary, since this will have significant implications in an *in vivo* model. If the effect is cytostatic on normal cells but cytotoxic on breast cancer cells then fisetin is expected to be effective as a therapeutic agent.

An interesting observation was that HUVEC exhibited a significant decrease in number at all fisetin treatment concentrations and time points (Figure 6). Although HUVEC were initially investigated as a "normal" cell, they have also been used to study the effect of various compounds on angiogenesis. Fisetin has previously been demonstrated to mitigate angiogenesis *in vitro* (Fotsis, Pepper et al. 1997) and *in vivo* in a mouse model (Touil, Seguin et al. 2010). These studies, however, utilized other types of vascular cells whereas the observation made in HUVEC in the current study is preliminary. Future directions in this area of research should include an evaluation of the effect of fisetin on angiogenesis *in vitro* using a three-dimensional collagen gel and

measuring penetration and sprout length of vascular cells in the absence or presence of fisetin (Fotsis, Pepper et al. 1997); a "scratch"/wound healing assay to evaluate the ability of fisetin-treated HUVEC to move and reclaim disrupted area (Malinda, Sidhu et al. 1998); the effect of fisetin on growth factors like vascular endothelial growth factor (Sung, Pandey et al. 2007) and signaling pathways that regulate angiogenesis such as the PI3K pathway (Zhong, Chiles et al. 2000). Another option would be to investigate effects of fisetin on the vasculature of zebrafish, as discussed below.

The results of the cell quantity assay led me to focus on the MDA-MB-468 and MDA-MB-231 breast cancer cell lines in order to further investigate the specific effects of fisetin on cell death and cell cycle progression. These particular cell lines were selected because they exhibited a range of dose- and time-related responses to fisetin, which suggests that different underlying processes might be responsible for the observed decreases in cell quantities (Figure 3). In addition, both breast cancer cell lines exhibited the triple negative phenotype, which eliminated receptor status as a variable. Also, triple negative breast cancer cells are of great clinical relevance, as systemic treatment of triple-negative breast cancers is limited to chemotherapy.

4.2 Fisetin reduces cell proliferation

The results from the experiments with the MTT, crystal violet and acid phosphatase assays only represent changes in the quantity of cells. Thus, the next step was to delineate whether this effect was the result of anti-proliferative effects due to restricted cell division.

When cells undergo mitosis, the dye will be equally distributed in both daughter cells and the number of cell divisions can therefore be counted. Fisetin-treated MDA-MB-468 and MDA-MB-231 breast cancer cells demonstrated a significant decrease in the number of cell divisions (Figure 7) when assessed with Oregon Green 488®; these results supported results obtained from the cell quantification assays, which demonstrated that the MDA-MB-468 breast cancer cells were more sensitive than MDA-MB-231 breast cancer cells to the effect of fisetin.

To further investigate this anti-proliferative effect of fisetin, clonogenic assays were completed on the two breast cancer cell lines (Figure 8) to determine the effect of fisetin over a longer period of time with shorter exposure periods. The assessment is based on the understanding that cells may still be viable and metabolically active after exposure to compounds but can no longer divide or form colonies (i.e., they are senescent) (Tannock, Hill et al. 2005). After being exposed to fisetin for a 24 hour period, viable cells were plated. All cells at this time point were viable (i.e., no cells that were examined had allowed the trypan blue stain to enter the cell). This observation suggests that the decrease in the quantity of cells at this point in time was due to a fisetin-induced decrease in proliferation as opposed to cell death. Another interesting observation was that MDA-MB-231 breast cancer cells formed significantly more colonies than MDA-MB-468 breast cancer cells; this observation might reflect the aggressive characteristics inherent to MDA-MB-231 breast cancer cells.

4.3 Fisetin induces cell specific cell cycle arrest

Fisetin has anti-proliferative effects on several cancer cell lines; the mechanism of action of these effects was found to be specific to a given cell-line. In addition, fisetin causes cell cycle arrest at different phases in a variety of other cancer cells; it causes a G_2/M arrest in prostate cancer cells (Haddad, Venkateswaran et al. 2006; Haddad, Fleshner et al. 2010) and a G_0/G_1 arrest in bladder cancer cells (Li, Cheng et al. 2011). Fisetin has also been shown to bind and inhibit CDKs, including CDK1, CDK2, CDK4 and CDK6 in colon cancer cells (Lu, Chang et al. 2005; Lu, Jung et al. 2005), which would affect all phases of the cell cycle. The mechanism of the inhibitory effect of fisetin on breast cancer cell proliferation was the relevant question for my research.

