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Cholesteryl Ester Transfer Protein (CETP) as a Possible Drug-Resistance Marker in Breast Cancer

MSc Research Dissertation

Liang Gu (683077)

Research Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in the fulfilment of the requirements for the Degree of Masters in Science.

Signed on 24 May 2018 in Johannesburg:



Declaration

I, Liang Gu (683077), registered for the degree of Masters in science (MSc) by dissertation in the academic year 2016-2017 at the University of the Witwatersrand, declare the following:

- I confirm that the work submitted for assessment is my own, unaided work.
- I have not submitted this dissertation before for any other degree or examination at any other Universities.
- It is submitted to the University of the Witwatersrand, Johannesburg.
- I understand that the University of the Witwatersrand may take disciplinary actions against me if there is plagiarism involved and if there is belief that this is not my own work or acknowledged the source of the ideas.

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.

Signature

(Signature of Candidate)

24th day of May 2018 at Johannesburg

Abstract

Hormone responsive breast cancer (BC) is the most common BC and relies on steroid hormones for cell proliferation and survival, therefore endocrine therapy; Tamoxifen (TAM), serves as the main therapeutic strategy in treating hormone receptor positive BC. Despite the use of TAM for over four decades, overcoming drug resistance remains challenging. Due to the fact that steroid hormones are derived from cholesterol and evidently, increased level of cholesterol is associated with cancer progression, in this research, we investigated the effect of reducing intracellular cholesterol as a possible alternative method to induce cell death in BC cells. An important protein involved in cholesterol homeostasis is the Cholesteryl Ester Transfer Protein (CETP). CETP maintains cholesterol homeostasis by storing cholesteryl esters (CEs) in lipid droplets. The intracellular accumulation of CEs leads to aggressive cancer development and drug resistance. Therefore, we aim to investigate whether *CETP* can possibly be used as a drug-resistance marker in BC. This study has shown that knocking-down *CETP* resulted in increase in apoptosis in MCF-7 cells when treated with TAM (by 10-40%) and various other drugs. Furthermore, *CETP* knock-down with the addition of a cholesterol-depleting agent increased apoptosis by 10 fold when compared to the non-transfected MCF-7 cells, possibly due to a decrease in CE content. Similar results were observed in MDA-MB-231 cells. Therefore, it was concluded that *CETP* could thus serve as a potential drug-resistance marker in cancer cells, more specifically BC. Furthermore, strategies targeting *CETP* could be used as a potential combination treatment for treating cancer.

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List of Symbols

β	Beta
µ	Micro
™	Trademark
°C	Degree Celsius
%	Percentage
©	Copyright
®	Registered Trademark

Abbreviations

40S	Small Ribosomal RNA subunits
60S	Large Ribosomal RNA subunits
5-FU	5-Fluorouracil
ABCA1	ATP-binding Cassette Transporter
ACAT	Acyl-Coenzyme A: Cholesterol Acyltransferase
Acetyl-CoA	Acetyl-coenzyme
Akt	Protein Kinase B
AMPK	Activated Protein Kinase
AP	Acetyl – Plumbagin
AP-1	Activator Protein
APO%	APOPercentage
ApoA-I	Apolipoprotein A-I
APS	Ammonium Persulphate
BC	Breast cancer
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
Caspase	Cysteine-Aspartic Proteases

cDNA	Complementary Deoxyribonucleic Acid
CE	Cholesteryl Ester
CETP	Cholesteryl Ester Transfer Protein
CETP	Cholesteryl Ester Transfer Protein (Gene)
CO_2	Carbon Dioxide
Cu^{2+}	Copper Sulphate
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
ESR1	Estrogen Receotor gene
FBS	Foetal Bovine Serum
Fig.	Figure
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HDL	High-Density Lipoprotein
HEK 293	Human Embryonic Kidney 293 cells
HER2	Epidermal Growth Factor Receptor
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A
HRP	Horseradish Peroxidase
IDT	Integrated DNA Technologies
IGF	Insulin-like Growth Factor
KPO_4	Potassium Phosphate
LCAT	Lecithin: Cholesterol Acyltransferase

LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LXR	Liver X Receptor
MAPK	Mitogen-Activated Protein Kinase
MCF-7	Michigan Cancer Foundation – 7
MDA-MB-231	M.D. Anderson Metastatic Breast Cancer - 231
MDR	Multi-Drug Resistance
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide
MβCD	Methyl-β-Cyclodextrin
NaCl	Sodium Chloride
NF-κB	Nuclear Factor Kappa B
NIH	National Institute of Health
ns	Not Significant
OD	Optical Density
PBS	Phosphate Buffered Saline
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol 3 Kinase
PL	Plumbagin
PR	Progesterone Receptor
P-S	Penicillin-streptomycin
P-value	Statistical/ Null Hypothesis
PVDF	Polyvinylidene Fluoride/ Difluoride
qPCR	Quantitative Polymerase Chain Reaction
RCT	Reverse Cholesterol Transport
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

S.D	Standard Deviation
SCAP	SREBP Cleavage-Activating Protein
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SERM	Selective ER Modulator
siRNA	Small Interfering Ribonucleic Acid
SR-B1	Scavenger Receptor Class B Type 1
SREBP	Sterol Regulatory Element-Binding Protein
TAM	Tamoxifen
TEMED	Tetramethylethylenediamine
TNF-R	Tumour Necrosis Factor-R
VLDL	Very-Low-Density Lipoprotein
WHO	World Health Organisation

1. Introduction

1.1 Prevalence of Cancer and BC

Worldwide, cancer is the second leading cause of death with an estimated increase of 14 million new cases reported yearly (WHO, 2018). In 2015, 8.8 million deaths were caused by cancer of which 521 000 deaths were caused by BC specifically (WHO, 2018). Shockingly, nearly 70% of BC cases and BC-related deaths occur in developing countries (WHO, 2018). In South Africa, a total of 3206 deaths were documented in the year 2000, and in 2012 the cancer registry estimated that one in every eight South African women are at risk of developing BC, thus ranking BC the leading cause of cancer deaths amongst other common cancers (Cansa, 2013). In addition, 8132 BC deaths were reported in 2013 making up a total of 22.22% of all cancer-related deaths (Cansa, 2013).

1.2 Hallmarks of Cancer and BC

Cancer is a well-known challenging disease that involves the abnormal or unrestricted cell proliferation that progress into neoplasms (Cansa, 2013). The various infamous hallmarks that define cancer include: over-proliferation, metastasis or invasion, and circumvention of apoptosis (Hanahan and Weinberg, 2011). BC is amongst the top five lethal cancers (cervical, colorectal, uterine and lung cancer) in South African women (Cansa, 2013). BC is a malignant tumour that develops in or around the various ducts and tissues in the breast (American Cancer Society, 2016). The metastatic nature and resistance to apoptosis makes BC one of the most difficult diseases to treat (Mazars et al., 1992). Apoptosis is a well-established process of programmed cell death that leads to physiological and morphological changes in cells (Fig. 1.1) (Hanahan and Weinberg, 2011). There are two apoptotic pathways namely; intrinsic and extrinsic pathways. These pathways activate different cysteine-aspartic proteases (caspases) through various tumour suppressor proteins and genes to eliminate mutated cells (Putcha et al., 2002). In cancer, BC included, these pathways are suppressed thus leading to unhindered cell proliferation. Additionally, various proliferative pathways in BC; such as PI3K (phosphatidylinositol 3 kinase) or Akt (protein kinase B) pathway (Paplomata and O'Regan, 2014), Wnt and sonic hedgehog signalling pathway (Luchetti et al., 2016; Sheng et al., 2014) and MAPK (mitogen-activated protein kinase) pathway (Derynck and Zhang, 2003; Moustakas and Heldin, 2005), are up-regulated through the interaction between steroid hormones and its respective cell surface receptors making BC an aggressive

cancer type. Therefore, emphasising the importance of advancing BC therapies (American Cancer Society, 2016).

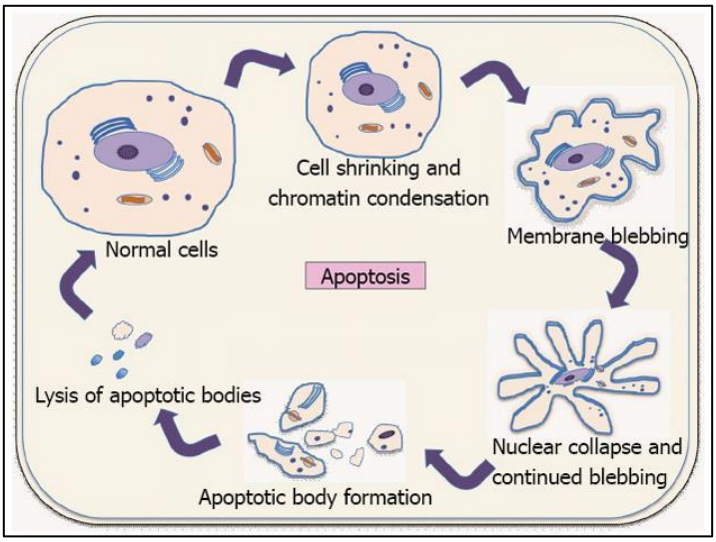


Figure 1.1: Morphological changes to cells during apoptosis

Apoptosis may occur through various internal and external stimuli (such as gene mutations or drugs respectively). During early apoptosis, cells undergo a membrane flip and begin to shrink. Thereafter, membrane blebbing occurs in apoptotic cells and starts to collapse and forms apoptotic bodies, which are digested by macrophages. (Figure adopted from: (Larrubia et al., 2013) .

1.3 Hormone Induced BC

Steroid hormones such as estrogen and progesterone synthesised from cholesterol serves as significant regulators of cell proliferation and differentiation in both normal cells as well as cancer cells (Ghayee and Auchus, 2007).

In normal breast cells, steroid hormones maintain normal physiological and behavioural responses of breast cells such as reproduction and development (Anderson and Clarke, 2004). BC cells, however, can be identified or recognised based on the presence of surface receptors that are responsible for the binding of steroid hormones for cell proliferation (Wang et al., 2015), and thus serve as a useful biomarker to define various cancer types. BC cells can, therefore, be estrogen or progesterone receptor positive (ER+, PR+), estrogen receptor negative (ER-), and triple negative. ER- BC cells are independent of steroid hormones and as a result, they are dependent on alternate means such as human epidermal growth factor (EGF) receptor (HER2) for cell proliferation (Cleator et al., 2007). Triple negative BC cells,

however, lack both the hormone receptors (ER and PR)and HER2 (Cleator et al., 2007) but rather responds to insulin-like growth factors (IGF) for proliferation and cell survival (Davison et al., 2011). Despite the various BC types, ER+ BC still accounts for the majority (75%) of BC cases (Fig. 1.2) where these hormone-induced BC cells are reliant on hormones for promoting proliferative signals involved in cell proliferation, differentiation, migration and survival (Breastcancer.org, 2016). For this reason, increased expressions of hormone receptors and elevated levels of estrogen are frequently associated with breast neoplasia. Therefore, it can be said that there is a positive correlation between estrogen levels or synthesis and the presence of hormone receptors. Furthermore, surface receptors are thus often used as a clinical biomarker to predict effectiveness of potential therapeutic strategies (Cianfrocca and Goldstein, 2004).

