ANTICANCER RESEARCH *42*: 565-579 (2022)
doi:10.21873/anticanres.15514

Cholesterol Depletion Modulates Drug Resistance Pathways to Sensitize Resistant Breast Cancer Cells to Tamoxifen

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Abstract. *Background: Cancer drug resistance poses a significant risk of relapse and mortality. Adjuvant tamoxifen use has significantly reduced breast cancer mortality; however, many patients relapse due to acquired resistance. We aim to assess the potential of a cholesterol depletor (acetyl plumbagin) combined with tamoxifen to reduce cholesterol accumulation and cancer drug resistance. Materials and Methods: Cell viability, apoptosis and cholesterol staining was assessed following combination treatment. Gene and protein expression in cancer drug resistance and lipoprotein signalling pathways were assessed using RT^2 Profiler™ PCR arrays and STRING networks. Results: Combined treatment led to an increase in apoptosis and reduced intracellular cholesterol in MCF-7 and long-term estrogen deprived (LTED) cells compared to single compound treatments. Furthermore, the combination treatment perturbed several cholesterol-related and cancer-drug resistance pathways. Conclusion: The present study demonstrates the efficacy of tamoxifen combined with acetyl plumbagin in potentially disrupting the PI3K/Akt/PKB and Akt/mTORC1 signalling pathways in MCF-7 cells, reducing breast cancer cell proliferation and resistance.*

Breast cancer is the second most common malignancy worldwide with approximately 70% of cases being estrogen receptor positive (ER+) (1). The activity and signalling of the ER is modulated by genomic and non-genomic pathways contributing to ER-targeted treatment (2, 3). Selective estrogen receptor modulators (SERMs) act as estrogen antagonists by competing with estrogen to bind to the ER, blocking estrogen's ability in stimulating breast cancer cell growth. The most widely used SERM is the gold-standard adjuvant drug, tamoxifen (TAM), which has been the primary ER+ breast cancer treatment for over 40 years. Although mortality rates have decreased by 25% since 1990 from breast cancer due to improved use of adjuvant TAM and chemotherapy (4, 5), patients often relapse with acquired resistance after prolonged use or in initial phases (de novo resistance) of TAM treatment (3).

Several mechanisms responsible for acquired TAM resistance in breast cancer cells have been proposed. These include: the dysregulation of the ER signalling pathway; mutations and/or post-translational modifications of the ER; alterations in signalling that control cell cycle, survival, proliferation, and inhibition of apoptosis; and the activation of several pathways providing tumours with alternative proliferative signals (2). When treated with TAM, breast cancer cells use alternative growth factor receptor pathways, such as the PI3K/Akt/MAPK pathway (6) reducing survival rates in breast cancer patients. Furthermore, increased mitogen-activated protein kinase (MAPK) activity has been observed in long-term estrogen deprived (LTED) breast cancer cell lines (6, 7). Studying LTED cells is important to elucidate mechanisms in post-menopausal women who have low levels of estrogen, but still have the ER present. LTED cells are usually unaffected by TAM treatment and are therefore inherently resistant to TAM by employing alternative growth factor receptor signalling pathways to activate ER (8, 9). Additionally, resistant LTED cells have increased expression of genes encoding enzymes within the cholesterol synthesis pathway which are associated with acquired TAM resistance (10). Cholesterol is linked to cancer progression by an increased rate of cholesterol biosynthesis and excess cholesterol accumulation in lipid rafts for sufficient proliferative abilities (11-14). Currently, there are two approaches targeting cholesterol as a possible therapy for breast cancer. The first approach involves the blocking of cholesterol synthesis using statins (15-17) and the second treatment involves the depletion of excess membrane cholesterol using cholesterol depleting agents (10, 18, 19). A detailed viewpoint of these approaches has been discussed

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Key Words: Breast cancer, drug resistance, cholesterol, tamoxifen, acetyl plumbagin.

in detail (14). The focus of the present study is depleting excess membrane cholesterol levels thereby inhibiting breast cancer growth along with reduction of drug resistance in cancer cells.

The administration of cholesterol depleting agents such as methyl-β-cyclodextrin (MβCD) and acetyl plumbagin (AP) has been effective in the promotion of cell death in breast cancer cells (14, 20, 21). These compounds deplete excess membrane cholesterol in breast cancer cells thus sensitising cells to chemotherapeutic drugs, such as TAM (19, 21, 22). This is accomplished by disrupting lipid raft integrity, which increases membrane permeability for chemotherapeutic drug passage (22). AP has anticancer and cholesterol depleting activities as it inhibits various cancer signalling and cholesterol-related pathways (21). A previous study found that the depletion of cholesterol using AP resulted in increased cancer cell death via mitochondrial-mediated apoptosis (21), demonstrating AP's ability to induce apoptosis by depleting cellular cholesterol. Therefore, we hypothesized that combining a cholesterol depleting agent (AP) with a chemotherapeutic drug (TAM) could effectively treat cholesterol-rich resistant ER+ breast cancer. Thus, this study investigated the molecular connection between cholesterol accumulation and TAM resistance by identifying the pathways that are perturbed through the modulation of cholesterol content in breast cancer cell line models.

Materials and Methods

Cell culture. The MCF-7 cell line was procured from the European Collection of Authenticated Cell Cultures and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% FBS (Celtic Molecular Diagnostics, Cape Town, SA; Biowest, Nuaillé, France) and 1% penicillin-streptomycin (Thermo Fisher Scientific). LTED cells were derived from MCF-7 cells and cultured in DMEM (phenol red-free) supplemented with 5% dextran charcoal stripped FBS (DC FBS) (Celtic Molecular Diagnostics; Biowest) and 1% penicillin-streptomycin. Both cell lines were cultured at 37˚C and 5 % CO_2.

Cell proliferation experiments. MCF-7 and LTED cell growth was observed with varying concentrations of estradiol (E_2) (Sigma-Aldrich, St. Louis, MO, USA) over time. Cells were seeded for 24 h at 37˚C in the presence of 1 nM and 10 nM E_2 respectively (7, 23). Cells were detached using 1 X trypsin/EDTA (Celtic Molecular Diagnostics; Biowest) and counted daily for 6 days using a Neubauer haemocytometer.

Growth inhibition. Growth inhibition was estimated using the MTT assay (Sigma-Aldrich) as previously described (21). A range of concentrations of TAM (Sigma-Aldrich), AP [synthesized as described (20)], and a combination of TAM and AP was tested as indicated in the results. The appropriate concentrations for downstream assays were selected based on the results of the MTT assay. Control wells had equivalent volumes of 40 μM plumbagin (PL) (Sigma-Aldrich). The optical density (OD) was subsequently measured at 570 nm using a Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ software).

Cell death experiments. Cells were treated with appropriate concentrations of compounds and controls as indicated in the results for 2 h, with 40 μM PL as a positive control. Media was removed and cells were stained with an APOPercentage™ (Biocolor, Carrickfergus, UK) dye as previously described (24). OD was measured at 550 nm.

A mitochondrial outer membrane potential (MOMP) assay was performed as described (24). Cells were treated for 24 h and stained with a JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Sigma-Aldrich) for 30 min, with 40 μM PL as a positive control. Analysis using the BD Accuri™ C6 flow cytometer and software (BD Biosciences, Franklin Lakes, NJ, USA) was performed by plotting FL2-A *vs* FL1-A and applying a quadrant gate to determine JC-1 aggregates and monomers.

Cholesterol staining. Cells were treated as indicated in results. Positive control wells had equivalent volumes of 1 mM MβCD (Sigma-Aldrich). Following treatment, cholesterol was stained with several fluorescent dyes and quantified. Namely, filipin (Sigma-Aldrich) for free cholesterol, CholEsteryl BODIPY™ FL C12 (Thermo Fisher Scientific) for cholesteryl esters (CEs) and the Vybrant™ Alexa Fluor™ 594 Lipid Raft Labelling Kit (Invitrogen™ , Thermo Fisher Scientific) for lipid rafts. Nuclei were stained with either DAPI (Sigma-Aldrich) or NucRed® Dead 647 ReadyProbes® Reagent (Thermo Fisher Scientific). Cells were visualised using the FLoid™ Cell Imaging Station (Thermo Fisher Scientific) and densitometry analysis was performed using the ImageJ1 Software (NIH, Bethesda, MD, USA). Sample values obtained were normalized based on the average values of the negative control.