After identifying a fisetin-induced decrease in cell quantity as well as an antiproliferative effect, the next step was to determine if this was the result of cell cycle arrest in MDA-MB-468 and MDA-MB-231 breast cancer cells versus fisetin-induced cell death, or a combination of the two. The cell cycle analysis was performed using flow cytometry to quantify DNA stained by the fluorescent compound PI after breast cancer cells were exposed to fisetin. MDA-MB-468 breast cancer cells demonstrated an increased number of cells in G_2/M and subG_1 phases, which corresponded to a significant decrease in the number of cells in G_1 phase (Figure 9A). Apigenin treatment on MDA-MB-468 breast cancer cells have demonstrated a similar arrest in G_2/M by decreasing CDK1, CDK4, cyclin B, cyclin D and A (Yin, Giuliano et al. 2001). DNA in cells undergoing apoptosis becomes fragmented and therefore has less than a total of 2N, falling into the subG_1 category. Although SubG_1 phase represents apoptosis, it can also represent necrotic cells, nuclear fragments, clumps of chromosomes, micronuclei, and

nuclei with normal DNA quantity but abnormal structure that does not allow PI to bind (Riccardi and Nicoletti 2006).

Two potential confounders that could affect the parameters of analysis are (i) that breast cancer cells can display aneuploidy, which might falsely suggest an increase in S-phase and (ii) the introduction of error in the control group caused by two cells in G_o/G_1 proceeding through the flow cytometry at the same time, which would suggest a 4N quantity of DNA or G_2/M). These two confounders were minimized by giving each cell line its own control (constant rate of aneuploidy) and cells were processed by flow cytometry at a slow rate (no greater than 200 cells/sec). It should also be noted that MDA-MB-468 breast cancer cells were only analyzed following exposure to 50 μM fisetin, as it was impossible to analyze cells treated with 100 μM fisetin because of an inability to identify individual peaks, which suggests significant damage to the cells at this concentration and time point (48 hours). An analysis of cells exposed to less than 50 μM fisetin would be a consideration for future studies.

Fisetin-treated MDA-MB-231 breast cancer cells demonstrated a different pattern of cell cycle arrest (Figure 9B), namely, a significant increase in the S-phase cell population with a corresponding decrease in the proportion in G_1 phase. In the literature a S-phase cell cycle arrest has been demonstrated in MDA-MB-231 breast cancer cells with exposure to quercetin. The arrest has been attributed to decreased levels of thymidylate synthase, cyclin A and cyclin D (Chien, Wu et al. 2009). This suggests that the cells are not progressing through the cell cycle and the decrease in quantity by fisetin could be cytostatic. There was no significant increase in the population of cells in subG_1, suggesting no increase in the number of cells undergoing apoptosis. There was, however,

a trend toward an increase in the number of cells in this population, and further studies could utilize a higher concentration of fisetin or longer time period of exposure (>48 hours) to further evaluate this finding.

The above data provide a possible explanation for the decrease in cellular proliferation observed following fisetin treatment of both breast cancer cell lines. However, the mechanism of action will require further evaluation of fisetin's effect on CDKs, CAKs, retinoblastoma, cyclins, and inhibitors of the cell cycle (i.e., Kip and INK4).

4.4 Fisetin induces cell death in breast cancer cells

In the cell cycle analysis, the proportion of MDA-MB-468 breast cancer cells in subG_1 phase was significant following fisetin treatment and although the results in the MDA-MB-231 breast cancer cells were not significant, it did demonstrate a trend toward increasing number of cells in subG_1 with increased fisetin exposure. Since there are limitations to the use of subG_1 as an indicator of apoptosis, the next step was to perform Annexin-V-FLUOS/PI assay. This assay detects phosphotidylserine, an anionic phospholipid that is normally located on the inner leaflet of healthy cells but is translocated to the outer leaflet during apoptosis. Annexin-V-FLUOS is a calcium-dependent phospholipid-binding protein, with high affinity for phosphatidylserine, which allows for the localization of phosphatidylserine in the cells being studied (Tannock, Hill et al. 2005).

