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Investigating the Potential of Acetyl Plumbagin to Reduce Cancer-Related Drug Resistance in Various Breast Cancer Cell Lines

MSc Research Dissertation

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DECLARATION

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NRF Declaration

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged.

Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be contributed to the NRF.

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And finally I'd like to thank my close friends and family who have supported and encouraged me to finish my research.

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LIST OF SYMBOLS

β	Beta
©	Copyright
°C	Degrees Celsius
g	Grams
kDa	Kilodaltons
µ	Micro
µg	Micrograms
µL	Microlitres
µM	Micromolar
mg	Milligrams
mL	Millilitres
mm	Millimetres
nm	Nanometres
nM	Nanomolar
pg	Picograms
®	Registered
™	Trademark
U/mL	Unit per millilitre
x *g*	Centrifugal force

LIST OF ABBREVIATIONS

5-FU	5- Fluorouracil
ACAT1	Acetyl-coenzyme A acetyltransferase 1
ADP	Adenosine diphosphate
AIs	Aromatase inhibitors
AP	Acetyl Plumbagin
APS	Ammonium persulfate
BC	Breast cancer
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Cav-1	Caveolin-1
CEs	Cholesterol esters
CETP	Cholesteryl ester transfer protein
CO_2	Carbon dioxide
CRE	Cholesterol response element
Cu^{2+}	Copper cation
DC FBS	Dextran charcoal stripped foetal bovine serum
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E_2	Estradiol
ECACC	European Collection of Authenticated Cell Cultures

EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ER+	Estrogen receptor- positive
ERK	Extracellular signal-regulated kinases
FBS	Foetal bovine serum
Fig.	Figure
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HER2	Human epidermal growth factor receptor 2
HMG-CoA	Hydroxy-methyl glutaryl-coenzyme A
HRP	Horseradish peroxidase
hrs	Hours
IGF1-R	Insulin-like growth factor receptor
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide
KPO_4	Potassium phosphate buffer
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LTED	Long-term estrogen deprived
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase

MβCD	Methyl-β-cyclodextrin
MCF7	Michigan Cancer Foundation-7
miRNA	MircoRNA
min	Minutes
MOMP	Mitochondrial outer membrane potential
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MTT	3-(4,5-Dimethylthiazol-z-yl)-2,5diphenyltetrazolium bromide
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide
NP-40	Nonidet P-40
OD	Optical density
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kextin type- 9
pH2AX	Phosphorylated histone H2AX
PI3K	Phosphoinositide 3-kinase
PL	Plumbagin
PR	Progesterone receptor
P-S	Penicillin-streptomycin
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RT	Room temperature
S.D	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERMs	Selective estrogen receptor response modulators
SRE	Sterol regulatory element
SREBP	Sterol regulatory element-binding protein
Tam	Tamoxifen
TBST	Tris-buffered saline with Tween-20
TEMED	Tetramethylethylenediamine
V	Volts
VLDL	Very low-density lipoproteins
WT	Wild-type

ABSTRACT

Breast cancer (BC) is the second most common malignancy comprising of 21.78% of all cancers in women. Death rates have decreased significantly from BC due to improved use of adjuvant Tamoxifen (Tam) and chemotherapy. However, given the improvement of survival rates due to endocrine therapies, many patients relapse with acquired resistance at later times after initial response. Thus a greater understanding of the mechanisms involved in cancer-related drug resistance is needed along with the identification of new compounds that could help overcome drug resistance in cancer cells. The aim of this investigation was to test Acetyl Plumbagin (AP) as an adjuvant compound in various estrogen receptor positive (ER+) BC cell lines to reduce cholesterol accumulation and cancer-related resistance. This study observed the changes in pathways affected in two BC cell lines when AP was added alone and when AP was added in combination with Tam. This allowed for the observation of enhanced efficacy of Tam in inhibiting cell growth in MCF7 and long-term estrogen deprived (LTED) cells by 500% and 300% respectively. A significant increase in early-stage apoptosis was observed when cells were treated with the combination therapy as compared to single treatments. Cholesterol depletion was observed in both cell lines indicating that Tam and AP work in a synergistic manner in inducing apoptosis via cholesterol depletion in cell membranes. AP significantly enhanced the sensitivity of ER+ BC cells to Tam treatment. This indicates that AP could act as an anti-cancer lead compound for future drug development, targeting cholesterol accumulation and Tam resistance in BC.

1. LITERATURE REVIEW

1.1 Prevalence of cancer and breast cancer occurrence

Cancer is the second leading cause of death worldwide with an estimated 14 million new cases reported yearly and 9.6 million deaths occurring in 2018 (WHO, 2018). Breast cancer (BC) is the second most common malignancy worldwide and is the most common cancer in women comprising of 21.78% of all cancers (Herbst, 2018). It has been reported that there have been 2.09 million breast cancer cases worldwide with 627 000 deaths in 2018 (WHO, 2018). BC is ranked as the leading cause of cancer deaths in South African women (Herbst, 2018). It is estimated that the burden of cancer will increase to 23.6 million new cases yearly by 2030 (WHO, 2018). This indicates a significant increase in cancer-related deaths, highlighting the urgent need for novel chemotherapeutic strategies in order to improve current cancer treatment.

1.2 BC characteristics

BC is a malignant tumour that begins to develop in various ducts and tissues in the breast (American Cancer Society, 2018). The hallmarks that govern the transformation of normal cells to malignant cells include: unlimited proliferative potential, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis, self-sufficiency in growth signals, and insensitivity to anti-growth signals (Hanahan and Weinberg, 2000). BC is an aggressive cancer due to its metastatic potential as well as its potential to acquire resistance to treatment. BC also has remarkable abilities to evade apoptosis, which classifies it as one of the most difficult cancers to treat (Hanahan and Weinberg, 2000).

Apoptosis is a process of programmed cell death which leads to physiological and morphological changes in the cell (Hanahan and Weinberg, 2000). The two apoptotic pathways include intrinsic and extrinsic pathways. Both of these pathways induce apoptosis via the activation of caspases through the degradation of proteins as seen in Fig. 1 below (Putcha *et al.*, 2002). These pathways are suppressed in BC, which leads to the up-regulation of several proliferative pathways leading to uncontrolled proliferation. These pathways are up-regulated through the interaction between steroid hormones and cell surface receptors, leading to a more resistant and aggressive tumour. These characteristics of BC make treatment difficult and emphasises the importance of novel treatments.

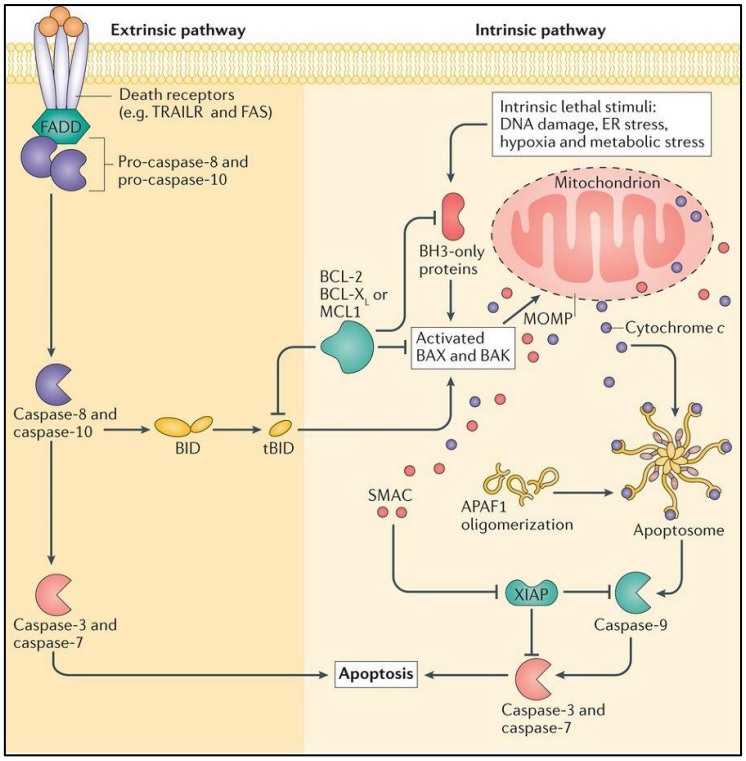


Figure 1: The extrinsic and intrinsic pathways involved in apoptosis

Apoptosis occurs through these main pathways which includes the activation of several caspases. In the extrinsic pathway, a signal is received from outside of the cell instructing it to undergo programmed cell death. The intrinsic pathway is triggered by internal stress or damage to the cell which then undergoes apoptosis (Lowe and Lin, 2000).

Figure adopted from: [(Biologydictionary.net, 2018) Accessed: 20.10.18]

1.3 Hormone induced BC

Steroid hormones such as estrogen and progesterone are essential for normal breast cell growth and development (Anderson and Clarke, 2004). These hormones are synthesised from cholesterol and are fat-soluble, meaning they can pass through the cell membrane and bind to nuclear steroid hormone receptors bringing about a change in the cell (Heffner and Schust, 2010). BC is linked to the dysregulated expression of these endogenous steroid hormones (Esau *et al.*, 2016). The three main hormones associated with BC include estrogen, progesterone and human epidermal growth factor. BC is therefore classified based on the presence of the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) or those that lack these three receptors, termed triple negative (Baek and Nelson, 2016). Of all BC cases reported, approximately 70% of these cases are ER positive (ER+) (Kohler *et al.*, 2015). ER+ BC cells rely on the estrogen hormone and ER-mediated pathway in order to promote cell proliferation, migration and survival.

Estrogen is a key driver in BC proliferation and works by binding to the ER, which promotes the dimerisation and conformational change in the ligand-binding domain of the ER. This change then leads transcriptional co-activators to be recruited for further gene expression (Rosenfeld and Glass, 2001). The aberrant expression of ER in BC cells exerts proliferative, metastatic and survival effects (Clemons and Goss, 2001). Estrogen directly initiates tumour development by activating oncogenes involved in nucleic acid synthesis and indirectly promotes tumour development by stimulating several transcription factors involved in abnormal cell proliferation (Clemons and Goss, 2001). Therefore, abnormal expression of estrogen and ER are regularly associated with BC cases. Hormone receptors are therefore important prognostic and predictive factors for potential chemotherapeutic strategies (Cianfrocca and Goldstein, 2004). Endocrine therapies target the hormone activation of the ER signalling pathway thereby reducing BC cell proliferation (Wong and Chen, 2012) and are therefore one of the most common forms of BC treatment.

The ER is part of the nuclear receptor family of ligand-activated transcription factors and is required for cell growth in ER+ BC cells (Viedma-Rodríguez *et al.,* 2014). After passing through the cell membrane, estrogen binds to the nuclear bound ER which undergoes conformational changes (phosphorylation and dimerisation). Following these changes, the ER binds to the estrogen response elements upstream of estrogen-dependent genes. These genes are involved in cell proliferation and survival (Viedma-Rodríguez *et al.*, 2014). The ER can be phosphorylated by membrane receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGF1-R) (Viedma-Rodríguez *et al.*, 2014). Crosstalk between growth factor receptors and ER signalling pathways has been well established. These growth factor receptors activate the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signalling pathways which down-regulate ER expression. ER has been found not only in the nucleus but also in the membrane and cytoplasm, working through non-genomic pathways such as the PI3K/Akt and MAPK pathways (Viedma-Rodríguez *et al.,* 2014). In conclusion, ER activity and signalling is modulated by several pathways which could contribute to ER-targeted treatment.

1.4 Current BC treatments

There have been many interventions and chemotherapeutic approaches developed in an effort to treat BC cases. However, many approaches fail due to the aggressive and resistant nature of BC cells. The most common forms of treatment of BC include: surgery, radiation, chemotherapy and hormone or endocrine therapies (American Cancer Society, 2018).

Although these therapies are effective they are invasive and can lead to several complications. Therefore, the preferred treatment for BC is endocrine therapy as many BC cases are induced by hormones and it is a non-invasive treatment.

Endocrine therapy, also called anti-estrogen therapy, works by lowering the levels of estrogen circulating in the body or by blocking estrogen from binding to the ER (Breastcancer.org, 2018). Triple negative BC cells do not respond to endocrine therapy as they do not have hormone receptors. Endocrine therapy is used as an adjuvant therapy often after surgery to reduce the recurrence of BC tumours. The main forms of endocrine therapy include: selective estrogen receptor modulators (SERMs), aromatase inhibitors (AIs), ER down-regulators, luteinizing hormone-releasing hormone agent, and prophylactic ovary removal (Breastcancer.org, 2018).

The most common endocrine therapy is the use of SERMs which blocks the effects of estrogen's ability to stimulate the growth of BC cells. SERMs serve as estrogen antagonists by competing with estrogen to bind to the ER and ultimately block estrogen from binding to the ER. SERMs also mimic estrogen effects (serve as estrogen agonists). This means that a SERM blocks estrogen effects in breast tissue but acts like estrogen in other tissues such as the uterus and bone (Breastcancer.org, 2018). Examples of SERMs include: Tamoxifen (Tam), Raloxifene and Toremifene. Other anti-estrogen drugs such as Fulvestrant act in a similar way to SERMs; however, they have no estrogen agonist effects and are often used to treat advanced BC in post-menopausal women (Kohler *et al.*, 2015). Although there are currently many endocrine treatments on the market, the gold-standard drug, Tam has been used for more than 40 years as the primary source of treatment for ER+ BC.

As previously mentioned, Tam is a SERM that acts as an antagonist to estrogen on the ER. Tam is initially metabolised by a family of cytochrome P450 enzymes into active metabolites called endoxifen and 4-hydroxy-tamoxifen, whereby both metabolites competitively bind to the ERs of BC cells (Lu *et al.*, 2012). The active metabolites are internalised and bind to estrogen-dependent genes thus reducing gene expression through the recruitment of co-repressors as seen in Fig. 2 below (Hodges *et al.*, 2003). Consequently, this prevents the activity of various transcription factors that promote cellular replication (Osborne, 1998). The inhibition of ER activity using Tam is important in managing and reducing ER+ BC recurrence (Cuzick *et al.*, 2010; Lin *et al.*, 2015).

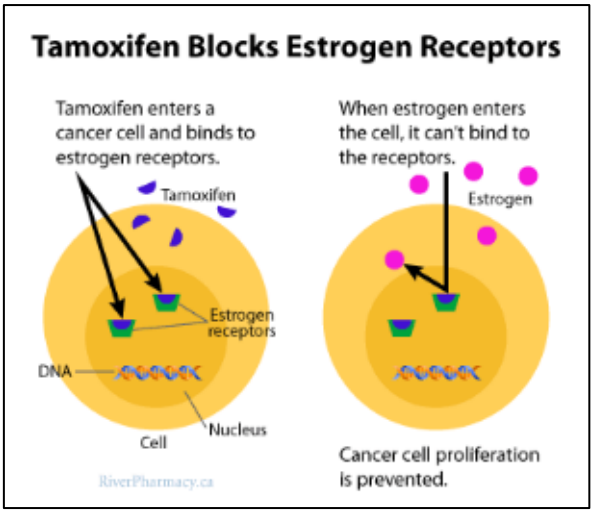


Figure 2: The mode of action of Tam in a cancer cell

Tam competes with estrogen to bind to the ER. Co-repressors are subsequently recruited resulting in cancer cell proliferation prevention and growth inhibition.

Figure adopted from: [https://astro-metrics.com/tamoxifen-mechanism/ Accessed: 20.10.18]

Death rates have decreased by 25% since 1990 from BC and this is due to improved use of adjuvant Tam and chemotherapy (Karami-Tehrani and Salami, 2003; Parton *et al.*, 2001). Although an improvement in survival rates has been observed, these patients acquire resistance to treatment after prolonged use after initial response. Additionally, after metastatic relapse, disease is not curable even with other available therapies such as AIs, and consequently many patients die. Currently, there are no targeted therapies for this stage of BC after relapse and therefore an aggressive form of chemotherapy has to be used (Karami-Tehrani and Salami, 2003). Thus further research is required to help minimise the side effects of Tam by reducing its toxicity and inherent resistance. Furthermore, a greater understanding of the mechanisms involved in cancer-related drug resistance is needed along with the identification of new compounds that could help overcome drug resistance in cancer cells.

1.5 Cancer-related drug resistance

Although Tam has been used as the first-line adjuvant endocrine therapy for ER+ BC for several years, patients have developed resistance to this therapy resulting in aggressive, metastatic tumours (Wang *et al.*, 2018). There are two forms of drug resistance: *de novo* and acquired resistance. *De novo* resistance occurs when patients do not initially respond to treatment and acquired resistance occurs when patients who initially respond to treatment, acquire resistance over prolonged use of the treatment. There have been several investigations

that have taken place in order to elucidate and understand the mechanisms involved in acquired Tam resistance and how to overcome this resistance.

Several mechanisms responsible for acquired Tam resistance in BC cells have been proposed. These include: the deregulation of the ER signalling pathway; loss of ER expression; mutations and/or post-translational modifications of the ER; pharmacological mechanisms; alterations in signalling that control cell cycle, survival, proliferation, stress response, and inhibition of apoptosis; alterations in microRNA (miRNA) expression; and the activation of escape pathways that provide tumours with alternative proliferative signals (Musgrove and Sutherland, 2009, Viedma-Rodríguez *et al.,* 2014). Aberrant expression of molecules involved in cell survival (c-Myc, Bad and Bcl-2) are also associated with Tam resistance (Riggins *et al.,* 2007). It has been proposed that in the presence of Tam, BC cells use an alternative growth regulatory pathway (Knowlden *et al.,* 2003). The MAPK pathway is a key mediator of cell proliferation and increased activation of this pathway is associated with a decreased survival time in ER+ BC patients (Knowlden *et al.,* 2003). Furthermore, increased MAPK activity has been demonstrated in long-term estrogen deprived (LTED) and Tam-resistant BC cell lines (Shim *et al.,* 2000, Knowlden *et al.,* 2003). Therefore, it is necessary to understand the role of Tam resistance in BC by studying ER-related pathway mechanisms.

The loss of ER expression is one of the major hallmarks associated with Tam resistance. There have been several mechanisms proposed to explain the loss of ER expression and its effect in Tam resistance. These mechanisms include: epigenetic changes such as methylation of the ER promoter and histone deacetylation; ER gene mutations leading to loss of ER expression; and the activation of the PI3K/Akt and MAPK signalling pathways inducing ER phosphorylation altering the response of ER to Tam (Fig. 3) (Viedma-Rodríguez *et al.,* 2014). These mechanisms lead to acquired Tam resistance in ER+ BC cells over time. Several key pathways and regulators involved in Tam resistance have been highlighted below.

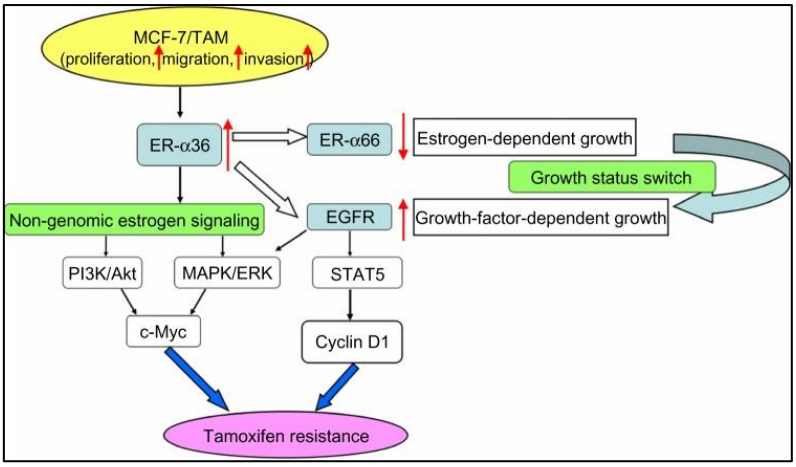


Figure 3: Mechanisms associated with Tam resistance

Several non-genomic and genomic signalling pathways are involved in the development of Tam resistance in ER+ BC cells. These pathways are involved in many cellular processes such as cell survival, proliferation, migration, invasion and stress response.

Figure adopted from: [(Su et al., 2014). Accessed: 13.11.18]

The PI3K/Akt pathway is a key signalling pathway linked to several cellular functions and plays a role in promoting Tam resistance (Fig. 3). PI3K is activated in BC cells by growth factor receptor tyrosine kinases or G-protein-coupled receptors (Viedma-Rodríguez *et al.*, 2014). The activation of Akt through PI3K-mediated processes is linked to ER phosphorylation and ligand-independent ER transcriptional activity (Viedma-Rodríguez *et al.*, 2014, Riggins *et al.*, 2007). Therefore, signalling events triggered by PI3K promote cell growth and survival in BC cells.

Other important growth factors associated with Tam resistance include EGFR, HER2 and IGF1-R which have been found to stimulate cell growth. It has been found that phosphorylated IGF1-R which interacts with EGFR and membrane bound ER is up-regulated in Tam-resistant tumours (Viedma-Rodríguez *et al.*, 2014). Overexpression of EGFR and HER2 in ER+ BC cells leads to increased downstream PI3K/Akt activity (Fig. 3) (Riggins *et al.*, 2007). Therefore, EGFR could mediate Tam resistance in ER+ BC despite suppression of the genomic function of ER by Tam.

The IGF1-R signalling pathway is important in several functions such as cell proliferation, programmed cell death and controlling ER expression. Genomic and non-genomic actions of ER can activate mitogen-inducing signals of the IGF1-R pathway (Viedma-Rodríguez *et al.*, 2014). BC cells may evade Tam-induced apoptosis via IGF-mediated activation of Akt as well

as the phosphorylation and activation of ER (Viedma-Rodríguez *et al.,* 2014). *In vitro* studies have found that the inhibition of IGF1-R reduced Tam-resistant BC cell growth (Viedma-Rodríguez *et al.,* 2014) and could be a possible therapeutic approach in reducing acquired Tam resistance.

Cyclins such as cyclin D1 are major cell cycle regulators involved in promoting cell progression through G1 to S phase (Viedma-Rodríguez *et al.,* 2014). ER signalling regulates cyclin D1 expression and is a direct target of estrogen signalling. Chemotherapeutic agents such as Tam therefore reduce cyclin D1 expression and inhibits cell progression from G1 to S phase (Viedma-Rodríguez *et al.,* 2014). Overexpression of cyclin D1 is sustained in Tam-resistant BC cells, resulting in a conformational change of ER that induces receptor activation in the presence of Tam which promotes BC cell growth (Fig. 3) (Viedma-Rodríguez *et al.,* 2014).

Recent studies have shown the role of miRNAs conferring drug resistance in cancer cells. In BC, the role of miRNAs in Tam resistance through cell cycle regulatory proteins has been suggested. In particular miR-101, miR-206, miR-221 and miR-222 were found to render ER+ BC cells resistant to Tam (Viedma-Rodríguez *et al.,* 2014). In Tam-resistant BC cells, miR-221/222 play roles in developing resistance to treatment by targeting cell cycle inhibitors such as p27/kip1. When these miRNAs down-regulate p27/kip1, it allows for cell progression and facilitates cell growth even when ERs are blocked by Tam (Viedma-Rodríguez *et al.,* 2014). miRNA-mediated targeting of ER co-factors has also been linked to reduced chemotherapeutic response (Viedma-Rodríguez *et al.,* 2014). Therefore, targeting miRNAs is a novel approach in reducing drug resistance in BC cells.

Resistance to endocrine therapies arise through several distinct mechanisms. For this reason, many strategies for overcoming this resistance have been in the spotlight. One such strategy is the use of combination treatments which targets multiple pathways at once thereby delaying the onset of acquired resistance in tumours. Combination treatments can also be used to re-sensitise tumours to chemotherapeutic agents for which they are resistant (Riggins *et al.,* 2007). Treatments such as inhibiting the above growth factor receptors such as EGFR, IGF1-R, PI3K or MAPK in combination with endocrine therapy can block pro-proliferative activities of the ER and survival pathways activated by these growth factor receptors (Riggins *et al.,* 2007).

Treating ER+ BC in post-menopausal women is achieved by estrogen deprivation or blocking estrogen-regulated pathways. This is accomplished through ovarian ablation, Tam treatment to

block ER action, and direct inhibition of estrogen production by the use of AIs (Wong and Chen, 2012). While the use of anti-estrogens and AIs is effective at first, acquired resistance towards these treatments occurs at later times. Therefore, it is also necessary to develop a novel therapeutic approach in reducing cancer-related resistance in ER+ BC cells that have been deprived of estrogen for long periods of time (LTED).

1.6 LTED BC cells

LTED cells are ER+ BC cells that have undergone long-term estrogen deprivation (i.e. >180 days) and are therefore termed resistant LTED cells. The purpose of studying LTED cells in this current investigation was for two reasons. The first was to mimic the mechanisms at play in post-menopausal women who have low levels of estrogen circulating the body, but still have the ER present. The second was to mimic the surgical removal of ovaries for reduced estrogen production once patients were diagnosed with ER+ BC. Characterising resistant LTED cells sought to identify pathways involved in the mechanisms of Tam resistance of this cell line.