Western blotting. Cells were treated for 24 h as indicated in the results and protein lysates were quantified using a bicinchoninic acid assay. Normalised whole-cell lysate was subjected to SDS-PAGE, transferred to PVDF membranes and probed with antibodies to cholesteryl ester transfer protein (CETP), ER, and sterol regulatory element-binding protein (SREBP) (Novus Biologicals, Centennial, CO, USA). Anti-β-Actin and anti-β-Tubulin (Sigma-Aldrich) were used as loading controls. Densitometry analysis was performed using the Image Lab™ 4.0 Software (Bio-Rad, Hercules, CA, USA).

RT^2 Profiler™ PCR arrays. Human Cancer Drug Resistance and Human Lipoprotein Signalling & Cholesterol Metabolism RT^2 Profiler™ PCR arrays (Qiagen, Hilden, Germany) were used post combination treatment. RNA was extracted and cDNA synthesis was performed using the RT^2 First Strand Kit (Qiagen) as per the manufacturer's protocol. Synthesized cDNA samples were mixed with the RT^2 SYBR Green qPCR Mastermix (Qiagen) and added to the appropriate array of interest, after which RT-qPCR was performed, with appropriate controls using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Analysis was performed using the RT^2 Profiler™ PCR Array Data Analysis v3.5 software (Qiagen). Relative changes in gene expression were analysed using the $2^{-\Delta\Delta CT}$ method, comparing the treated to the untreated group. Differentially expressed genes were identified as those with a $|\log_2$ (fold-regulation) $| \geq 1$ against the untreated group.

Heatmaps were generated using MultiExperiment Viewer (MeV 4.9.0) (J. Craig Venter Institute, La Jolla, CA, USA) (25) to visualise differentially expressed genes with $\log_2$ fold-regulation. OmicsNet (Institute of Parasitology, Quebec, Canada) was used to identify protein-protein interactions of the differentially expressed genes (26) and networks of molecular interactions and biological pathways were created using Cytoscape (version 3.7.2) (Institute for Systems Biology, Seattle, WA, USA) (27).

Statistical analyses. Statistical analysis was performed using Microsoft Office Excel© and GraphPad Prism version 5 (CA, USA). The statistical significance of differences between control and treated sample cells were calculated using the student's *t*-test and one-way analysis of variance (ANOVA) followed by a Bonferroni *post-hoc* test. A *p*-Value of <0.05 (*) was the critical value for significance. A Z-factor was also calculated for each assay (above >0.6) performed in 96-well plates, indicating good to excellent robustness (28).

Results

Development and characterization of the LTED cell line. The LTED cell line was developed from MCF-7 cells (Supplementary Figure 1) as explained previously (8). *In vitro* studies have revealed that estrogen deprived breast cancer cells pass through three phases. These phases include: a quiescent phase (phase 1) where cell proliferation is decreased and ER expression is low within the first 90 days; a hypersensitive phase (phase 2) where cell growth is stimulated by the addition of exogenous estrogen within 180 days; and an independent phase (phase 3) in which exogenous estrogen has no effect on cell growth after 180 days and there is a 2-4 fold increase in ER binding sites (29).

Following the development of the LTED cell line, the effects of varying concentrations of E_2 on the growth of MCF-7 and LTED cells were observed (Figure 1A). Increasing concentrations of E_2 had stimulatory effects on MCF-7 cell growth, whereas in LTED cells this effect was not observed within 90 days. Low concentrations of E_2 (1 nM) stimulated LTED growth after 90 days, however at higher concentrations (10 nM), cell growth decreased, indicating that LTED cells grow independently of E_2. It is evident that E_2 did not have stimulatory effects on LTED cell growth, and these cells grow independently of E_2 after 180 days of adaptation.

Western blots confirmed ER expression of LTED cells during the three phases of adaptation (Figure 1B). Within 90 days of culturing in estrogen-free conditions (quiescent phase), ER expression of LTED cells was significantly downregulated compared to MCF-7 cells. Within 180 days (hypersensitive phase), ER expression in LTED cells was still lower in comparison to MCF-7 cells. However, ER expression in LTED cells was increased from phase 1 to phase 2. After 180 days (independent phase), ER expression was up-regulated in LTED cells compared to MCF-7 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.

TAM and AP induces apoptosis in MCF-7 and LTED cells
Upon treatment with TAM and AP, MCF-7 cells had a ~65% greater increase in growth inhibition when compared to single TAM treatments. Similarly, LTED cells had a ~79% greater increase as seen in Figure 2A-F. Cells were treated with several single TAM and AP treatments (data not shown) however, no treatment surpassed 50% growth inhibition therefore, IC 50 values could not be accurately calculated. The combination treatment of 5 μM TAM + 10 μM AP was most effective at inhibiting MCF-7 cell growth and 5 μM TAM + 20 μM AP in LTED cells, indicating that AP greatly enhances TAM's efficacy at low concentrations.

Based on the MTT assay, a range of concentrations of TAM, AP, and a combination of the two were selected for measuring the level of apoptosis using an APOPercentage™ assay (21). MCF-7 and LTED cells experienced a higher rate of apoptosis when treated with combination treatments than single treatments (~40% increase in MCF-7 cells and ~30% increase in LTED cells) as seen in Figure 2G-L and Supplementary Figure 2. TAM's efficacy in inducing early-stage apoptosis significantly increased when combined with AP, indicating an additive effect. These experiments show that the combination treatment of 1 μM TAM + 10 μM AP was effective at inducing apoptosis in breast cancer cells *in vitro*. Therefore, further experiments were performed using the 1 μM TAM + 10 μM AP combination.

In order to observe the molecular changes associated with treatment, a MOMP assay was performed using JC-1 (30). When MCF-7 and LTED cells were treated with combinations, there was a significant increase in mitochondrial membrane potential disruption in MCF-7 and LTED cells, indicating increased apoptosis as seen in Figure 2M-N. The interaction of co-treatment is effective in inducing cell death in both MCF-7 and LTED cells.

TAM and AP reduces cholesterol content in MCF-7 and LTED cells. When comparing untreated LTED and MCF-7 cells, LTED cells had more cholesterol due to these cells being more resistant in nature. TAM and AP co-treatment led to a significant reduction in free cholesterol in both MCF-7 (~80% reduction) and LTED (~22% reduction) cells compared to untreated cells (Figure 3A). Similarly, co-treatment led to a significant reduction in CEs in both MCF-7 and LTED (~80% reduction) cells compared to untreated cells (Figure 3B). Finally, co-treatment also significantly reduced the amount of lipid rafts in MCF-7 (~55% reduction) and LTED (~75% reduction) cells compared to untreated cells (Figure 3C). This confirms that TAM and AP treatment is effective at depleting excess cholesterol accumulation within MCF-7 and LTED cells thereby