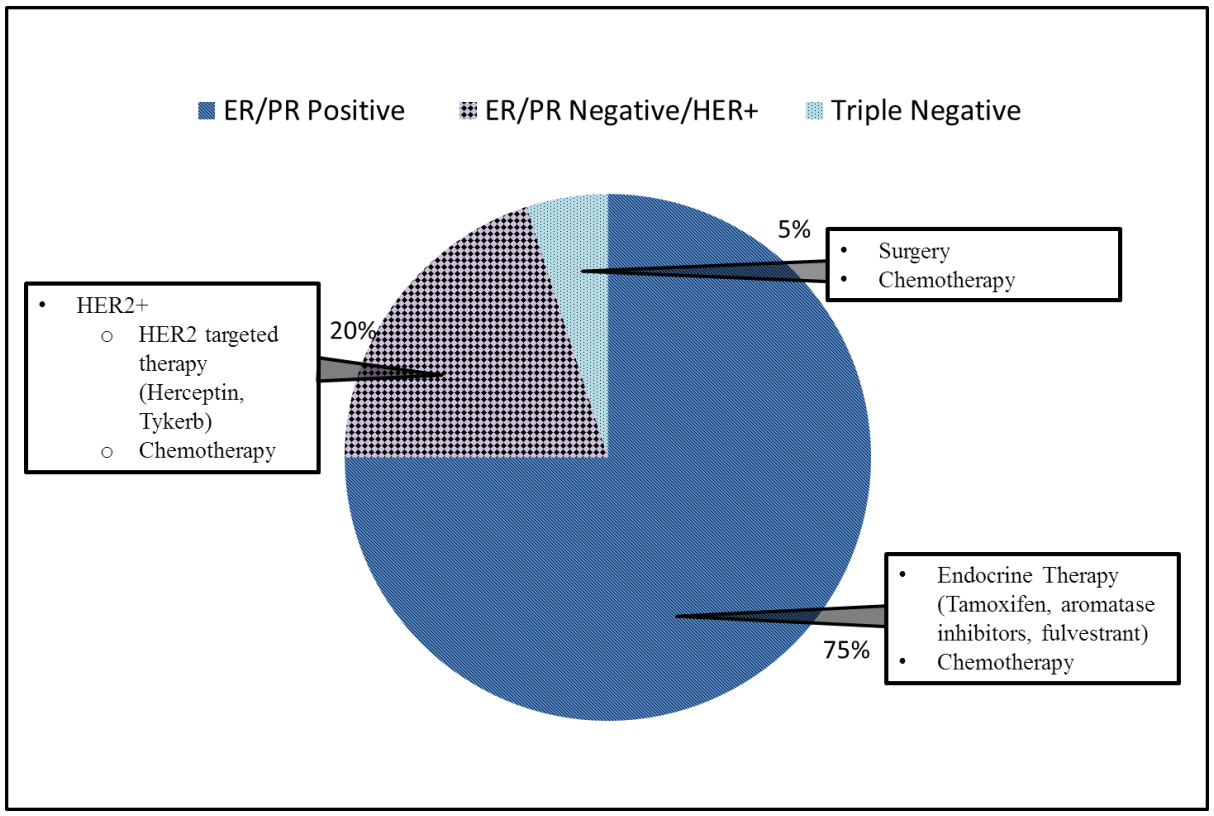


Figure 1.2: Representative of prevalence of hormone induced BC types with the respective currently available drugs

Receptor positive BC is the most common BC, accounting for about 75% of hormone induced BC. Currently, endocrine therapy is the most effective strategy in treating ER+ BC. In rare cases where BC cells are independent of hormones for cell growth and proliferation, treatment options become limited. These include: drugs targeting HER2 for hormone negative BC and chemotherapy and surgery for triple negative BC cells.

1.4 Available treatment for BC

Limitations in BC therapies have been a burden for women world-wide hence the increasing death rate statistics. Chemotherapy is often used simultaneously with BC medications to treat both early stage and late stage BC (Breastcancer.org, 2016). Hormone therapy or endocrine therapy is the most widely used strategy that involves the use of selective ER modulators (SERMs), such as TAM, which has been the first-line or gold standard treatment for hormone responsive BC for over four decades (Maughan et al., 2010). TAM has anti-estrogen properties, playing an antagonistic role to the hormone estrogen (Hodges et al., 2003; Mourits et al., 2001). The National Surgical Adjuvant Breast and Bowel Project proposed that using TAM reduces or slows down 45% ER+ BC recurrences (Mourits et al., 2001). TAM is initially metabolised by a family of cytochrome P450 enzymes (Cronin-Fenton et al., 2014) into various active metabolites namely: endoxifen and 4-hydroxy-tamoxifen, both which competitively bind to the ERs of BC cells (Lu et al., 2012). The principle metabolite, endoxifen, was found to be produced from *N*-desmethyl-tamoxifen by an enzyme known as CYP2D6 (Lu et al., 2012), The metabolite is then internalised and binds to estrogen-dependent genes thus reducing gene expression possibly through the recruitment of corepressors (Hodges et al., 2003). Consequently, this prevents the activity of various transcription factors thus impeding growth promoting signals for cellular replication (Hodges et al., 2003). Interestingly, TAM has also been found to have agonist properties, in which it promotes transcription by initiating the activator protein (AP-1) pathway in the uterine endometrium, bone and cardiovascular system (Hodges et al., 2003; Webb et al., 1995). However, long-term use of TAM has been reported to cause adverse side effects such as: early onset of menopausal symptoms, blood clots, endometrial cancer (Acconcia et al., 2006) and relapse (Breastcancer.org, 2016; Mourits et al., 2001). In addition, the continual use of TAM leads to a build-up of resistance against TAM and ultimately become ineffective in preventing BC and other cancers (Ring and Dowsett, 2004). It was found that at least 40% of patients eventually relapse after ±5 years of using TAM despite its therapeutic activities (Ring and Dowsett, 2004). Other popular drugs used to treat ER+ BC patients include the aromatase inhibitors. Aromatase inhibitors blocks the aromatase enzymes involved in the production of estrogen and thereby reducing the proliferation of ER+ cells (Breastcancer.org, 2016). Another chemotherapeutic drug is 5-Fluorouracil (5-FU). 5-FU has been a widely studied chemotherapeutic drug as it is closely related to uracil, which is preferentially used by tumour cells for proliferation (Cohen et al., 1958). 5-FUis thus incorporated into RNA by replacing the uracil (Cohen et al., 1958). In addition, it also inhibits DNA synthesis by disrupting thymine production (Cohen et al., 1958). However, despite the use of these drugs,

drug resistance still remains a significant problem with regards to clinical use. Therefore, further research is needed to improve the response and decrease resistance to currently available dugs and therapeutic strategies.

1.5 BC and link to cholesterol

Steroid hormones are produced by the endocrine glands; adrenal cortex, testes, ovaries and peripheral tissues, such as the adipose tissues and the brain (Ghayee and Auchus, 2007). Hormone production is a highly complex process that involves a number of different pathways and enzymes (Fig. 1.3).

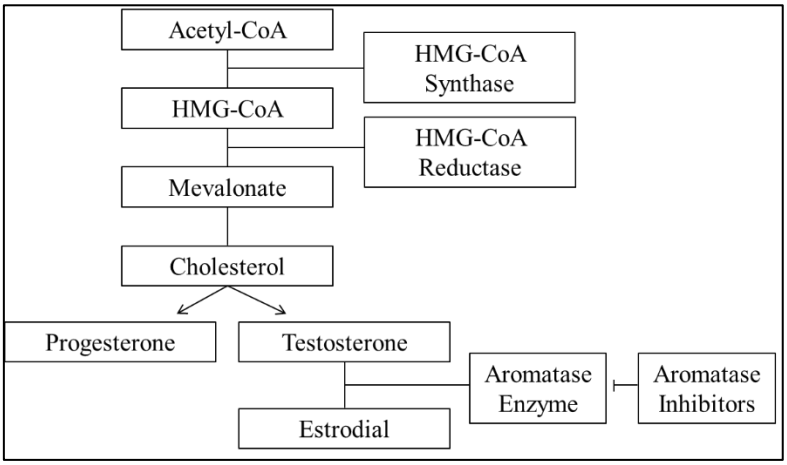


Figure 1.3: The biosynthesis of cholesterol and the production of steroid hormones from cholesterol

The biosynthesis of cholesterol initiates from acetyl-CoA (acetyl-coenzyme A), a by-product from glycolysis, and it is converted to HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A), which is then reduced by HMG-CoA reductase to form mevalonate. Through a cascade reaction, cholesterol is ultimately produced. The cholesterol is then used in the essential synthesis of various hormones (estrogen, progesterone and testosterone) for maintaining internal homeostasis. One enzyme that inhibits the synthesis of estrogen specifically is the aromatase inhibitor, which inhibits the aromatase that transitions testosterone into estrogen. Estrogen, as aforementioned, is involved in cell proliferation and differentiation. Therefore by inhibiting the synthesis of estrogen, it is in-turn inhibiting cell proliferation and induces cell death (Billig et al., 1993; Lippman et al., 1976).

Since, it has been established that cholesterol is the precursor of various hormones including estrogen and progesterone (Evans, 1988). Consequently, cholesterol concentrations are higher in BC cells compared to normal cells (Cruz et al., 2013; Mostaghel et al., 2012). The excess

cholesterol is used to maintain the rapid production of estrogen for the continual support of cancer cell proliferation (Buchwald, 1992; dos Santos et al., 2014). Cholesterol is naturally produced by the body through the mevalonate pathway (Fig. 1.3). It is a lipid molecule that is exclusively biosynthesised by all animals (WHO, 2018). In humans, cholesterol poses three main functions. Firstly, as previously mentioned, cholesterol is involved in the synthesis of various steroid sex hormones, and cortisone-like hormones in response to stress (Isaacsohn, 1992). In addition, it is involved in the production of bile in the liver for the digestion of fats (Isaacsohn, 1992). Most importantly, cholesterol is found in all animal cell membranes and maintains cell stability and shape by forming lipid rafts (Isaacsohn, 1992). Since cholesterol is semi-soluble in aqueous conditions it is thus restricted from circulating throughout the body in the blood stream (hydrophilic) (Isaacsohn, 1992). Therefore, cholesterol molecules are encapsulated in water-soluble protein coats, known as lipoproteins that aid in the distribution of cholesterol as well as the reverse cholesterol transport (RCT) process (Fielding and Fielding, 1995). There are four types of lipoproteins namely: chylomicrons, very low-density lipoprotein (VLDL), low density lipoproteins (LDL), and high density lipoprotein (HDL), where LDLs and HDLs are the main cholesterol carriers (Cruz et al., 2013; Isaacsohn, 1992).