Initial ER+ BC treatment includes SERMs and reduction of estrogen secretion by surgical removal of the ovaries (oophorectomy) (Jeng *et al.,* 1998). Patients initially respond well to these treatments which last approximately 12-18 months (Yue *et al.,* 2003). Among patients with relapsing disease, 50% experience further tumour regression through secondary therapies via further inhibition of circulating estrogen through the use of AIs (Yue *et al.,* 2003). This suggests that BC cells that survive estrogen deprivation can adapt and re-grow whilst being resistant to treatment. After LTED, ER+ BC cells become more sensitive to the mitogenic effects of estrogen (Masamura *et al.,* 1995, Yue *et al.,* 2003). Enhanced sensitivity to estrogen may explain re-growth and resistance of BC cells in a low estrogen environment (Yue *et al.,* 2003). Understanding the mechanisms involved in LTED adaptation and resistance will help improve current endocrine treatments for hormone-dependent ER+ BC as well as the development of new chemotherapeutic drugs.

In vitro studies have revealed that BC cells that have been deprived of estrogen for long periods pass through three phases. These phases include: a quiescent phase where cell proliferation is decreased and ER expression is low, a hypersensitive phase where cell growth is stimulated by the addition of exogenous estrogen, and an independent phase in which exogenous estrogen has no effect on cell growth and there is a 2-4 fold increase in ER binding sites (Martin *et al.,* 2003). The exact mechanisms underlying these adaptive phases are not well understood; however, increased secretion of growth factors plays a major role (Jeng *et al.,* 1998). Thus anti-

estrogens such as Tam block growth factor actions as a mechanism for inhibiting cellular proliferation. The beneficial actions of existing endocrine treatments are attenuated by the ability of BC cells to circumvent the need for estrogen while still retaining the nuclear ER (Martin *et al.*, 2003). Therefore, the identification of the factors and pathways responsible for the development of these resistance conditions is crucial for the development of new therapeutic strategies. The development of LTED adaptation and resistance mechanisms are highlighted below.

Previous *in vitro* investigations by Shim *et al* (2000) suggested that LTED cells adapt to allow maximum cell growth in response to minute amounts of residual estrogen. When residual estradiol (E_2) is completely removed or antagonised by anti-estrogens, LTED cells can then be stimulated to grow with exogenous E_2 (Santen *et al.*, 2003, Shim *et al.*, 2000). Under these conditions, cells respond to much lower concentrations of E_2 than wild-type (WT) MCF7 cells, which demonstrates hypersensitivity (Shim *et al.*, 2000). In pre-menopausal women with ER+ BC, tumours initially require a range of 50-600 pg/mL of E_2 for growth. However, the removal of ovarian estrogens leads to the adaptation of low E_2 levels and tumours then only require 10-20 pg/mL of E_2 for growth. A further reduction of E_2 levels using AIs to 1-2 pg/mL causes additional tumour regression (Shim *et al.*, 2000). This is evidence that adaptive hypersensitivity is observed in LTED cells, which induces a shift to the left in the E_2 dose-response curve (Shim *et al.*, 2000). Although there is evidence for hypersensitivity, the precise mechanism of adaptation and hypersensitivity to E_2 in LTED cells is unknown and therefore several investigations have taken place in order to elucidate potential adaptive mechanisms.

ER+ BC cells have numerous receptors for hormones and growth factors. These cells produce many growth factors which are modulated by E_2 and anti-estrogens like Tam. It was therefore predicted that LTED of ER+ BC cells causes an increased secretion of growth factors or increased growth factor receptor production (Katzenellenbogen *et al.*, 1987, Martin *et al.*, 2003, Masamura *et al.*, 1995, Shim *et al.*, 2000) and crosstalk between growth factor and steroid hormone-stimulated signalling pathways (Jeng *et al.*, 1998). This suggests that the high proliferation rate of LTED cells cannot be further increased by exogenous E_2. It also suggests that Tam or other anti-estrogens inhibiting cell growth functions via the decrease of growth factor production.

When a BC cell is responsive to endocrine therapy, estrogen and ER-mediated signalling pathways are responsible for cell growth. However, during LTED, the estrogen-induced ER

pathway is no longer viable, resulting in ER activation through alternative mechanisms (Wong and Chen, 2012). These alternative mechanisms are activated through increased expression, secretion and activation of growth factors such as HER2, Akt and MAPK which can phosphorylate ER. The phosphorylation of ER by MAPK or Akt results in increased cancer cell growth (Wong and Chen, 2012). *In vitro* studies have stipulated that the interaction between ER-mediated functions and the activation or up-regulation of MAPK and its related pathways are responsible for the adaptive mechanisms observed in LTED cells as seen in Fig. 4 below (Jeng *et al.,* 1998, Santen *et al.,* 2003, Shim *et al.,* 2000, Yue *et al.,* 2003, Zheng *et al.,* 2007).

A possible mechanism explaining MAPK activation in LTED BC cells could be through non-genomic effects of estrogen acting through membrane-bound ER which could make use of growth factor pathways (Shim *et al.,* 2000). Growth factor binding to their receptors initiate a cascade of events that includes receptor phosphorylation and MAPK activation (Shim *et al.,* 2000). Therefore, it is possible that an increase in growth factor receptor levels could activate MAPK in LTED cells. Findings revealed that growth factor inhibitors blocked LTED cell proliferation. Thus the activation of growth factor pathways is involved in MAPK activation in LTED cells (Shim *et al.,* 2000, Yue *et al.,* 2003). Genomic and non-genomic effects are responsible for LTED cell growth; however, activation of the non-genomic pathway (membrane-related ER-mediated process) is more heavily involved in inducing hypersensitivity in LTED cells (Santen *et al.,* 2003).

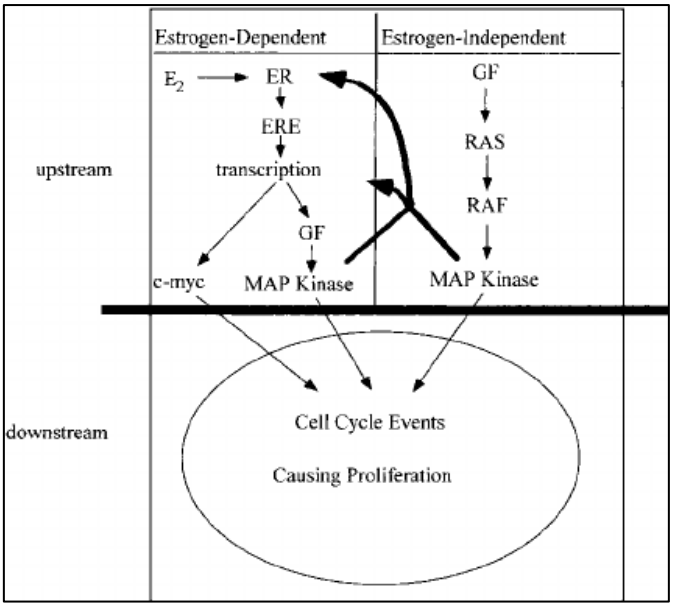


Figure 4: The mechanisms involved for E_2 hypersensitivity and adaptation in LTED cells

LTED cells adapt to low levels of estrogen through estrogen-independent pathways, which involve increased growth factor receptor secretion. MAPK is activated which leads to a cascade of signalling events leading to cell proliferation and survival of LTED cells in low levels of estrogen.

Figure adopted from: [(Jeng et al., 2000). Accessed 13.11.18]

Although it has been found that the MAPK pathway is one of the most important mechanisms necessary for E_2 hypersensitivity; it is not the only mechanism involved. *In vitro* studies by Yue *et al* (2003) revealed that blocking MAPK activation reduced E_2 hypersensitivity but did not reverse it in LTED cells. The PI3K/Akt pathway as well as HER2 and IGF1-R were also found to be up-regulated in LTED cells and are important mechanisms in E_2 hypersensitivity (Santen *et al.*, 2003, Wong and Chen, 2012, Yue *et al.*, 2003). Inhibiting both pathways resulted in the reversion of hypersensitivity of LTED cells (Yue *et al.*, 2003). Therefore, several mechanisms are involved in the hypersensitivity to E_2 observed in LTED cells with particular reference to MAPK activation and targeting this pathway could be a potential therapeutic strategy in the treatment of LTED BC cells.

LTED cells are usually unaffected by Tam treatment which indicates that they are resistant to Tam. Cells begin as estrogen-dependent cells which are responsive to Tam but during prolonged treatment, cells become less dependent on estrogen and develop alternative pathways to activate ER (Wong and Chen, 2012). In these resistant cells, estrogen-mediated ER signalling is blocked; however, Tam functions as a weak estrogen, driving cell growth. This is the first stage of resistance. Over time, cell growth becomes more estrogen-independent and

relies on several growth factor signalling pathways. Finally, once the cells have achieved total estrogen-independence, they no longer respond to any Tam treatment (Wong and Chen, 2012). Previous *in vitro* studies that made use of inhibitors against growth factor receptors observed reduced activation of these proteins that led to reduced cell growth (Wong and Chen, 2012). In addition, these findings showed that a combination treatment of inhibitors and Tam can lead to LTED cells regaining sensitivity to Tam which shows these cells are capable of overcoming acquired resistance (Wong and Chen, 2012).

In addition to enhanced growth factor receptor signalling pathways, resistant ER+ cells with LTED have a significant increase in genes encoding enzymes within the cholesterol biosynthesis pathway (Simigdala *et al.,* 2016). These enzymes may be associated with acquired resistance to endocrine therapy (Simigdala *et al.,* 2016). Therefore the up-regulation of the cholesterol biosynthesis pathway has been identified to play a role as a resistance mechanism in ER+ LTED BC cells (Simigdala *et al.,* 2016) and requires further investigation.

1.7 Cholesterol accumulation linked to BC incidences

Cholesterol is an important molecule in the human body as it is a main component of lipid rafts, which mediates cell membrane proteins, receptor transportation, and signal transduction (Silvius, 2003). Cholesterol and its metabolites act as hormone precursors for the production of various steroid hormones, including estrogen which promotes ER+ BC progression. Cholesterol is linked to cancer progression mainly by increased cholesterol synthesis in cancer cells and cholesterol accumulation in lipid rafts (Clendening and Penn, 2012; Li *et al.,* 2006; Nelson *et al.,* 2014). Interestingly, BC cells are found to be approximately 2-3 times more rich in cholesterol than normal cells (Hu *et al.,* 2010). Considering the enhanced cholesterol accumulation in BC cells, the synthesis of steroid hormones has also been shown to be notably increased in these cells. Therefore, cholesterol dyshomeostasis in BC cells not only causes enhanced intracellular cholesterol accumulation, but can also be indirectly linked to apoptosis evasion and uncontrolled proliferation in BC cells (Silvente-Poirot and Poirot, 2014).

Cancer cells often increase the rate of cholesterol biosynthesis in order to have sufficient cholesterol for proliferation. Oncogenes dysregulate cell growth by activating anabolic and biosynthetic pathways leading to *de novo* cholesterol synthesis in order to produce energy (Antalis and Buhman, 2012). This is done by an increased influx of glucose into proliferating cells and can also be done through the glycolytic pathway. Decreasing plasma cholesterol levels disrupts the formation of lipid rafts thereby inhibiting BC development.

1.8 Cellular cholesterol homeostasis

Cholesterol biosynthesis is a natural process that occurs in all cells and occurs through the mevalonate pathway (Silvente-Poirot and Poirot, 2014). Acetyl-CoA, which is produced during glycolysis, acts as a precursor molecule for cholesterol synthesis. In the first step of the mevalonate pathway, two acetyl-CoA molecules are condensed to yield acetoacetyl-CoA (Martín *et al.,* 2014). Following this, a second condensation step occurs, forming hydroxy-methyl glutaryl-coenzyme A (HMG-CoA). The reduction of HMG-CoA then produces mevalonate, which is finally converted to cholesterol (Martín *et al.,* 2014).

Cholesterol is a lipid molecule that is encapsulated in a water-soluble protein coat, called a lipoprotein (Didichenko *et al.,* 2016). Lipoproteins are complex amphiphilic molecules that act as carriers for the internal transportation of cholesteryl esters (CEs) and the transport of free cholesterol at the surface of these molecules (Tosi and Tugnoli, 2005). There are four principal classes of lipoproteins. These include: chylomicrons, high-density lipoproteins (HDLs), low-density lipoproteins (LDLs) and very low-density lipoproteins (VLDLs) (Cruz *et al.,* 2013). Intracellularly, cholesterol is taken up by the cell via binding of LDL to the LDL receptor (LDLR). LDLs transport cholesterol from the liver, which is the site of synthesis to peripheral tissues (Cruz *et al.,* 2013). Endosomes are then formed due to internalisation of LDL. Lysosomal degradation releases cholesterol into the cytosol of cells, where it is used for various purposes or exported directly out of the cell via HDL and excreted by the liver. HDLs maintain cholesterol homeostasis by eliminating excess cholesterol from cells through the direct transport of cholesterol to the liver or by reverse cholesterol transport (Cruz *et al.*, 2013). In the process of reverse cholesterol transport, HDLs shuttle CEs to the LDLs and then to the liver where it is ultimately converted to bile salts (Tosi and Tugnoli, 2005). CEs are a form of esterified cholesterol, which allow for more efficient transport of free cholesterol by lipoproteins to the liver and thus aids in the maintenance of cholesterol homeostasis (Tosi and Tugnoli, 2005).

Cholesterol accumulation in BC cells occurs via hyper-activation of the PI3K/Akt/mTOR pathway which activates the sterol regulatory element-binding protein (SREBP). This esterifies and compartmentalises cholesterol into lipid droplets allowing for fatty acid uptake leading to increased proliferation of cancer cells (Peck and Schulze, 2014). There is increased PI3K expression in resistant ER+ LTED cells which shows cholesterol accumulation in BC cells (Simigdala *et al.,* 2016). In addition, several other proteins, enzymes and gene regulatory elements help maintain cholesterol homeostasis. One such protein is the cholesteryl ester

transfer protein (CETP); a plasma glycoprotein that promotes the exchange of neutral lipids, triglycerides and CEs between distinct lipoprotein classes (Chirasani *et al.,* 2016). CETP has a binary function, acting intra- and extracellularly to maintain cholesterol homeostasis. Therefore, targeting these proteins or using cholesterol depleting compounds can be an alternative therapeutic approach in the treatment of BC. Studying the molecular mechanisms involved in cholesterol accumulation and how it affects resistance in BC cells is also important.

1.9 Targeting cholesterol for the treatment of BC

Currently, there are two approaches targeting cholesterol as a possible treatment therapy for BC. The first treatment includes the blocking of cholesterol synthesis using drugs such as statins and the second treatment involves the depletion of excess membrane cholesterol using cholesterol depleting agents.

Statins, also known as HMG-CoA reductase inhibitors are the most widely used lipid-lowering drugs on the market. Statins are cholesterol synthesis inhibitory drugs and there has been extensive research on the use of these drugs. The primary function of statins is to inhibit HMG-CoA reductase which plays a major role in cholesterol production (Mostaghel *et al.,* 2012). This inhibition impedes the mevalonate pathway as the conversion of Acetyl-CoA to HMG-CoA does not occur. Consequently, cholesterol levels decrease thus being an effective treatment in atherosclerosis and coronary heart disease (Stancu and Sima, 2001). There have been numerous debates regarding the use of statins in cancer treatments. Several studies have concluded that statins possess anti-cancer properties (Campbell *et al.,* 2006, Chan *et al.,* 2003) whereas other studies have made contradictory conclusions suggesting that statins actually promote cancer cell growth (Boudreau *et al.,* 2010, Duncan *et al.,* 2005). Therefore, it is important to further expand on the knowledge of statin use as a therapeutic approach on the treatment of cancer and BC.

The administration of cholesterol depleting agents such as, Methyl-β-cyclodextrin (MβCD) and Acetyl Plumbagin (AP) has been shown to be effective in promoting cell death in BC cells (Esau *et al.,* 2016, Sagar *et al.,* 2014,). These agents deplete excess cholesterol or deplete cholesterol in the lipid rafts thereby disrupting cell membranes and have been extensively researched in recent years as an alternative method in the treatment of BC and cholesterol-related diseases. The depletion of excess cholesterol within BC cell membranes induces apoptosis and induces chemo-sensitivity to current chemotherapeutic drugs, such as Tam (Esau *et al.,* 2016, Mandal and Rahman, 2014, Mohammad *et al.,* 2014).

The cholesterol depleting agent, AP has recently emerged to have a promising effect on BC cells. AP is a derivative of Plumbagin (PL) (5-hydroxy-2-methyl-1,4-naphthoquinone), which is a naturally occurring yellow pigment found in the plants of the Plumaginaceae, Ancestrocladaceae and Dioncophyllaceae families (Manu *et al.,* 2011). It is a naphthoquinone derivative from the roots of the plant *Plumbago zeylanica* (Jamal *et al.,* 2014). PL has many pharmacological properties and has shown anti-proliferative and pro-apoptotic activities in different cancer cells (Manu *et al.,* 2011). It is especially well known for its anti-cancer activities as it has many inhibitory effects on various cancer signalling pathways; however, the exact molecular interactions of these pathways have not yet been elucidated (Jamal *et al.,* 2014).

PL has been reported as a 'lead' molecule in developing novel anti-cancer agents (Sagar *et al.,* 2014). However, novel derivatives of PL to enhance its activity and reduce toxicity have been developed in order to maximise therapeutic potential (Sagar *et al.,* 2014). The addition of an acetyl group at the 5-C position, which produces AP (Fig. 5) has been shown to reduce the toxicity of PL towards normal cells and induces apoptosis in ER+ BC cell lines (Sagar *et al.,* 2014).

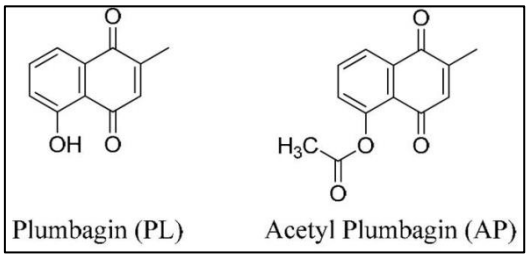


Figure 5: The molecular structures of PL and AP

The addition of an acetyl group at the 5-C position of PL significantly increases cytotoxicity in cancer cells while reducing toxicity in normal cells (Sagar et al., 2014).

Figure adapted from: [(Sagar et al., 2014) Accessed: 20.10.18]

AP has the potential to reduce toxicity and increase efficacy of known chemotherapeutic drugs such as Tam. It may enhance the usage of these drugs at lower concentrations with increased cancer killing effects. Preliminary data has shown that when AP is used in combination with chemotherapeutic drugs such as 5-Fluorouracil (5-FU), Paclitaxel and Tam, cell viability of normal cells increases while at the same time significantly reducing the viability of cancer cells as seen in Fig. 6 (previous unpublished work of Professor Kaur). Another important observation was found that AP is a cholesterol depletor and could be acting by altering the cholesterol-related mechanisms (Esau *et al.,* 2016). Although the exact mechanisms of AP have

not yet been well studied, the depletion of cholesterol using AP resulted in a significant increase in mitochondrial-mediated apoptosis (Esau *et al.*, 2016), which indicates that AP could act as a novel lead molecule for further drug development targeting cholesterol accumulation in ER+ BC. It also has the potential to overcome the resistance associated with Tam.

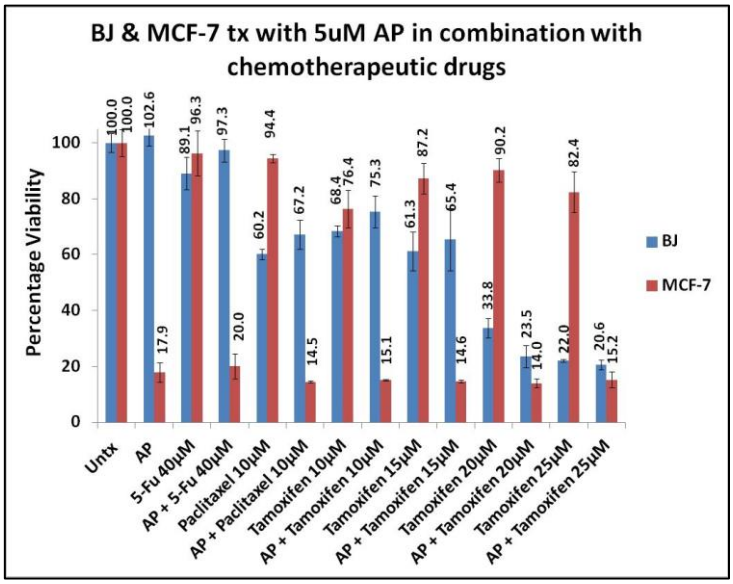


Figure 6: The effect of AP on viability of normal and BC cells

When AP is combined with a range of concentrations of various chemotherapeutic drugs, such as Tam, 5-FU and Paclitaxel, the viability of normal cells is unaffected whereas the viability of BC cells is decreased.
Figure adopted from: [Previous unpublished work of Professor Kaur. Accessed: 20.10.18]

It can be postulated that cholesterol depletion in the membranes of BC cells disrupts lipid raft integrity, which increases membrane permeability for chemotherapeutic drug passage (Mohammad *et al.*, 2014). Therefore, the use of a cholesterol depleting agent (AP) in combination with a chemotherapeutic drug (Tam) can be an effective treatment in combatting cholesterol-rich resistant ER+ BC. This therapeutic combination strategy also has the potential to increase the efficacy of chemotherapeutic drugs and reduce cancer-related drug resistance. However, this is a novel concept which requires the basic understanding of the role of AP in combination with Tam and the molecular mechanisms involved. In this study, we have attempted to study the effects of AP and Tam combination treatments on cell growth, apoptosis and cholesterol depletion in different BC cell lines.

1.10 Introduction to the current study

This study observed the changes in pathways affected in different BC cell lines when AP was added alone and when AP was added in combination with Tam. This allowed for the

observation of enhanced efficacy of Tam in different BC cell lines which can help in overcoming resistance. Observations were also made on the cholesterol accumulation pathways and which proteins were up- or down-regulated in BC cells once treated.

The cell lines that were used in this study included ER+ MCF7 BC cells and ER+ LTED BC cells derived from WT MCF7 cells. The purpose of using LTED cells in this study were to compare effects of treatments on cells that did not require estrogen for growth to those cells that required estrogen. This allowed for the observation of how AP acts on the ER in the absence of estrogen and how AP affects downstream pathways with ER activation.

Several proteins involved in cholesterol accumulation were observed in order to identify molecular mechanisms related to resistance in BC cells. These proteins are mainly involved in cholesterol transport, efflux, absorption, and homeostasis and also participate in multi-drug resistance. By understanding which pathways are involved in mediating resistance and cholesterol depletion, AP can become the future anti-cancer lead molecule for possible treatment of BC. It is therefore predicted that AP plays a role in altering the expression of these proteins in BC cells hence decreasing cholesterol accumulation. It is also predicted that AP reduces toxicity and enhances efficacy of Tam in various BC cell lines.

2. AIM AND OBJECTIVES OF THE STUDY

2.1 Aim

To test AP as an adjuvant compound in MCF7 and LTED cell lines to reduce cholesterol accumulation and cancer-related resistance.

2.2 Objectives

To fulfil the aim of the study, the following objectives were set:

- To develop an LTED cell line.
- To treat MCF7 and LTED cells with different concentrations of Tam in combination with AP.
- To observe pathway changes in MCF7 and LTED cells involved in cholesterol depletion and cancer-related resistance.
- To assess the potential of AP as an adjuvant compound for endocrine therapy with increased efficacy.

3. MATERIALS AND METHODS

The following methods were performed on both MCF7 and LTED cell lines. Each experiment was performed repeatedly at least two to three times for accurate and reproducible results. The compounds that were tested included: Tam (Sigma Aldrich, UK) and AP (synthesised at the University of the Witwatersrand in the School of Chemistry according to the methods of Sagar *et al* (2014) (1µM, 5 µM, 10 µM, 20 µM) and control compound: PL (Sigma Aldrich, UK) (40 µM).

Stock concentrations (30 mM in dimethyl sulfoxide (DMSO)) of the above compounds were initially prepared. Several dilutions (1 mM and 500 µM) were further prepared in dH_2O for appropriate selected concentrations. A range of concentrations of Tam, AP, and PL were tested prior to the selection of chosen concentrations (data not shown). The concentration of each compound was carefully selected due to the efficacy of these compounds at inducing apoptosis at selected concentrations in BC cell lines as well as having little cytotoxic effects in normal cells (Sagar *et al.*, 2014).

3.1 Cell Culture

The MCF7 cell line was procured from the European Collection of Authenticated Cell Cultures (ECACC). All cells were cultured in phenol red or phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, UK and Gibco: Life Technologies, UK) with varying concentrations of foetal bovine serum (FBS) or dextran charcoal stripped FBS (DC FBS) (Celtic Molecular Diagnostics, SA; Biowest, UK), and 1% penicillin-streptomycin (P-S) (Sigma Aldrich, UK) according to Table 1 below, incubated at 37 °C and 5% carbon dioxide (CO_2) to mimic *in vivo* conditions.