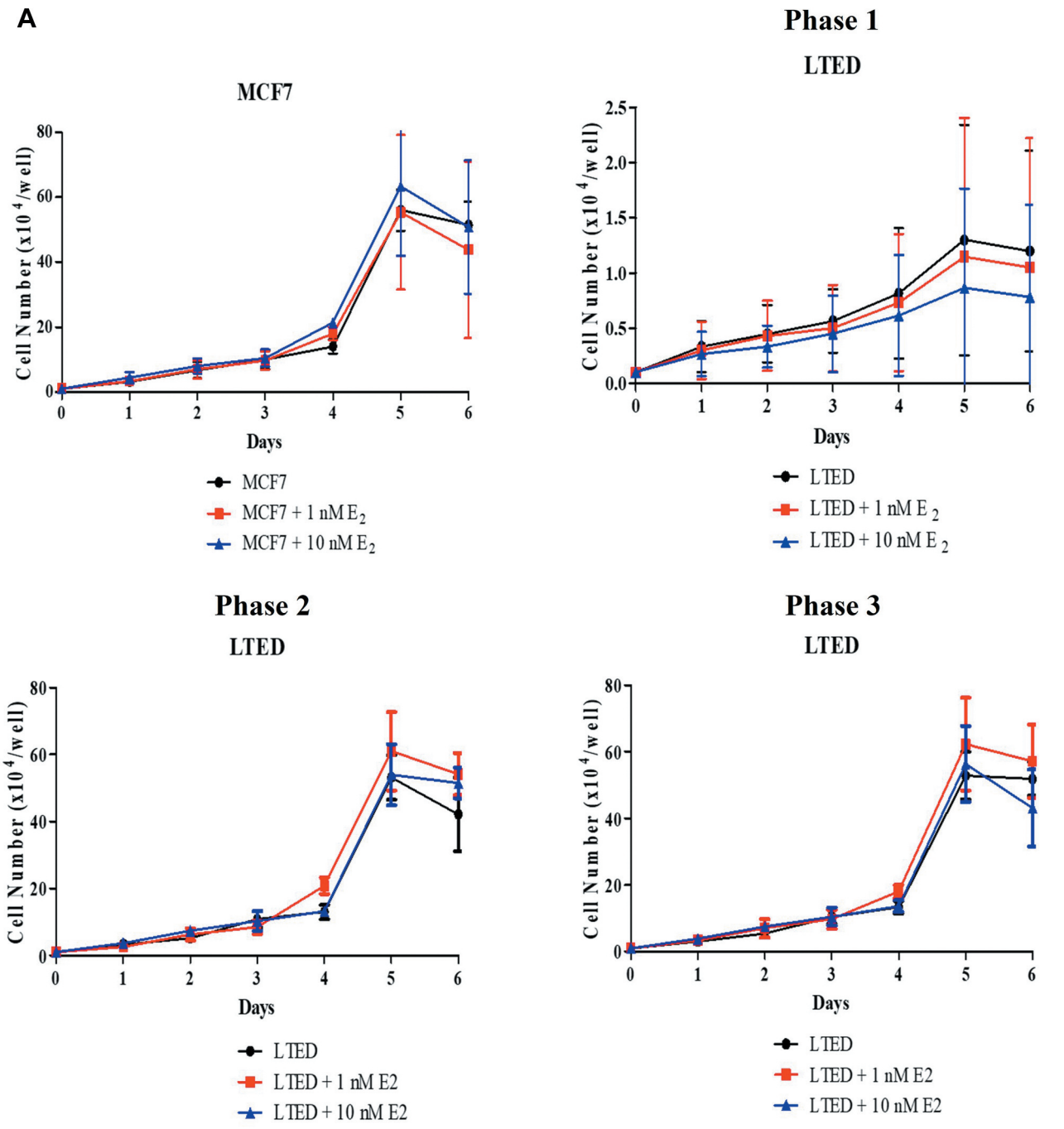

Figure 1. *Continued*

inducing apoptosis and possibly lowering cancer-related drug resistance in breast cancer cells.

TAM and AP alters cholesterol-related protein expression in MCF-7 and LTED cells. The expression of several proteins was observed to elucidate molecular mechanisms of resistance in breast cancer cells. Basal ER (66 kDa) expression levels were increased in LTED cells in comparison to MCF-7 cells (Figure 4A) due to the adaptation of LTED cells to low levels of estrogen as seen in Figure 1. However, ER expression was reduced upon co-treatment, indicating the efficacy of this treatment on resistant breast cancer cells. CETP (77 kDa) expression levels were lower in LTED cells than in MCF-7 cells because LTED cells have slightly lower intracellular

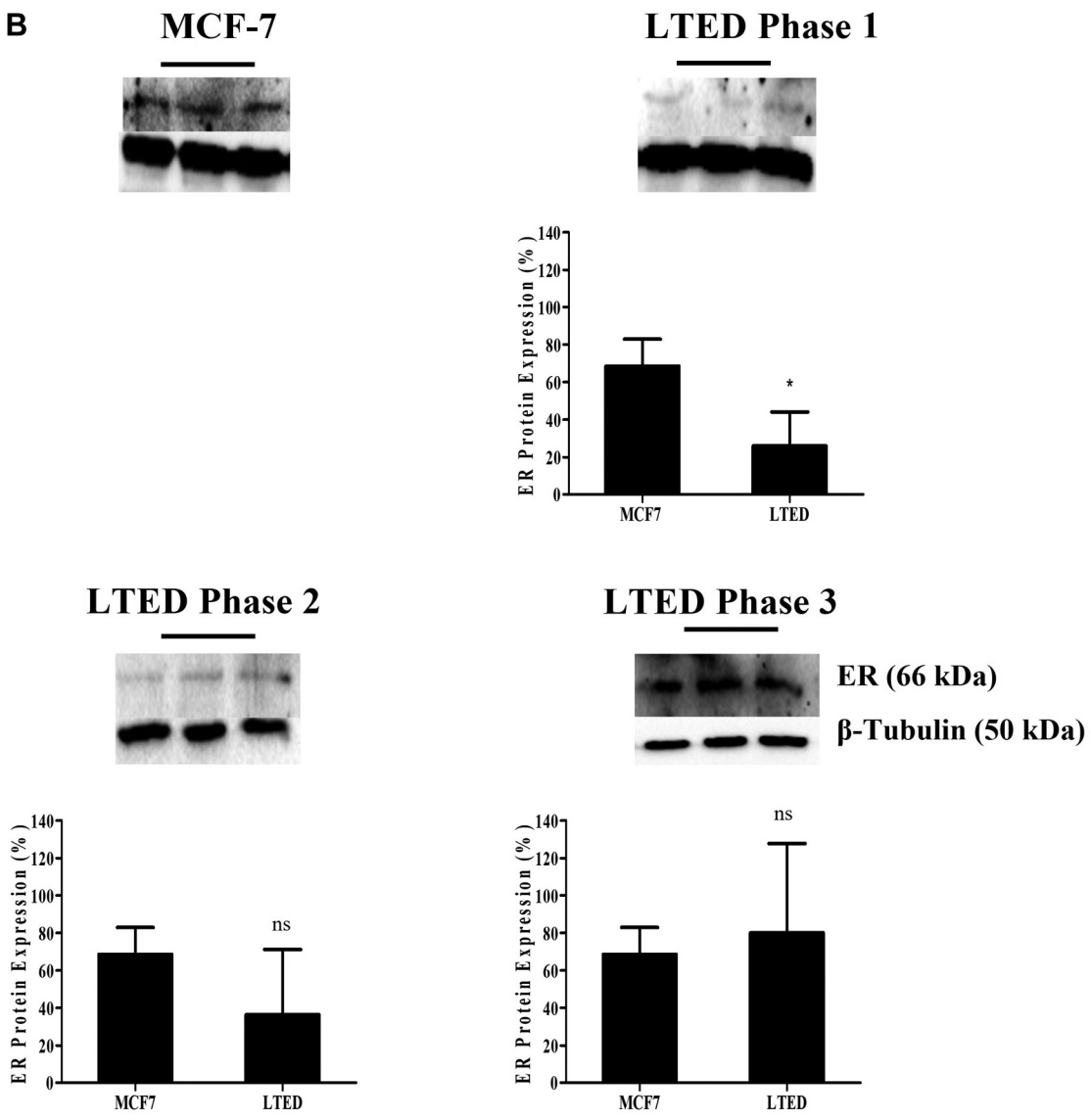

Figure 1. *E_2 effects on MCF-7 and LTED cell growth. (A) Representative graphs indicating the effects of increasing concentrations of E_2 (1 and 10 nM) on MCF-7 and LTED (phases 1-3) cell growth over time. Cells were grown and treated with E_2 at the indicated concentrations for a period of 6 days and cell numbers were counted; (B) Western blots indicating ER expression (66 kDa) in LTED cells in the 3 different phases of adaptation compared to parental MCF-7 cells. A Precision Plus Protein™ Unstained standard was used as a molecular weight marker. Anti-β-Tubulin (50 kDa) was used as a loading control indicating equal loading of lysate. Data are mean±S.D (n=3) from raw data, where *p<0.05 indicates significant difference to untreated control.*

cholesterol content than MCF-7 cells. LTED cells had slightly more SREBP (50 kDa) expression than MCF-7 cells because of lower cholesterol levels.