1.6 Intracellular and Extracellular Cholesterol Homeostasis

Cholesterol homeostasis (Fig. 1.4) is a complex process regulated by several proteins; intracellular (proteins and enzymes) and extracellular (lipoproteins and CETP mechanisms) (Fig. 1.4) (Cruz et al., 2013). Extracellularly, free cholesterol synthesized from liver hepatocytes is transported to various cells via LDLs to cell surface receptors; LDLRs, where it is used to maintain cell membrane stability (Fielding and Fielding, 1995). Intracellular excess cholesterol is then converted to CEs by acyl-CoA: cholesterol acyltransferase (ACAT) in the endoplasmic reticulum and stored as lipid droplets (Fig. 1.4) (Fielding and Fielding, 1995; Lin et al., 1999; Willner et al., 2003). In addition, intracellular cholesterol levels are maintained by the sterol regulatory element-binding proteins (SREBPs) (Fig. 1.4). SREBPs are endoplasmic reticulum membrane proteins that are part of the transcription factor family involved in cholesterol synthesis (Weber et al., 2004). SREBP forms a complex with the SREBP cleavage-activating protein (SCAP) (Horton et al., 2002; Ikonen, 2008; Nelson et al., 2014), which acts as both a cholesterol sensor as well as a chaperone. In a cholesterol-poor environment, SCAP transports SREBP to the golgi body and undergoes several proteolytic activities and subsequently signals for cholesterol synthesis (Horton et al., 2002; Iddon et al.,

2001). On the contrary, if cholesterol levels rise, SCAP prevents the movement of SREBPs and confines it in the endoplasmic reticulum (Horton et al., 2002; Iddon et al., 2001). An additional protein involved in intracellular cholesterol homeostasis is CETP. The human *CETP* gene, encodes a 74 kDa CETP protein, containing approximately 476 amino acids (Morton and Izem, 2014; Takahashi et al., 1993). *CETP* is found on chromosome 16q13 with a total of 17 exons (NCBI, 2018). The main function of CETP is CE and triglyceride transport in the plasma, however intracellularly, CETP protein has been shown to translocate CEs from endoplasmic reticulum to storage droplets or lipid droplets where CEs are stored. This is due to the fact that free cholesterol is cytotoxic to cells (Esau et al., 2016; Lagrost, 1994). Moreover, *CETP*-deficient cells have been found to have lower levels of cholesterol, which could possibly be due to the accumulation of CEs within the endoplasmic reticulum. This accumulation could possibly signal for the down-regulation of proteins involved in cholesterol synthesis and possibly increase or upregulate proteins involved in the cholesterol clearance pathways (Esau et al., 2016; Lagrost, 1994).

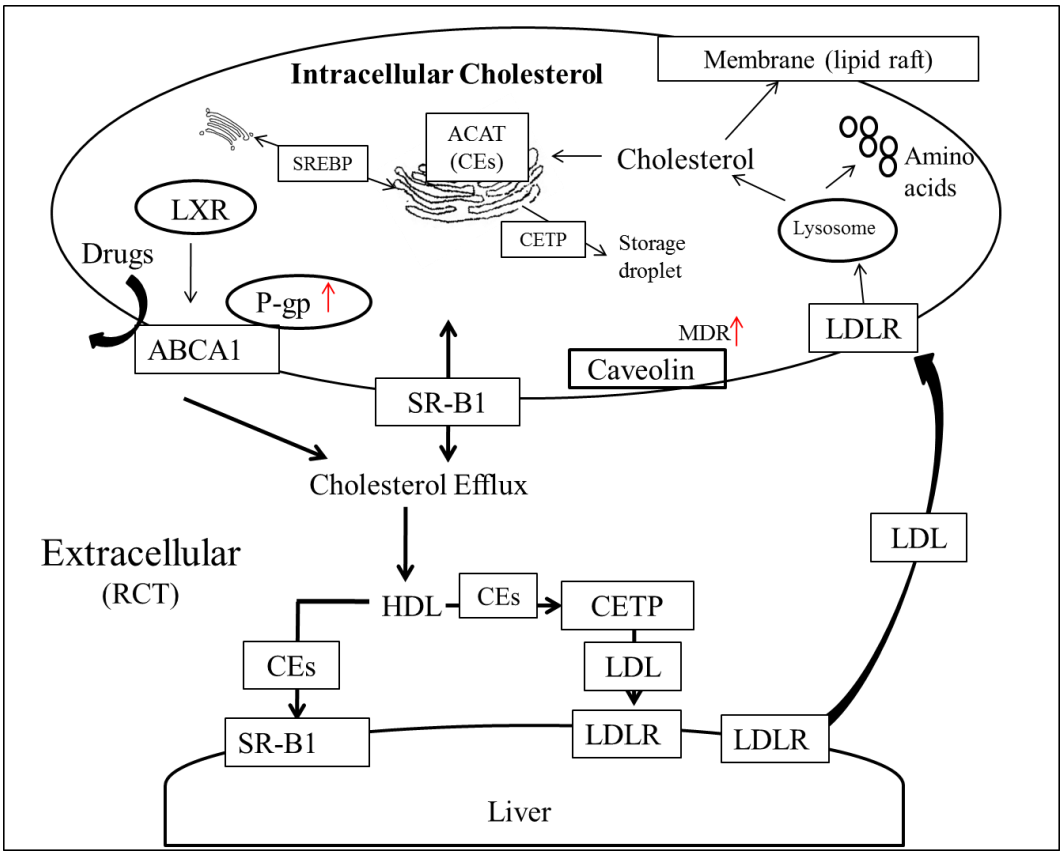


Figure 1.4: Intracellular and extracellular transportation of cholesterol

Free cholesterol is transported to cells via LDL and LDLR interactions. LDLs are then broken down by lysosomes into cholesterol and amino acids. The cholesterol is then used in

cell membrane stability or converted to CEs by ACAT and stored as lipid droplets mediated by CETP. Furthermore, SREBP regulate intracellular cholesterol synthesis and uptake between the endoplasmic reticulum and golgi body. Several proteins and receptors such as ATP-binding cassette transporters (ABCA1), scavenger receptor class B type 1 (SR-B1) and caveolins maintain intracellular cholesterol homeostasis. Internal or cellular toxicity is regulated through the coupling of p-glycoprotein (P-gp) to ABCA1, which transports drugs to the extracellular matrix. Therefore, ABCA1 is an important protein involved in multi-drug resistance (MDR). In addition MDR occurs when there is an increase in caveolin (red arrow), which increases membrane cholesterol. Extracellular cholesterol homeostasis is maintained by lipoproteins; HDL and LDLs. Cholesterol is cleared either directly through HDLs which interacts with SR-B1 to be excreted by the liver or indirectly, whereby CEs are shuttled by CETP from HDLs to the LDLs, which bind to LDLRs on hepatocytes for cholesterol clearance.

One key player in intracellular cholesterol homeostasis is ABCA1 (family of ATP-binding cassette transporter) (Kang et al., 2013; Yvan-Charvet et al., 2010). ABCA facilitates the transfer of excess cholesterol (in the presence of P-gp) from the inside of cells to HDLs and apolipoprotein A-I (ApoA-I) that are outside of the cells, where cholesterol esterification occurs through lecithin: cholesterol acyltransferase (LCAT) (Fig. 1.4) (Tall et al., 2008). More importantly, overexpression of ABCA1 protein is involved in MDR, where it assists in the export of various hydrophobic compounds such as TAM (Dean et al., 2001), therefore reducing intracellular cytotoxicity. Overexpression of ABCA1 can be stimulated by LXR (liver X receptor), a nuclear membrane receptor that contains DNA-binding and ligand binding domains (Li et al., 2007). The interaction between a ligand (lipophilic molecules) and the receptor is followed by a conformation change in LXRs, which initiates coactivators for transcription of various target genes, one of which includes the gene encoding for ABCA1 (Li et al., 2007). Therefore, the activation of LXR is directly proportional to cholesterol availability; where increased intracellular cholesterol enhances LXR activity and subsequently escalates ABCA1 expression and thus aids in the RCT process (Li et al., 2007). Furthermore, MDR is associated with high levels of caveolin-1 protein (a membrane protein specialising in lipid raft formation that are rich in signalling molecules, receptors, effector proteins, transducers) as increased level of this protein enhances cholesterol efflux (Fu et al., 2004; Ho et al., 2008; Lavie and Liscovitch, 2000). In contrast to the unidirectional cholesterol transport of ABCA1 and caveolin-1, SR-B1 is an additional membrane protein that enables bidirectional, gradient-dependent transfer of free cholesterol (both cholesterol

efflux as well as influx) (Ohashi et al., 2005; Rosenson et al., 2012; Yancey et al., 2003). Moreover, SR-B1 mediates selective uptake of CEs, phospholipids and triglycerides in the RCT system, thereby maintaining cholesterol homeostasis both intracellularly and extracellularly (Yancey et al., 2003).

Cholesterol clearance in the plasma is mostly maintained through lipoproteins in the RCT process. Excess cholesterol in the plasma is removed via HDLs either directly or indirectly (Fig. 1.4). Directly, HDLs transport CEs to the SR-B1 located on the liver to be excreted (Fig. 1.4). Indirectly, RCT is mediated by an important protein; CETP, where CETP aids in the shuttling of CEs and triglycerides from HDLs to LDLs and ultimately to the liver through the LDLRs (Fig. 1.4) (Agerholm-Larsen et al., 2000; Lagrost, 1994; Ohashi et al., 2005). The equilibration of cholesterol through CETP is crucial as lipid imbalance may lead to the development of cholesterol-related diseases and may affect normal brain functions. As previously mentioned, *CETP* deficient cells decreases CE levels inside the cell, extracellularly however, HDL levels appear to increase while LDLs decrease in the absence of CETP (Clark et al., 2004; de Grooth et al., 2004). Subsequently, the raised HDL levels in the plasma suggest enhanced direct removal of plasma cholesterol (Esau et al., 2016; Lagrost, 1994). Therefore, the concept of inhibiting CETP has been investigated as a possible therapeutic strategy in treating arthrosclerosis, cardiovascular disease and possibly Alzheimer's disease (Bisgaier and Newton, 2001; de Grooth et al., 2004; Masson, 2009; Shinkai, 2012). Torcetrapib is one of the most well-known CETP inhibitors studied as a potential drug to treat artherosclerosis and lipid-related diseases by inhibiting CETP and increasing HDL levels (Bots et al., 2007; Clark et al., 2004). Despite the increase in HDL levels, the negative effects of torcetrapib have been shockingly concerning, these included: increased blood pressure, increased cardiovascular events and no significant decrease in coronary artherosclerosis risks (Barter et al., 2007; Bots et al., 2007; Forrest et al., 2008; Nissen et al., 2007). Due to the toxicity and off-target effects of torcetrapib, further research was terminated, which warrants alternative strategies to treat cholesterol-related diseases. In addition, the relationship of *CETP* in the context of cancer has never been investigated. Therefore, in this study, we aimed to knock-down *CETP* with small interfering RNA (siRNA) to observe the effect on cancer cells (due to positive correlation between cancer and cholesterol content). As aforementioned, it was found that *CETP*-deficient cells have lower CE levels hence our hypothesis whereby *CETP* knock-down and the use of a cholesterol

depleting agent after chemotherapeutic drug treatment could possibly be an alternative therapeutic advancement in reducing cancer-related drug resistance.