MDA-MB-468 breast cancer cells (Figure 10C) and MDA-MB-231 breast cancer cells (Figure 10D) showed localization of phosphatidylserine to the outer leaflet after

exposure to fisetin, indicating the induction of apoptosis. The MDA-MB-231 breast cancer cells were again relatively resistant to fisetin's effect, which is consistent with assay results described above. The main reason for conducting this assay over several time points, in addition to identifying time-dependent differences of fisetin treatment, was to ensure eventual progression to cell death. Ultimately, cell death is heralded by cell membrane perforation and PI entrance into the cell. This may represent an apoptotic cell death since cells will undergo eventual breakdown of the cell membrane in the absence of phagocytes to ingest apoptotic bodies in tissue culture (i.e., MDA-MB-468 breast cancer cells showing apoptosis at 24 hours and 48 hours progressing to late apoptosis at 72 hours in Figure 10C), or a necrotic cell death. This former hypothesis (apoptotic cell death) was confirmed by the absence of LDH release by fisetin-treated cells (Figure 11), which demonstrated that the MDA-MB-468 and MDA-231 breast cancer cells underwent cell death predominantly by apoptosis.

The original description of apoptosis was by direct observation of cells showing characteristic morphological changes. This was attempted in my study by way of phase contrast microscopy and Hoechst staining (data not shown), but with minimal success. The Hoechst stain emits bright blue fluorescence when bound to double-stranded DNA within apoptotic cells (Araki, Yamamoto et al. 1987). Although a few pertinent characteristics were observed by microscopy, conclusive data will have to be elucidated in future studies. Possible options to further substantiate fisetin-induced apoptosis could include showing DNA laddering (Sellins and Cohen 1987) and using terminal deoxynucleotidyl transferase-mediated dUPT-biotin nick end labeling (TUNEL) assay (Gavrieli, Sherman et al. 1992).

4.5 Fisetin induces mitochondrial destabilization and caspase-dependent apoptosis

After demonstrating that fisetin caused MDA-MB-468 cells to die predominantly by apoptosis, I next determined the mechanism. Apoptosis involves the caspase cascade, which includes both initiator caspases (capase-8, -9 and -10) and executioner caspases (caspase-3, -6, -7); the caspase cascades are initiated by two main pathways, the DR-pathway and mitochondrial pathway (See section 1.3 for further details), which are not mutually exclusive.

Because most apoptotic pathways eventually converge on the caspase cascade, the annexin-V-FLUOS/PI assay with two different pan-caspase inhibitors (Z-VAD-FMK and BOC-D-FMK) were used to determine caspase involvement in fisetin-induced apoptosis. These pan-caspase inhibitors affect both initiator and executioner caspases. This study was performed at 72 hours of fisetin treatment of MDA-MB-468 breast cancer cells. The 100 μM fisetin plus pan-caspase inhibitor demonstrated a significant decrease in total cytotoxicity and apoptosis when compared with 100 μM fisetin alone, suggesting that fisetin-induced apoptosis occurs through caspase activation (Figure 13A). Fisetin has been demonstrated to induce apoptosis through caspase activation in leukemia cell lines (Lee, Shen et al. 2002) and hepatocellular carcinoma (Chen, Shen et al. 2002).

It is noteworthy that breast cancer cells treated with 50 μM of fisetin in the presence of a pan-caspase inhibitor revealed no significant difference in total cytotoxicity or apoptosis when compared to the vehicle control (Figure 13A), despite the fact that this concentration of fisetin had previously demonstrated a significant increase in total cytotoxicity (Figure 10C). Breast cancer cells treated with 100 μM fisetin plus pan-

caspase inhibitor showed substantial total cytotoxicity and late apoptosis/necrosis compared to the vehicle control, suggesting that in the presence of pan-caspase inhibitors, the dominant form of cell death is necrosis rather than apoptosis.