Interestingly, LTED cells displayed almost no difference in CETP expression when treated with TAM, AP, or their combinations (Figure 4B-C), whereas CETP was down-regulated in MCF-7 cells after treatments. These results demonstrate that different molecular mechanisms are operative in different cell types. This observation led us to investigate the dynamics of molecular pathways operative in these cell types.

TAM and AP alters gene expression involved in lipoprotein signalling and cancer drug resistance pathways in breast cancer cells. Using the Human Cancer Drug Resistance and the Human Lipoprotein Signalling & Cholesterol Metabolism RT^2 Profiler™ PCR Arrays, the effects of the

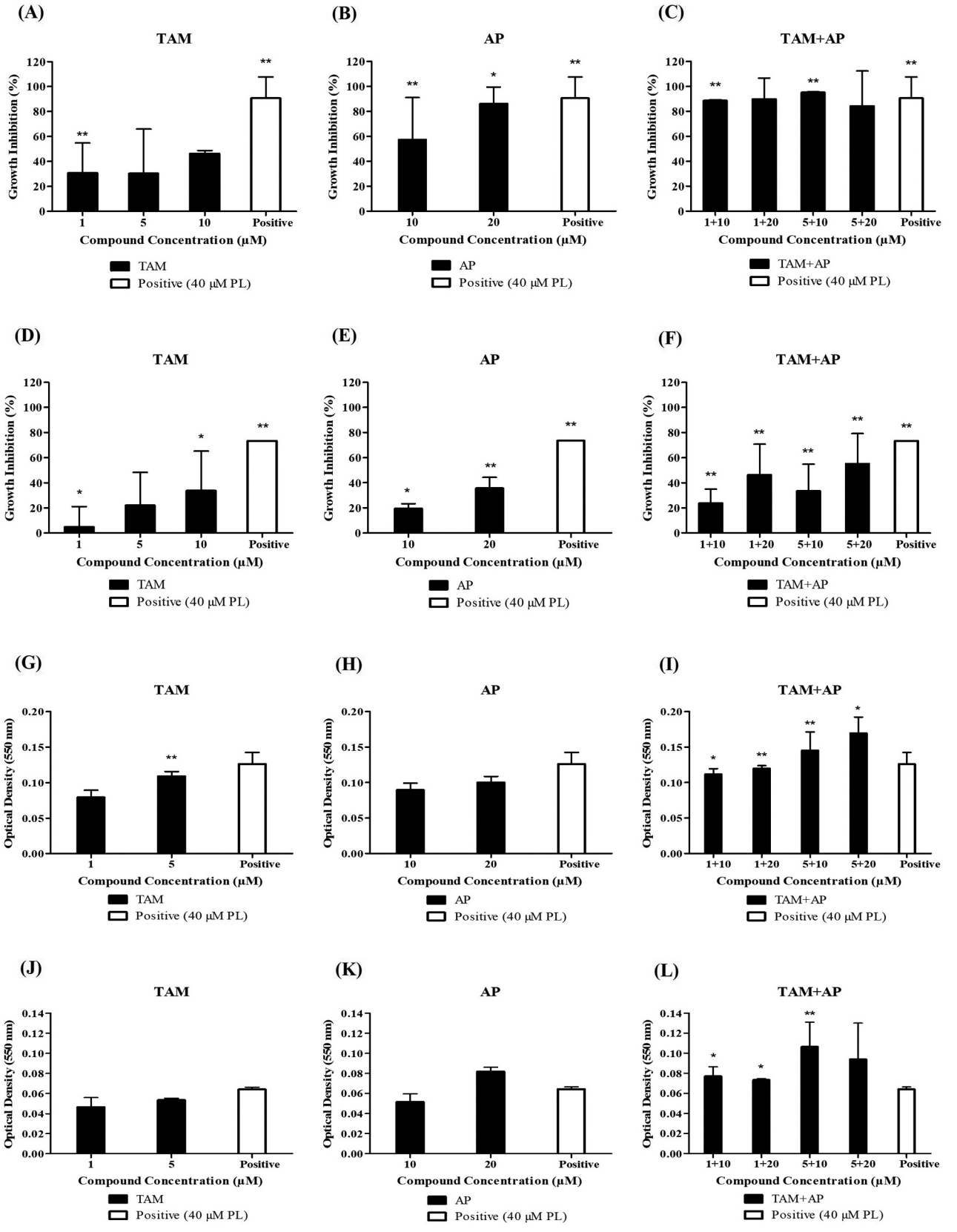

Figure 2. *Continued*

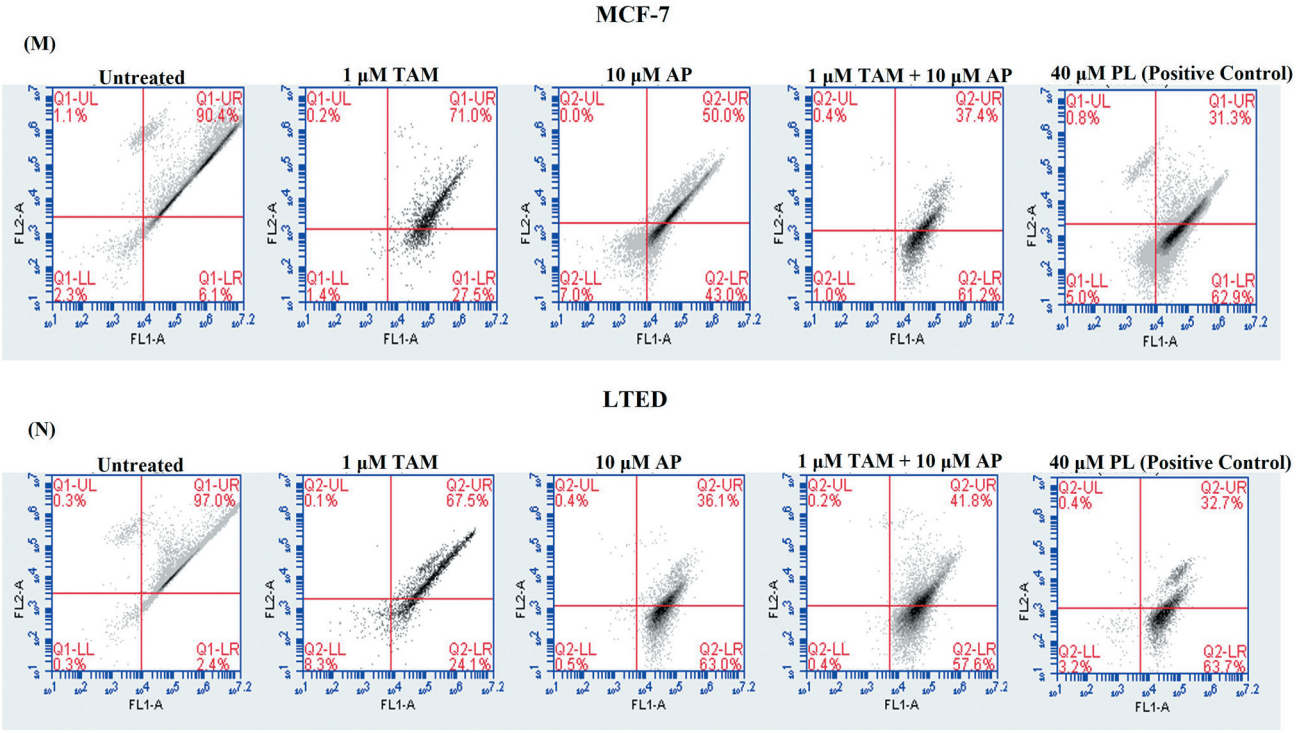

Figure 2. *Increased growth inhibition and apoptosis in MCF-7 and LTED cells post TAM and AP treatment. Representative graphs of the percentage growth inhibition of MCF-7 (A-C); and LTED (D-F) cells at selected concentrations of TAM, AP and a combination of TAM and AP. Representative graphs of the optical density (550 nm) in comparison to the untreated control observed in MCF-7 (G-I); and LTED (J-L) cells undergoing apoptosis at selected concentrations of TAM, AP and a combination of TAM and AP after treatment for 2 h. Representative gated flow cytometry analysis with plotted FL2-A vs. FL1-A parameters observed in MCF-7 (M); and LTED (N) cells at selected concentrations of test compounds TAM, AP and a combination of TAM and AP. Bottom left, bottom right, top right and top left quadrants indicate unstained cells, early-stage apoptotic cells, live cells and necrotic cells, respectively. The percentage indicated in the bottom right quadrant represents the amount of MOMP loss observed. PL (40 μM) was used as a positive control for all assays above. Data are mean±S.D (n=3) from raw data, where *p<0.05 and **p<0.01 indicates significant difference to untreated control.*

combination of 1 μM TAM and 10 μM AP was observed on MCF-7 and LTED cells. The genes, their mode of action and their fold-regulation are summarised in Supplementary Table I and Supplementary Table II. Not all the genes tested from several biological pathways involved in drug resistance and metabolism, cell cycle control, DNA damage repair, transcription factors, cholesterol efflux, metabolism, catabolism, biosynthesis, homeostasis, and transport, were perturbed by the treatment (Figure 5). Functional STRING networks of lipoprotein and cancer drug resistance associated protein-protein interactions were created for both cell lines post co-treatment (Supplementary Figure 3 and Supplementary Figure 4), indicating the complex interactions between cholesterol and drug resistance pathways.