Table 1: Description of cell lines used for the current study

	MCF7	**LTED**
Full Name	Michigan Cancer Foundation-7	Long-term estrogen deprived MCF7
ER expression	ER+ (constantly)	Decreased ER expression within first 90 days Increased ER after first 90 days
E_2 effects	Stimulates growth	Hampers growth within first 90 days

Morphology	Epithelial, polygonal	Elongated, fibroblast-like
Disease	Adenocarcinoma, breast mammary gland	Adenocarcinoma, breast mammary gland
Growth mode	Adherent	Adherent
Media	DMEM, 10% FBS, 1% P-S	Phenol red-free DMEM, 5% DC-FBS, 1% P-S

The LTED cell line was created by culturing cells in specific hormonal-free conditions for a minimum of 180 days in order to deprive the cells of estrogen (Santen *et al.*, 2005). This was accomplished because DC FBS selectively removes hormones without non-specific loss of other serum components (Sigma-Aldrich, UK).

3.1.1 Development of the LTED cell line

LTED cells were derived from existing MCF7 cells in the laboratory. A timeline of LTED cell line development is depicted in Fig. 7 below representing the dates and phases of development. LTED cells required growth media free of hormones thus a change of media conditions was implemented from 10% FBS in phenol red 1 X DMEM to 5% DC FBS in phenol red-free 1 X DMEM without sodium pyruvate (LTED media). Two concentrations of FBS were initially used (5 and 10%) however cells adapted better to a lower serum percentage. First, MCF7 cells were washed 3 x with 1 X PBS in order to get rid of any residual phenol red media in the culture flask. Cells were trypsinised and placed in a fresh culture flask with fresh LTED media. This was unsuccessful as cells were under too much stress and could not adapt to estrogen-free media. Following this, cells were washed 3 x with 1 X PBS and trypsinised. Cells were then placed in a new culture flask with a combination media of 3:1 (3 parts phenol red media: 1 part LTED media). Cells were left to grow for 3 days to acclimatise. Following this, cells were washed with 1 X PBS and media was changed to a 1:1 combination for 3 days. Media was then changed to a 1:3 combination. Eventually cells were grown in LTED phenol red-free media after 30 days with a slow proliferation rate. After 60 days, cell proliferation started to increase and within 90 days, doubling time was equal to that of MCF7 cells. Cells have been growing in these conditions for 12 months and appear to be robust.

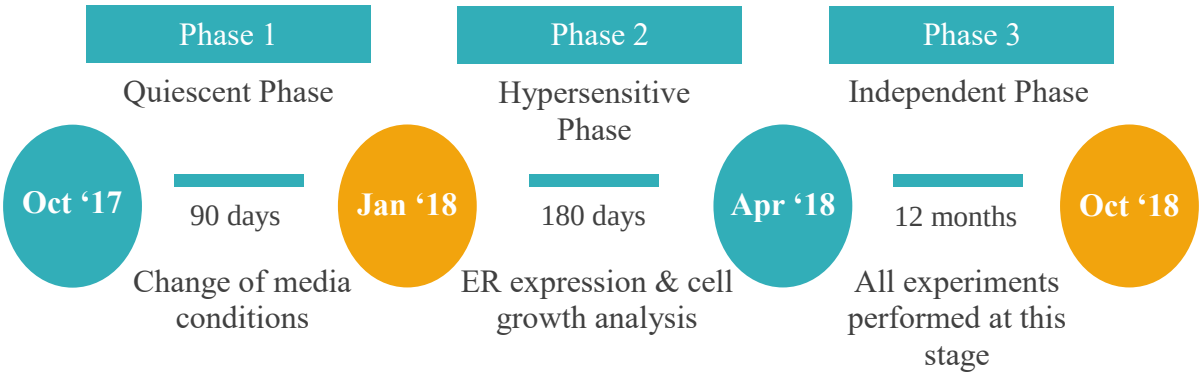


Figure 7: A representative timeline of LTED cell line development

3.2 Cell counting

A Neubauer haemocytometer was used to count cells in order to seed accurate numbers of cells in each well. A haemocytometer is a counting chamber that uses a grid method on a glass slide to predict an estimated total number of cells in the culture flasks. Prior to cell counting, media was removed and 2 mL of 1 X trypsin/EDTA (ethylenediaminetetraacetic acid) (Sigma Aldrich, UK; Celtic Molecular Diagnostics, SA) was added into the culture flask and incubated at 37 °C for the activation of trypsin to allow cells to be detached. Once cells were detached, 100 µL of media containing cells was added to equal parts of 0.4% trypan blue (Sigma Aldrich, UK) and mixed thoroughly. Trypan blue is the most commonly used dye that stains dead cells blue due to membrane disruption, leaving live cells unstained due to the intact cell membrane which prevents trypan blue from entering the cells. Thereafter, 10 µL of the mixture was added onto the haemocytometer and cells were counted under a Nikon Phase Contrast Microscope.

Cellular viability was calculated as follows:

$$\%\, Cell\ Viability\ = \frac{no.\,of\ live\ cells\ (unstained)}{Total\ no.\,of\ cells\ counted\ (dead\ and\ live)} \times 100$$

Cellular viability measures the number of viable cells in the culture flask that can be used in further experiments and cell viability above 95% was used for further experiments.

Cells were then seeded at a desired density as follows:

$$45\ \mu L\ \times total\ no.\,of\ wells\ = total\ volume\ of\ media\ required\ (\boldsymbol{a})$$

$$5000\ cells/well \times total\ no.\,of\ wells\ = total\ no.\,of\ cells\ required$$

$$\frac{Total\ volume\ in\ flask\ (\mu L) \times total\ no.of\ cells\ required}{Total\ no.of\ cells\ counted\ in\ flask} = volume\ of\ cells\ required\ from\ flask\ (\boldsymbol{b})$$

$$\boldsymbol{a} - \boldsymbol{b} = volume\ of\ media\ to\ be\ added\ to\ cells$$

45 µL was then pipetted into each well with approximately 5000 cells per well.

3.3 Growth curve using cell counts

The growth of MCF7 and LTED cells was observed with varying concentrations of E_2 (Sigma-Aldrich, USA) over time. This was performed in order to compare the response of MCF7 and LTED cells to different concentrations of E_2. A 6-well plate was seeded with 10 000 cells per well and incubated for 24 hours (hrs) at 37 °C in the presence of 1 nM and 10 nM E_2 respectively as previously described by Shim *et al* (2000) and Katzenellenbogen *et al* (1987). Cells were detached and counted daily as mentioned above for 6 days using a Neubauer haemocytometer. Relevant graphs were produced in GraphPad Prism5® indicating the cell growth in numbers of MCF7 and LTED cells over time with and without the addition of E_2.

Concentrations of 1 nM and 10 nM E_2 were selected as reports have shown that LTED cells have an increased stimulation to growth in the presence of minute amounts of exogenous E_2 (<10 nM) (Santen *et al.,* 2003, Shim *et al.,* 2000). Therefore, these concentrations were selected in order to observe the changes in LTED cell growth in comparison to MCF7 cells.

3.4 Growth inhibition assay (primary screen)

Cell viability was estimated by using a 3-(4,5-Dimethylthiazol-z-yl)-2,5diphenyltetrazolium bromide (MTT) (Sigma Aldrich, UK) assay. A range of concentrations of Tam and AP was tested and the appropriate concentrations for downstream assays were selected based on the results of the MTT assay.

3.4.1 MTT assay

The MTT assay is a colorimetric assay that measures the reduction of tetrazolium salts by metabolically active cells and results in the formation of formazan crystals. This assay was performed in a similar manner to that of Sagar *et al* (2014), where the effect of each compound with varying concentrations of Tam and AP as well as combination treatments of Tam with AP on cell viability for 24 hrs was measured.

Cells were seeded into a 96-well plate at a density of 5000 cells/well and incubated at 37 °C for 24 hrs. After incubation, 5 µL of Tam, AP, and a combination of both Tam and AP at concentrations of 1, 5, 10 and 20 µM was added to the cells for 24 hrs. Following incubation, 5 µL of sterile 5 mg/mL MTT (Sigma-Aldrich, USA) dissolved in 1 mL 1 X phosphate buffered saline (PBS) was added to each well and the cells were incubated for 2 hrs after which the formazan crystals were subsequently dissolved in solubilising solution (10% sodium dodecyl sulfate (SDS) (Sigma Aldrich, UK) with 10 mM hydrochloric acid (HCl)) for at least 16 hrs. The optical density (OD) of each well was subsequently measured at 570 nm using a microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ software). The percentage viability was determined and relevant graphs produced accordingly in GraphPad Prism5®.

3.5 Apoptosis assays (secondary screen)

3.5.1 APOPercentage™ assay

To further elucidate if cancer cells were dying via apoptosis post compound treatment, a dye inclusion calorimetric assay called APOPercentage™ (Biocolor, UK) was used. APOPercentage™ is a dye-uptake assay that detects the early stages of apoptosis in adherent cells. The APOPercentage™ dye enters cells that are undergoing apoptosis during which the scramblase enzyme inactivates flippases causing a membrane flip as seen in Fig. 8 below (Biocolor, UK). Under normal circumstances flippases maintain the membrane structure.

Relevant cells (5000 cells/well) were seeded into a 96-well plate and incubated at 37 °C for 24 hrs. Thereafter, cells were treated with appropriate concentrations of compounds for 2 hrs as the assay detects the beginning stages of apoptosis within this time period. Compound treatment for more than 2 hrs may lead to detachment of cells and thus risk being washed away resulting in inaccurate readings. Media was removed from each well and 65 µL of dye mix (0.5 µL of APOPercentage™ dye and 64.5 µL 1 X PBS) was added to each well and incubated at 37 °C for 20-30 minutes (min) as previously performed by Esau *et al* (2016). Subsequently, each well was washed gently with 100 µL of 1 X PBS. Lastly, 100 µL of 1 X PBS was added to prevent the cells from drying out. The OD of each well was measured at 550 nm using a microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ software). The percentage of cells undergoing apoptosis was calculated according to the formula below and relevant graphs produced accordingly in GraphPad Prism5®.

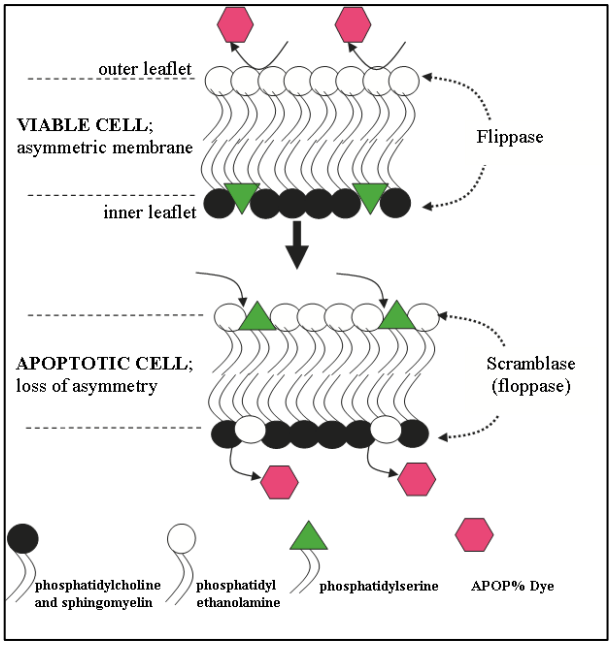


Figure 8: The mode of action of a membrane flip seen in apoptotic cells

Apoptotic cells undergoing a membrane flip due to the scramblase enzyme inhibiting the action of flippase, thereby causing a membrane flip. Phosphatidylserine transmembrane movement results in the uptake of the dye. Figure adopted from: [www.biocolor.co.uk Accessed 20.10.18]

$$Average\ of\ absorbance\ values\ of\ test\ samples - Average\ of\ absorbance\ values\ of\ blanks\ (\boldsymbol{a})$$

$$\%\ Cell\ death\ = \frac{[(\boldsymbol{a})of\ experimental\ - (\boldsymbol{a})of\ untreated]}{(\boldsymbol{a})of\ untreated} \times 100$$

3.5.2 Mitochondrial Outer Membrane Potential (MOMP) assay

In order to further confirm early-stage apoptosis in cells post compound treatment, a JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide) (Sigma-Aldrich, USA) inclusion assay called MOMP was used. The JC-1 dye is a membrane-permeant dye that is used to monitor mitochondrial health in cells. A feature of early-stage apoptosis involves the disruption of active mitochondria which includes changes in the membrane potential and alterations to the oxidation-reduction potential of the mitochondria (Thermo Fisher Scientific). The JC-1 dye is positively charged which accumulates in the electronegative interior of the mitochondrion which is measured using flow cytometry. Early-stage apoptosis in the cell causes a fluorescence emission shift from green (529 nm) to red (590 nm). This is due to concentration-dependent formation of red fluorescent J-aggregates.

Relevant cells (10 000 cells/well) were seeded into a 96-well plate and incubated at 37 °C for 24 hrs. Thereafter, cells were treated with appropriate concentrations of compounds for 24 hrs as above. The cells were then lifted using 12 µL of 15 mM EDTA (Sigma-Aldrich, USA) at 37 °C for 40 min as previously performed by Kaur and Esau (2015). After incubation, 15 µL of the 2 µM JC-1 dye diluted in serum-free media was added to the cells and incubated at 37 °C for 30 min. The cells were analysed by a BD Accuri™ C6 flow cytometer (BD Biosciences). Analysis using the BD Accuri™ C6 software was performed by plotting FL2-A vs. FL1-A and applying a quadrant gate to determine JC-1 aggregates and monomers. Relevant graphs were produced accordingly in GraphPad Prism5®.

In addition, both untreated and positive controls were included in the above growth inhibition and apoptosis assays. Cells that were treated with test compounds were compared to cells treated with PL (40 µM) as the positive control. PL is a known compound that causes growth inhibition in BC cell lines (Esau *et al.*, 2016). The comparison of test samples to the positive control ensured that cells were undergoing apoptosis and growth inhibition and therefore confirmed the authenticity of the obtained results. Furthermore, the untreated control provided a useful reference for apoptosis that naturally occurs in untreated BC cells. The repetition of each assay multiple times ensured the reproducibility of the obtained results.

3.6 E_2 supplementation

In order to assess whether varying concentrations of E_2 affected growth inhibition or apoptosis in both cell lines, cells were supplemented with E_2 prior to compound treatment and apoptosis measurement.

Cells were pre-treated with either 1 nM or 10 nM E_2 24 hrs prior to seeding. Cells were then seeded into a 96-well plate at a density of 5000 cells/well and incubated at 37 °C for 24 hrs. After incubation, cells were treated with compounds as above and incubated at 37 °C for 24 hrs. The MTT, APOPercentage™ and MOMP assays were then performed as above.

3.7 Cholesterol quantification assay

A cholesterol quantification assay was performed to measure the cholesterol content in mass (µg) in MCF7 and LTED cells with compound treatment, which was used to confirm whether this treatment affected the cholesterol level in the cell.

This method involves the hydrolysis of CEs by cholesterol esterase into cholesterol as shown in Fig. 9 as previously described by Robinet and colleagues (2010). Cholesterol is then oxidised by cholesterol oxidase that results in two products: cholest-4-ene-3-one and hydrogen peroxide

(H_2O_2). H_2O_2 is detected with a highly specific colorimetric probe (Ampliflu™ Red - Sigma Aldrich, USA), where horseradish peroxidase (HRP) catalyses the reaction between H_2O_2 and Ampliflu™ Red that binds in a 1:1 ratio. This reaction results in the development of a pink colour, which is read at 570 nm (Sigma Aldrich, UK).

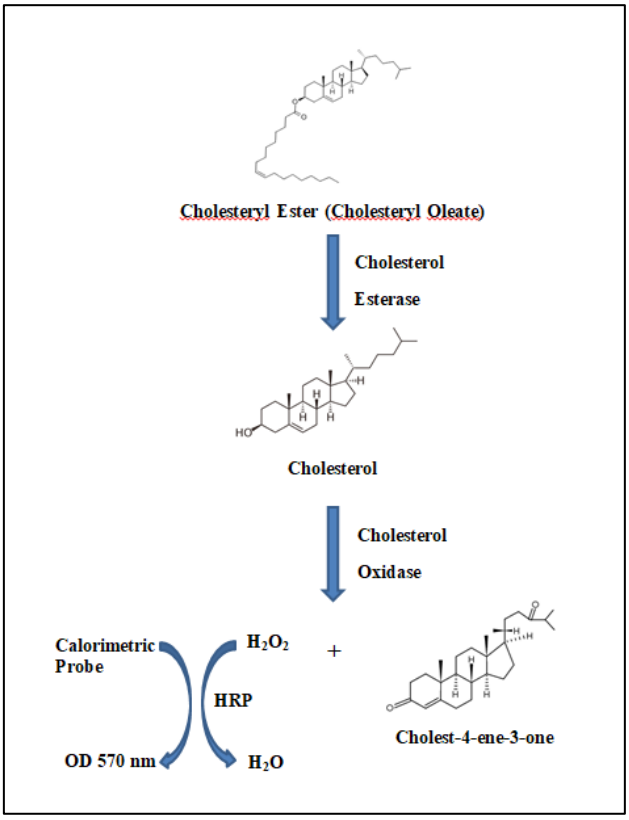


Figure 9: The principle of the cholesterol quantification assay

Cholesterol esters are hydrolysed via cholesterol esterase into cholesterol, which is then oxidised by cholesterol oxidase into the ketone cholest-4-en-3-one plus H_2O_2. H_2O_2 is then detected with a highly specific colorimetric probe, where HRP catalyses the reaction between the probe and H_2O_2, which bind in a 1:1 ratio.
Figure adapted from: [https://www.bioscience.co.uk/userfiles/pdf/STA-384-total-cholesterol-colorimetric-assay-kit.pdf Accessed: 20.10.18]

MCF7 and LTED cells were seeded into a 96-well plate at a density of 5000 cells/well in duplicates in two sets: one testing total cholesterol and the other testing free cholesterol. The seeded cells were incubated at 37 °C for 24 hrs and treated with compounds for 24 hrs. The media was then removed from each well and washed with 100 µL 1 X PBS. Subsequently, 10 µL of 100 U/mL catalase (Sigma Aldrich, UK) was added to each well and incubated at 37 °C for 15 min. Catalase is an enzyme that is known to degrade excess H_2O_2 into water and thus

protects cells from reactive oxygen species (ROS). It also prevents background noise when measuring the absorbance. Reagent A was prepared according to Table 2 below.

Table 2: Components for Reagent A per well

Reagent A	Total Cholesterol (with esterase) (µL/well)	Free Cholesterol (without esterase) (µL/well)
1 M Potassium phosphate buffer (KPO_4) (Sigma Aldrich, UK)	2.5	2.5
1 M Sodium chloride (NaCl) (Sigma Aldrich, UK)	6.25	6.25
100 mM Cholic acid (Sigma Aldrich, UK)	1.25	1.25
100% Triton-X-100 (Sigma Aldrich, UK)	0.25	0.25
20 U/mL cholesterol oxidase (Sigma Aldrich, UK)	2.5	2.5
150 U/mL HRP (Sigma Aldrich, UK)	1.44	1.44
25 U/mL cholesterol esterase (Inqaba Biotec™, SA)	3.33	-
Isopropanol: NP-40 (Nonidet-P40) (9:1) (Merck, SA)	6.48	9.81
10 mM Ampliflu™ Red (Sigma Aldrich, UK)	1	1

Following the 15 min incubation with catalase, 25 µL of Reagent A (with esterase and without esterase, respectively) was added to the respective wells and incubated in the dark at 37 °C for 30 min. The OD of each well was subsequently measured at 570 nm using a microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ software).

Unknown cholesterol concentrations were determined using the standard curve of known cholesterol standards (0, 5, 10, 15, and 20 µg/mL) in Fig. 10 below.

The inclusion of PL as a positive control was necessary for this assay as PL functions in part via cholesterol depletion. MβCD was also included as a positive control (data not shown) as it is a well-studied cholesterol depleting agent (Esau *et al.*, 2016). This ensured and confirmed that cells were undergoing cholesterol depletion when treated with test compounds. An untreated control was used as a reference in order to compare cholesterol depletion in untreated BC cells to treated apoptotic BC cells.

CEs were calculated as follows:

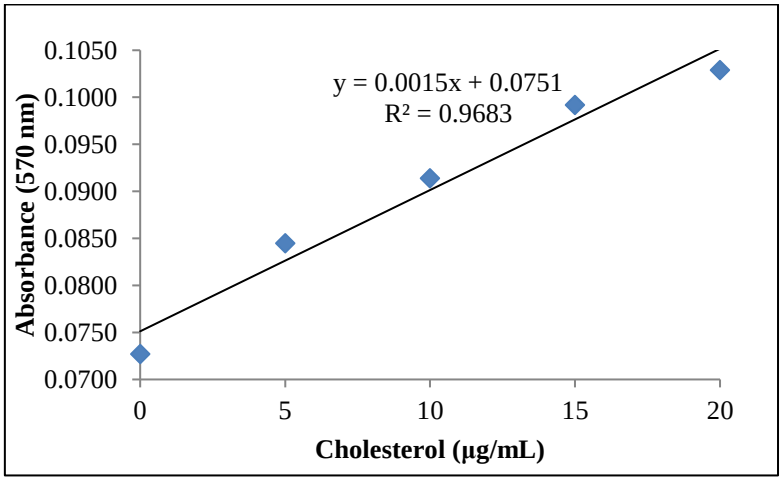


Figure 10: A representative cholesterol standard curve

$$Average\ of\ samples\ -Average\ of\ blanks\ = (\boldsymbol{a})$$

$$Substituted\ y-value\ with\ (\boldsymbol{a})\ in\ y = mx + c\ formula\ in\ standard\ curve\ above$$

$$\therefore\ x-value = cholesterol\ concentration\ (\mu g/mL)$$

$$\mu g/mL\ converted\ to\ \mu g/\mu L$$

$$x-value\ \div 1000\ \times total\ volume\ in\ well = cholesterol\ concentration\ (\mu g/\mu L)$$

$$Total\ cholesterol - Free\ cholesterol = Cholesterol\ esters$$

3.8 SDS-PAGE and Western blot

Western blots were performed in order to observe the alteration of expression of specific proteins in both cell lines once treated with compounds. It was an important step to elucidate whether AP plays a role in decreasing cholesterol accumulation and cancer-related resistance in BC cells.

In order to harvest cells, treated and non-treated MCF7 and LTED cells (10^6 cells/well) were detached from the wells by using 2 mL 1 X tryspin/EDTA. Harvested cells were then pelleted at 4000 x *g* for 5 min. The supernatant was removed and each pellet was re-suspended in 1 X Ripa or lysis buffer (prepared according to Table 3 below) (100 μL/10^6 cells) on ice for 30 min. Lysis buffer disrupts the cell membrane thus releasing the internal proteins and components of the cell. The protein concentrations of each cell sample were measured using a bicinchoninic acid (BCA) assay.

Table 3: Lysis buffer components

	Components for 100 mL
1 X Ripa/Lysis buffer	300 mM NaCl = 0.876 g 1% Triton-X-100 = 1 mL 1% Deoxycholate = 1 g 0.1% SDS (1 mL of 10% SDS) 50 mM Tris = 1.21 g 10 mM EDTA = 1 mL (All reagents from Sigma Aldrich, UK)

3.8.1 Protein quantification: BCA assay

The BCA assay is a technique based on the reduction of copper sulfate Cu^{2+} to Cu^{+} in the presence of protein, which interacts with BCA resulting in the development of a purple colour that is measured spectrophotometrically (Smith *et al.,* 1985). Protein concentrations of unknown samples may then be determined by comparing with a known protein standard (example shown in Fig. 11 below). Protein quantification is essential to ensure equal loading during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

A bovine serum albumin (BSA) (Amresco; Inqaba Biotec™, SA) standard curve was generated (Fig. 11) using the following BSA concentrations; 1, 0.8, 0.6, 0.4, 0.2 and 0 mg/mL. Twenty-five microlitres of each standard concentration was added into a 96-well plate in triplicates. The protein concentration of each lysate was determined by the addition of 5 µL of protein lysate and 20 µL of 1 X lysis buffer into each well in triplicates. Thereafter, 200 µL of BCA reagent mix (196 µL of BCA (Sigma Aldrich, UK) and 4 µL of Cu^{2+} (Sigma Aldrich, UK)) was added into each well and the plate was incubated at 37 °C for 30 min. The OD of each well was subsequently measured at 562 nm using a microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ software). The protein concentration of each lysate was calculated using Microsoft Office Excel©.