This particular study was only performed on MDA-MB-468 breast cancer cells because they demonstrated significant induction of apoptosis under multiple fisetin treatment conditions (Figure 10C). It would be interesting to determine whether the alternate form of cell death that occurs in the presence of the pan-caspase inhibition is the same form of cell death induced by fisetin in MDA-MB-231 breast cancer cells. Since late apoptosis/necrosis seemed to occur more prominently in the MDA-MB-231 breast cancer cell line, this might explain the death of MDA-MB-468 breast cancer cells treated with fisetin in the presence of pan-caspase inhibitors.

This alternate form of cell death necrosis/oncosis has been described in the literature, as cells can be sensitized for necrotic cell death in the presence of caspase inhibitors (Vercammen, Beyaert et al. 1998; Vandenabeele, Berghe et al. 2006). A possible pathway is through the PARP-1-mediated caspase-independent cell death pathway. PARP-1 is a DNA repair enzyme, and PARP-1-mediated cell death is due to the depletion of NAD and ATP, resulting in energy depletion and subsequent necrosis. Future studies could attempt to examine the effect of pan-caspase inhibitors plus fisetin on PARP-1 cleavage in breast cancer cells.

Since PARP-1 is a cornerstone of apoptosis and a substrate of the caspase cascade, this molecule was investigated to further evaluate caspase activation (Herceg and Wang 2001). PARP-1 cleavage in MDA-MB-468 breast cancer cells was assessed after treatment with 50 μM fisetin for 24 hours; the results demonstrated an increase in

the cleavage product of PARP-1 as well as a corresponding decrease in intact PARP (Figure 14), which supports the presence of activated caspase in fisetin-treated breast cancer cells.

I next studied the effect of fisetin on mitochondrial stabilility in breast cancer cells, since this is a target of the intrinsic pathway of apoptosis. Mitochondrial destabilization is also involved in the DR-pathway with the crossover of caspase-8 activation. The mitochondrial membrane can therefore be destabilized at the end of the caspase cascade, regardless of the apoptosis initiation pathway. A fluorescent dye compound, $DiOC_6$, which enters the cell and binds to membrane-bound organelles, was used to detect mitochondrial membrane destabilization. It was necessary to use a 40 nM concentration of the dye in order to have preferential binding to the mitochondria (Koning, Lum et al. 1993). This experiment demonstrated a loss of the mitochondrial membrane potential in both MDA-MB-468 and MDA-MB-231 breast cancer cells at 72 hours of fisetin treatment (Figure 12A and 12B).

When a mitochondrion loses its membrane potential, transmembrane pores open and release pro-apoptotic molecules such as cytochrome-*c*, SMAC/Diablo, AIF and endonuclease G (Chinnaiyan 1999; Gupta 2001). Western blotting showed an increase in cytosolic cytochrome-*c* at 24 hours of fisetin treatment (Figure 12C), which is consistent with destabilization of the mitochondrion. The timeline of these events, however, is still not clear. The $DiOC_6$ assessment was performed at 72 hours and Western blotting was performed at 24 hours. This suggests that there may be both an early and late role of the mitochondria in the cell death process that results from breast cancer cell exposure to

fisetin.. An alternate explanation is that the two assays have differing sensitivities for identifying the change in mitochondria membrane integrity.

4.6 ROS production is not involved in fisetin-induced cytotoxicity

ROS production is a normal product of cellular metabolism; ROS can act as both a signaling pathway for, and be produced by, death stimuli. Necrosis and/or apoptosis may be the result of ROS production. Necrosis occurs in the setting of high oxidative stress, whereas apoptosis typically occurs in the setting of moderate oxidative stress due to upregulation of different signaling pathways (Verheij, Bose et al. 1996; Hampton and Orrenius 1997; Yin, Terauchi et al. 1998). Preliminary studies were therefore undertaken in order to investigate whether ROS production was involved in fisetin-induced breast cancer cell death.