Cancer drug resistance. Using a gene expression threshold of $|\log_2$ (fold-regulation) $| >= 1$ relative to untreated cells, 14 out of 84 genes represented by the Human Cancer Drug Resistance RT^2 Profiler™ PCR Array were identified as having increased gene expression in MCF-7 cells and 16 genes in LTED cells upon co-treatment, with two genes being down-regulated in both cell lines. From the biological network analysis, cell cycle, hormone receptors, transcription factors and drug resistance pathways were affected upon treatment in MCF-7 cells, whereas drug metabolism, DNA damage and repair, and cell cycle pathways were mostly affected in LTED cells upon co-treatment.

In general, *CYP450* genes, which metabolize TAM, were up-regulated, demonstrating that co-treatment increased sensitivity of cancer cells towards TAM. This is also contributed by increased expression of estrogen receptor 1 (*ESR1*), especially in LTED cells, thus sensitizing these cells towards TAM treatment (Figure 1 and Figure 5). Genes such as xeroderma pigmentosum, complementation group C (*XPC*) (DNA damage and repair), ATP binding cassette subfamily C member 3 (*ABCC3*), B-cell lymphoma 2 (*BCL2*), and nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-kB*) were significantly up-regulated in

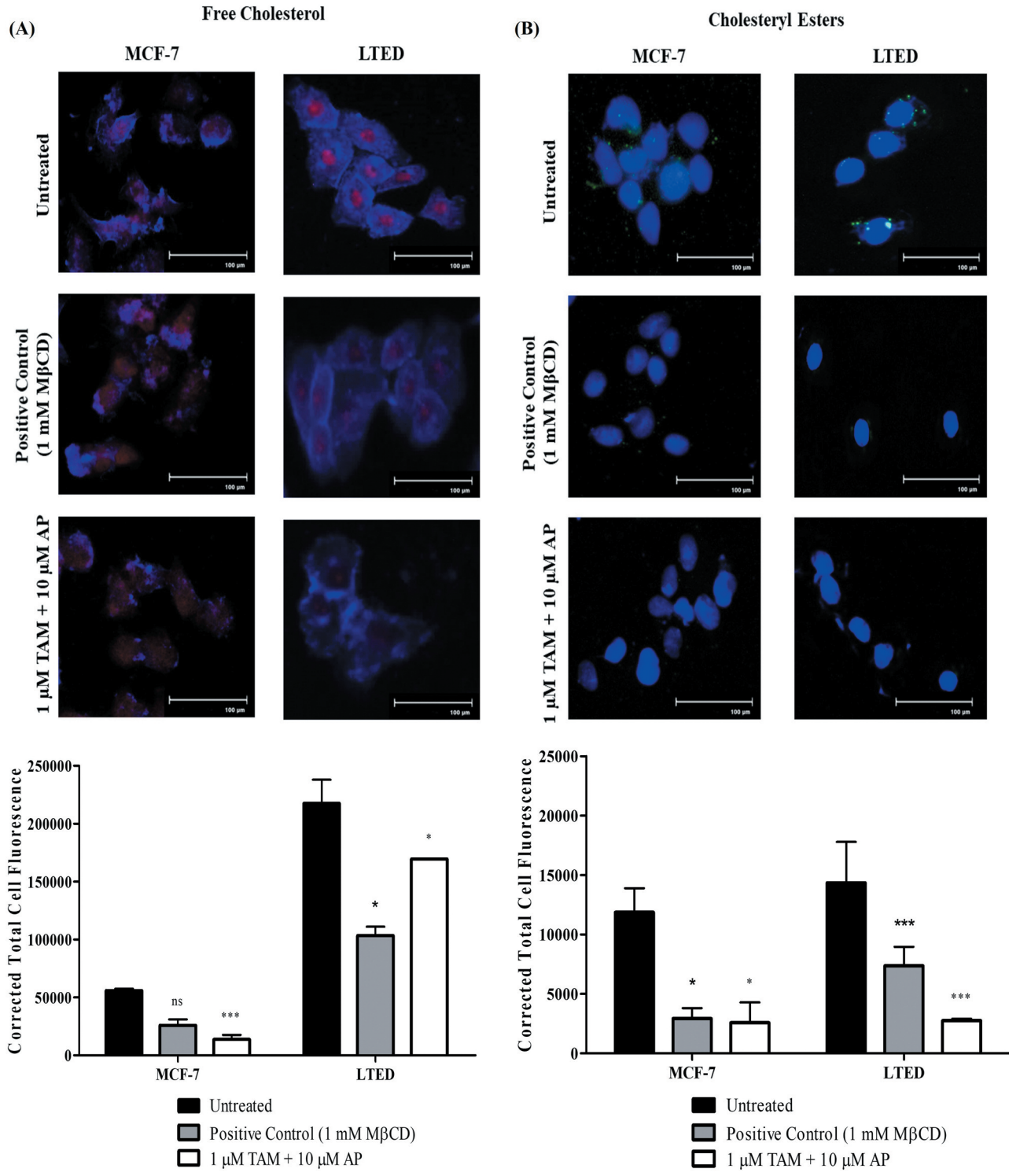

Figure 3. *Continued*

LTED cells. Therefore, suggesting that the interplay of different pathways aid cancer cells in acquiring resistance to chemotherapeutic drugs.

The aberrant expression of several transcription factors such as FOS proto-oncogene, AP-1 transcription factor subunit (*FOS*) can have a significant impact on cancer cells'