1.7 Targeting Cholesterol for Cancer Treatment

There are currently two approaches that target cholesterol as a possible therapy to treat BC; 1) to block cholesterol synthesis using drugs such as statins, bisphosphonates, Lapaquistat and Lamisil (Fig. 1.5), and 2) to deplete excess membrane cholesterol using cholesterol-depleting agents (Fig. 1.5).

1.7.1 Cholesterol synthesis inhibitory drugs

In light of the accumulation of excess cholesterol in cancer cells for cell proliferation and survival, a vast number of studies have investigated the use of cholesterol synthesis inhibitory drugs (Isaacsohn, 1992; Mostaghel et al., 2012; NIH, 2016). The most well known of these drugs includes the family of statins (Mostaghel et al., 2012). Statins are HMG-CoA reductase inhibitors that impede the function of this enzyme involved in the conversion of acetyl-CoA to HMG-CoA, thereby stunting the mevalonate pathway (Stancu and Sima, 2001). This consequently, decreases cholesterol levels and thus have shown to be effective in the treatment of arthrosclerosis and coronary heart disease (Stancu and Sima, 2001). Moreover, the hindrance of cholesterol synthesis negatively impacts on cancer cells by halting the production of growth hormones (Hoque et al., 2008). Evidently, statins have been observed to have the ability to impair cell growth by down-regulating growth-promoting signals involving RAS, PI3K/AKT, Wnt/β-catenin signalling cascades and induce proapoptotic signalling pathways in various cancer cells to increase apoptotic potential (Ampuero and Romero-Gomez, 2015; Tsubaki et al., 2011). Despite the significant anticancer properties, there have been several adverse side effects associated with statin use. These side effects may be fatal and include: myopathy, idiopathic polyneuropathy and at times, the onset of developing dementia as cholesterol is vital for normal brain functions (Moosmann and Behl, 2004; Sinzinger et al., 2002). The use of statins and its effects on cancer development have been controversially discussed for decades (Boudreau et al., 2010; Hindler et al., 2006). Several studies have shown that statins possess anticancer properties (Campbell et al., 2006; Chan et al., 2003; Goard et al., 2010; Van Wyhe et al., 2017). However, numerous studies have also emerged that suggest statins actually promote the growth of cancer (Boudreau et al., 2010; Duncan et al., 2005; Fujimoto et al., 2015; Goldstein et al., 2008; Ravnskov et al.,

2015). Therefore, it is crucial to improve the knowledge on the various effects of statins. An alternative to statins are bisphosphonates, which also blocks the synthesis of cholesterol in the mevalonate pathway (Fig. 1.5). However, bisphosphonates have been associated with the development of osteonecrosis (Kennel and Drake, 2009). Therefore, it is crucial to study the feasibility of blocking cholesterol synthesis as a therapeutic approach.

1.7.2 Cholesterol-depleting agents

On the contrary, due to the high level of cholesterol in cancer cells, especially in ER+ BC cells, depleting the excess cholesterol or disrupting the lipid rafts has been anticipated as an alternative or better method in treating BC, as well as cholesterol-related diseases. Increasing evidence has proposed that depleting cholesterol in the lipid rafts disrupt cell membranes and thus initiates apoptosis (Esau et al., 2016). Additionally, the removal of excess cholesterol within cell membrane induces chemosensitivity to certain drugs, such as TAM (Mandal and Rahman, 2014; Mohammad et al., 2014).

Methyl-β-cyclodextrin (MβCD) is amongst the most extensively studied cholesterol-depleting agents, which has shown significant anticancer activity in a number of cancers including BC (Fig. 1.5) (Mohammad et al., 2014), by effectively decreasing membrane cholesterol through lipid raft disruption (Ahern et al., 2014). MβCD is a well-studied cyclodextrin compound that possesses a hydrophobic cavity, which allows the extraction of cholesterol from cell membranes (Zidovetzki and Levitan, 2007). Studies performed on BC cells also showed that cholesterol-depletion by MβCD induced apoptosis in BC cells through down regulation of caspase-3 activation as well as Akt activity (Li et al., 2006). In addition, MβCD was shown to increase the efficacy of TAM treatment by increasing its cholesterol uptake (Mohammad et al., 2014). The structures of membrane microdomains are essential for the modulation of the influx and efflux of cancer drugs (Mohammad et al., 2014). Therefore, cholesterol-depletion in the membrane disrupts lipid raft integrity and simultaneously increases membrane permeability for drug passage (Mohammad et al., 2014). Although MβCD has been shown to be less toxic as compared to most drugs, it is not cancer specific and thus it negatively impacts on normal cell proliferation (Mahammad and Parmryd, 2015).

Recently, a new cholesterol depleting agent: acetyl-plumbagin (AP) has emerged and currently being investigated (Fig. 1.5). AP is a derivative of the highly toxic cholesterol-lowering agent: plumbagin (PL) (Sagar et al., 2014). In several studies, PL has been found to induce apoptosis by a number of pathways. These include; signalling the production and

release of radical oxygen species (ROS) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), thereby inhibiting the activation of cyclin D to induce apoptosis (Sagar et al., 2014; Sandur et al., 2006; Srinivas et al., 2004; Wang et al., 2008). Due to the high toxicity of this molecule, various derivatives of PL were developed, one being AP (Sagar et al., 2014; Sakao et al., 2009). AP was observed to be several fold less cytotoxic compared to PL, especially in normal fibroblasts both *in-vitro* and *in-vivo* (Esau et al., 2016). Though the exact mechanisms of AP have not been well studied, the depletion of cholesterol using AP resulted in a decrease in *CETP* mRNA expression, as well as a significant increase in mitochondrial mediated apoptosis (Esau et al., 2016). However, due to a decrease in *CETP* mRNA expression that was observed, we thus speculate that by knocking-down *CETP* along with the use of a cholesterol-depleting agent (such as AP) in combination with a chemotherapeutic drug could possibly increase cytotoxicity in cancer cells. Thus, this could serve as an alternate therapeutic strategy in treating BC or cholesterol-related diseases. It would also be interesting to see if *CETP* knock-down could result in increased efficacy of chemotherapeutic drugs and reduce resistance. However, this is a completely novel concept and thus requires understanding of the basic role of CETP in cancer. In this study, we have attempted to study the effects of *CETP* knock-down on cell growth and apoptosis with or without drug treatment.

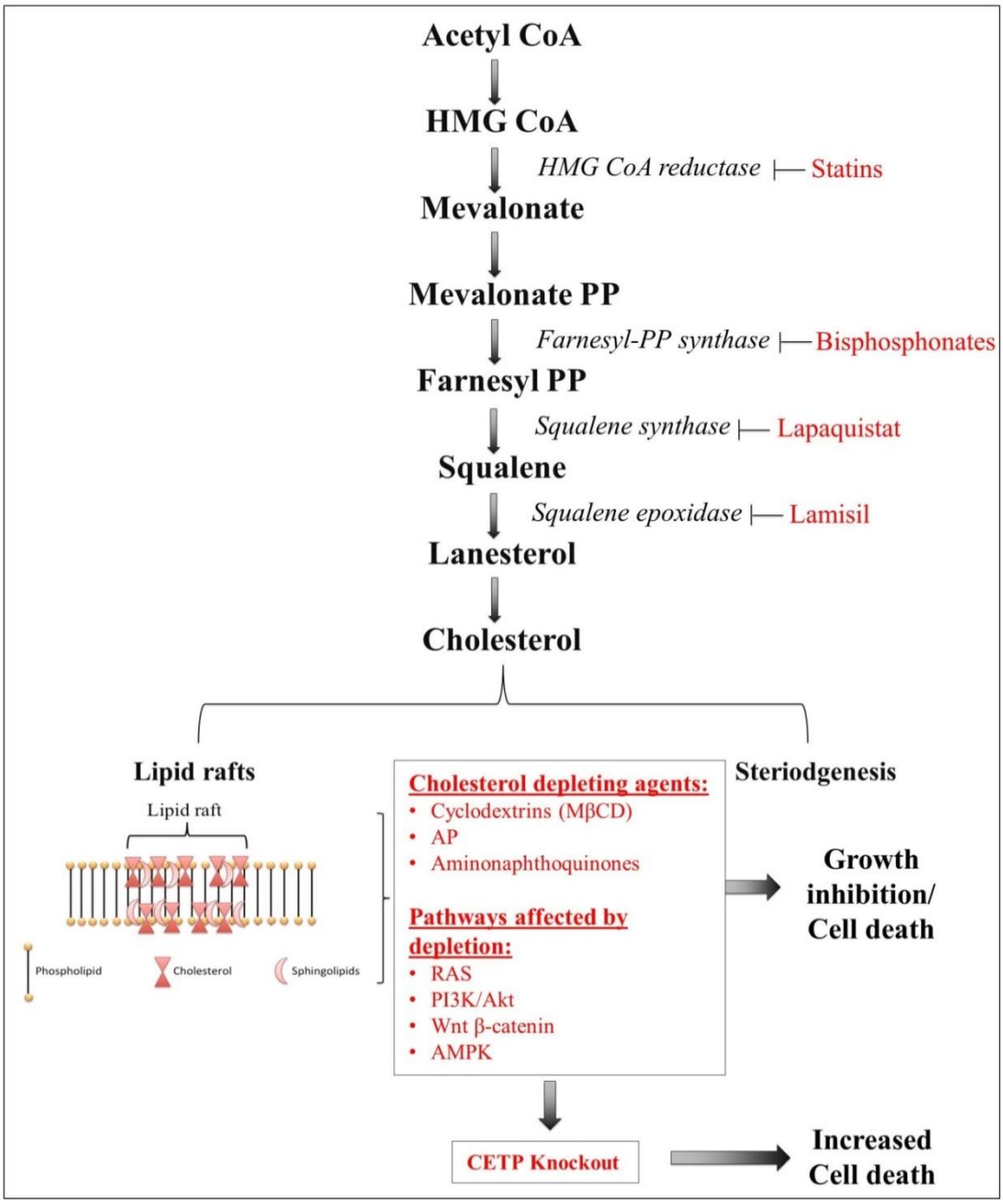


Figure 1.5: Targeting cholesterol as a possible therapeutic approach

Currently, several drugs targeting cholesterol synthesis pathways have been developed. These drugs include: statins, bisphosphonates, lapaquistat and lamisil. In addition, the cholesterol-depleting agents such as MβCD, AP and aminonaphtoquinones (unpublished results) have been verified to target excess cholesterol to cause cell death. Furthermore, the proliferative pathways, that have been mentioned previously; RAS, PI3K/Akt, Wnt/SHH pathway and AMPK (activated protein kinase) pathway, could have been affected. Moreover, CETP with the use of a cholesterol-depleting agent, simultaneously, resulted in an increased cell death. (Esau et al., 2016).

1.8 Preliminary Studies on CETP

Preliminary results from Professor Kaur's previously published work (Esau et al., 2016) showed that downregulation of *CETP* mRNA expression and the use of cholesterol depleting agents (AP, MβCD) resulted in the inhibition of growth and induced an increase in mitochondrial-mediated apoptosis of cancer cells. In addition, it was also shown that caspase-3/7 activity in MCF-7 cells (TAM-treated) increased significantly when *CETP* was knocked-down (Esau et al., 2016). Therefore, using the preliminary results, we hypothesised that *CETP* mRNA expression can possibly be used as a therapeutic marker, more specifically as a drug-resistance marker for monitoring and modifying the chemotherapeutic effects of drugs such as: TAM and 5-FU.