DHE is a non-polar, non-fluorescent compound that diffuses into the cell and, in the presence of superoxide anions, becomes converted to fluorescent ethidium (Fink, Laude et al. 2004); DHE was therefore used in order to investigate the role of ROS production in fisetin-induced cell death. Although there was a shift in fluorescence during flow cytometry analysis that appeared to suggest ROS production, no conclusions can be drawn from this assay because fisetin is also fluorescent (Figure 15). I subsequently performed a preliminary experiment using MDA-MB-486 cell line that was more sensitive to fisetin-induced cell death. If ROS production was a major component in fisetin-induced cell death, the presence of an antioxidant should reduce the effect of fisetin on cell number. However, the fisetin-treated breast cancer cells continued to show a significant decrease in cell quantity in the presence of GSH (Figure 16). This finding is

consistent with the literature, which indicated that that fisetin has antioxidant effects in several physiological conditions. The molecular structure of fisetin with its 4 phenolic rings makes it an excellent free radical scavenger (Shia, Tsai et al. 2009). Paradoxically, fisetin in low concentrations (0 to 25 μM) protects against free radicals but in high concentrations (50 to 250 μM) induces apoptosis (Kim, Jeon et al. 2010).

Interestingly, I observed that in the presence of antioxidants the potency of fisetin was increased (Figure 16), perhaps because fisetin maintains its parent form when at a higher concentration. The reactivity of fisetin may be required for its underlying interaction with the cell such as the demonstrated intercalation of DNA by fisetin (Sengupta, Banerjee et al. 2005). This area will require further study.

4.7 MDR breast cancer cells are resistant to fisetin

Resistance of tumours to chemotherapy can be a devastating problem because tumours that become chemoresistant generally have the ability to progress, often resulting in increased patient mortality. The study of MDR breast cancer cells is important, since the sensitivity of these cells to a new compound might provide alternate options for therapy. It is also important to investigate whether phytochemicals interact with mechanisms that govern chemoresistance in breast cancer cells.

Two cell lines that were developed by growing MCF-7 breast cancer cells in the presence of mitoxantrone (MCF-7 MITX) and paclitaxel (MCF-7 TX400) were used to examine breast cancer cell resistance to fisetin. Both of these cell lines demonstrated a relative resistance to fisetin (Figure 17). Chemoresistance was confirmed by an increased expression of BCRP in MCF-7 MITX breast cancer cells (Figure 18A), while MCF-7

TX400 demonstrated a relative resistance to paclitaxel compared with MCF-7 native cells (Figure 18B). These findings suggest that the mechanisms of mitoxantrone resistance and paclitaxel resistance may also impart fisetin resistance to breast cancer cells. Alternatively, although BCRP was expressed on MCF-7 MITX breast cancer cells, it does not necessarily mean that resistance to fisetin is also mediated by this protein. Mitoxantrone resistance can also occur through altered topoisomerase II activities (Harker, Slade et al. 1991; Errington, Willmore et al. 1999), or over-expression of the drug efflux pump P-glycoprotein (p-Gp) (Consoli, Van et al. 1997). Since fisetin also inhibits topoisomerase II (Olaharski, Mondrala et al. 2005) and increases the effectiveness of p-Gp for its drug substrates (Chung, Sung et al. 2005), further investigation is necessary to identify the specific mechanisms of fisetin resistance. For MCF-7 TX400, the mechanism of resistance is thought to be through overexpression of p-Gp but again, further study is required to understand the mechanisms of fisetin resistance in this cell line.

4.8 Fisetin enhances cytotoxicity of radiation and chemotherapeutic agents

Any novel therapy must be compared to the established gold standards. Not infrequently, new therapies are used in conjunction with current therapies in an attempt to improve outcomes. It is clear that fisetin has a cytotoxic effect on breast cancer cells, so the next step was to investigate its use in combination with radiation or different chemotherapeutic agents. It not only important to identify potential benefits of these treatment combinations, but also to ensure that current effective treatments are not impeded by the addition of a new compound.

Preliminary investigations examined the combined use of radiation and fisetin. Radiation therapy is used extensively throughout clinical practice for both palliative and curative treatment of breast cancer. Of interest is the possibility that fisetin's antioxidant potential might protect cells from the radiation-induced free radicals, which are necessary for the intended cytotoxic effect of radiation treatment. In addition, the body has naturally occurring mechanisms (e.g., thiol compounds, glutathione, cysteine, and manganese superoxide dismutase to counter excessive ROS ((Tannock, Hill et al. 2005). Combining fisetin with radiation therefore has three potential outcomes: increased cytotoxicity, no effect or increased protection from radiation-induced cell death.