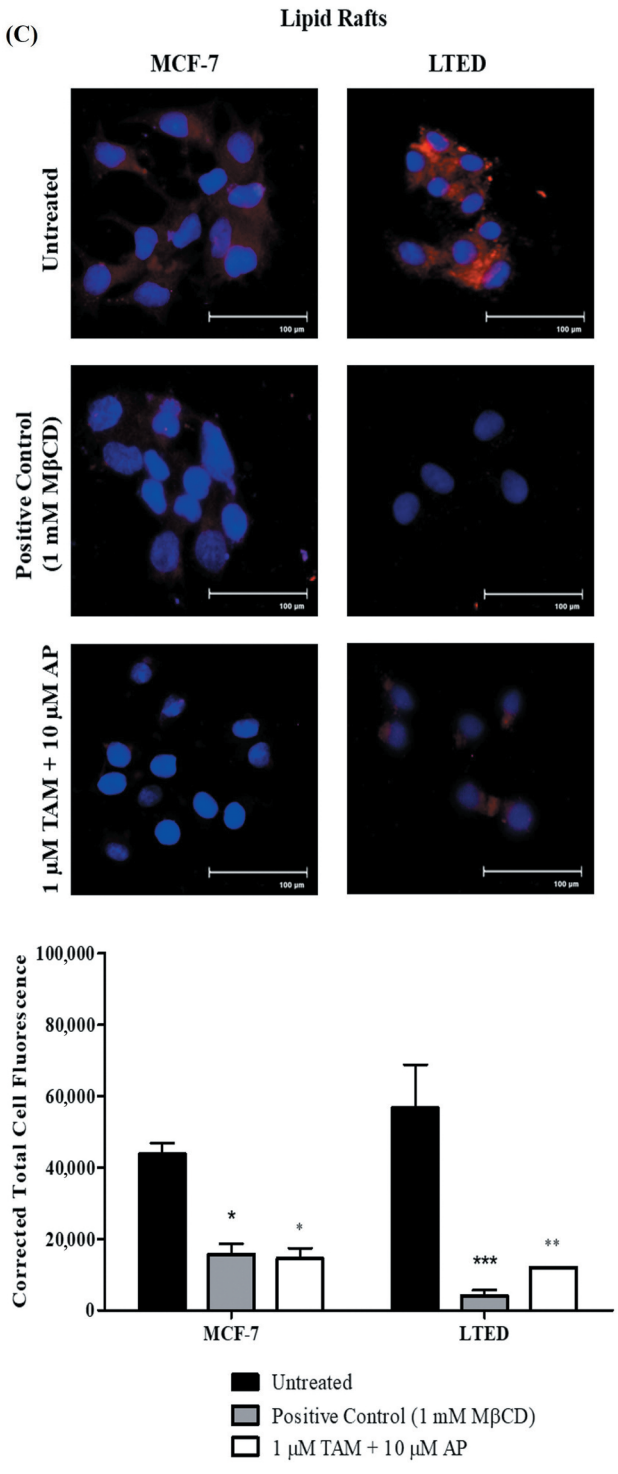

Figure 3. *Reduced cholesterol in MCF-7 and LTED cells post TAM and AP treatment. Representative fluorescent images and graphs from untreated and TAM + AP treated MCF-7 and LTED cells with a positive control (1 mM MβCD). (A) Free cholesterol is shown in blue, and respective nuclei are shown in red; (B) CEs are shown in green, with respective nuclei in blue and (C) Lipid rafts are shown in red and respective nuclei in blue. Treated groups presented an overall reduction in free cholesterol, CEs, and lipid rafts in comparison to untreated control. Images were taken using the EVOS FLoid™ Cell Imaging Station (Invitrogen, ThermoFisher Scientific). Scale bars indicate 100 μM. Bar graphs depict corrected total cell fluorescence in untreated and treated cells with a positive control. ImageJ software was used for fluorescence quantification. Data were analysed by one-way ANOVA followed by a Bonferroni post-hoc test to compare individual differences. The data are the means±SD *p<0.05, **p<0.01, ***p<0.001; ns non-significant compared to untreated; n=3 per group.*

response to therapies. It was observed that *FOS* was down-regulated in MCF-7 cells and *ESR1* was up-regulated, thus contributing to TAM sensitivity observed in Figure 2 as overexpression of *FOS* has been linked with low ER expression and TAM resistance (31). Hypoxia-inducible factor 1-alpha (*HIF-1α*) and MYC proto-oncogene, BHLH transcription factor (*MYC*) were down-regulated in LTED cells, which further affected several genes involved in pathways relating to drug resistance, cell cycle, DNA damage and repair, drug metabolism, and hormone and growth factor receptors.

The increased expression of the ATP-binding cassette (ABC) transporter family proteins is one of the major factors contributing to multidrug resistance (MDR) due to increased drug efflux. The genes *ABCC3* and *ABCC5*, which serve as markers for MDR in many cancers, had increased gene expression upon treatment in LTED cells. Genes involved in the drug resistance pathway had higher expression post treatment in LTED cells than in MCF-7 cells, indicating inherent TAM-resistance of LTED cells.

Lipoprotein signalling and cholesterol metabolism. Using the Human Lipoprotein Signalling & Cholesterol Metabolism RT^2 Profiler™ PCR Array, 17 out of 84 genes were up-regulated in MCF-7 cells upon treatment, with 7 genes being down-regulated. Whereas, in LTED cells, 12 genes were up-regulated upon treatment and 3 genes down-regulated (Figure 5). Genes involved in the cholesterol biosynthesis, transport, metabolism and homeostasis pathways were affected in both MCF-7 and LTED cells upon treatment, with MCF-7 cells displaying more down-regulated genes across all pathways. This indicates that TAM and AP work additively in dysregulating these pathways therefore reducing cell proliferation and survival, as well as MDR. Many lipoprotein genes were unaffected by treatment in LTED cells, possibly due to their resistant nature.

There was significant up-regulation in cholesterol transport gene expression (such as ATP binding cassette subfamily G member 1 (*ABCG1*)) in LTED cells compared to MCF-7 cells. Interestingly, TAM and AP did not affect the cholesterol biosynthesis pathway in LTED cells but did have

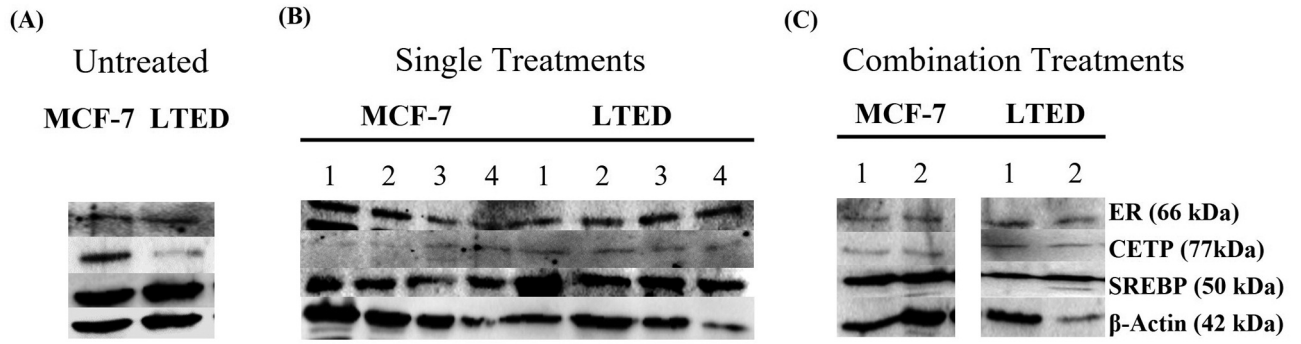

Figure 4. *Protein expression changes in MCF-7 and LTED cells upon single and combination treatments. Western blots comparing the basal expression levels of different proteins in (A) untreated MCF-7 and LTED cells; and MCF-7 and LTED cells treated with (B): single treatments; and (C): combination treatments of TAM and AP. A Precision Plus Protein™ Unstained standard was used as a molecular weight marker. Anti-β-Actin (42 kDa) was used as a loading control. B: Lane 1: 1 μM TAM, Lane 2: 5 μM TAM, Lane 3: 10 μM AP, and Lane 4: 20 μM AP. C: Lane 1: 1 μM TAM + 10 μM AP, Lane 2: 1 μM TAM + 20 μM AP.*

effects in MCF-7 cells. This indicates that the use of cholesterol biosynthesis inhibitors (statins) may not work on resistant breast cancer cells.

Apolipoprotein F (*APOF*) was up-regulated in both cell lines post co-treatment. Reduced expression of *APOF* is associated with a poor survival rate in breast cancer patients (Supplementary Figure 5), therefore, increasing this gene's expression leads to a higher survival rate due to increased cholesterol efflux and enhanced clearance from the cell. This shows that TAM and AP are effective in altering cholesterol metabolism and transport within resistant breast cancer cells.

Discussion

Combating TAM resistance by targeting cholesterol accumulation in breast cancer cell lines is a novel concept especially for resistant LTED cells. We have previously found that AP functions by depleting cholesterol in the cells, possibly by binding to and blocking CETP function. AP was found to regress tumour growth *in vivo* with no associated toxicity and has selective activation of caspases and apoptotic pathways in ER+ breast cancer cells (21). In this study, we have demonstrated the efficacy of a TAM and AP combination in inhibiting cell growth, inducing early-stage apoptosis and disrupting mitochondrial membrane potential in both MCF-7 and LTED cells. These findings indicate that the efficacy of TAM is significantly increased from single treatments (1 μM), when combined with AP (10 μM). This is significant, as the use of low doses in a clinical setting reduces toxicity in normal cells and aids in the reduction of acquired TAM resistance. This combination (1 μM TAM + 10 μM AP) is also within the safer limits of AP's cytotoxicity potential as well as its ability to counteract TAM resistance in breast cancer cells.