2. Aims and Objectives

2.1 Aim

- To test *CETP*'s role as a potential drug-resistance marker during the treatment of BC cells with commonly used chemotherapeutic drugs.

2.2 Objectives

- To perform *CETP* knockdown on BC cells to observe how it enhances the effectiveness of chemotherapeutic drugs (TAM and 5-FU).

- To measure changes in cholesterol content, more specifically CEs, before and after *CETP* knockdown in ER+ and ER- BC cell lines.

3. Methods and Materials

The following methods were performed on both the transfected and non-transfected cell lines. Each experiment was performed repeatedly (at least three times) for accurate and reproducible results. The drugs that were tested included- test compounds: TAM (Sigma Aldrich, UK) and 5-FU (Sigma Aldrich, UK), and control compounds: PL (Sigma Aldrich, UK) or AP (synthesized from the Chemistry Department, University of the Witwatersrand) and MβCD (Sigma Aldrich, UK) in four different concentrations (5 µM, 10 µM, 20 µM and 40 µM).

3.1 Cell Culture

All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, UK and Gibco: Life Technologies, UK) with varying concentrations of foetal bovine serum (FBS) (Celtic Molecular Diagnostics, SA; Biowest, UK) (Table 1), 1% penicillin-streptomycin (P-S) (Sigma Aldrich, UK) and incubated at 37 °C and 5% carbon dioxide (CO_2) incubator to mimic *in-vivo* conditions.

Table 1: Cell lines used for the research with the respective FBS concentrations

	MCF-7	MDA-MB 231	HEK 293
Full Name	Michigan Cancer Foundation-7	M.D. Anderson Metastatic Breast Cancer	Human embryonic kidney cells
Receptors	BC cell (ER+)	Triple Negative BC cells	ER negative
Morphology/ Type	Epithelial	Epithelial	Epithelial
Disease	Adenocarcinoma, breast mammary gland	Adenocarcinoma, breast mammary gland	-
Growth mode	Adherent	Adherent	Adherent
Media	DMEM,10%FBS, 1% P-S	DMEM, 5%FBS, 1% P-S	DMEM, 10%FBS, 1% P-S

3.2 Cell Counting

A Neubauer haemocytometer was used to count cells in order to seed the accurate number 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 flasks. Prior to cell counting, media was removed and 2 ml of 1 X trypsin/EDTA (Sigma Aldrich, UK; Celtic Molecular Diagnostics, SA) was added into the 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 the same amount of 0.4% trypan blue (Sigma Aldrich, UK) and mixed thoroughly. Trypan blue is the most commonly used dye that stains the dead cells blue due to disrupted membrane and leaves the live cells unstained as intact cell membrane prevents the trypan blue from entering the cells. Thereafter, 10 µl of the mixture was added onto the haemocytometer and cells were counted under the microscope. Both live and dead cells were counted in the four outer quadrants (A, B, C and D in Fig. 3.1). In addition, cells on the top and right lines (indicated in red lines) of the outer edges of the 16 squares were also included in the count.

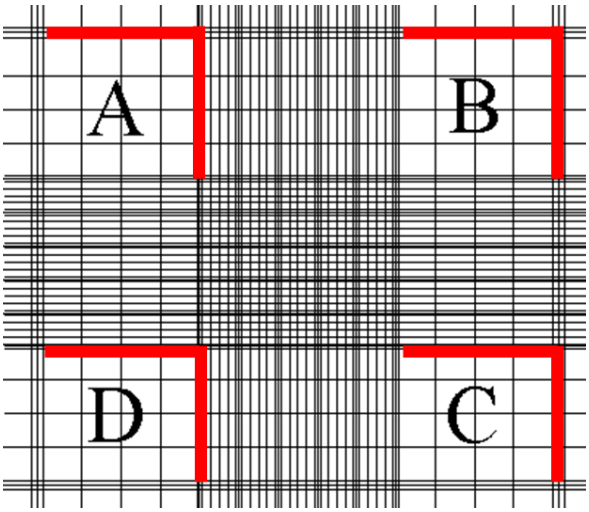


Figure 3.1: Haemocytometer; Cell Counting Chamber

Cellular viability was calculated as follows:

$$\% \text{ cell viability} = \frac{\text{number of dead cells (unstained)}}{\text{Total cells counted (dead and live)}} \times 100$$

Cellular viability measures the number of viable or live cells in the 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 µl x total number of wells = total volume of media required (a)

5000 cells/ well x total number of wells = total number of cells required

$$\frac{\text{Total volume in flask (µl)} \times \text{total number of cells required}}{\text{Total number of cells counted in flask}} = \text{volume of cells required from flask (b)}$$

a - b = media to be added to cells

45 µl was then pipetted into each well with approximately 5000 cells/well

3.3 Growth Inhibition Assay

MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich, UK) assay was performed to measure the effect of each drug or compound with varying concentrations (5 µM, 10 µM, 20 µM and 40 µM) as well as combination treatment of 40µM TAM with AP (5 µM, 10 µM, 20 µM) on cell viability (Sagar et al., 2014) for 24 hours (hrs). PL (40 µM) was used as a positive control as it is a well-known compound to cause growth inhibition (Esau et al., 2016). MTT assay is a colorimetric assay that measures the reduction of MTT/tetrazolium salt by metabolically active cells and results in the formation of formazan crystals. Cells were seeded into a 96-well plate at a density of 5 000 cells/well and incubated at 37 °C for 20 - 24 hrs. MTT (5mg/ml in phosphate buffered saline (PBS) (Sigma Aldrich, UK)) was added for 2 hrs and the formazan crystals were subsequently dissolved in solubilising solution (10% SDS (sodium dodecyl sulphate) (Sigma Aldrich, UK) with 10mM hydrochloric acid (HCl)) for at least 16 hrs. The optical density (OD) was subsequently measured at 570 nm using a microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ software) and the percentage of cell growth inhibition was calculated in Microsoft Office Excel© (formula shown below).

Average of absorbance values of test samples - Average of absorbance values of blanks (a)

$$\% \text{ Cell Viability} = [(a) \text{ of test} \div (a) \text{ of untreated}] \times 100$$

$$\% \text{ Growth Inhibition} = 100 - \% \text{ Cell Viability}$$

3.4 Apoptosis Assay

APOPercentage (APO%) assay was performed using the APO% assay kit (Biocolor, UK) to observe apoptosis post drug treatment (same treatments as in MTT assay). APO% assay detects the early stages of apoptosis in adherent cells. The APO% dye enters cells that are undergoing apoptosis where during this process the scramblase enzyme inactivates flippases (under normal circumstances flippases maintains the membrane structure) causing a membrane flip (Fig. 3.2) (Biocolor, UK).

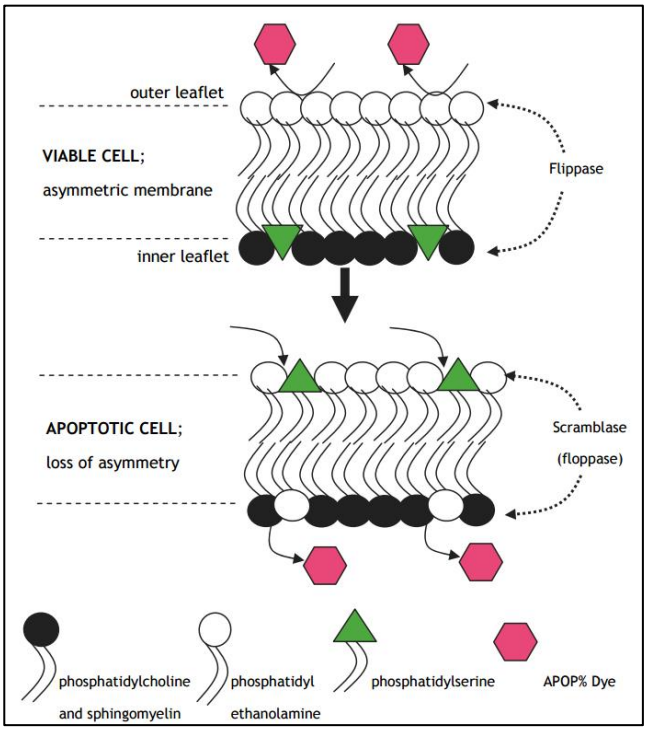


Figure 3.2: APO% Assay; Mode of Action

(Image adopted from: www.biocolor.co.uk)

Cells (5 000 cells/well) were seeded into 96-well plate and incubated at 37 °C for 20 - 24 hrs. Thereafter, cells were treated with drugs/compounds for 2 hrs as the assay detects the beginning stages of apoptosis. Drug treatment for more than 2 hrs may lead to detachment of cells and thus risk of being washed off resulting in inaccurate readings. Media was removed from each well and 65 µl of dye mix (0.5 µl of APO% dye and 64.5 µl 1 X PBS) was added to each well. The plate was then incubated at 37 °C for 20 - 30 minutes (min). Subsequently, each well was washed gently with 100 µl of 1 X PBS. Lastly, 100 µl of 1X PBS was added to prevent the cells from drying out. The plate was then measured at 550 nm using a microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ software) and the percentage of cells undergoing apoptosis was calculated in Microsoft Office Excel© (formula shown below).

Average of absorbance values of test samples - Average of absorbance values of blanks (a)

$$\% \text{ Cell Death} = [(a) \text{ of experimental} - (a) \text{ of untreated}] \div (a) \text{ of untreated} \times 100$$

3.5 Transfection (*CETP* knock-down)

Transfection is the process of manipulating the expression of a particular gene by introducing foreign nucleic acids such as plasmid DNA or RNA (siRNA) into eukaryotic cells. The siRNA technique is most commonly used for reducing gene expression post-transcriptionally by degrading mRNA. The siRNAs are small interfering RNAs that are approximately 20 - 25 base pairs. They are double stranded RNAs that interfere with gene expression of target sequences. The siRNA bind to argonaute or RNA-induced silencing complex. Argonaute prevents elongation factors from binding to the RNA and recruits RNA-induced silencing complex (RISC), which prevents interaction between 60S and 40S (RNA subunits). The siRNA-RISC complex then binds its complementary sequence and silences target genes (Fig. 3.3) (Dharmacon™, GeLifesciences, SA).