My initial experiments used clinically relevant doses of radiation (0 to 4 Gy); a standard daily fraction in clinical use is 2 Gy (Whelan, MacKenzie et al. 2002). Figure 19 illustrates the results of the use of a crystal violet assay of breast cancer cells treated with various concentrations of fisetin and radiation dose combinations for 72 hours, and shows that fisetin alone, and radiation alone, both induce cell death. The results of the combination treatment are difficult to assess but the slope of both MDA-MB-468 and MDA-MB-231 breast cancer cells suggest that there is likely a beneficial effect.

Only limited conclusions can be drawn from the analysis of this data, as the time period for study was relatively short (maximum of 72 hours post treatment). Radiated cells either died immediately following treatment or maintained their viability but lost their ability to replicate indefinitely. Possible loss of immortality can only be assessed over longer periods of time, and requires such tests as clonogenic assays. The literature confirms that significant findings can be observed after hours to days, depending on the cell line (Tannock, Hill et al. 2005). In addition, there are issues with correlating the data

from MTT assays with clonogenic assays (Tannock, Hill et al. 2005). Clonogenic assays and spheroid assays could further evaluate the effect of these combinations of fisetin and other therapies. These assays could be used to study the combination of fisetin and radiation longer time periods or radiation given over time with multiple exposures as observed in clinical therapy (multiple daily fractions over several weeks) (Whelan, MacKenzie et al. 2002).

The combination of fisetin with chemotherapeutic agents, in a preliminary study, demonstrated a trend toward benefit, although possible complications were also observed. The pattern that emerged with the crystal violet assay over 72 hours demonstrated that fisetin did not significantly inhibit the chemotherapeutic drug's action and may in fact have a beneficial effect (Figures 20, 21, 22, 23, and 24). Further experiments are required, such as the clonogenic assay discussed above to confirm theses findings, but one interesting pattern was observed in this data. Figure 20B demonstrated that fisetin in combination with cisplatin had increased effectiveness in a stepwise fashion, suggesting potentially additive beneficial effects. All of these studies will require further evaluation with clonogenic assays in order to generate survival curves and produce isobolograms, which are a method of identifying whether two compounds act in a synergistic (the effect of two or more drugs/treatments are greater than the sum of their individual effects), additive (the effect of two or more treatments are equivalent to the sum of their individual effects), or antagonistic (a treatment that partially or totally prevent the effects the other treatment) manner (Hollinger 2003).

4.9 Toxicity of fisetin

The goal of *in vitro* research that investigates novel therapeutic compounds is to transition tissue culture findings to an *in vivo* model and, eventually, to humans. In preparation for use of a novel compound in an *in vivo* model, toxicity must first be evaluated. Flavonoids have been linked to liver failure, contact dermatitis, hemolytic anemia, and estrogenic-related concerns that include effects on sexual reproduction (Galati and O'Brien 2004). One fisetin-specific concern is that topoisomerase II inhibition has been shown to induce some forms of leukemia (Olaharski, Mondrala et al. 2005).

Initial investigations into fisetin's effect on normal cells in culture were completed as discussed earlier in this section. Subsequently, the *in vivo* effect of fisetin on cell mortality was investigated using an emerging model for cancer research, zebrafish. Because fisetin is hydrophobic and insoluble in cold water, and only minimally soluble in warm water (Sando and Bartlett 1918), concentrations of fisetin higher than 200 μM were not possible. Zebrafish embryos are usually maintained in a 28.5°C environment, but in preparation for xenograft, after being treated the embryos were maintained at 37°C. The temperature limitations might represent a significant barrier to the use of this model and alternative routes of fisetin administration may be required. Embryo viability was not affected at these concentrations during 72 hours of exposure (Figure 25).

Bioavailability is another further area of interest for fisetin because fisetin has limited solubility in water (hydrophobic) (Guzzo, Uemi et al. 2006). In my research, the solubility problems of fisetin were also demonstrated by the inability to have concentrations greater than 200 μM in Zebrafish egg water without crystallization.