We also showed that co-treatment led to an overall reduction in cholesterol in both MCF-7 and LTED cells, providing evidence that cholesterol depletion sensitises cells towards enhanced TAM-mediated apoptosis in these cells. This was further confirmed, where CETP expression was down-regulated upon AP treatment in MCF-7 cells, indicating AP's function in halting CE transport, and possibly inducing DNA damage in these cells. This finding also demonstrates that the LTED cell line's resistance to therapy may be mediated by alternative intracellular cholesterol storage mechanisms independent of CETP.

Our gene expression analysis revealed that co-treatment altered a variety of drug resistance and lipoprotein signalling pathways in MCF-7 and LTED cells. This enabled us to identify several differentially expressed genes in both cell lines. The dysregulation of several pathways provides evidence that TAM and AP work additively at depleting excess cholesterol in breast cancer cells *in vitro* and derails several pathways such as drug efflux, cholesterol transport and cholesterol metabolism, leading to apoptosis and reduced cancer-related drug resistance.

Studies have revealed that ER+ breast cancer cells acquired resistance to TAM when *HIF-1α* was overexpressed, which is associated with poor survival in breast cancer patients undergoing endocrine therapy (Supplementary Figure 5) (32, 33). Studies demonstrated that *HIF-1α* inhibition restored TAM sensitivity in breast cancer cells (33). *HIF-1α*-induced TAM resistance involves crosstalk between several transcription factors, including epidermal growth factor receptor (EGFR) and MAPK, which may serve as therapeutic targets, alongside *HIF-1α* for TAM-resistant breast cancer patients (34, 35). A study showed that the EGFR-linked PI3K/Akt pathway drives cholesterol uptake through the up-regulation of low-density lipoprotein receptor (LDLR), a

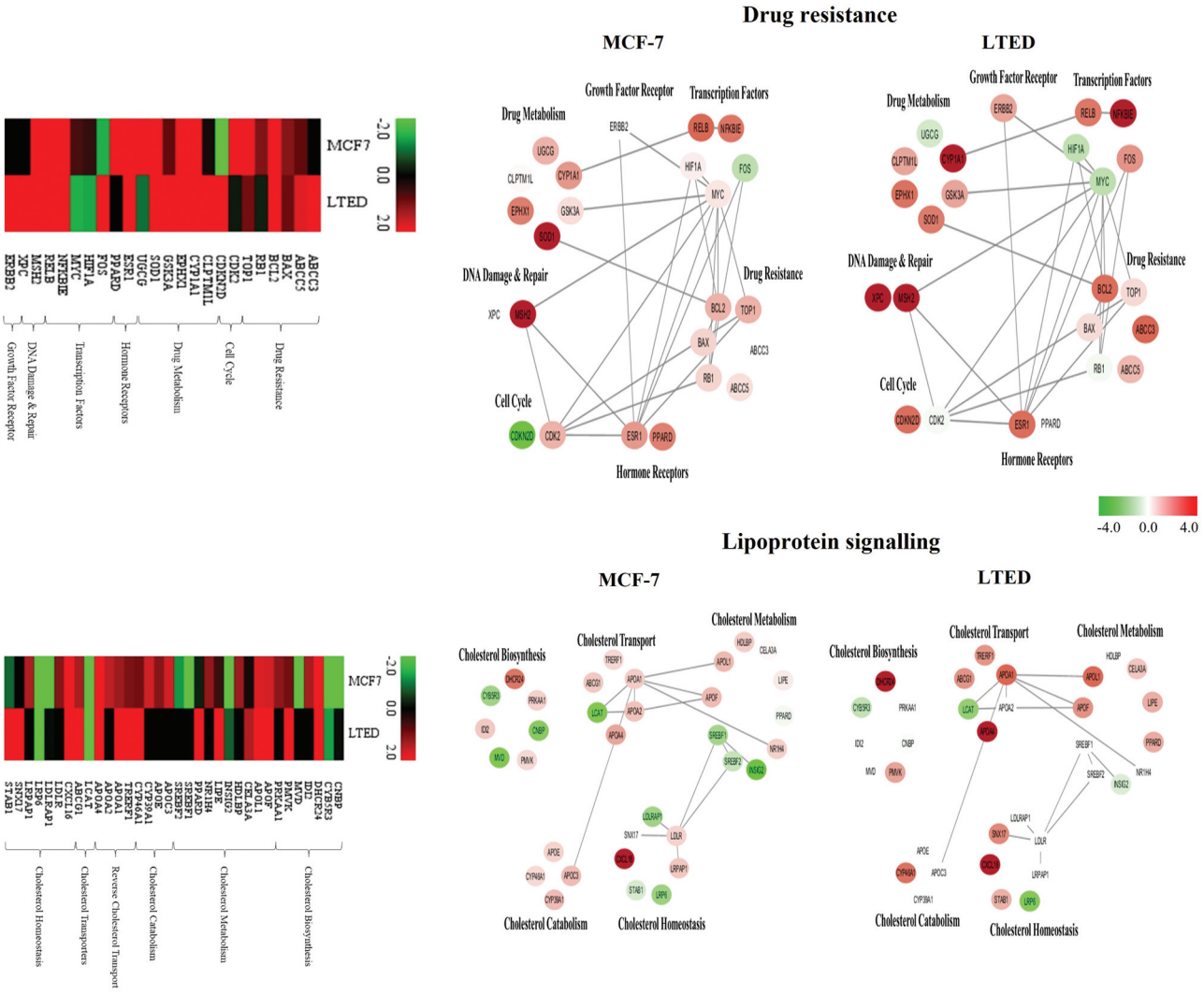

Figure 5. *Differential gene expression in drug resistance and lipoprotein signalling pathways post TAM and AP treatment. Heatmap and network visualisation of MCF-7 and LTED cell lines analysed using Human Cancer Drug Resistance and Human Lipoprotein Signalling & Cholesterol Metabolism RT² Profiler™ PCR Arrays. Heatmap: red (positive log2 fold-regulation) represents increase, black represents no change and green (negative log2 fold-regulation) represents decrease in gene expression of a sub-set of genes in TAM and AP treated MCF-7 and LTED cell lines compared to respective untreated controls. Similarly, in the network: red (positive log2 fold-regulation) represents increase, white represents no change and green (negative log2 fold-regulation) represents decrease. Only protein coding genes with |expression| >1 from selected pathways in at least one of the comparisons are displayed. Nodes represent genes and edges represent interactions. Genes were separated based on the biological pathways they are involved in.*

receptor involved in intracellular cholesterol uptake (36). Therefore, the reduction of *HIF-1α* via co-treatment could be a potential therapeutic approach in reducing breast cancer cell growth, resistance and possibly stemness.

Cholesterol transport is an important event in shuttling CEs to desired locations. The cholesterol efflux protein, ABCG1, depletes lipid rafts and mediates cholesterol efflux (37, 38). Excess cellular cholesterol is exported from the cell by ATP-binding cassette subfamily A member 1 (ABCA1) and ABCG1 or converted to less toxic CEs and then stored in lipid droplets or secreted within lipoproteins (39). Reduced *ABCG1* expression results in intracellular cholesterol accumulation leading to enhanced cell proliferation (37). A study revealed that ABCG1 gene and protein expression levels were enhanced upon liver X receptor (LXR) activation leading to increased cholesterol efflux to isolated HDLs (40). This deprives MCF-7 cells of cholesterol, resulting in inhibition of cell proliferation and enhanced apoptosis (40). High levels of apolipoprotein E (APOE) present in HDL particles as well as apolipoprotein