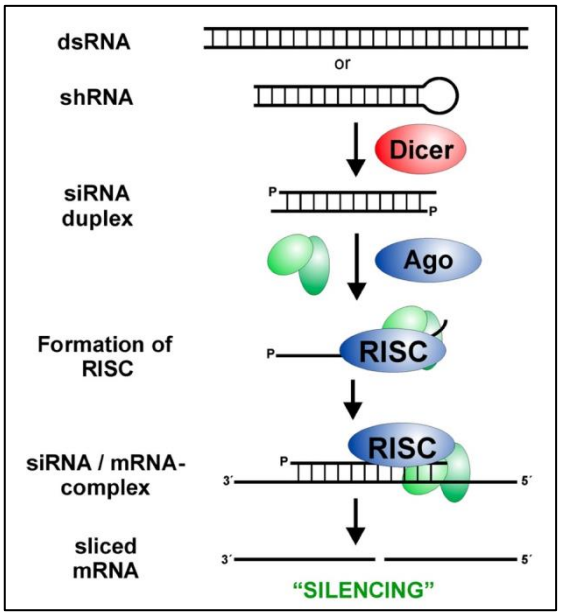


Figure 3.3: siRNA; Mode of Action

(Image adopted from: http://mcmanuslab.ucsf.edu/node/268)

For this project, *CETP* was knocked-down using the Silencer® Select Pre-designed *CETP* siRNA (Ambion®, Life Technologies, USA) and the lipid-mediated DharmaFECT™ Transfection Reagent (Dharmacon™, GE Healthcare, UK; Inqaba Biotec™). Cells were seeded into a 6-well plate at a density of 50 000 cells/well (MCF-7) and 10 0000 cells/well (HEK293 and MDA-MB 231) in triplicates for at least 20 hrs. HEK 293 and MDA-MB 231

cells were plated at different cell densities to account for cells that do not re-attach on plate surfaces as they are highly sensitive. The cells were then transfected as follows:

Table 2: Volumes of siRNA and transfection reagent per well to make up a final siRNA concentration of 25 nM

			Tube 1: siRNA/ siRNA negative control (µl/well)		Tube 2: DharmaFECT™reagent (µl/well)		
Cell Type	Cell Density	Complete medium (µl)	Volume of 5 µM siRNA (µl)	Serum-free medium (µl)	Volume of DharmaFECT™ reagent (µl)	Serum-free medium (µl)	Total volume per well (µl)
MCF-7	5×10^4	1 600	10	190	1	199	2 000
MDA-MB-231	1×10^5	1 600	10	190	2	198	2 000
HEK293	1×10^5	1 600	10	190	2	198	2 000

In tube 1, the required amount of 5 µM siRNA/siRNA negative control was added to serum-free media and in tube 2, DharmaFECT™ reagent was added to the recommended serum-free media as shown in Table 2. The tubes were allowed to sit at room temperature for 5 min and thereafter, contents of tube 2 were added and mixed to tube 1 making up a total volume of 400 µl. The siRNA and DharmaFECT™ reagent mix in tube 1 was then incubated at room temperature for 20 min to allow the transfection reagent to encapsulate the siRNA. Subsequently, this mixture was then added to each well making up a total volume of 2 000 µl/well. Cells were transfected for 72 hrs before harvesting/ plating of cells for downstream applications.

3.6 To confirm successful transfection

Western blotting and quantitative polymerase chain reaction (qPCR) was carried out to confirm successful knock-down of *CETP*.

3.6.1 Western Blotting

Transfected and non-transfected cells were harvested (multiple wells were combined with at least 10^6 cells/well) and pelleted at 4 000 x *g* for 5 min. The supernatant was removed and 1 X Ripa or lysis buffer was added (100 µl/10^6 cells). 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 BCA (bicinchoninic acid) assay.

Table 3: Lysis Buffer component

	Component (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)

- **Protein Quantification: BCA Assay**

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 purple colour that is measured spectrophotometrically (Smith et al., 1985; Walker, 1994). Protein concentrations of unknown samples may then be determined by comparing with a known protein standard (example shown below in Fig. 3.4). Protein quantification is essential to ensure equal loading during SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). A BSA (bovine serum albumin) (Amresco; Inqaba Biotec™, SA) standard curve was generated using the following BSA concentrations; 1 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml, 0.2 mg/ml and 0 mg/ml as blank and 25 µl of each concentration was added to 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 plate was then measured at 562 nm using a microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific,

SkanIt™ software) and the protein concentration of each lysate was calculated in Microsoft Office Excel©.

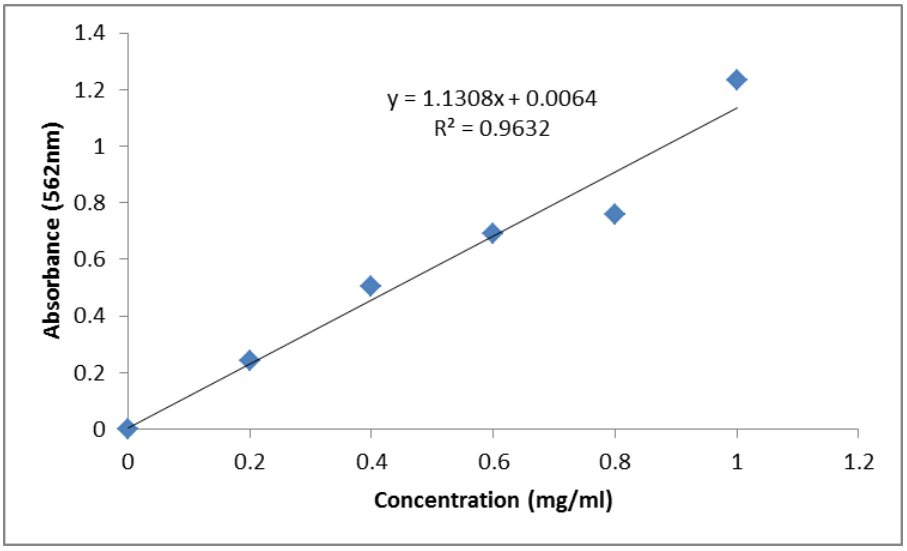


Figure 3.4: BSA Protein Standard

BSA Protein Standard was created using 1 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml, 0.2 mg/ml of BSA and 0 mg/ml as blank

The concentrations of protein lysate were calculated as follows:

Average of samples – average of blank = (a)
Substituted y-value with (a) in the y = mx + c formula above in BSA protein standard
∴ Calculated x-value
x-value × 5 (for dilution) = protein lysate concentration (mg/ml)

Table 4: SDS-PAGE gel (10%, 1 mm) 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	200 µl	30 µl

(APS) (Sigma Aldrich, UK)		
Tetramethylethylenediamine (TEMED) (Sigma Aldrich, UK)	20 µl	3 µl

Table 5: 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 (Sigma Aldrich, UK)	• 30 g Acrylamide • 0.8 g Bisacrylamide • 0.1 g SDS • Make up to 100 ml dH_2O
10% APS (Sigma Aldrich, UK)	• 100 mg APS in 1 ml dH_2O

Lysates of equal volume were loaded onto the gel with the Precision Plus Protein™ unstained standards (Bio-Rad, USA) and run at a voltage of 120 V for 1 hr. Proteins were then transferred onto a 0.22 µM PVDF (Polyvinylidene fluoride or polyvinylidene difluoride) membrane using the Trans-Blot®Turbo™ Transfer System (Bio-Rad, USA). The membrane (containing the proteins) was then transferred into a 3% BSA blocking solution for 1 hr to prevent non-specific binding. Primary antibodies (anti-CETP or anti-β-tubulin; loading control) were then added and incubated overnight at 4 °C. Thereafter, 5 X 5 min PBS washes were performed to wash off unbound antibodies followed by the addition of specific secondary antibodies and incubated at room temperature on the shaker for 1 hr. Subsequent PBS washes were performed and ECL substrate (horseradish peroxidase conjugated substrate – 1ml of reagent A and 1 ml of reagent B) was added. Membranes were viewed on the chemidoc (Bio-Rad, USA) and densitometry was performed using Image Lab™ 4.0 Software (Bio-Rad, USA).

3.6.2 Reverse Transcription - Quantitative Polymerase chain reaction (RT-qPCR)

An alternative method, RT-qPCR, to confirm successful knock-down was employed. This method is a rapid and sensitive technique in determining and quantifying of nucleic acid biological samples after amplification, in this case mRNA expression in transfected and non-transfected cells.

- **RNA Extraction**

RNA extraction was performed using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Inqaba Biotec™, SA). TRIzol® reagent (300 µl) (Ambion®, Life Technologies, USA) was added to the cell pellet and mixed thoroughly for 5 min at room temperature; Trizol is a commonly used chemical reagent that breaks cell membranes for the isolation or extraction of intact RNA, DNA and proteins (Simms et al., 1993; Simões et al., 2013). Equal amount of 99.9% ethanol was added to the samples to precipitate the nucleic acids. The samples were then transferred to Zymo-Spin™ IIC Column in a collection tube and centrifuged at 16000 x *g* for 30 seconds (s); as well as for subsequent centrifugation steps. The flow through was discarded and the column was washed with 400 µl RNA wash buffer. Thereafter, a DNase step (5 µl DNase I with 75 µl DNA digestion buffer) was added, and column was incubated at room temperature for 15 min to prevent DNA contamination during RNA extraction procedure. The sample in the column was then washed with 400 µl of Direct-zol™ RNA PreWash and again washed with 700 µl of RNA wash buffer. Finally, RNA was eluted and suspended in 50µl of DNase/RNase-Free water and stored at -80 °C (Direct-zol™ RNA MiniPrep kit; Zymo Research, Inqaba, SA). RNA quantification was performed using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) to ensure that the RNA extracted was pure and intact for downstream applications. RNA samples with a 260/280 ratio between 1.9 and 2 were used for further experiments.

- **cDNA synthesis**

The RevertAidFirst Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) was used to convert isolated RNA to cDNA in preparation for qPCR. The following components were added to RNA template:

Table 6: Following reagents were involved in the cDNA synthesis process for a 20 µl reaction

Reagent	Volume (µl)
Template RNA	2µg (volume dependent on RNA concentration)
Oligo $(dT)_{18}$ primer	1
Nuclease-free water	Up to 12
Total Volume	12
Master Mix	
5X Reaction Buffer	4
RiboLock RNase Inhibitor	1
10mM dNTP Mix	2
RevertAid M-MuLV RT (200U/µl)	1
Total Volume	20

The samples were mixed and centrifuged briefly prior to incubation at 42 °C for 1 hr using the MultiGene PCR machine (Labnet International, UK). The reaction was then terminated at 70 °C for 5 min and stored at -20 °C indefinitely.

- **RT-qPCR**

The SensiFAST™ SYBR® No-ROX kit was used to determine *CETP* mRNA expression in transfected and non-transfected cells. SYBR green is a cyanine dye that intercalates into double stranded DNA during the annealing and extension step. Consequently, SYBR green detection (Cq- initial detectable signal of SYBR green) is directly proportional to the amount of template and extension during RT-qPCR. The reaction was reduced to half therefore final volume of reaction mix is 10 µl and the components are shown below in Table 7.

Table 7: qPCR reagent composition

Reagent	Volume (µl)	Final Concentrations
2X SensiFAST™ SYBR® No-ROX mix	5	1X
10µM Forward Primer	0.4	400 nM
10µM Reverse Primer	0.4	400 nM
cDNA Template	2	-

dH_2O	2.2	-
Final Volume	10	-

Primers were synthesised by Integrated DNA Technologies (IDT- Illinois, USA) and the sequences are shown in table 6.