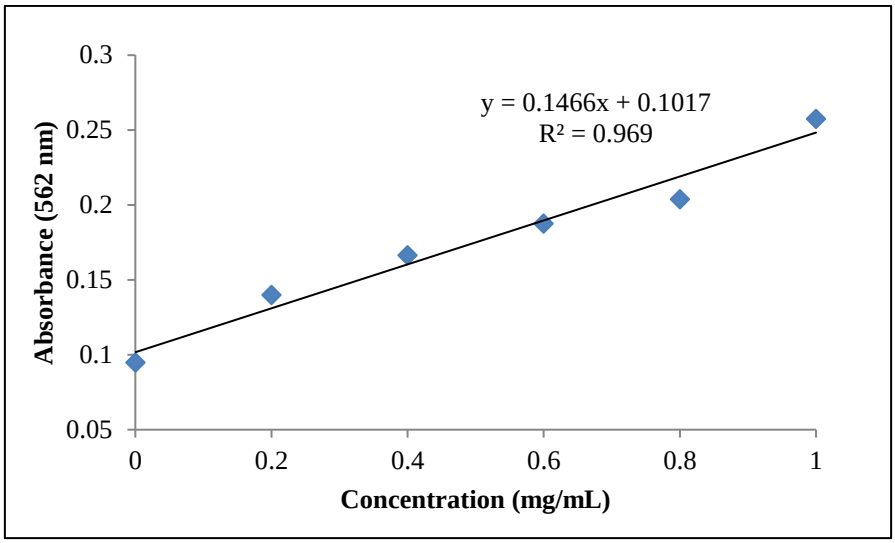


Figure 11: A representative BSA protein standard curve

Protein lysate concentrations were calculated as follows:

$$Average\ of\ samples - Average\ of\ blank = (\boldsymbol{a})$$

$$Substituted\ y - value\ with\ (\boldsymbol{a})\ in\ y = mx + c\ formula\ in\ standard\ curve\ above$$

$$\therefore x - value\ \times 5\ (dilution\ factor) = protein\ lysate\ concentration\ (mg/mL)$$

3.8.2 SDS-PAGE

The standard curve above (Fig. 11) allowed for the relevant calculations to be made in order to determine protein concentration in a sample, ensuring equal loading. The samples were boiled at 85 °C for 5 min and placed on ice immediately and 20 µL of normalised sample was loaded per well in the gel (prepared according to Table 4 and Table 5 below) for analysis and ran at 120 volts (V) for 1 hr. A protein molecular weight marker (Precision Plus Protein™ unstained standards (Bio-Rad, USA)) was included for size estimation.

Table 4: SDS-PAGE gel (10%, 1 mm) components

Components	Resolving gel	Stacking gel
Resolving gel buffer with SDS	3 mL	-
Stacking gel buffer with SDS	-	750 µL
30% Bis-acrylamide (Sigma Aldrich, UK)	3 mL	500 µL
Distilled water (dH_2O)	3 mL	1.75 mL
10% Ammonium persulfate (APS) (Sigma Aldrich, UK)	100 µL	30 µL
Tetramethylethylenediamine (TEMED) (Sigma Aldrich, UK)	10 µL	3 µL

Table 5: SDS-PAGE buffer components

	Components
Resolving gel buffer	36.2 g Tris 0.8 g SDS Dissolve in 150 mL dH_2O pH to 8.9 Make up to 200 mL with dH_2O
Stacking gel buffer	5.9 g Tris 0.4 g SDS Dissolve in 70 mL dH_2O pH to 6.8

	Make up to 100 mL with dH_2O
30% Bis-acrylamide	30 g Acrylamide 0.8 g Bis-acrylamide 0.1 g SDS Make up to 100 mL dH_2O
10% APS	100 mg APS in 1 mL dH_2O

3.8.3 Western blot

Resolved proteins were then transferred onto a 0.22 μM polyvinylidene fluoride (PVDF) membrane for 30 min using the Trans-Blot®Turbo™ Transfer System (Bio-Rad, USA). The membrane was then incubated in a 5% blocking solution (2.5 g milk powder in 50 mL 1 X Tris-buffered saline with Tween-20 (TBST)) for 1 hr to prevent non-specific binding. Incubation of the membrane with relevant primary antibodies was performed overnight at 4 °C according to Table 6 below. Thereafter, 5 x 5 min 1 X TBST washes were performed to wash off unbound antibodies followed by the addition of specific secondary antibodies (Table 7) incubated for 1 hr at room temperature (RT). Subsequent 1 X TBST washes were performed.

Table 6: Primary antibodies and dilutions

Protein	Source	Dilution	Company
β-Actin	Mouse monoclonal	1:2000	Sigma Aldrich, UK
β-Tubulin	Mouse monoclonal	1:2000	Sigma Aldrich, UK
CETP	Rabbit monoclonal	1:1000	Sigma Aldrich, UK
ER	Rabbit monoclonal	1:500	Sigma Aldrich, UK
PARP	Mouse monoclonal	1:1000	Sigma Aldrich, UK
pH2AX	Rabbit monoclonal	1:1000	Sigma Aldrich, UK
SREBP	Rabbit monoclonal	1:1000	Novus

Table 7: Secondary antibodies and dilutions

Target	Source	Dilution	Company
Mouse IgG-HRP conjugate	Goat	1:2000	Bio-Rad, USA
Rabbit IgG-HRP conjugate	Goat	1:2000	Bio-Rad, USA

Chemiluminescence was performed by using Clarity™ Western ECL Substrates (Bio-Rad, USA). The membrane was incubated in equal volumes of Peroxidase solution and Luminol/enhancer solution for 2 min in the dark before viewing on the ChemiDoc (Bio-Rad, USA). Densitometry analysis was performed using the Image Lab™ 4.0 Software (Bio-Rad, USA) by comparing proteins of interest to loading controls as well as comparing proteins of treated samples to that of untreated samples.

Monoclonal anti-β-Tubulin and anti-β-Actin antibodies were used as loading controls. Since these proteins are constitutively expressed in cells, the protein expression level of both proteins were used to normalise the results obtained from protein expression levels of all other proteins used in the current study. Moreover, the loading controls ensured that the primary antibodies used were indeed specific for the targeted proteins (CETP, ER, pH2AX, PARP and SREBP1).

3.9 Statistical analysis

A Z-factor of ≥0.6 was recorded for each assay performed in 96-well plates, indicating good to excellent robustness according to a previous study by Zhang *et al.,* 1999. A student's *t*-test was used to determine statistical significance where $p<0.05$. All statistics including mean and standard deviation calculations were performed using Microsoft Office Excel© or GraphPad Prism5®.

Percentage increase/decrease was calculated according to the formulae below:

$$\%\ Increase = \frac{(B - A)}{A} \times 100$$

$$\%\ Decrease = \frac{(A - B)}{A} \times 100$$

$$Where\ A = Initial\ value\ and\ B = Final\ value$$

4. RESULTS

4.1 Characterisation of LTED cells

4.1.1 Morphology of MCF7 and LTED cells

The comparison of morphology of MCF7 and LTED cells are visually represented below (Fig. 12). There were characteristic differences between MCF7 and LTED cells within the first 90 days of culturing in estrogen-free conditions (Fig. 12A and B). LTED cells appeared smaller in size and rounder in shape compared to MCF7 cells which are epithelial-like and appear polygonal in shape. After 90 days of adapting to low estrogen levels, LTED cell size and shape appeared to change (Fig. 12C). LTED cells became larger in size and elongated in shape, which appeared to be fibroblast-like. These results were similar to that of previous studies performed by Santen *et al* (2005). This difference can be attributed to the fact that these cells were grown in a stressful environment and led to the eventual adaptation to this environment.

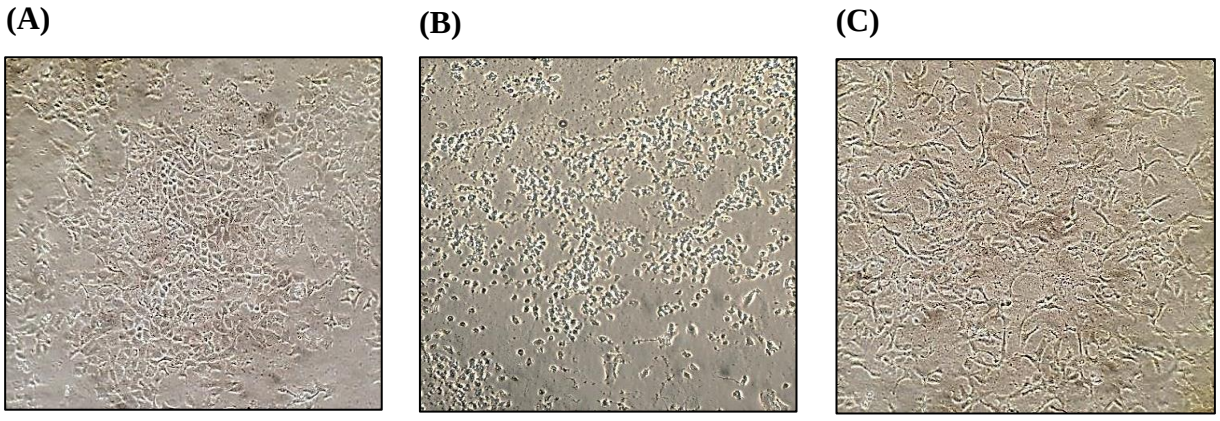


Figure 12: A visual representation of the morphological differences between MCF7 and LTED cells

*Representative photographs of untreated **(A):** MCF7 cells, **(B):** LTED cells within 90 days of culturing in estrogen-free conditions (phase 1), and **(C):** LTED cells after 90 days of culturing in estrogen-free conditions (phases 2-3). LTED cells retained this morphology from 90 days onwards. Cells were grown in conditions as described above.*

4.2 Growth curve using cell counts

Following the development and characterisation of the LTED cell line, a cell counting assay was performed to compare the effects of varying concentrations of E_2 on the growth of MCF7 and LTED cells over a period of 6 days (Fig. 13). The differences in the growth of MCF7 and LTED cells were not tested for statistical significance and therefore can only be used as a

qualitative observation. Further interpretation of these results will rely on subsequent experimental tests for validation of the growth patterns observed. These assays were performed before 90 days, where ER expression and cell growth was significantly lower in LTED cells compared to MCF7 cells and after 90 days, where cells had increased ER expression and cell growth. Increasing concentrations of E_2 had a stimulatory effect on the growth of MCF7 cells (Fig. 13A), whereas in LTED cells this effect was not observed within 90 days (Fig. 13B). MCF7 cells had a maximum of 600 000 cells/well (Fig. 13A), whereas LTED cells had a maximum of 12 500 cells/well (Fig. 13B). Cell growth was significantly retarded in LTED cells especially within the first 60 days of estrogen-free culturing conditions. Fig. 13B shows a ~100% decrease in cell growth compared to MCF7 cells, which indicates a stressful environment in which these cells were adapting to. This was calculated according to the formula in Materials and Methods above. An increase in growth of LTED cells after 90 days of adapting to estrogen-free conditions is shown in Fig. 13C below (phases 2-3). Cell numbers were similar to those of MCF7 cells indicating the adaptation of LTED cells to low levels of estrogen. Low concentrations of E_2 (1 nM) stimulated LTED growth after 90 days, however at higher concentrations (10 nM), cell growth started to decrease (Fig. 13C), indicating that LTED cells grow independently of E_2.

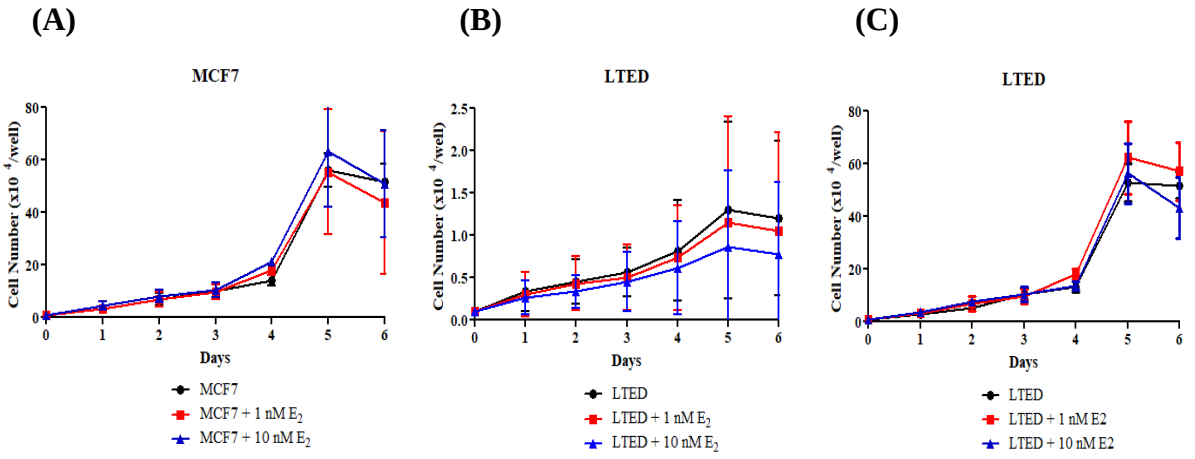


Figure 13: Effect of E_2 on the growth of MCF7 and LTED cells

*Representative graphs of the effect of E_2 on numbers of **(A):** MCF7 cells, **(B):** LTED cells within the first 180 days and **(C):** LTED cells after 180 days of culturing in estrogen-free conditions. Cells were grown and treated with E_2 at the indicated concentrations as described in Materials and Methods for a period of 6 days. Cell numbers were counted with a Neubauer haemocytometer. Data are mean ± standard deviation (S.D) (n=3) from raw data.*

It is evident from Fig. 13 above that E_2 does not have a stimulatory effect on LTED cell growth and these cells grow independently of E_2 after 180 days of adaptation.

4.3 Presence and absence of ER confirmed using Western blotting in MCF7 and LTED cells respectively

Based on previous *in vitro* findings by Martin *et al* (2003) that LTED cells pass through three phases of adaptation, Western blots were performed in order to confirm the different ER expression levels of LTED cells over time. Within the first 90 days of culturing in estrogen-free conditions (quiescent phase), ER expression of LTED cells was significantly down-regulated (~130% decrease) compared to MCF7 cells (Fig. 14). This was expected as cells were acclimatising and adapting to new growing conditions. There was a difference in expression levels between MCF7 cells and LTED cells in phases 2 and 3; however, the difference was not statistically significant possibly due to the low number of replicates. Within 180 days of estrogen-free conditions (hypersensitive phase), ER expression in LTED cells was ~47% less in comparison to MCF7 cells. There was an increase in ER expression in LTED cells from phase 1 to phase 2. The addition of exogenous estrogen stimulated LTED cell growth in this phase as these cells were hypersensitive to low levels of estrogen. After 180 days of culturing (independent phase), ER expression in LTED cells was up-regulated by ~17% compared to MCF7 cells. In the third phase, LTED cells had adapted fully and became hypersensitive to low levels of residual estrogen whereby cell growth was unaffected by exogenous estrogen.

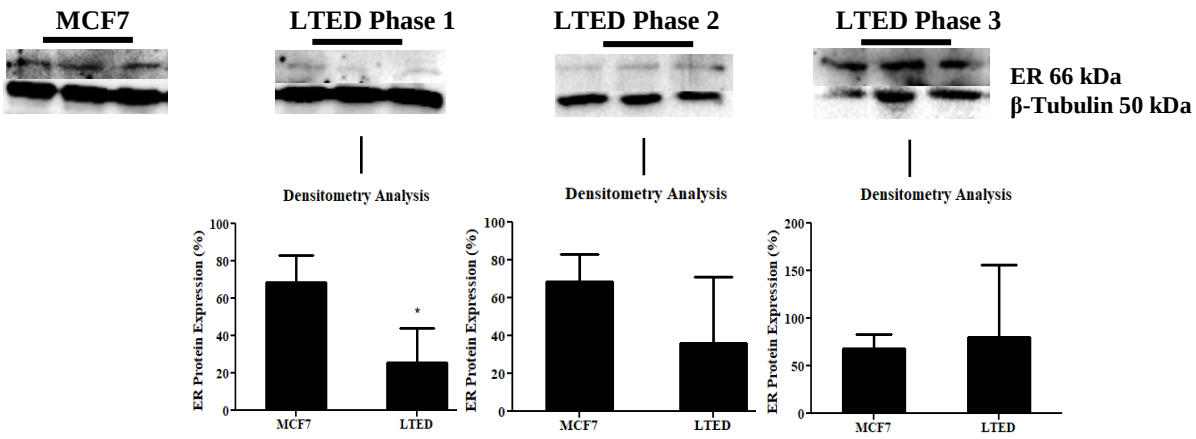


Figure 14: Confirmation of differences in ER expression in LTED cells over time

*Western blots confirming the presence of ER (66 kDa) expression in MCF7 cells and varying levels of ER expression in LTED cells in the 3 phases of adaptation. Relevant densitometry analyses show the increase or reduction of ER expression in LTED cells in different phases in comparison to MCF7 cells. A Precision Plus Protein™ Unstained standard was used as a molecular weight marker for size detection of proteins. Equal bands observed for β-Tubulin at 50 kDa indicated equal loading of lysate which confirms the different ER expression levels within LTED cells. Data are mean ± S.D (n=2) from raw data, where *p<0.05 significant difference to untreated control.*

4.4 Characterisation of MCF7 and LTED cells

The characteristics of LTED cells upon adaptation to low levels of estrogen are summarised in Table 8 below. These cells went through 3 phases of adaptation over time and differed from MCF7 cells regarding ER expression and the effects of E_2 on cell growth.

Table 8: Phases and characteristics of LTED cell line adaptation

Phase	Time (days)	ER expression	Doubling time	Addition of exogenous E_2
Quiescent phase	30-90	Down-regulated	Stunted compared to MCF7 cells	Hinders cell growth
Hypersensitive phase	90-180	Similar to MCF7 cells	Similar to MCF7 cells	Low E_2 stimulates cell growth
Independent phase	>180	Up-regulated	Similar to MCF7 cells	No effect on cell growth

4.5 Growth inhibition analysis in MCF7 and LTED cells

Following the confirmation that LTED cells had increased ER expression and had adapted to grow independently of E_2, MTT assays were performed with varying concentrations of E_2 in order to confirm the stimulatory and inhibitory effects on cell growth of MCF7 and LTED cells respectively. Assays were performed on LTED cells in phase 3 because the current study aimed at investigating ER+ cells that underwent estrogen deprivation for long periods of time (>180 days) in order to mimic the biological mechanisms observed in BC patients with low levels of estrogen circulating the body. In addition, MTT assays were also performed in order to compare the effects of various compound treatments (especially the combination treatments of Tam and AP) and E_2 treatments on growth inhibition in these cells. Combination treatments were performed in order to observe an increase in cell death in both cell lines as compared to single treatments. A range of concentrations of Tam, AP and a combination of Tam and AP was initially tested and the appropriate concentrations for downstream assays were selected based on the results of the MTT assays.

4.5.1 MCF7 cells

MCF7 cells were treated with and without E_2 supplementation in order to make direct comparisons of the effect of E_2 on cell growth inhibition in MCF7 cells. Low concentrations of Tam and AP were selected due to the increasing cytotoxicity of these compounds at higher concentrations based on previous work performed by colleagues in the laboratory. Combination treatments greatly increased Tam's efficacy at inhibiting growth inhibition in MCF7 cells without E_2 supplementation (Fig. 15A). There was a ~500% increase in growth inhibition when Tam and AP were combined compared to single Tam treatments, indicating a synergistic effect. The growth inhibition observed in AP treated cells and combination treatments seemed to stay the same. This indicates that AP significantly enhances Tam's efficacy in reducing BC cell growth. Interestingly, when E_2 was added to MCF7 cells, growth inhibition decreased (Fig. 15B and C). This shows that the effects of both compounds at inhibiting cell growth were stunted. This is expected as E_2 has stimulatory effects on MCF7 cells. However, combination treatments still greatly inhibited cell growth by at least ~500%. It is seen from Fig. 15 below that the combination treatment of 1 µM Tam + 20 µM AP was the most effective at inhibiting MCF7 cell growth with a minimum of 70% cell inhibition observed (Fig. 15B). This is a significant finding as AP greatly enhances Tam's efficacy even at low concentrations. This is useful as women with BC can be treated with low concentrations of compounds meaning resistance to Tam can be greatly reduced.

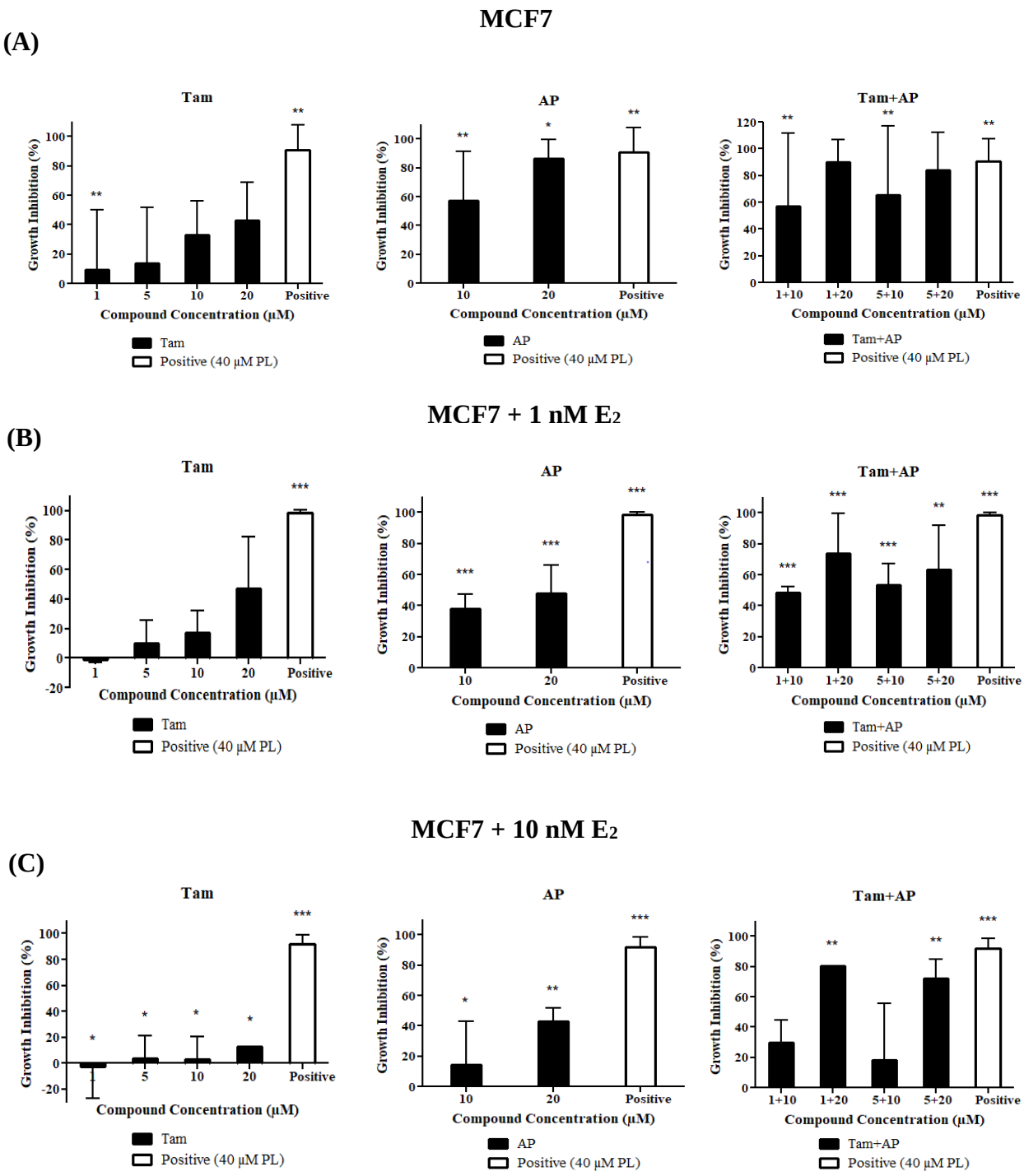


Figure 15: Growth inhibition observed in MCF7 cells treated with varying concentrations of E_2 and test compounds

Representative graphs of the percentage growth inhibition of ***(A):*** *MCF7 cells without E_2 supplementation (Z-factor of 0.95),* ***(B):*** *with 1 nM E_2 supplementation (Z-factor of 0.98), and* ***(C):*** *10 nM E_2 supplementation (Z-factor of 0.68) at selected concentrations of test compounds Tam, AP and a combination of Tam and AP. 40 µM PL was used as a positive control. Data are mean ± S.D (n=3) from raw data, where *p<0.05, **p<0.01 and ***p<0.001 significant difference to untreated control.*

4.5.2 LTED cells

Conditions for the growth inhibition assay in LTED cells were identical to those of MCF7 cells for statistical significance. As with MCF7 cells, AP greatly increased Tam's efficacy at inhibiting LTED cell growth without E_2 supplementation (Fig. 16A). There was a ~300% increase in growth inhibition when Tam and AP were combined compared to single Tam treatments. As compared with MCF7 cells, the effect of AP was not as prominent in LTED cells, which could be due to LTED cells being more resistant to treatment. When varying concentrations of E_2 were added to LTED cells, growth inhibition was increased by at least ~200% (Fig. 16B and C). This is the opposite effect seen in MCF7 cells. This indicates that E_2 has inhibitory effects on LTED cell growth. Although the overall growth inhibition seen in LTED cells was less than in MCF7 cells, combination treatments were still significantly higher than single treatments, once again solidifying that AP enhances Tam's efficacy in resistant BC cell lines.