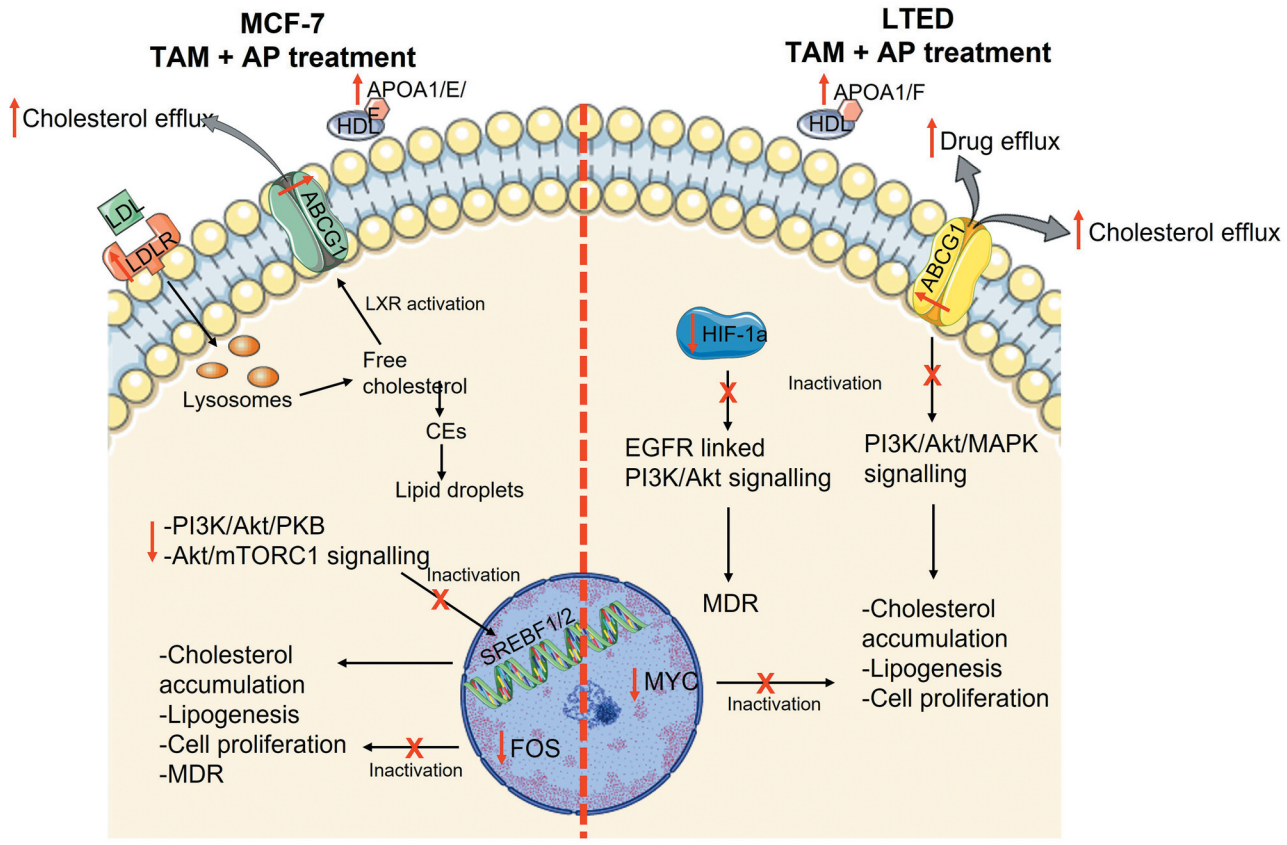

Figure 6. *Hypothetical model representing dysregulated events in MCF-7 and LTED cells upon TAM and AP treatment. Here, we propose that TAM and AP treatment mainly affects cholesterol metabolism in MCF-7 cells, whilst affecting cholesterol transport in LTED cells. In MCF-7 cells, we propose that co-treatment down-regulates the PI3K/Akt/PKB signalling pathway and the Akt/mTORC1 signalling axis, in turn inactivating the expression of SREBPF1/2. This leads to reduced cholesterol accumulation and lipogenesis within the cell, thereby reducing cell proliferation and MDR. TAM and AP treatment also up-regulates APOA-1/E/F expression to HDLs. Increased LDLR expression leads to increased free cholesterol which is fluxed out of the cell via ABCG1 due to LXR activation. Reduced FOS expression reduces cell proliferation, survival and MDR. We also propose that in LTED cells, the expression of ABCG1 is up-regulated upon co-treatment, causing an increase in CE and drug efflux due to reduced PI3K/Akt/MAPK signalling thereby reducing cholesterol accumulation and cell survival. TAM and AP treatment also up-regulates APOA-1/F expression to HDLs. Moreover, reduced HIF-1α expression inactivates EGFR-linked PI3K/Akt signalling which could restore TAM sensitivity thereby reducing TAM-related resistance in these cells. The down-regulation of MYC reduces cell proliferation, survival, and differentiation.*

A-1 (APOA-1) have important roles in driving cholesterol efflux via ABCG1 by promoting cholesterol esterification in HDL particles, especially in CETP-deficient environments (41). The current study revealed that TAM and AP increased both *APOA-1* and *APOE* expression leading to increased *ABCG1* expression therefore, reducing intracellular cholesterol accumulation, subsequently reducing breast cancer cell proliferation, survival, and resistance.

Increased PI3K/Akt/PKB signalling and overexpression of SREBP1 and SREBP2 is commonly activated in several cancers (42). One important downstream effector of Akt is mammalian target of rapamycin complex 1 (mTORC1), involved in the regulation of lipid synthesis (42). Interestingly, SREBP activation as a result of mTORC1 stimulates breast cancer cell proliferation and growth (43). The hyperactivation of Akt/mTORC signalling promotes breast cancer formation, indicating that SREBP-dependent lipogenesis is crucial in cell transformation. The present study demonstrates the efficacy of TAM and AP in disrupting the signalling cascade of the PI3K/Akt/PKB and Akt/mTORC1 signalling pathways in MCF-7 cells in turn reducing sterol regulatory element-binding transcription factors 1 and 2 (*SREBF1/2*) expression, leading to reduced breast cancer cell proliferation and resistance as seen in the hypothetical model Figure 6.

When breast cancer cells undergo resistance, there is a shift in gene expression. This study demonstrated that cholesterol and drugs are metabolised and transported quickly out of the resistant cell for survival and has provided an insight into

mechanisms in which TAM and AP exert their effects in breast cancer cells *in vitro*. The efficacy of a TAM and AP combination significantly improves the acquired resistance associated with breast cancer as lower concentrations of TAM are used. This will allow for a flexible dosage-increasing window over longer treatment periods and less harmful side effects in breast cancer patients.

The use of *in vitro* models to study molecular mechanisms involved in cancer-related drug resistance and to test novel compounds in the treatment of breast cancer is useful, although the limitation is that they are not true representations of the disease itself and does not account for several modifications present *in vivo*. However, baseline findings were needed in a cell line model before advancing into future *in vivo* studies.

Conclusion

This study sought to investigate AP's potential at enhancing TAM's efficacy at low concentrations by reducing cholesterol accumulation and cancer-related drug resistance in MCF-7 and LTED cells. The current study provided baseline results indicating that combination treatments of TAM and AP functioned effectively at lower concentrations in inducing cytotoxicity in both cell lines *in vitro*. AP significantly re-sensitised ER+ breast cancer cells towards enhanced TAM treatment, therefore reducing cancer-related drug resistance via cholesterol depletion. The current findings also suggest that AP has the potential to be an adjuvant compound as it targets cholesterol transport and metabolism, which could be used additively to reduce the toxicity of existing TAM treatment. Taken together, the combination of TAM and AP treatment could be a promising novel strategy in treating resistant LTED ER+ breast cancer by reducing cholesterol accumulation and cancer-related drug resistance.

Supplementary Material

The dataset and sources supporting the conclusions of this editorial are publicly available in the FigShare repository at: <https://figshare.com/projects/Cholesterol_Depletion_Modulates_Drug_Resistance_Pathways_to_Sensitize_Resistant_Breast_Cancer_Cells_to_Tamoxifen/126785>.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

GBHP: Data acquisition, data analysis and interpretation, and original draft preparation. MK: Conception and design of the study, acquired funding and resources, reviewed, and edited.

Acknowledgements

The financial assistance of the National Research Foundation (grant no: 113442; 118720) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF. The funding body did not play a role in any of the following: design of the study; collection, analysis, and interpretation of data; and in writing the manuscript.

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Received October 12, 2021
Revised November 19, 2021
Accepted November 23, 2021