Table 8: Primer sequences of CETP and GAPDH genes

Gene	Sequence (5′-3′)	Annealing Temperature (°C)	GC Content (%)	Ref.
CETP (Forward)	GAGTCCCATCACAAGGGTCA	57.1	55	Ding et al., (2015)
CETP (Reverse)	GGAAGACTCGCTCAGAGAACC		57.1	
GAPDH (Forward)	CGTGTCGGTTGTGGATCTGA		55	
GAPDH (Reverse)	TGACGAAGTGGTCGTTGAGG		55	

A 3-step cycling parameter was set on the AriaMx Real-time PCR System (Diagnostech; Agilent Technologies, SA) and samples were run in white, opaque 8-well PCR strips (BIOPlastics, Netherlands; Celtic Diagnostics, SA). The change in mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 9: The 3-step cycling parameters

Cycles	Temperature (°C)	Time	Step
1	95	2 min	Polymerase activation
40	95	5 s	Denaturation
	57.1	10 s	Annealing
	72	15 s	Extension

3.7 Cholesterol Assay

Cholesterol assay was performed to measure the cholesterol content in the transfected and non-transfected cells with and without drug treatment, which was used to confirm whether the transfection as well as the drug treatment affected the cholesterol level in the cell.

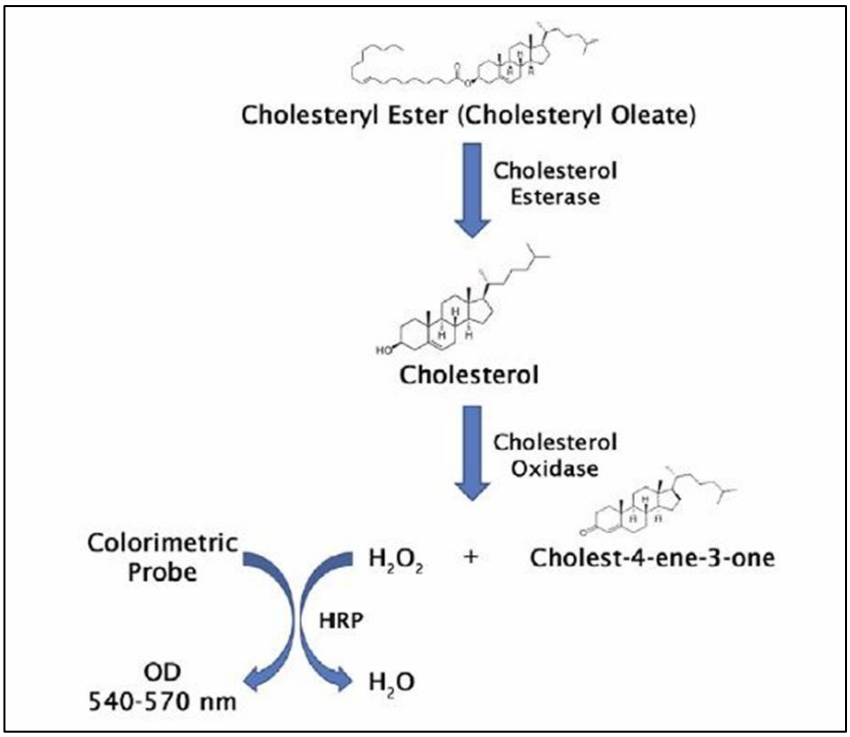


Figure 3.5: Principle of Cholesterol Assay

(Image Adopted from: https://www.bioscience.co.uk/userfiles/pdf/STA-384-total-cholesterol-colorimetric-assay-kit.pdf)

Briefly this method involves the hydrolysis of CEs by cholesterol esterase, which forms cholesterol. Cholesterol is then oxidised by cholesterol oxidase that results in two products: 1) cholest-4-ene-3-one and 2) hydrogen peroxide (H_2O_2). H_2O_2 is detected with a highly specific colorimetric probe (ampliflu red - Sigma Aldrich, UK), The 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).

Transfected and non-transfected cells were seeded in a 96-well plate at a density of 5 000 cells/well in duplicates (as reagents are expensive) in two sets; one testing total cholesterol and free cholesterol in the other. The seeded cells were incubated at 37 °C for 20-24 hrs and treated with drugs for 24 hrs. The media was removed from each well and washed with 50 µl 1 X PBS. Subsequently, 10 µl of 100 U/ml catalase (Sigma Aldrich, UK) to each well and incubated for 15 min at 37 °C. Catalase is an enzyme that is known to degrade excess H_2O_2 to water and thus protect cells from ROS also preventing background noise when measuring the absorbance. Reagent A was prepared using the following components:

Table 10: Components for reagent A per well in this order

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
100mM 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 (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, mixed using plate shaker at 500 rpm and incubated, covered with foil, for 30 min. at 37 °C. The absorbance of each well was measured with the microtiter plate reader-Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt^{TM} software) and cholesterol concentration was calculated in comparison to the known cholesterol standards (5, 10, 15, 20 and 0 µg/ml as blank). CEs were calculated as follows:

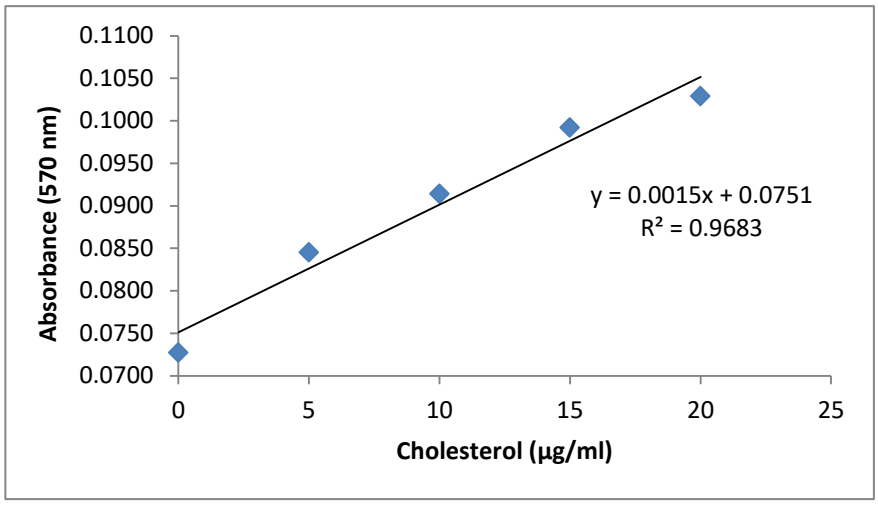


Figure 3.6: Cholesterol Standard Curve

Average of samples – average of blanks = (a)

Substituted y-value with (a) in the y = mx + c formula above in the cholesterol standard curve

∴ Calculated x-value for total cholesterol and free cholesterol

x-value = cholesterol concentration (µg/ml)

µg/ml converted to µg/µl → x-value ÷ 1000 × total volume in well = cholesterol concentration (µg/µl)

Total cholesterol – free cholesterol = CE

3.8 Statistical analysis

The statistical analyses were performed using Microsoft Office Excel©. The experimental procedures were performed in triplicates and repeated at least 3 times for reproducibility. Furthermore, the statistical significance was calculated using student's t-test. A p-value of less than 0.05 was used to estimate the significance of the observations. A Z-factor (Zhang et al., 1999b) was calculated for each 96-well plate and assays having Z-factor above > 0.5 were included in the statistical analysis as it is the acceptable range in which the technicality of the experiments was trustworthy.

3.9 Plan of work

Due to the complexity of the project, the flow chart (Fig 3.7) outlines my work flow for this study, where red represents MDA-MB-231 cell line, blue representing MCF-7 cell line and green representing HEK 293 cell line (Fig. 3.7). Due to time constraints, only a limited number of experiments were completed for MDA-MB-231 and HEK 293 cells. MTT and APO% assays were performed on MCF-7 and MDA-MD-231 cells in the absence or presence of TAM, MβCD, 5-FU, AP, and PL. Only MTT assay was performed on HEK 293 cells. *CETP* knock-down experiments were performed in MCF-7 and MDA-MB-231 cells and knock-down was confirmed or quantified using Western blotting (protein) and RT-qPCR (mRNA). The transfected cells were again subjected to TAM, MβCD, 5-FU, AP, and PL treatment where MTT and APO% assay was performed in both MCF-7 and MDA-MB-231 cells. Thereafter, the CE level was measured in transfected and non-transfected MCF-7 cells only. A combination treatment of various TAM concentrations with different concentrations of AP was performed in transfected and non-transfected MCF-7 and MDA-MB-231 cells to see if *CETP* knock-down would increase apoptosis in cancer cells. MTT and APO% assay for the combination treatment was performed in non-transfected MCF-7 and MDA-MB-231 cells. However, MTT and APO% assay for the combination treatment was only performed in transfected MCF-7 cells only. In addition, results will be presented according to cell line. Supporting data will also be presented in the appendix.

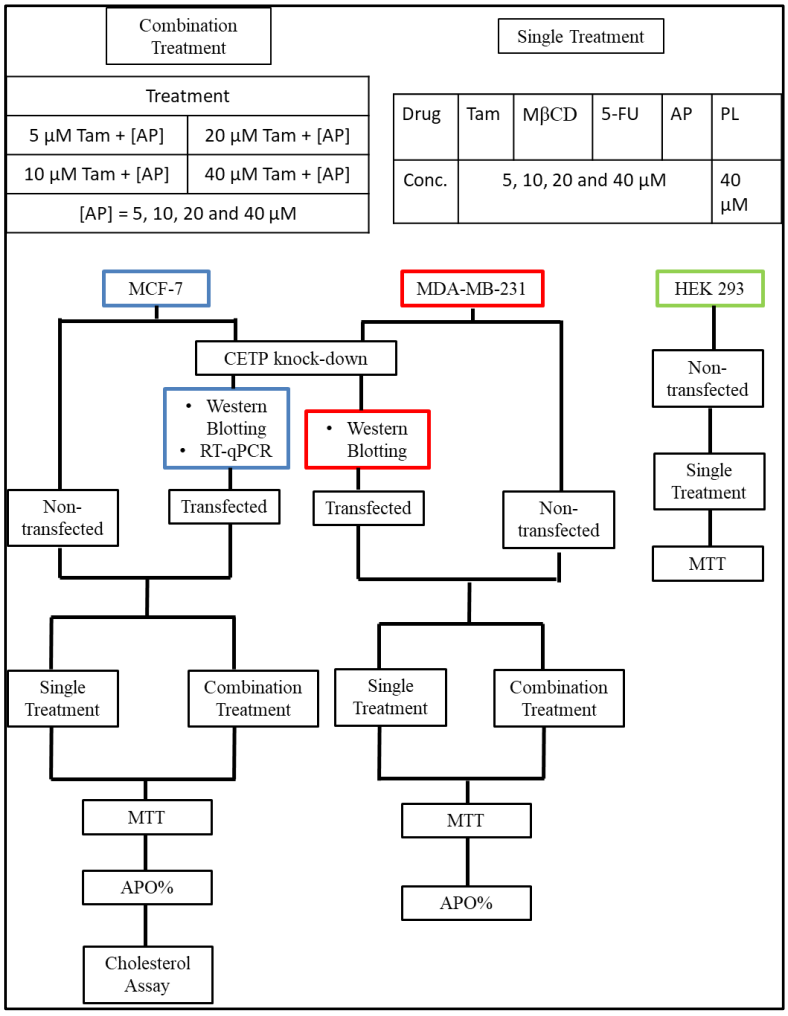


Figure 3.7: Flow Diagram of work plan

4. Results and Discussions

Targeting cholesterol as a possible therapeutic strategy to treat cancer, more specifically BC, is a relatively new concept. Previous research (Esau et al., 2016) reported that knocking down *CETP* resulted in promising outcomes where *CETP* knock-down combined with a cholesterol-depleting agent (AP) increased apoptotic activity in cancer cells significantly while depleting cholesterol. Therefore, in the present work, *CETP* knock-down was performed to observe the effect on current BC drugs such as TAM and 5-FU as well as cholesterol depleting agents such as MβCD and AP while using PL as positive control. Combination treatment of the cholesterol-depleting agent AP with TAM was also performed to observe the effect before and after transfection. This will thus serve as baseline results for further investigations.