From Figs. 15 and 16, it is evident that a concentration of 1 nM E_2 has similar effects on cell growth to that of 10 nM E_2; therefore, the following downstream assays were performed with a concentration of 1 nM E_2 supplementation. The selected concentrations of Tam for downstream assays were 1 and 5 µM as concentrations higher than these lead to high toxicity in cells.

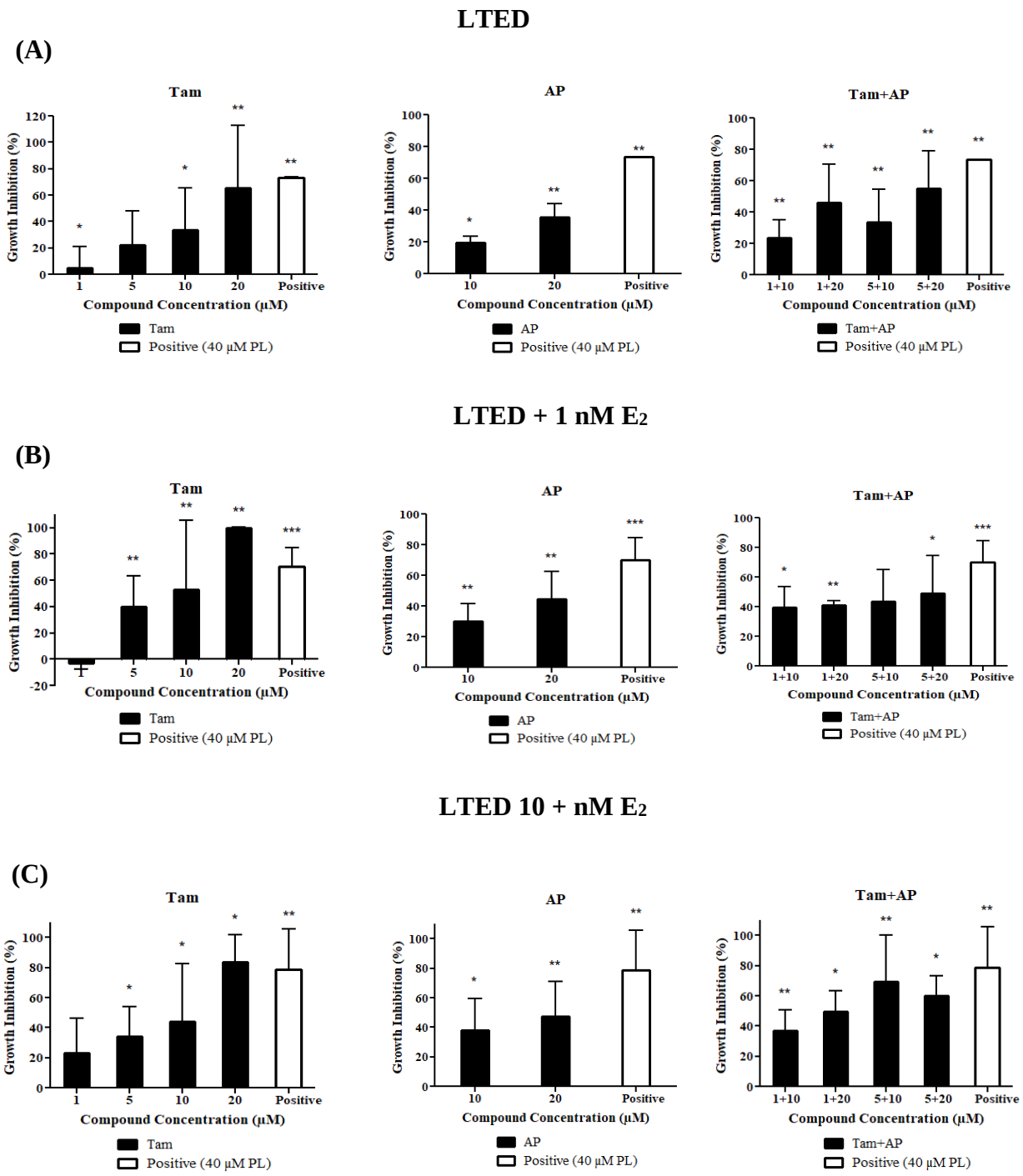


Figure 16: Growth inhibition seen in LTED cells treated with varying concentrations of E_2 and test compounds

Representative graphs of the percentage growth inhibition of ***(A):*** *LTED cells without E_2 supplementation (Z-factor of 0.93),* ***(B):*** *with 1 nM E_2 supplementation (Z-factor of 0.87), and* ***(C):*** *10 nM E_2 supplementation (Z-factor of 0.64) at selected concentrations of test compounds Tam, AP and a combination of Tam and AP. 40 µM PL was used as a positive control. Data are mean ± S.D (n=3) from raw data, where *p<0.05, **p<0.01 and ***p<0.001 significant difference to untreated control.*

4.6 Apoptosis assays (secondary screen)

A range of concentrations of Tam, AP and a combination of Tam and AP was selected from the above growth inhibition assay and these concentrations were tested on MCF7 and LTED cells. Apoptosis assays were performed in order to confirm the growth inhibition assay. These apoptosis assays confirm apoptosis at different stages and are resourceful ways of confirming the efficacy of the test compounds in both cell lines.

4.6.1 Early-stage apoptosis analysis using an APOPercentage™ assay in MCF7 and LTED cells

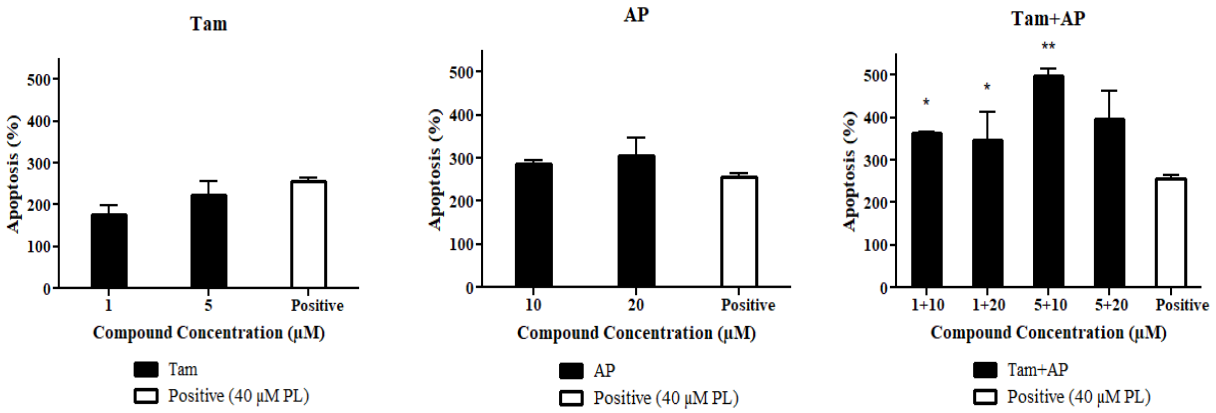
MCF7 and LTED cells were treated with selected concentrations of Tam, AP and a combination of Tam and AP. After treatment with the above compounds, the cells were treated with APOPercentage™ dye that is used to detect apoptotic cells during phospholipid phosphatidylserine transmembrane movement (Biocolor, UK). This assay shows that the cells undergoing apoptosis take up the pink stain which is representative of membrane flipping in cells undergoing apoptosis (Esau *et al.,* 2016). The percentage apoptosis seen in MCF7 and LTED cells once treated with the above compounds and dye is represented below.

4.6.1.1 MCF7 cells

This assay is an effective indicator of early-stage apoptosis and therefore cells were treated for two hrs with test compounds as Tam and AP are known to show apoptotic effects within hours of treatment. MCF7 cells underwent a higher rate of apoptosis when treated with combination treatments than single treatments (~100% increase in apoptosis) as seen in Fig. 17A. It was therefore confirmed that AP works synergistically with Tam in order to increase Tam's efficacy. Fig. 17B confirmed the findings seen above, that when MCF7 cells were treated with E_2, a decrease in apoptosis was observed. However, combination treatments had a significant increase in cell death compared to single treatments. The visual representation (Fig. 17C) shows a qualitative result of what was quantified. It is evident that with increasing concentrations of compounds, there was an increase in pink staining (increased dye uptake in apoptotic cells) compared to the untreated. Combination treatments retained more dye than single treatments. Combination treatments showed similar visual representations to the positive control, which shows great apoptosis induction of these compounds. PL was used as a positive control as it is a known apoptotic inducing compound.

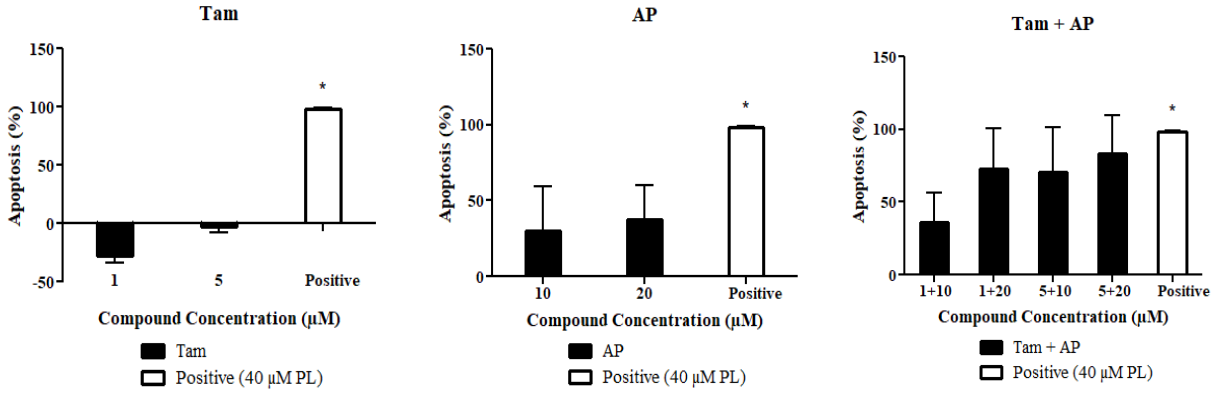
MCF7

(A)



MCF7 + 1 nM E_2

(B)



(C)

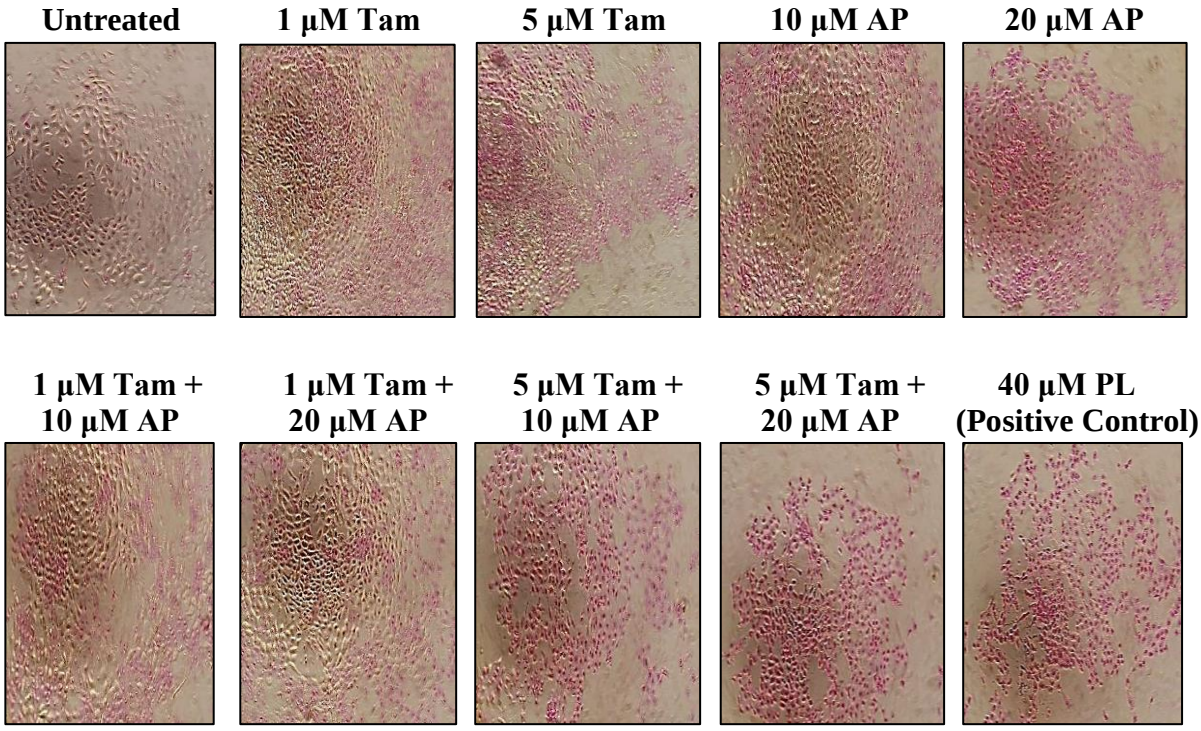


Figure 17: Apoptosis seen in MCF7 cells treated with E_2 and test compounds due to membrane flipping

Representative graphs of the percentage apoptosis seen in ***(A):*** *MCF7 cells due to membrane flipping at selected concentrations of test compounds Tam, AP and a combination of Tam and AP without E_2 supplementation and* ***(B):*** *with 1 nM E_2 supplementation.* ***(C):*** *Visual representation of the effects of various compound treatment concentrations in MCF7 cells without E_2 supplementation compared to untreated MCF7 cells. 40 µM PL was used as a positive control. Data are mean ± S.D (n=3) from raw data, where *p<0.05 and **p<0.01 significant difference to untreated control.*

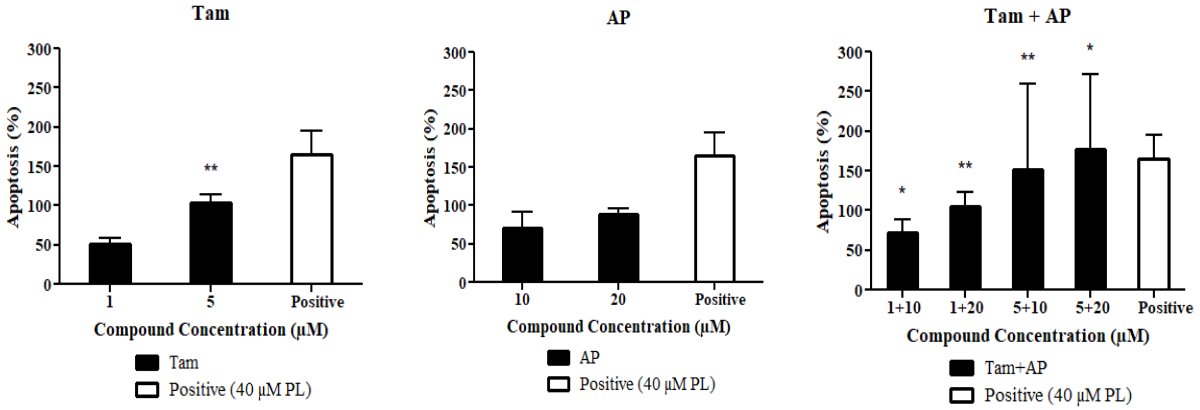
4.6.1.2 LTED cells

Apoptosis induced by single treatments and combination treatments were not as prominent as in MCF7 cells, which confirmed the above growth inhibition assay findings. However, combination treatments increased apoptosis of LTED cells by ~60% as compared to single treatments (Fig. 18A). Such a decrease in apoptosis compared to MCF7 cells further confirms that LTED cells are resistant to treatment. When LTED cells were supplemented with E_2 (Fig. 18B), apoptosis of these cells decreased significantly compared to cells without E_2 supplementation. However, Tam's efficacy in inducing apoptosis significantly increased when combined with AP. It can be seen that AP alone and in combination did not have a significant effect on cell death in LTED cells. The visual representation of apoptotic cells (Fig. 18C) shows

an increase in dye uptake with increasing compound concentration, however, not as significant as seen in MCF7 cells.

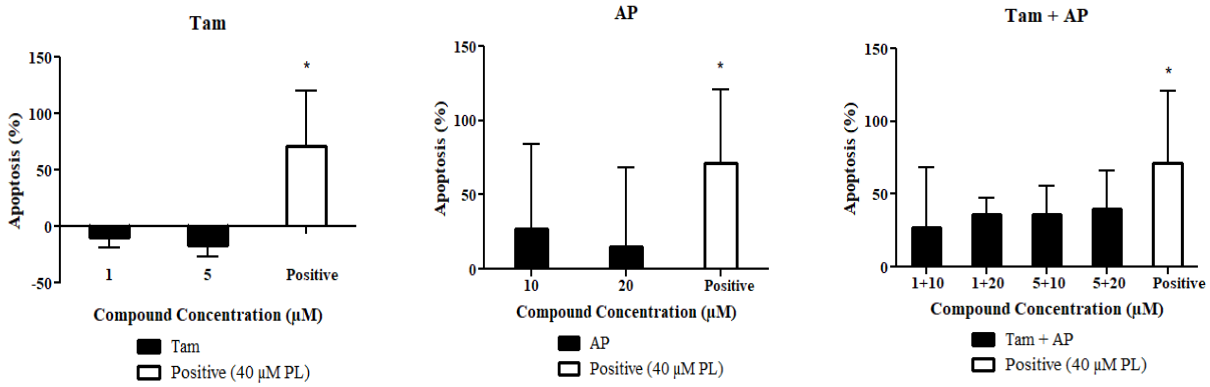
LTED

(A)



LTED + 1 nM E_2

(B)



(C)

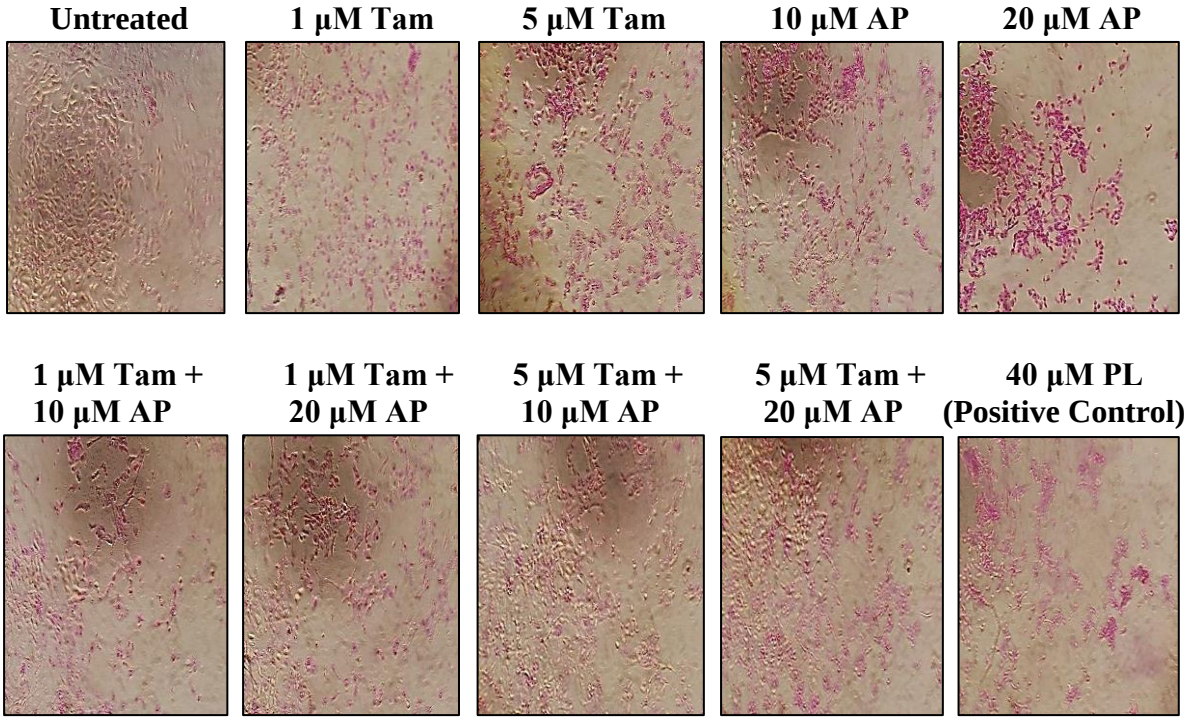


Figure 18: Apoptosis seen in LTED cells treated with E_2 and test compounds due to membrane flipping

*Representative graphs of the percentage apoptosis seen in (A): LTED cells due to membrane flipping at selected concentrations of test compounds Tam, AP and a combination of Tam and AP without E2 supplementation and (B): with 1 nM E2 supplementation. (C): Visual representation of the effects of various compound treatment concentrations in LTED cells compared to untreated LTED cells without E2 supplementation. 40 µM PL was used as a positive control. Data are mean ± S.D (n=3) from raw data, where *p<0.05 and **p<0.01 significant difference to untreated control.*

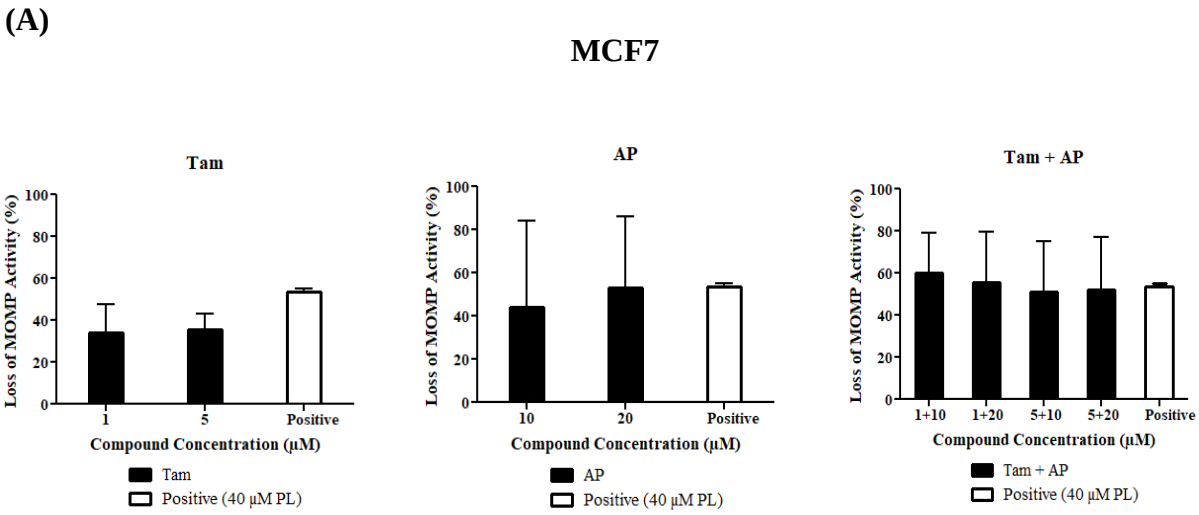
4.6.2 Further apoptosis analysis using a MOMP assay in MCF7 and LTED cells

MCF7 and LTED cells were treated with selected concentrations of Tam, AP and a combination of Tam and AP as above. After treatment with the above compounds, the cells were treated with JC-1 cyanine which is a membrane permeable dye that accumulates in the mitochondria as it is attracted to the high electrochemical-gradient maintained within the inner membrane space (Perelman *et al.,* 2012). This assay detects cells with dysfunctional mitochondria associated with early-stage apoptosis. The percentage of MOMP loss observed in MCF7 and LTED cells once treated with the above compounds and dye is represented below.

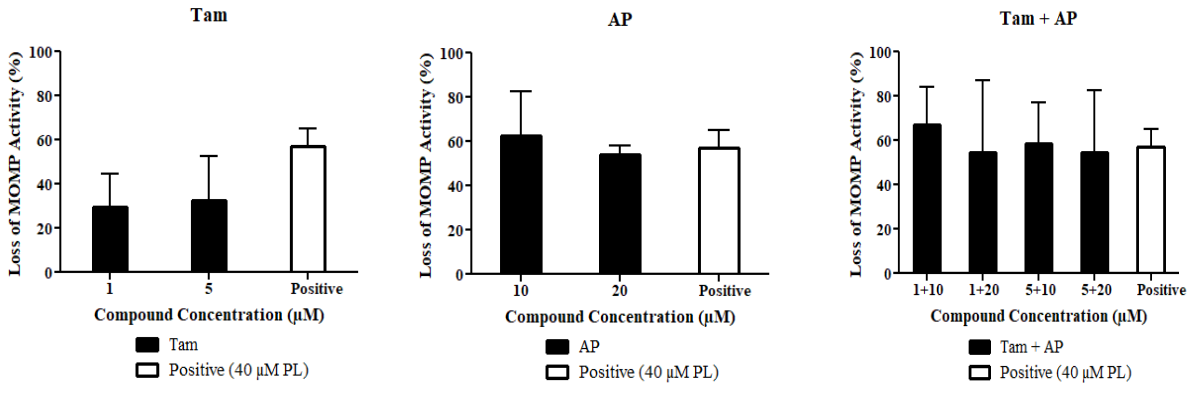
4.6.2.1 MCF7 cells

The MOMP assay is a good indicator of dysfunctional mitochondria and makes use of flow cytometry which is a reliable and effective technique in apoptosis quantification. It is seen that MCF7 cells had similar mitochondrial disruption when treated with single treatments of Tam and AP (Figs. 19A and C). However, no statistical significance difference was observed as this assay did not allow for the inclusion of technical replicates due to time constraints. Therefore, experimental procedures will need to be repeated with the careful inclusion of biological and technical replicates for statistical significance. Bottom left, bottom right and top right quadrants indicate unstained cells, early-stage apoptotic cells, and live cells respectively. However when cells were treated with combinations, there was a ~70% increase in mitochondrial disruption, indicating increased apoptosis. Figs. 19B and D showed similar effects with a ~100% increase in mitochondrial disruption in MCF7 cells supplemented with E_2. These findings showed that E_2 did not have a significant effect on mitochondrial disruption in these cells and further confirms the stimulatory effect it has on MCF7 cells.