Another concern is the concentration of fisetin available in its parent form. For instance, fisetin was involved in oxidation/reduction reactions, as demonstrated in the MTT control experiment. Fisetin may benefit from exploring alternate delivery methods such as liposomal formulations, nanoparticles, microemulsions and polymeric implantable devices as described in the literature (Bansal, Goel et al. 2011; Mignet, Seguin et al. 2012).

4.10 Future directions and conclusions

The research that I have presented focused on the initial understanding of fisetin's effect on breast cancer cells *in vitro*. The use of multiple cell lines, with emphasis on two triple negative cell lines (MDA-MB-468 and MDA-MB-231), have identified the potential therapeutic benefits of this compound, but also demonstrated relative resistance in breast cancer cell lines. One limitation of this research is that cell culture is a simulated model in an isolated environment; as such, the effects may not be entirely transferable to an *in vivo* model. Although the effect of fisetin on normal cells (i.e. HMEC, fibroblasts and HUVEC) have also been evaluated, further study regarding fisetin's interaction with these cells in the presence of breast cancer cells, as well as in the dynamic microenvironment of a solid tumour is still required; toward that end, several suggestions for future directions of research have been presented. A summary of future research directions, and potential barriers to their completion, are further discussed below.

First, additional studies to advance the understanding of fisetin's mechanism of action are of paramount importance. The focus of the current research on triple negative breast cancer cell lines has provided preliminary information on possible different

pathways of fisetin-induced cell death. In MDA-MB-468 breast cancer cells, fisetin induced apoptosis by triggering caspase activation. If, however, the caspase pathway was blocked, a different mechanism for cell death was employed that appeared to act in a similar fashion as the pattern of necrosis observed in fisetin-treated MDA-MB-231 breast cancer cells. These specific mechanisms should be further evaluated using individual caspase inhibitors and Western blot analysis of specific components such as the initiator and executioner caspases. Because fisetin also causes cell line-specific cell cycle arrest, the effects of fisetin on cell cycle inhibitors, checkpoints and promoters also need to be studied in more depth.

Secondly, the evaluation of other cell lines that are sensitive to fisetin (e.g., MCF-7 breast cancer cells) should also be undertaken. MCF-7 breast cancer cells express wild type p53 (Carlson, Dubay et al. 1996; Zhuang and Miskimins 2011), as well as estrogen and progesterone receptors (So, Guthrie et al. 1997; Zhuang and Miskimins 2011), but not caspase-3 (Zhuang and Miskimins 2011). Since fisetin induces apoptosis via p53 in colon cancer cells (Lim do and Park 2009) the use of MCF-7 breast cancer cells could reveal yet another mechanism of action of fisetin on breast cancer cells.

Finally, movement toward an *in vivo* model is important to take the research from a one-dimensional (monolayer) to a three-dimensional model. Limitations to the current monolayer model include the absence of stroma and hence the inability to assess the effect of fisetin on crucial stromal-tumour interactions (Kim, Stein et al. 2004). Preliminary studies examining fisetin's effect on normal cells have been completed, however, they were not performed in the presence of breast cancer cells. This area could be studied using spheroids cultured in different extracellular matrices (Kim, Stein et al.

2004). This model can be used to evaluate drug-penetrance, in addition to the effect of fisetin on angiogenesis and metastasis (Lee, Huh et al. 2009; Liao, Shih et al. 2009).

The *in vivo* zebrafish model that was explored in this research represents preliminary experiments to evaluate fisetin's effect on embryo viability. Zebrafish offer many advantages over a murine model, i.e. these fish are small and inexpensive, and have the ability to breed in large numbers with a short generation time, in addition to possessing optical clarity (Parng, Seng et al. 2002). Further study will also allow evaluation of potentially beneficial effects of fisetin on tumour-associated angiogenesis and metastasis; positive effects identified could then be substantiated using a xenograft murine model.

Fisetin has demonstrated cytotoxic effects on a variety of cancer cell types, and I have shown that this benefit can translate to breast cancer. In conclusion, fisetin shows promise for possible future use in the treatment of breast cancer.

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