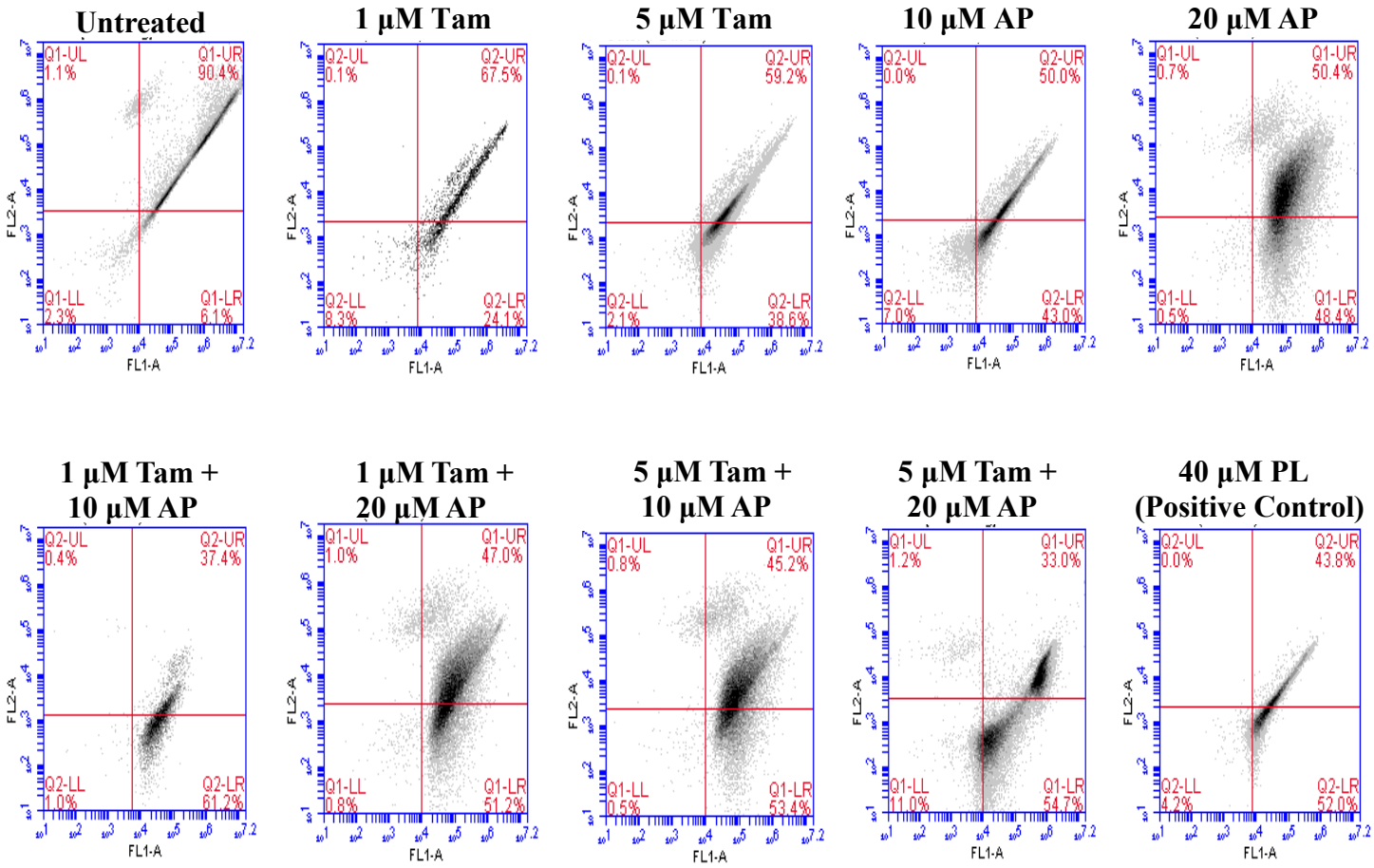
(A)



(B)



(C)


Untreated
Q1-UL 1.1%
Q1-UR 90.4%
Q1-LL 2.3%
Q1-LR 6.1%
1 µM Tam
Q2-UL 0.1%
Q2-UR 67.5%
Q2-LL 8.3%
Q2-LR 24.1%
5 µM Tam
Q2-UL 0.1%
Q2-UR 59.2%
Q2-LL 2.1%
Q2-LR 38.6%
10 µM AP
Q2-UL 0.0%
Q2-UR 50.0%
Q2-LL 7.0%
Q2-LR 43.0%
20 µM AP
Q1-UL 0.7%
Q1-UR 50.4%
Q1-LL 0.5%
Q1-LR 48.4%
1 µM Tam + 10 µM AP
Q2-UL 0.4%
Q2-UR 37.4%
Q2-LL 1.0%
Q2-LR 61.2%
1 µM Tam + 20 µM AP
Q1-UL 1.0%
Q1-UR 47.0%
Q1-LL 0.8%
Q1-LR 51.2%
5 µM Tam + 10 µM AP
Q1-UL 0.8%
Q1-UR 45.2%
Q1-LL 0.5%
Q1-LR 53.4%
5 µM Tam + 20 µM AP
Q1-UL 1.2%
Q1-UR 33.0%
Q1-LL 11.0%
Q1-LR 54.7%
40 µM PL (Positive Control)
Q2-UL 0.0%
Q2-UR 43.8%
Q2-LL 4.2%
Q2-LR 52.0%
FL1-A
FL2-A

(D)

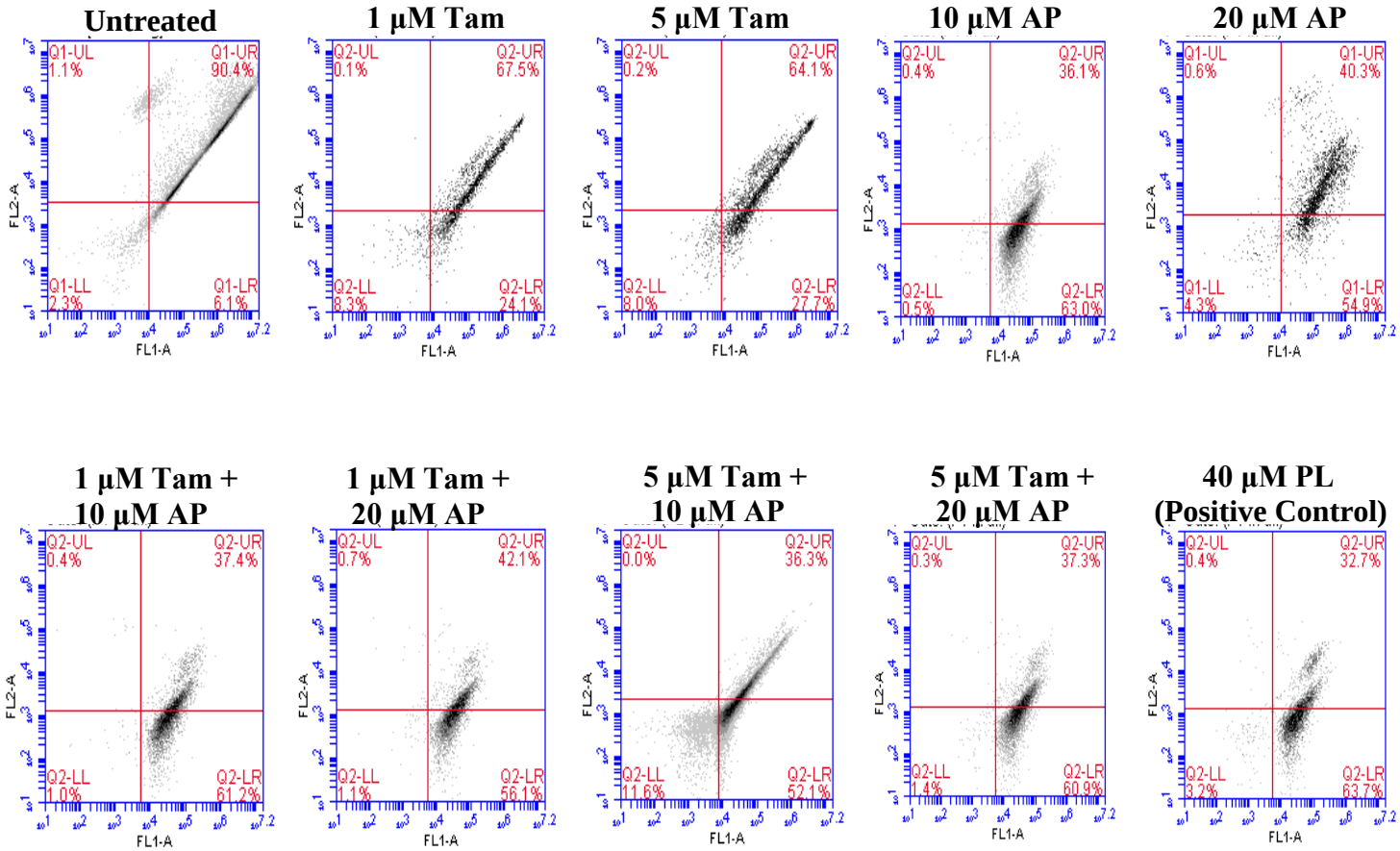

Untreated
Q1-UL 1.1%
Q1-UR 90.4%
Q1-LL 2.3%
Q1-LR 6.1%
1 µM Tam
Q2-UL 0.1%
Q2-UR 67.5%
Q2-LL 8.3%
Q2-LR 24.1%
5 µM Tam
Q2-UL 0.2%
Q2-UR 64.1%
Q2-LL 8.0%
Q2-LR 27.7%
10 µM AP
Q2-UL 0.4%
Q2-UR 36.1%
Q2-LL 0.5%
Q2-LR 63.0%
20 µM AP
Q1-UL 0.6%
Q1-UR 40.3%
Q1-LL 4.3%
Q1-LR 54.9%
1 µM Tam + 10 µM AP
Q2-UL 0.4%
Q2-UR 37.4%
Q2-LL 1.0%
Q2-LR 61.2%
1 µM Tam + 20 µM AP
Q2-UL 0.7%
Q2-UR 42.1%
Q2-LL 1.1%
Q2-LR 56.1%
5 µM Tam + 10 µM AP
Q2-UL 0.0%
Q2-UR 36.3%
Q2-LL 11.6%
Q2-LR 52.1%
5 µM Tam + 20 µM AP
Q2-UL 0.3%
Q2-UR 37.3%
Q2-LL 1.4%
Q2-LR 60.9%
40 µM PL (Positive Control)
Q2-UL 0.4%
Q2-UR 32.7%
Q2-LL 3.2%
Q2-LR 63.7%
FL1-A
FL2-A

Figure 19: Loss of MOMP seen in MCF7 cells treated with and without E_2 and test compounds due to dysfunctional mitochondria

*Representative graphs of the percentage of MOMP loss observed in **(A):** MCF7 cells without E_2 supplementation, **(B):** with 1 nM E_2 supplementation, **(C):** accompanying gated flow cytometry analysis with plotted FL2-A vs. FL1-A parameters without E_2 supplementation, and **(D):** accompanying gated flow cytometry analysis with plotted FL2-A vs. FL1-A parameters with 1 nM E_2 supplementation at selected concentrations of test compounds Tam, AP and a combination of Tam and AP. 40 µM PL was used as a positive control. Data are mean ± S.D (n=3) from raw data.*

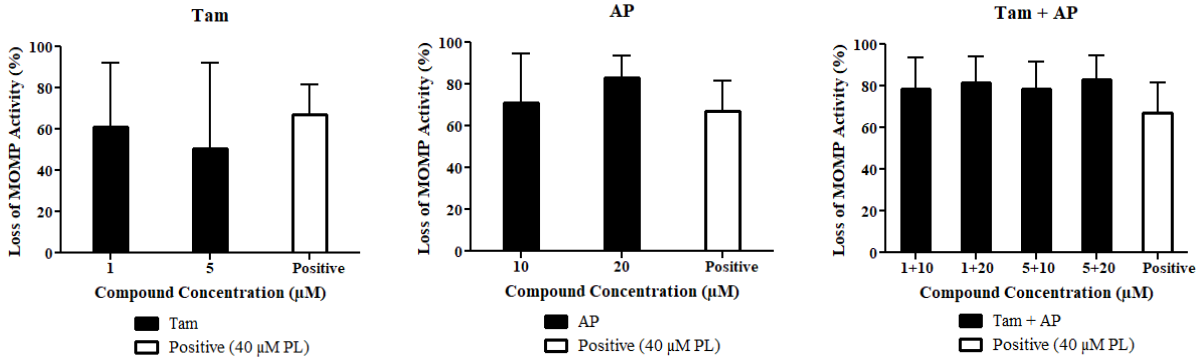
4.6.2.2 LTED cells

LTED cells had higher mitochondrial disruption than MCF7 cells without E_2 supplementation. Combination treatments had a ~50% increase in LTED apoptosis compared to single treatments of Tam and AP (Figs. 20A and C). The effective activity of AP in inducing apoptosis is shown in this assay and indicates that AP could work via mitochondrial dysfunctional pathways. However, no statistical significance difference was observed as this assay did not allow for the inclusion of technical replicates due to time constraints. Therefore, experimental procedures will need to be repeated with the careful inclusion of biological and technical replicates for statistical significance. Apoptosis decreased by ~100% when cells were supplemented with E_2 (Figs. 20B and D). Combination treatments did not have a significant increased apoptotic effect on these cells compared to single treatments in the presence of E_2, with only a ~10% increase in apoptosis. These results indicate that E_2 greatly induces apoptosis in LTED cells. It is clear that the synergy of Tam and AP combined is effective in inducing cell death in both MCF7 and LTED cells and can be used as a therapeutic treatment in reducing cancer-related drug resistance in BC cells.

These apoptosis analyses solidify the results of the growth inhibition assay and show the increased efficacy of Tam when in combination with AP. These findings also suggest that LTED cells are more resistant to treatment as they have lower apoptotic induction compared to MCF7 cells.

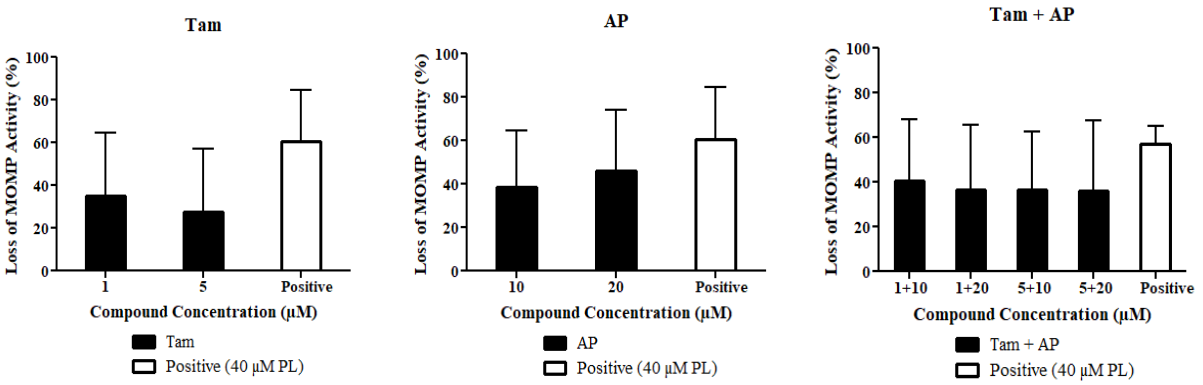
(A)

LTED

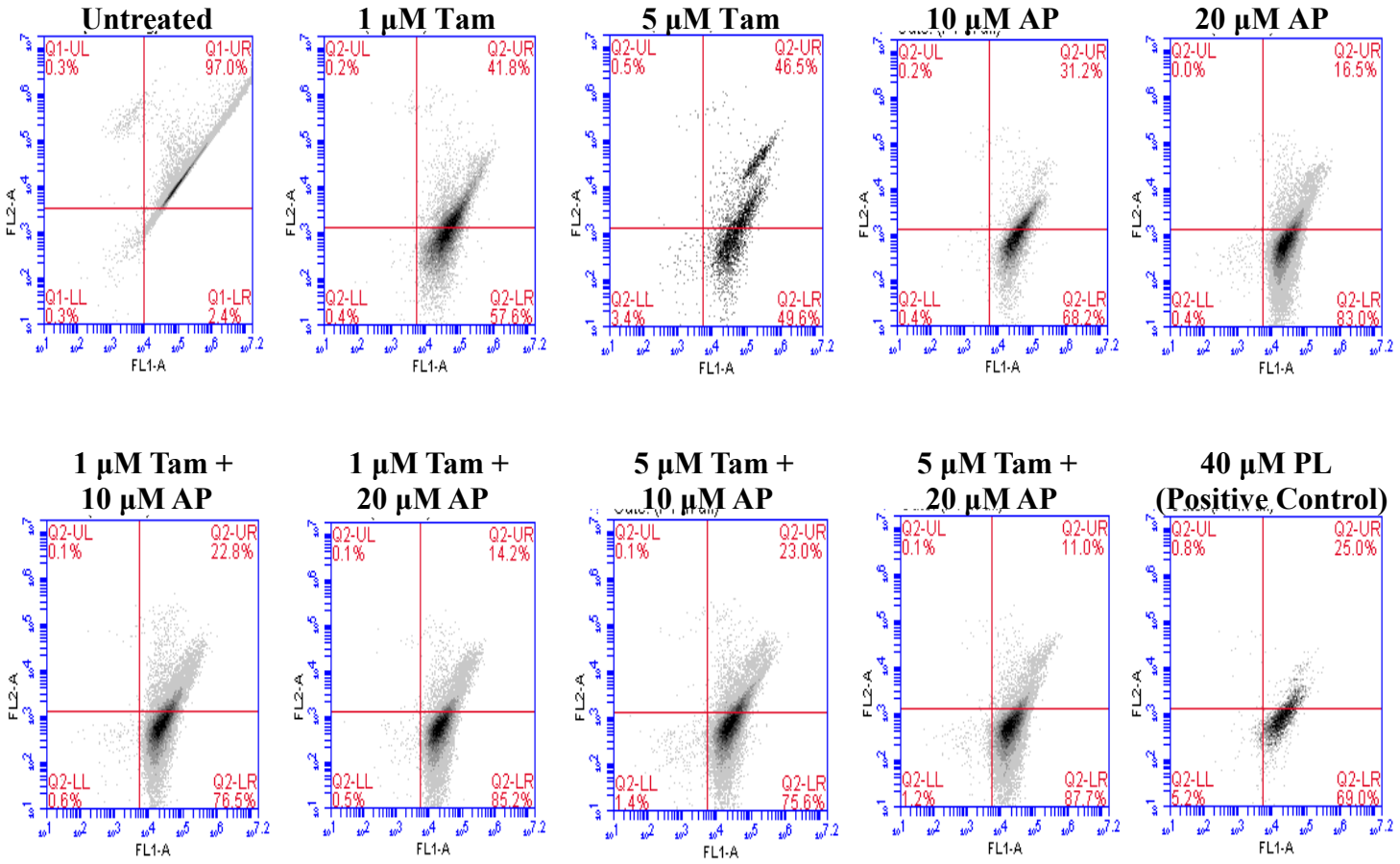


(B)

LTED + 1 nM E_2



(C)


Untreated
Q1-UL 0.3%
Q1-UR 97.0%
Q1-LL 0.3%
Q1-LR 2.4%
1 µM Tam
Q2-UL 0.2%
Q2-UR 41.8%
Q2-LL 0.4%
Q2-LR 57.6%
5 µM Tam
Q2-UL 0.5%
Q2-UR 46.5%
Q2-LL 3.4%
Q2-LR 49.6%
10 µM AP
Q2-UL 0.2%
Q2-UR 31.2%
Q2-LL 0.4%
Q2-LR 68.2%
20 µM AP
Q2-UL 0.0%
Q2-UR 16.5%
Q2-LL 0.4%
Q2-LR 83.0%
1 µM Tam + 10 µM AP
Q2-UL 0.1%
Q2-UR 22.8%
Q2-LL 0.6%
Q2-LR 76.5%
1 µM Tam + 20 µM AP
Q2-UL 0.1%
Q2-UR 14.2%
Q2-LL 0.5%
Q2-LR 85.2%
5 µM Tam + 10 µM AP
Q2-UL 0.1%
Q2-UR 23.0%
Q2-LL 1.4%
Q2-LR 75.6%
5 µM Tam + 20 µM AP
Q2-UL 0.1%
Q2-UR 11.0%
Q2-LL 1.2%
Q2-LR 87.7%
40 µM PL (Positive Control)
Q2-UL 0.8%
Q2-UR 25.0%
Q2-LL 5.2%
Q2-LR 69.0%
FL1-A
FL2-A

(D)

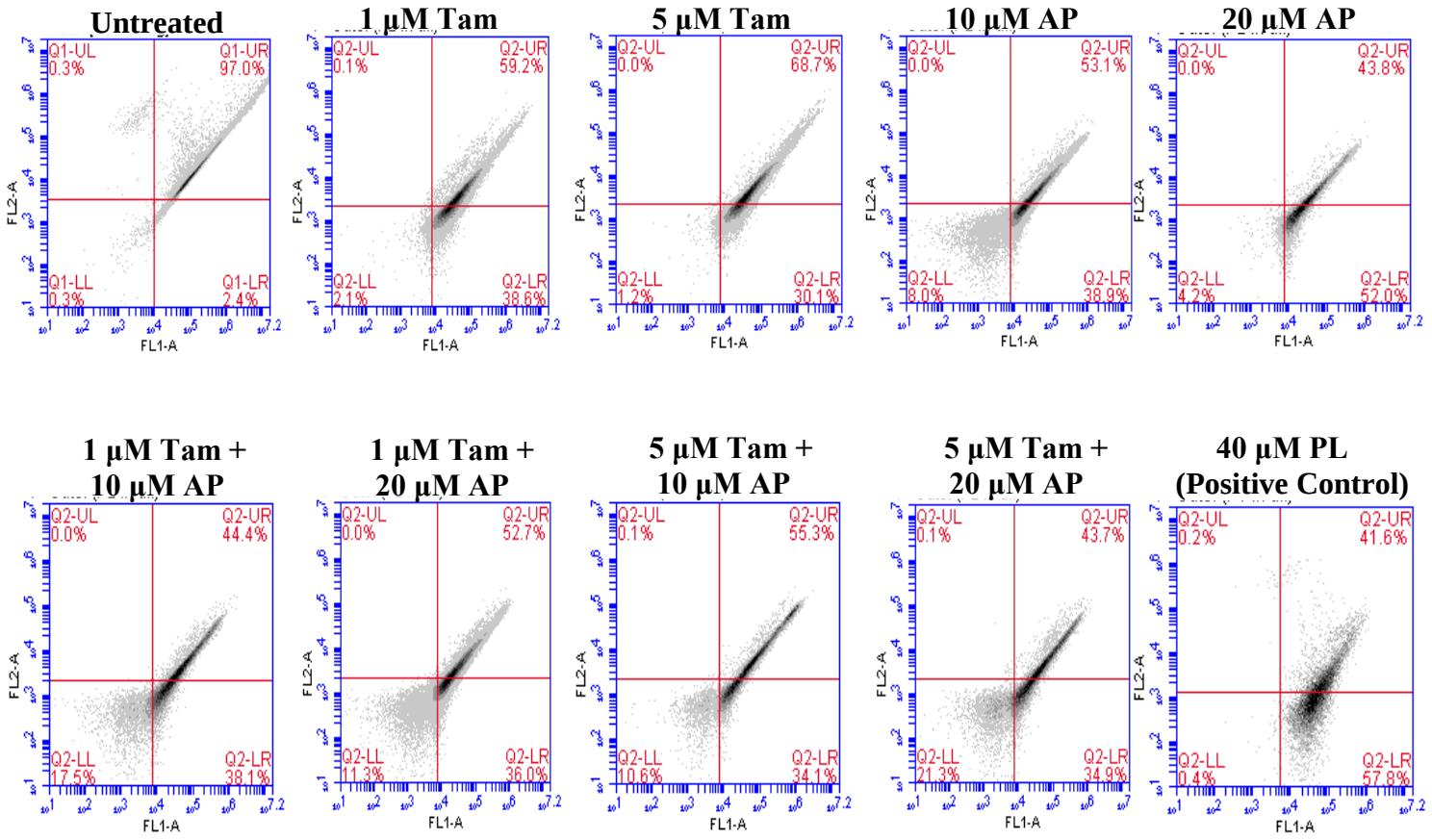

Untreated
Q1-UL 0.3%
Q1-UR 97.0%
Q1-LL 0.3%
Q1-LR 2.4%
1 µM Tam
Q2-UL 0.1%
Q2-UR 59.2%
Q2-LL 2.1%
Q2-LR 38.6%
5 µM Tam
Q2-UL 0.0%
Q2-UR 68.7%
Q2-LL 1.2%
Q2-LR 30.1%
10 µM AP
Q2-UL 0.0%
Q2-UR 53.1%
Q2-LL 8.0%
Q2-LR 38.9%
20 µM AP
Q2-UL 0.0%
Q2-UR 43.8%
Q2-LL 4.2%
Q2-LR 52.0%
1 µM Tam + 10 µM AP
Q2-UL 0.0%
Q2-UR 44.4%
Q2-LL 17.5%
Q2-LR 38.1%
1 µM Tam + 20 µM AP
Q2-UL 0.0%
Q2-UR 52.7%
Q2-LL 11.3%
Q2-LR 36.0%
5 µM Tam + 10 µM AP
Q2-UL 0.1%
Q2-UR 55.3%
Q2-LL 10.6%
Q2-LR 34.1%
5 µM Tam + 20 µM AP
Q2-UL 0.1%
Q2-UR 43.7%
Q2-LL 21.3%
Q2-LR 34.9%
40 µM PL (Positive Control)
Q2-UL 0.2%
Q2-UR 41.6%
Q2-LL 0.4%
Q2-LR 57.8%
FL1-A
FL2-A

Figure 20: Loss of MOMP seen in LTED cells treated with and without E_2 and test compounds due to dysfunctional mitochondria

*Representative graphs of the percentage of MOMP loss observed in **(A):** LTED cells without E_2 supplementation, **(B):** with 1 nM E_2 supplementation, **(C):** accompanying gated flow cytometry analysis with plotted FL2-A vs. FL1-A parameters without E_2 supplementation, and **(D):** accompanying gated flow cytometry analysis with plotted FL2-A vs. FL1-A parameters with 1 nM E_2 supplementation at selected concentrations of test compounds Tam, AP and a combination of Tam and AP. 40 µM PL was used as a positive control. Data are mean ± S.D (n=3) from raw data.*

4.7 Total cholesterol quantification within MCF7 and LTED cells

Cholesterol quantification of MCF7 and LTED cells was an important analysis in this study because it is known that cholesterol accumulation is significantly increased in BC cells. Measuring the decrease in cholesterol content in cells after treatment is important in confirming AP's apoptotic abilities through cholesterol depletion.

MCF7 and LTED cells were treated with Tam, AP and a combination of Tam and AP as above. After treatment with the above compounds, the total cholesterol mass (µg) in the cells were quantified using a cholesterol quantification assay as described in Materials and Methods. Free cholesterol and CEs are also measured by this assay (data not shown) and are important indicators of cholesterol accumulation within cells. It has previously been shown by a colleague in the laboratory that MCF7 cells have ~8 x higher total cholesterol content than normal cells. When comparing untreated LTED and MCF7 cells, cholesterol content is similar in both cell lines despite LTED cells growing in hormonal-free conditions. Therefore LTED cells have increased cholesterol accumulation compared to normal cells and depleting cholesterol within these cells could be effective at inducing cell death. Increasing concentrations of both compounds led to a decrease in total cholesterol mass and when combined led to even lower cholesterol content in both MCF7 cells (~25% reduction) and in LTED cells (~20% reduction) compared to single treatments (Fig. 21). As with the above MOMP assay, there was no statistical significance difference observed in this assay as technical replicates were not included due to time constraints. Therefore, experimental procedures will need to be repeated with the careful inclusion of biological and technical replicates for statistical significance. Tam is not a cholesterol depletor; however, there was a decrease in cholesterol content when cells were treated with Tam, indicating that Tam could have possible apoptotic inducing effects via certain cholesterol depleting pathways. The most effective treatment for both cell lines was 5 µM Tam + 20 µM AP which was expected as higher AP concentrations leads to higher depletion of cholesterol in BC cells. These results confirm the above findings indicating that

the combination treatment of Tam and AP is effective at depleting excess cholesterol within MCF7 and LTED cells thereby inducing apoptosis and possibly lowering cancer-related drug resistance in hormone-dependent BC cells.

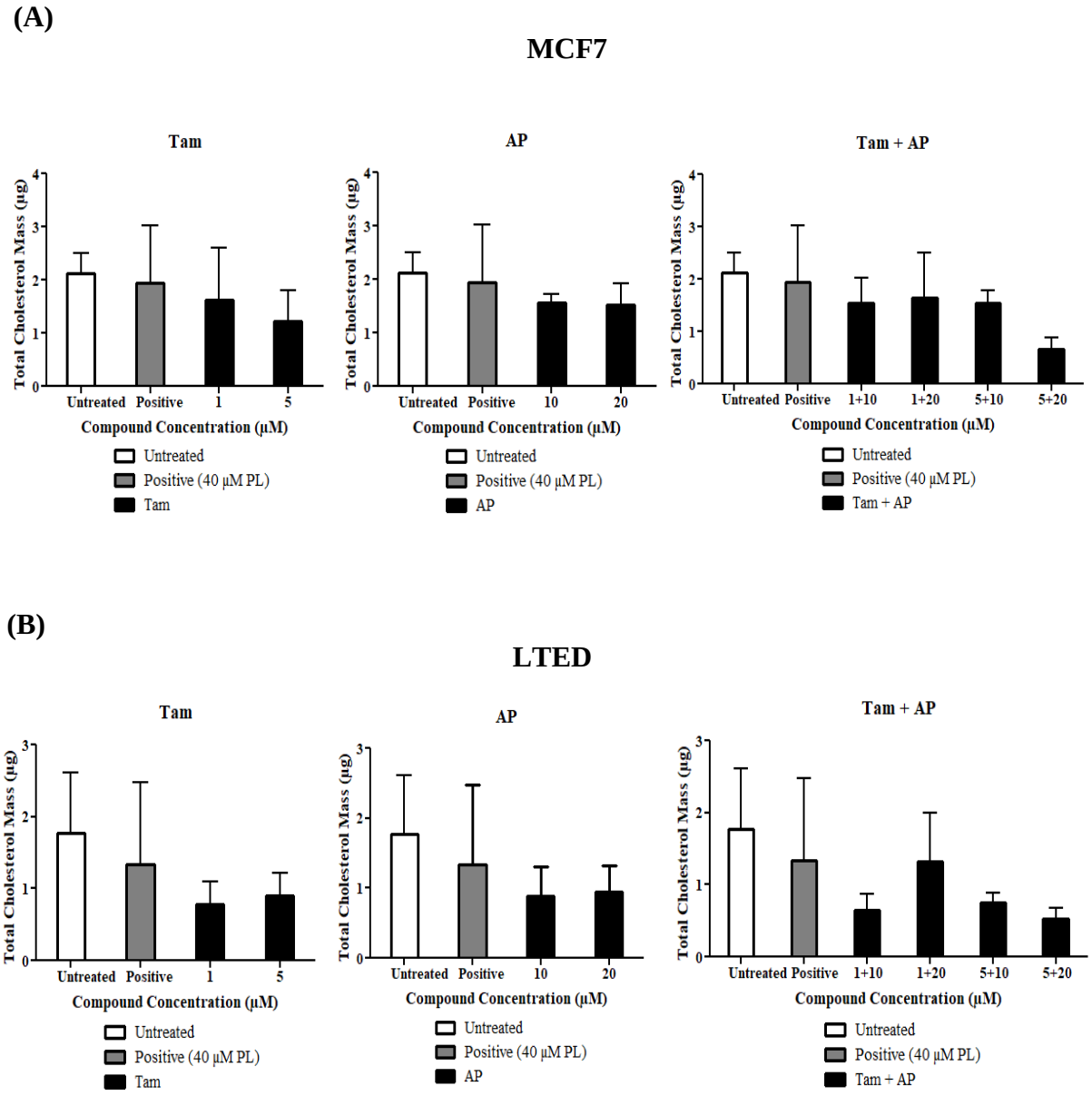


Figure 21: Total cholesterol mass quantified in MCF7 and LTED cells treated with varying concentrations of test compounds

Representative graphs of total cholesterol mass (µg) in ***(A):*** *MCF7 cells and in* ***(B):*** *LTED cells at selected concentrations of test compounds Tam, AP, and a combination of Tam and AP respectively. 40 µM PL was used as a positive control. Data are mean ± S.D (n=3) from raw data.*

4.8 Expression of proteins in MCF7 and LTED cells once treated with test compounds confirmed using Western blots

Several proteins involved in cholesterol accumulation and induction of apoptosis were observed in order to identify molecular mechanisms related to resistance in BC cells. These proteins included: cholesterol-related proteins CETP, SREBP and ER; DNA damage markers poly ADP ribose polymerase (PARP) and phosphorylated histone H2AX (pH2AX); and cytoskeletal damage markers and loading controls β-Tubulin and β-Actin. It was predicted that AP decreased cholesterol accumulation in BC cells and played a role in altering the expression of these proteins.

MCF7 and LTED cells were treated with Tam, AP and a combination of Tam and AP. Western blots were then performed in order to confirm whether the following proteins were affected (up/down regulated) via cholesterol depletion which leads to inhibitory effects seen in MCF7 and LTED cells.

4.8.1 Untreated MCF7 and LTED cells

Western blots were first performed on untreated MCF7 and LTED cells (>180 days) in order to make comparisons in basal expression levels of proteins. The basal expression of different proteins in MCF7 and LTED cells were observed in Fig. 22 below. ER expression levels (66 kDa) were increased in LTED cells compared to MCF7 cells and this was due to the adaptation and sensitivity of LTED cells to low levels of estrogen. Densitometry and percentage increase/decrease analysis was performed according to Materials and Methods mentioned above. It is shown that CETP (77 kDa) expression levels were significantly lower in LTED cells than in MCF7 cells (~57% reduction) and this is attributed to the fact that LTED cells have slightly lower intracellular cholesterol content than MCF7 cells. LTED cells have ~5% more SREBP (40 kDa) expression than MCF7 cells because of lower cholesterol levels. When sterol levels are low in the cell, this protein is activated in order to stimulate cholesterogenesis, indicating a compensatory mechanism to increase cholesterol content.

The DNA damage marker pH2AX has a molecular weight of 15 kDa which was observed in Fig. 22 below. Phosphorylation in the histone H2AX is induced by double-stranded breaks in DNA. This histone is implicated in DNA repair mechanisms following these breaks. Basal expression levels of this protein were slightly reduced in LTED cells compared to MCF7 cells. PARP is a DNA repair marker with a full length size of 116 kDa and is cleaved at 89 kDa. PARP is involved in the base excision repair pathway involved in chromatin architecture and

DNA metabolism. The basal expression levels of PARP were slightly less in LTED cells than in MCF7 cells.

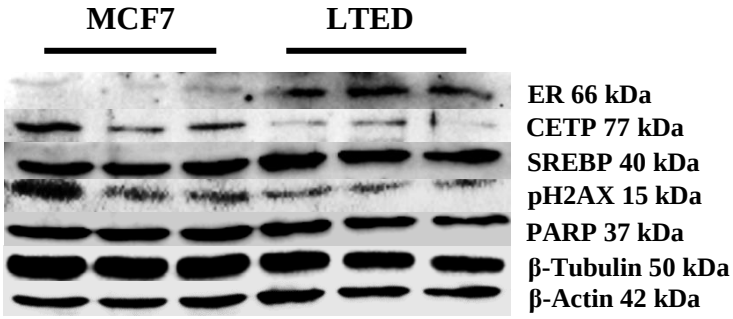


Figure 22: Basal expression of proteins in untreated MCF7 and LTED cells

A Western blot comparing the basal expression levels of different proteins in untreated MCF7 and LTED cells. β-Tubulin and β-Actin were used as loading controls confirming equal loading. A Precision Plus Protein™ Unstained standard was used as a molecular weight marker for size detection of proteins.

4.8.2 Single treatments of MCF7 and LTED cells

MCF7 and LTED cells (>180 days) were treated with 1 μM Tam, 5 μM Tam, 10 μM AP and 20 μM AP respectively (single treatments). The expression of different proteins in MCF7 and LTED cells post compound treatment are represented in Fig. 23 below. β-Actin was used as a loading control as opposed to β-Tubulin due to the fact that these compound treatments affected the microtubules of cells therefore equal loading could only be confirmed using β-Actin. Interestingly, MCF7 and LTED cells treated with 20 μM AP had less β-Actin expression levels compared to other treatments. This could be due to the fact that when AP depletes cholesterol (in cell membranes); damage to the cytoskeletal components is done due to actin's interaction with cellular membranes. ER expression was similar in both cell lines. Although it is unclear in Fig. 23 below, cells treated with AP had less CETP expression levels than cells treated with Tam. This is expected as AP functions via the cholesterol pathway in which CETP's role is heavily involved. LTED cells had an increase in SREBP expression when treated with Tam. This is due to the fact that these cells are resistant to Tam treatment thus compensatory mechanisms were activated in order to synthesise more cholesterol for increased survival and proliferation. PARP expression levels were significantly reduced in LTED cells treated with AP indicating AP's ability in inducing DNA damage and programmed cell death.

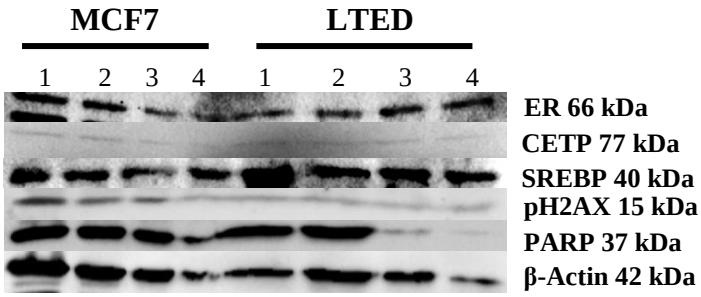


Figure 23: Expression of proteins in MCF7 and LTED cells with single treatments

A Western blot comparing the expression levels of different proteins in MCF7 and LTED cells treated with Tam and AP. β-Actin was used as a loading control confirming equal loading. A Precision Plus Protein™ Unstained standard was used as a molecular weight marker for size detection of proteins. ***Lane 1:*** *1 μM Tam,* ***Lane 2:*** *5 μM Tam,* ***Lane 3:*** *10 μM AP, and* ***Lane 4:*** *20 μM AP.*

4.8.3 Combination treatments of MCF7 and LTED cells

MCF7 and LTED cells (>180 days) were treated with combination treatments: 1 μM Tam + 10 μM AP, 1 μM Tam + 20 μM AP, 5 μM Tam + 10 μM AP, and 5 μM Tam + 20 μM AP respectively. The expression of different proteins in MCF7 and LTED cells post combination compound treatment are represented in Fig. 24 below. β-Actin was used as a loading control to confirm equal loading. The expression levels of ER was the same throughout both cell lines with the exception of 5 μM Tam + 10 μM AP and 5 μM Tam + 20 μM AP treatments in LTED cells. In LTED cells, the expression levels of CETP decreased when cells were treated with 5 μM Tam + 10 μM AP and 5 μM Tam + 20 μM AP. These were the highest concentrations of compounds therefore it was expected that apoptosis was induced via cholesterol depletion in LTED cells and CETP expression was down-regulated. When comparing combination treatments to single treatments, SREBP had lower expression levels in the combination treatment because Tam was combined with AP which is a cholesterol depletor, therefore cholesterol synthesis could not take place as SREBP was not activated. There was significantly less PARP expression in LTED cells compared to MCF7 cells. This indicates treated LTED cells were undergoing DNA damage when treated with Tam and AP.

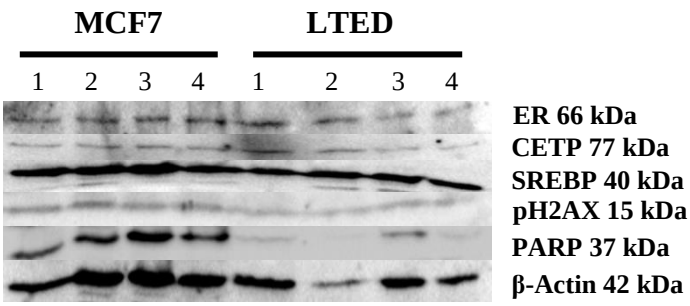


Figure 24: Expression of proteins in MCF7 and LTED cells with combination treatments

A Western blot comparing the expression levels of different proteins in MCF7 and LTED cells treated with combinations of Tam and AP. β-Actin was used as a loading control confirming equal loading. A Precision Plus Protein™ Unstained standard was used as a molecular weight marker for size detection of proteins. ***Lane 1:*** *1 μM Tam + 10 μM AP,* ***Lane 2:*** *1 μM Tam + 20 μM AP,* ***Lane 3:*** *5 μM Tam + 10 μM AP, and* ***Lane 4:*** *5 μM Tam + 20 μM AP.*

5. DISCUSSION

Combating the resistance associated with Tam by targeting cholesterol accumulation in BC cell lines is a novel concept. Preliminary data has shown that when the cholesterol depleting agent AP is used in combination with chemotherapeutic drugs such as Tam, cytotoxicity of normal cells decreases while increasing the cytotoxicity of cancer cells through cholesterol depletion (Esau *et al.*, 2016). Therefore, in the present study, a combination treatment of Tam with AP was performed to observe the changes in cholesterol accumulation pathways affected in different BC cell lines, while using PL as a positive control. This allowed for the observation of enhanced efficacy of Tam which can help in overcoming cancer-related drug resistance.

The results from this study confirm previous findings that hormone-dependent ER+ BC cells deprived of estrogen for long periods of time, go through three distinct phases (Martin *et al.*, 2003) as seen in Figs. 12-14. Initial growth and proliferation of LTED cells is slow for 90 days in which cells are adapting to low levels of estrogen. Once cells have acclimatised to this environment, cell growth starts to increase rapidly, where doubling times are similar to that of WT MCF7 cells. The final phase includes significantly increased levels of ER expression where exogenous E_2 is unable to further stimulate proliferation in LTED cells; however, anti-estrogens have inhibitory effects (Shim *et al.*, 2000). These findings confirm that LTED cells adapt to low levels of estrogen by becoming hypersensitive to E_2 (Jeng *et al.*, 1998, Masamura *et al.*, 1995, Santen *et al.*, 2004, Shim *et al.*, 2000, Santen *et al.*, 2003, Katzenellenbogen *et al.*, 1987). This hypersensitivity leads to increased ER expression in LTED cells which was evident in Fig. 14. These cells are therefore resistant to treatment and exogenous estrogen thus finding alternative therapies in reducing drug resistance and cell proliferation in BC cells is essential.

ER expression levels increased by ~17% in LTED cells (from 68% in MCF7 cells to 85% in LTED cells) that were deprived of estrogen for more than 180 days in comparison to MCF7 cells. These findings are similar to those of other *in vitro* studies where it was reported that cellular ER levels increased by 3- to 10-fold (Katzenellenbogen *et al.*, 1987, Santen *et al.*, 2003). However, the exact mechanism of this increase is currently unknown. It was reported that ER expression levels did not differ between LTED and MCF7 cells within one week of deprivation but only over a longer period of time (>180 days) therefore it was suggested that LTED cells undergo a long-term regulated change leading to adaptation of low estrogen levels (Katzenellenbogen *et al.*, 1987). Increased ER expression provides LTED cells with the advantage of escaping estrogen requirement for increased cellular proliferation during

adaptation (Jeng *et al.*, 1998). It can therefore be said that the development of hypersensitivity to E_2 serves as an adaptive mechanism in LTED cells.

The most common first-line endocrine therapy for ER+ BC patients is the use of Tam. Many patients benefit from this treatment, however, almost all tumours relapse due to acquired Tam resistance (Knowlden *et al.*, 2003). Tam acts through the ER by modulation of gene expression. At lower concentrations (<1 μM), Tam induces cell-cycle arrest and at higher pharmacological concentrations (>5 μM), Tam induces apoptosis of BC cells (Zheng *et al.*, 2007). Cancer-related drug resistance is currently heavily researched as tumour relapse is difficult to treat. A combination treatment of chemotherapy and AIs are then implemented in treating the aggressive tumour, which many times is unsuccessful and leads to death in patients. This study therefore investigated the potential of a combination treatment in order to reduce drug resistance in BC cells. Since cholesterol is a precursor for estrogen production and recently cholesterol accumulation has been shown to contribute to drug resistance, a better understanding of cholesterol processes in hormone-responsive ER+ BC cells is required. The exact mechanism of the function of AP is yet unknown but it is known that it works via the cholesterol pathway (possibly by binding to CETP and blocking its function) by depleting cholesterol in the cells. BC cells have increased levels of cholesterol accumulation and targeting this mechanism has proven successful in the reduction of BC cell proliferation (Esau *et al.*, 2016). Also, AP was found to regress tumour growth *in vivo* with no associated toxicity (Esau *et al.*, 2016) and it is less toxic than its parent compound, PL. AP is known to have selective activation of caspases and apoptotic pathways in ER+ BC cells (Esau *et al.*, 2016). For this reason AP was selected for this study as it can be a potential anti-cancer molecule (Esau *et al.*, 2016). Tam blocks the ER and suppresses estrogen production leading to even lower levels of estrogen within the cell. It was therefore predicted that treating resistant MCF7 and LTED cells with a combination therapy (Tam + AP); cell growth would significantly decrease by decreasing cholesterol accumulation in BC cells. It was also predicted that this combination therapy could greatly reduce drug resistance and induce apoptosis in these cells.

In this study, we have demonstrated the efficacy of Tam in combination with AP in inhibiting cell growth in both MCF7 and LTED cells (Figs. 15 and 16). Significant findings of >300% growth inhibition was seen indicating the synergistic effects of Tam and AP in combination and the effect that AP can increase Tam's efficacy significantly. It is interesting to note that when LTED cells were treated with increasing E_2 concentrations, cell inhibition was increased. This led us to believe that these cells adapted to low estrogen levels and the addition of estrogen

led to stress response in these cells thus leading to cell growth reduction (Santen *et al.*, 2005). The inverse was seen in MCF7 cells where increasing concentrations of E_2 led to increased cell growth. This was expected as E_2 has stimulatory effects on MCF7 cell growth. It is unknown how E_2 affects the biological action of AP and further investigations are required.

The exact mechanisms of acquired Tam resistance are yet to be elucidated; however, Tam-resistant BC cells remain responsive to anti-estrogen therapy while maintaining ER content. Thus the loss of ER signalling is unlikely to contribute to Tam resistance (Knowlden *et al.*, 2003). It has therefore been proposed that BC cells in the presence of anti-estrogens such as Tam use an alternative growth regulatory pathway. Additionally, it has been demonstrated that in the presence of Tam, there is crosstalk between ER and kinases which results in ER activation in the absence of estrogen. As mentioned previously, the inhibition of growth factor receptors and signalling pathways in combination with chemotherapeutic drugs could be an effective treatment in resistant BC cell lines by reverting these cells back to initial estrogen dependent ER-mediated pathways in order to promote cell growth (Wong and Chen, 2012). Several laboratories have investigated the use of these combination therapies and have proven to be successful. Examples of these studies are highlighted below.

A study found that the combination therapy of Tam and an EGFR tyrosine kinase inhibitor helped in overcoming *de novo* resistance and delayed acquired resistance in BC cells (Wong and Chen, 2012). *In vitro* studies revealed that LTED cells showed re-sensitisation to Tam and estrogen deprivation when treated with lapatinib (inhibitor of HER2 and EGFR) (Wong and Chen, 2012). In addition, clinical trials showed the combination therapies were superior to just single therapies alone in terms of the progression-free survival and clinical benefit rate (Wong and Chen, 2012). Several other studies have shown significant results when MAPK and PI3K inhibitors were used in the treatment of Tam-resistant BC cell lines (Viedma-Rodríguez *et al.*, 2014). The present study shows the effective treatment of combination therapy where AP depletes excess cholesterol in BC cells leading these cells to become re-sensitised to Tam treatment, thereby reducing resistance and inducing cell death. However, further studies to elucidate the exact synergistic mechanisms of Tam and AP are required.

The confirmation of early-stage apoptotic induction once cells were treated with combinations of Tam and AP was performed using an apoptosis assay called APOPercentage™ assay. An increase in early-stage apoptosis when cells were treated was observed in Figs. 17 and 18. Combination treatments showed a higher induction of early-stage apoptosis rate as compared

to single treatments in both cell lines (>60%) which confirmed the MTT assay above. This assay detects early-stage apoptosis and it has been shown that cells can recover from early apoptotic induction. Therefore further assays confirming later apoptotic stages were needed. For this reason, a MOMP assay was performed. An increase in mitochondrial disruption or MOMP collapse which leads to early-stage apoptosis in cells was shown in Figs. 19 and 20. An increase in caspase-3/7 activity was also seen in MCF7 cells which led to early-stage apoptosis in these cells (data not shown). These findings show that the efficacy of Tam is significantly increased in inducing early-stage apoptosis when combined with AP at low concentrations. This suggests that both compounds work in a synergistic manner, however, the mechanism of action is unknown. Further studies and apoptosis assays at later stages are required to elucidate the exact mechanism of action of combination treatments.

A study performed by Mohammad and colleagues (2014) demonstrated that the combinatorial effect of Tam and MβCD (a cholesterol depletor) significantly suppressed tumour growth of mice with no toxicity to vital organs. Tumour regression occurred due to enhanced anti-proliferative and anti-angiogenic effects of the combination treatment (Mohammad *et al.,* 2014). The reduction of tumour size was due to increased Tam levels in tumours of mice which therefore reduced the accumulation of Tam in vital organs such as kidney and liver tissues. This led to the conclusion that Tam is likely to be less toxic to vital organs in the presence of a cholesterol depletor, which is one of the major concerns in chemotherapeutic treatment (Mohammad *et al.,* 2014). The results obtained from the current study are in agreement with this previous report and solidifies that combination treatments do in fact lead to cell death in BC cells.

Lipid rafts of plasma membranes are responsible for many biological processes in the cell and the disruption of signalling molecules on these lipid rafts leads to impaired signalling events (Mohammad *et al.,* 2014). Therefore many components of plasma membranes of cells have been targeted for alternative therapeutic strategies in the treatment of cancer. It is known that cholesterol is a component of lipid rafts and maintains stability of cell membranes. The accumulation and dysregulation of cholesterol has recently been reported in various cancers, including BC and has been linked to BC aggressiveness and progression (Di Vizio *et al.,* 2008, Duncan *et al.,* 2004, Esau *et al.,* 2016). Drug trafficking of cell membranes is influenced by the amount of cholesterol present in lipid rafts (Mohammad *et al.,* 2014). Therefore cholesterol depleting compounds such as AP are an obvious choice and are under investigation due to their efficacy at enhancing the action of current chemotherapeutic drugs such as Tam. As AP

depletes cholesterol from the plasma membrane, this leads to cell membrane permeability and disruption thereby altering the transport and signalling of molecules within cells (Mohammad *et al.*, 2014, Esau *et al.*, 2016). It also induces apoptosis which could be due to cholesterol depletion (Sagar *et al.*, 2014).

A previous study revealed that lipid rafts were disrupted in MCF7 cells treated with AP while normal cells remained unaffected (Esau *et al.*, 2016). Quantitative measurements of cholesterol content revealed a 35% reduction of cholesterol in MCF7 cells treated with AP (Esau *et al.*, 2016). Therefore quantifying cholesterol content in cells was crucial in order to confirm the cholesterol depleting mechanisms of AP. In addition, it was also important to observe the changes in cholesterol levels when cells were treated with a combination therapy. The decrease in total cholesterol mass in both MCF7 (~25% reduction) and LTED (~20% reduction) cells once treated with Tam and AP are shown in Fig. 21. Increasing concentrations of the compounds led to a decrease in cholesterol content indicating that this combination treatment works in part via the cholesterol pathway by depleting cholesterol in cell membranes. This leads to lipid raft disruption and the eventual induction of apoptosis within BC cells. The present study provides evidence that cholesterol depletion sensitises cells towards Tam-mediated apoptosis. It also showed that AP enhanced Tam's cytotoxic effects in MCF7 and LTED cells.

The molecular mechanisms of the effects of Tam and AP in combination to treat BC cells are currently unknown. In previous findings by Li *et al* (2006), cholesterol depletion from the plasma membrane resulted in anoikis-like apoptosis which is specifically seen in prostate and BC cells that naturally possess higher levels of membrane cholesterol. Another study hypothesised that the decrease in expression of the protein Caveolin-1 (Cav-1) acts by deactivating Akt and extracellular signal-regulated kinases (ERK) growth promoting signals in tumourigenic cells (Mohammad *et al.*, 2014). Akt and ERK are major regulators of cell survival pathways governed by growth factors. The activation of PI3K/Akt and ERK pathways are linked to cell proliferation and resistance to programmed cell death in many cancer cells. Cav-1 is involved in the regulation of cholesterol homeostasis in the cell, which controls cholesterol accumulation on cell membranes. Therefore, AP in combination with Tam could possibly decrease Cav-1 expression which leads to the deactivation of growth stimulatory pathways thus leading to apoptosis; however, further investigations are required. Increased membrane cholesterol contributes to lipid raft expansion which potentiates oncogenic pathways of cell signalling. Therefore targeting lipid rafts or caveolae in plasma membranes of BC cells could

be a therapeutic treatment of BC by lowering cell membrane cholesterol content (Li *et al.,* 2006).

Several proteins involved in cholesterol accumulation and apoptosis induction were observed in this study to identify the molecular mechanisms involved in cancer-related resistance. Although many proteins are involved in the cholesterol pathway, a select few were chosen for this study. Of particular interest was the protein CETP. CETP shuttles insoluble CEs and triglycerides between HDL and LDL to maintain cholesterol homeostasis both intracellularly and extracellulary (Esau *et al.,* 2016). It can be seen in Fig. 22 that untreated LTED cells had ~57% less basal CETP expression than in MCF7 cells. This is due to slightly lower cholesterol levels in these cells as they are grown in hormone-free conditions. When cells were treated with single treatments, the expression levels of CETP were down-regulated in both MCF7 and LTED cells treated with varying concentrations of AP (10 μM and 20 μM) (Fig. 23). This was expected as AP is a cholesterol depletor and binds to CETP thus CETP's function is halted (Esau *et al.,* 2016). CETP expression was particularly down-regulated in LTED cells with combination treatments of the highest concentrations of Tam and AP (Fig. 24). The exact mechanism of the synergistic effects of Tam and AP reducing CETP expression is unknown. It can be seen that Tam and AP work at low concentrations via cholesterol depletion in both MCF7 and LTED cells in order to induce apoptosis.

CETP is involved in reverse cholesterol transport which facilitates CE and triglyceride uptake from the endoplasmic reticulum into storage droplets of the adipocytes as well as maintaining homeostasis intra- and extracellularly (Esau *et al.,* 2016). Extracellularly, CEs are taken up from HDLs and transferred to the liver to be eliminated from the body via the LDLR. *In vitro* studies have found that cells lacking CETP expression have lower intracellular cholesterol levels due to the possible accumulation of CEs at the synthesis site (endoplasmic reticulum) (Esau *et al.,* 2016). Since CE transport is impeded, there is inefficient removal of CEs from the endoplasmic reticulum, signalling sterol abundance. This leads to the down-regulation of pathways that synthesise cholesterol and the up-regulation of pathways involved in cholesterol removal (Esau *et al.,* 2016). In the absence of CETP, LDL levels decrease while HDL levels increase extracellularly. Increased HDL levels suggest an enhanced clearance of cholesterol in the plasma. Intracellularly, cholesterol is taken up by the cell via binding of LDL to LDLR, which is produced by the endoplasmic reticulum. Endosomes are then formed due to internalisation of LDL. Lysosomal degradation releases cholesterol into the cytosol of cells, where it used for various purposes or exported directly out of the cell via HDL and excreted by

the liver (Chouinard *et al.,* 1998). Cholesterol can also be removed by reverse cholesterol transport through the action of CETP, where HDLs shuttle cholesterol to LDLs, which is ultimately converted to bile salts in the liver. Therefore AP treatment reduces CETP expression which disrupts CE transport therefore leading to induced cell death in BC cells.

SREBP was another protein of interest in the current study and was found that LTED cells had a ~5% increase in basal SREBP expression compared to MCF7 cells (Fig. 22). This was due to the fact that LTED cells had slightly lower intracellular cholesterol levels than MCF7 cells. When LTED cells were treated with Tam, there was an increase in SREBP compared to AP treatments (Fig. 23). The increase in SREBP expression observed could be attributed to the fact that LTED cells are resistant to Tam and therefore compensatory mechanisms are up-regulated in order to increase cholesterol biosynthesis in these cells, aiding in cancer cell survival, progression and aggressiveness. When Tam and AP were added in combination to the cells, there was a decrease in SREBP expression in both cell lines compared to single treatments (Fig. 24). This is due to AP being a cholesterol depletor therefore SREBP cannot be activated and expressed. Recent studies have found that SREBP is heavily involved in cancer cell survival, stress response, protein homeostasis, and lipid metabolism (Griffiths *et al.,* 2013). Increased expression of SREBP is correlated with cancer progression and metastasis in several cancer types which are hormone-responsive such as colorectal, BC and prostate cancers (Li *et al.,* 2012). Cancer cells activate SREBP expression leading to lipid biosynthesis as a response to low levels of lipids within the tumour microenvironment (Griffiths *et al.,* 2013). The depletion of SREBP induces apoptosis in BC cell lines due to low levels of lipoproteins. Therefore targeting these processes could be a novel therapeutic approach for BC cancer treatment.

SREBP-1 is a basic helix-loop-helix transcription factor expressed during adipose differentiation (Chouinard *et al.,* 1998). It activates LDLR gene expression under sterol depletion conditions in cell culture. *In vivo* experiments revealed when SREBP-1 was overexpressed, mice had fatty livers due to increased cholesterol and triglyceride synthesis (Chouinard *et al.,* 1998). Another study showed that moving glioblastoma cells into low lipid conditions resulted in a significant up-regulation of *de novo* cholesterol biosynthesis which is SREBP dependent (Guo *et al.,* 2011). The current study confirms these studies where low hormone growing conditions resulted in up-regulation of SREBP in LTED cells. A study by Bao *et al* (2016) also revealed increased levels of SREBP-1 expression in BC tissues with the promotion of cell migration and invasion *in vitro*. Increased SREBP-1 signalling is therefore a

critical feature for cancer cell growth; however, the mechanism by which SREBP supports tumour growth is unclear. Targeting SREBP regulatory proteins could provide a viable therapeutic approach for the regulation of SREBP activity in tumours (Williams *et al.*, 2013).

Based on the functions of SREBP-1, it is evident that it plays a significant role in maintaining cholesterol homeostasis. CETP, a central protein in the intracellular cholesterol pathway, has been shown to be associated with SREBP-1 expression, since it is also involved in cholesterogenesis. SREBP-1 contributes to CETP gene expression under basal conditions (Chouinard *et al.,* 1998). The target genes of SREBP-1 include: LDLR, HMG coA synthase, and HMG coA reductase, all of which are involved in cholesterol synthesis (Shimano, 2001). Previously, CETP was not considered a target gene for SREBP-1, since it was not known to contain a sterol regulatory element (SRE) in its promoter region (Gauthier *et al.,* 1999). However, studies have emerged revealing that CETP contains a tandem repeat region known as the cholesterol response element (CRE) that is homologous to the SREs seen in SREBP-1's target genes (Gauthier *et al.,* 1999). Reduced levels of CETP lead to a cascade of signalling events where SREBP is subsequently up-regulated in order to synthesis cholesterol for increased cell survival and proliferation. Furthermore, a study conducted by Gauthier *et al* (1999) revealed that SREBP-1 is involved in the up-regulation of CETP, in a sterol-rich environment. Under these conditions, up-regulation of CETP may facilitate reverse cholesterol transport, where CEs are taken up from HDLs and transferred to the liver to be eliminated from the body via LDLR (Gauthier *et al.,* 1999).

Due to their higher proliferative capacity as compared to the normal cells, it is postulated that cancer cells maintain a high level of cholesterol in order to rapidly synthesise cell membranes for their quick growth and division (Cruz *et al.,* 2013; Tosi and Tugnoli, 2005). However, little is known about how cancer cells sustain these cholesterol levels. The study by Guo *et al* (2014) showed that the EGFR-linked PI3K/Akt pathway drives cholesterol uptake through the up-regulation of LDLR, an important receptor involved in intracellular cholesterol uptake in cells. It was elucidated that the PI3K/Akt/mTOR pathway plays a critical role in driving SREBP activity downstream of receptor tyrosine kinase growth receptors.

A link between CE accumulation and hyper-activation of the PI3K/Akt/mTOR pathway via SREBP and LDLR activation has been made which leads to cell proliferation, invasion and migration of aggressive cancer cells as these cells have a need for increased lipogenesis (Guo *et al.,* 2011, Yue *et al.,* 2014). Up-regulation of the PI3K/Akt/mTOR pathway leads to SREBP

activation which initiates esterification of cholesterol into lipid droplets, allowing for fatty acid uptake thus leading to increased cell proliferation (Peck and Schulze, 2014). SREBP and the PI3K/Akt/mTOR pathway are associated in two parts. Firstly, activated Akt can stabilise the nuclear form of SREBP-1 and sequentially promote its target gene expression by down-regulating an E3 ubiquitin enzyme that regulates SREBP-1 N-terminus degradation (Guo *et al.,* 2014). Secondly, PI3K/Akt signalling via the mammalian target of rapamycin complex 1 (mTORC1) regulates a phosphatidic acid phosphatase that controls SREBP-1 transcriptional activity and nuclear localisation (Guo *et al.,* 2014; Wen *et al.,* 2018). However, SREBP-1 regulation by the PI3K/Akt/mTOR pathway appears to be much more complex in cancer cells. mTORC1, PI3K, Akt and EGFR regulate SREBP-1 levels in the nucleus (Guo *et al.,* 2014). Recent studies show that high SREBP-1 expression is directly implicated with tumour metastasis and predicts poor prognosis in BC patients (Wen et al., 2018). Furthermore, SREBP-1 activation as a result of mTORC1 is shown to induce BC cell proliferation and growth (Wen *et al.,* 2018).

In vitro and *in vivo* studies have shown that growth factor receptor signalling pathways (PI3K and MAPK) can mediate resistance to all forms of endocrine therapy (Miller *et al.,* 2011). Overexpression of oncogenes that activate PI3K/Akt signalling (HER2, IGF1-R and activated mutant Akt1) confer resistance to Tam, Fulvestrant and estrogen deprivation in ER+ BC cells. Thus estrogen deprivation may suppress ER+ BC cell growth by decreasing PI3K/Akt/mTOR signalling. *In vivo* studies have found that Tam resistant ER+ MCF7 xenografts showed increased IGF1-R, HER2 and EGFR expression. LTED ER+ BC cell lines showed up-regulated PI3K/Akt/mTOR signalling and hyper-activation of IGF1-R (Miller *et al.,* 2011). A study by Simigdala and colleagues (2016) revealed an up-regulation of the cholesterol biosynthesis pathway in LTED models that retained ER expression. Therefore up-regulation of the cholesterol biosynthesis pathway may be related to estrogen signalling, as cholesterol is a precursor of the cholesterol synthesis pathway. In conclusion, SREBP has been shown to be heavily implicated in BC cell progression due to its role in cholesterol biosynthesis and its association with the PI3K/Akt/mTOR pathway and targeting this protein along with various other molecules involved in cancer resistance should be investigated further.

Another protein of interest in this current study was the DNA repair mechanism marker, PARP, which binds to single or double-stranded DNA breaks in response to DNA damage (Holleman *et al.,* 2005). At the breakage site, PARP catalyses the transfer of ADP-ribose polymers from the respiratory coenzyme NAD+ to nuclear acceptor proteins involved in chromatin structure,

DNA repair and DNA metabolism (Holleman *et al.,* 2005). PARP has been found to have an increased expression in cancer cells compared to normal cells. PARP expression promotes cancer relapse by regulating the metastatic process in a DNA repair-independent manner (Choi *et al.,* 2016). However, when resistant LTED cells are treated, PARP expression decreases as can be seen in Figs. 23 and 24. AP reduced the expression levels of PARP in these cells and when in combination with Tam, it reduced resistance to the treatment thus increasing cell death. Several studies have found when PARP expression is reduced or inhibited; a significant amount of double-stranded breaks occur (Kim *et al.,* 2017). The inhibition of PARP expression disrupts several mechanisms in the cell such as interfering with the promotion of metastasis thereby leading to suppressed invasion and colonisation of distal organs (Rodríguez *et al.,* 2013), anoikis-like apoptosis, and decreased self-renewal of cancer cells (Choi *et al.,* 2016). The inhibition of PARP also leads to anti-tumour activity and anti-angiogenic properties (Quiles-Perez *et al.,* 2010). Currently, there are several clinical trials underway making use of PARP inhibitors in decreasing cancer cell growth and resistance in these cells. Therefore the combination treatment of Tam and AP leads to inhibited PARP expression in resistant LTED cells (Figs. 23 and 24), possibly resulting in significant DNA damage and cell death.

This preliminary study provided baseline results in treating resistant ER+ BC cells with a cholesterol depletor and chemotherapeutic drug. The reduction of cancer-related drug resistance through depletion of excess cholesterol in BC cells is a novel concept and therefore requires much investigation. The baseline results indicated that the combination treatments can be effective at inhibiting cell growth, inducing early-stage apoptosis and depleting cholesterol content within these cells. Therefore the current findings suggest that AP has the potential to be an adjuvant compound for endocrine therapy with increased efficacy.

6. CONCLUSIONS

This study sought to investigate AP's potential at enhancing Tam's efficacy at low concentrations by decreasing cholesterol accumulation and cancer-related drug resistance in both MCF7 and LTED cells. The key findings of this study are highlighted as follows. ER+ BC cells (MCF7 cells) that were deprived of estrogen for long periods of time (>180 days) underwent three phases of adaptation to estrogen-free conditions. This led to the development of enhanced sensitivity or hypersensitivity to E_2 which led to increased ER expression in LTED cells. This hypersensitivity served as an adaptive mechanism in LTED cells, which is associated with the increased level of activation of MAPK and PI3K/Akt pathways. LTED cells were used in this study to mimic two mechanisms observed biologically. These include: low levels of estrogen circulating the body in post-menopausal women with ER+ BC, and the surgical removal of ovaries for reduced estrogen production once patients were diagnosed with ER+ BC. Although estrogen levels are low in these patients, tumours adapt to these conditions via several growth factor pathways and relapse occurs due to acquired drug resistance to current chemotherapeutic drugs. Therefore novel strategies for the treatment of LTED ER+ BC are crucial. This study found that combination treatments of Tam and AP at lower concentrations worked effectively in a synergistic manner inducing cytotoxicity via apoptosis in both MCF7 and LTED cells *in vitro*. AP significantly re-sensitised ER+ BC cells towards enhanced Tam treatment, therefore reducing cancer-related drug resistance via cholesterol depletion. In addition, it was found that increasing concentrations of combination treatments reduced cholesterol content in both cell lines indicating AP's function as a cholesterol depletor. The findings of this current study show that LTED cells have slightly lower cholesterol levels than MCF7 cells due to the conditions in which these cells were grown (hormone-free conditions). Therefore having reduced expression of CETP levels and increased expression of SREBP levels in order to synthesise cholesterol for increased proliferation and survival. PARP expression was inhibited in LTED cells when treated with AP and combination treatments, indicating AP's possible action in inducing DNA damage and cell death in resistant LTED cells. Taken together, the combination treatment of Tam and AP could be a promising novel strategy in treating resistant LTED ER+ BC by reducing cholesterol accumulation and inhibiting PARP expression as well as cancer-related drug resistance.

7. FUTURE WORK

The biological mechanisms for the observations made in the current study are still unknown as literature is limited. Therefore future work will be focused on observing and understanding the effects of combination treatments on cholesterol-related proteins. These proteins include: acetyl-coenzyme A acetyltransferase 1 (ACAT1) which prevents accumulation of toxic free cholesterol in cell membranes; LDLR which is involved in the uptake of cholesterol by the cell; liver X receptor (LXR) which maintains cholesterol homeostasis by decreasing cholesterol levels when activated; and proprotein convertase subtilisin/kextin type- 9 (PCSK9) which is involved in negatively regulating LDLR expression targeting it for lysosomal degradation. Western blots as well as real-time PCR will be used in order to analyse expression levels. Additionally, the caspase-3/7 apoptotic assay will be performed on MCF7 and LTED cells in order to confirm late-stage apoptosis in these cells. Lipid-raft staining will also be performed in order to visually represent the depletion of cholesterol within cell membranes. Further, elucidating the synergistic mechanisms between Tam and AP will be performed. The effects of changes in miRNA expression on genes involved in cholesterol metabolism and cancer drug resistance pathways will also be observed. Subsequently, *in vivo* studies should be performed to observe tumour regression in response to combination therapy. Studies on blocking growth factor signalling pathways in order to delay the development of E_2 hypersensitivity in LTED cells and acquired resistance should also be investigated in order to prolong disease-free survival.

8. LIMITATIONS OF THE STUDY

The use of *in vitro* models to study the molecular mechanisms involved in cancer-related drug resistance and to test novel compounds in the treatment of BC is important. Although these models are useful the limitation in this is that they are not true representations of the disease itself and does not account for other modifications present *in vivo.* This does not allow for the complete understanding of BC and resistance.

Testing chemotherapeutic drugs in cell models is not an accurate representation observed in the whole body system. A compound may show great efficacy *in vitro* but will not have the same effect on the tumour *in vivo*. Additionally, compounds may show great efficacy *in vivo* due to high toxicities which then renders it from advancing it to clinical trials. Animal models allow us to take the tumour microenvironment and whole system of the organisms into consideration whereas cell line models cannot allow this to happen. Using cell line models alone is not enough in a study dealing with so many factors of resistance in a tumour microenvironment, therefore animal studies needs to be conducted in order to compare to the results of cell line models. However the current study is a novel concept where baseline findings were needed in a cell line model before advancing into animal studies. The current findings provided us with insightful information in order to design future *in vivo* experiments and clinical trials going forward.

9. TROUBLESHOOTING

All experiments were standardised in MCF7 and LTED cells and assays were performed in several ways for optimal results.

MTT assay

The MTT assay was standardised with different concentrations of PL (5 μM, 10 μM, 20 μM and 40 μM) as a positive control. In addition, MTT solution was added in compound treated and non-treated cells and incubated at 37 °C for varying times (2, 3 and 4 hrs). It was found that 40 μM PL gave best results with MTT incubation of 2 hrs and formazan crystals were dissolved in 10% SDS with 10 mM HCl solution.

APOPercentage™ assay

This assay was standardised by a colleague in the lab. Cells were treated for various times (2, 4, and 24 hrs) and the most effective time was found to be 2 hrs as cells treated for longer started detaching from wells and were washed away. Different volumes of APOPerctange™ dye (0.5 and 1 μL) were also tested against the cells and 0.5 μL was the most effective volume as the dye became too toxic at high concentrations.

MOMP assay

The MOMP assay was standardised by a colleague in the lab. Varying volumes and concentrations of the dye, serum-free media and EDTA were tested. Varying incubation times were also tested in order for cells to be detached using EDTA and cells were seen to be completely lifted from the wells at 40 min. During analysis using flow cytometry, cells were kept on ice and in the dark due to the light sensitive nature of the JC-1 dye.

Cholesterol quantification assay

The cholesterol quantification assay was standardised due to limited funds preventing the purchase of kits. Therefore components of the kit were purchased separately and varying concentrations and volumes of cholesterol oxidase, cholesterol esterase, catalase, HRP and Ampliflu™ Red were tested. Different buffer solutions (PBS, Isopropanol: NP-40 and dH_2O) were also tested. It was determined that Isopropanol: NP-40 was the most effective producing more readable results. The addition of catalase greatly reduced background noise and was therefore chosen to be added to cells 15 min prior to the addition of Reagent A. Amplex Red was kindly synthesized by another laboratory and it was found that it had similar effects to that

of Ampliflu™ Red, but was more accessible to our laboratory. Different volumes of Reagent A were tested to add to cells and it was determined that the lowest, most effective volume was 25 µL to each well. Reagent A preparation was also performed and then stored at -20 °C for future use, however activity was lost due to multiple freeze-thaw cycles and the exposure of Reagent A to light at multiple times. Therefore Reagent A was made fresh for each run.

SDS-PAGE and Western blotting

SDS-PAGE was standardised by running gels at various concentrations (8, 10 and 12%). The most common concentration was a 10% gel, however based on the specific proteins required, percentage of gels differed. Various concentrations of sample lysates were loaded (5, 10 and 20 mg/mL) in order to obtain equal loading. Gels were run at different speeds (100–130 V) and it was shown that 120 V was the most effective voltage at running the gel.

Western blots were standardised by testing various blocking times (1-2 hrs or overnight) and it was found that a 1 hr blocking incubation was sufficient. In addition, two concentrations of blocking solutions were tested (5 and 10%) and it was found that 5% blocking solution greatly reduced the background of the blots once viewed and there was less non-specific binding. Various concentrations and dilutions of both primary and secondary antibodies were tested. Primary antibody dilution factors varied from 1:500 to 1:2000, whereas secondary antibodies varied from 1:1000 to 1:5000. The incubation periods of antibodies were also tested and it was found that an overnight incubation of the primary antibody at 4 °C and a 1 hr incubation of the secondary antibody at RT led to cleaner blots with protein of interest easily detected. Differing volumes and times of chemiluminescence substrates were also performed and it was found that 2 mL of each substrate incubated with the membrane for 5 min led to high detection of proteins.

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