4.1 CETP protein knock-down was confirmed using Western blotting and pPCR in MCF-7 cells

In Fig. 4.1, a successful *CETP* knock-down was observed using Western blotting and densitometry.

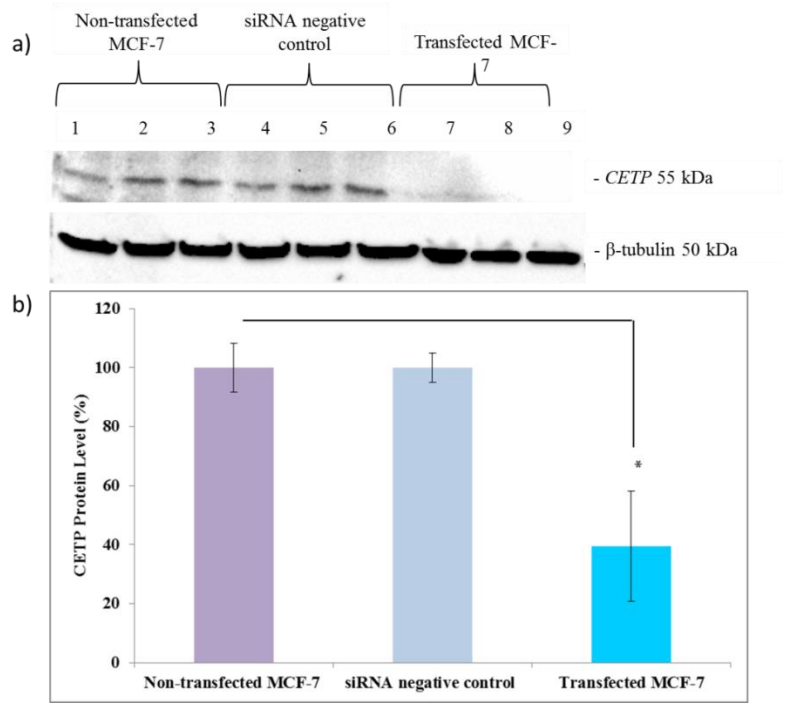


Figure 4.1: CETP protein was successfully knocked-down and confirmed by Western blotting in MCF-7 cells

*Fig. 4.1a illustrating protein bands from Western blotting using Chemidoc and Fig. 4.2b depicts the densitometry analysis in MCF-7 cells. Data represents the mean ± standard deviation S.D. (n=3), * P < 0.05.*

Equal bands observed for β-tubulin at 50 kDa indicating equal loading of lysate which confirms knock-down of *CETP* (faint bands as compared to the non-transfected and siRNA negative control) (Fig. 4.1a). 4.1b) Densitometry revealed a significant knock-down of CETP; decreasing protein level by 60.5% relative to the non-transfected MCF-7 cells.

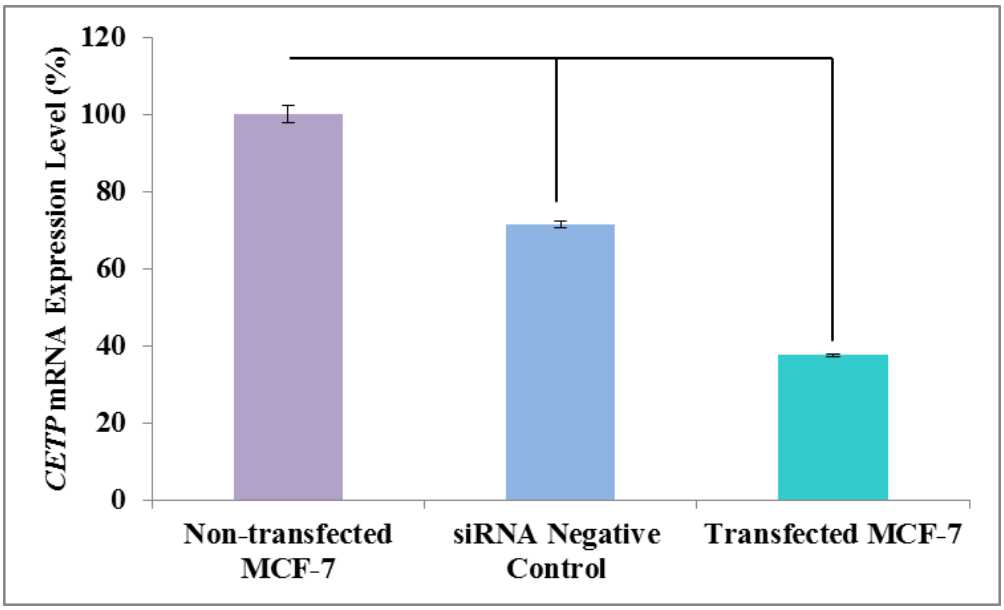


Figure 4.2: CETP mRNA expression level was successfully knocked-down and confirmed by qPCR

In addition, CETP knock-down was also confirmed using qPCR. Data represents the mean ± S.D. (n=2).

CETP mRNA expression level was successfully knocked-down by 63%.

4.2 Growth Inhibition and Apoptosis analysis in transfected and non-transfected MCF-7 cells

Following the successful knock-down of *CETP*, MTT assay was then performed on transfected and non-transfected MCF-7 cells to compare the effects of the various drug treatments on growth inhibition (Fig. 4.3). In addition, to observe whether *CETP* knock-down would cause increased cell death with reduced drug concentration (especially TAM) in cancer cells in the absence or presence of AP (Fig. 4.5).

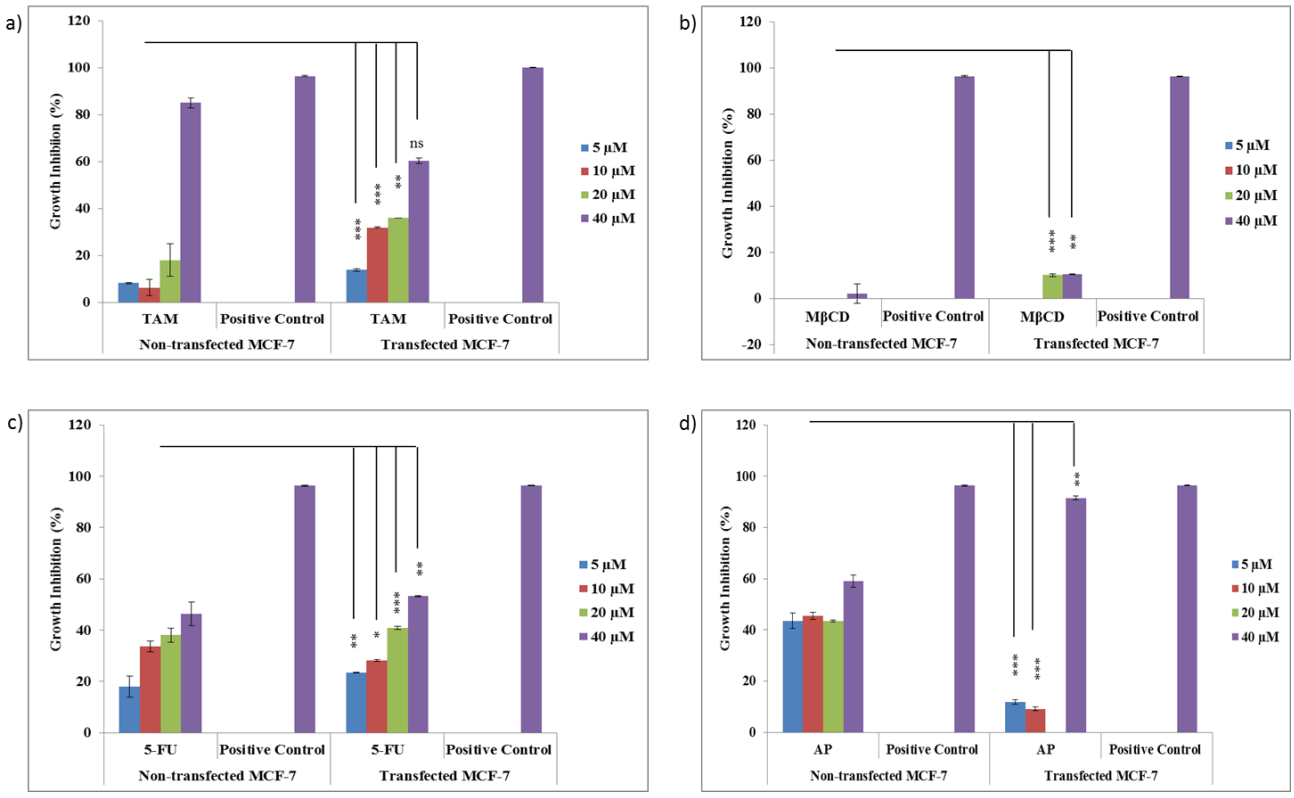


Figure 4.3: CETP knock-down significantly increased growth inhibition in transfected MCF-7

*Fig. 4.3 depicts the comparison of growth inhibition, through the analysis of MTT assay. The transfected MCF-7 cells were compared to non-transfected MCF-7 cells after treatment with various concentrations (5, 10, 20 and 40 µM) of a) TAM, b) MβCD, c) 5-FU and d) AP. 40 µM PL was used as a technical positive control. Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z-factor > 0.5.*

CETP knock-down resulted in an increase in growth inhibition by 6%, 25% and 18% with respective TAM concentrations (5 µM, 10 µM and 20 µM) in transfected MCF-7 cells when compared to non-transfected MCF-7 cells (Fig. 4.3a). However, at 40 µM TAM decreased in growth inhibition. b) Growth inhibition also increased when treated with 20 µM and 40 µM MβCD (by 10% and 8% respectively) in transfected MCF-7 cells compared to non-transfected cells. c) Furthermore, *CETP* knock-down also resulted in a significant increase in growth inhibition when treated with all concentrations, except 10 µM of 5-FU. d) Interestingly, *CETP* knock-down resulted in a significant increase in growth inhibition when treated with 40 µM AP only whereas at lower concentrations, *CETP* knock-down lead to a decrease in growth inhibition when compared to the non-transfected MCF-7 cells.

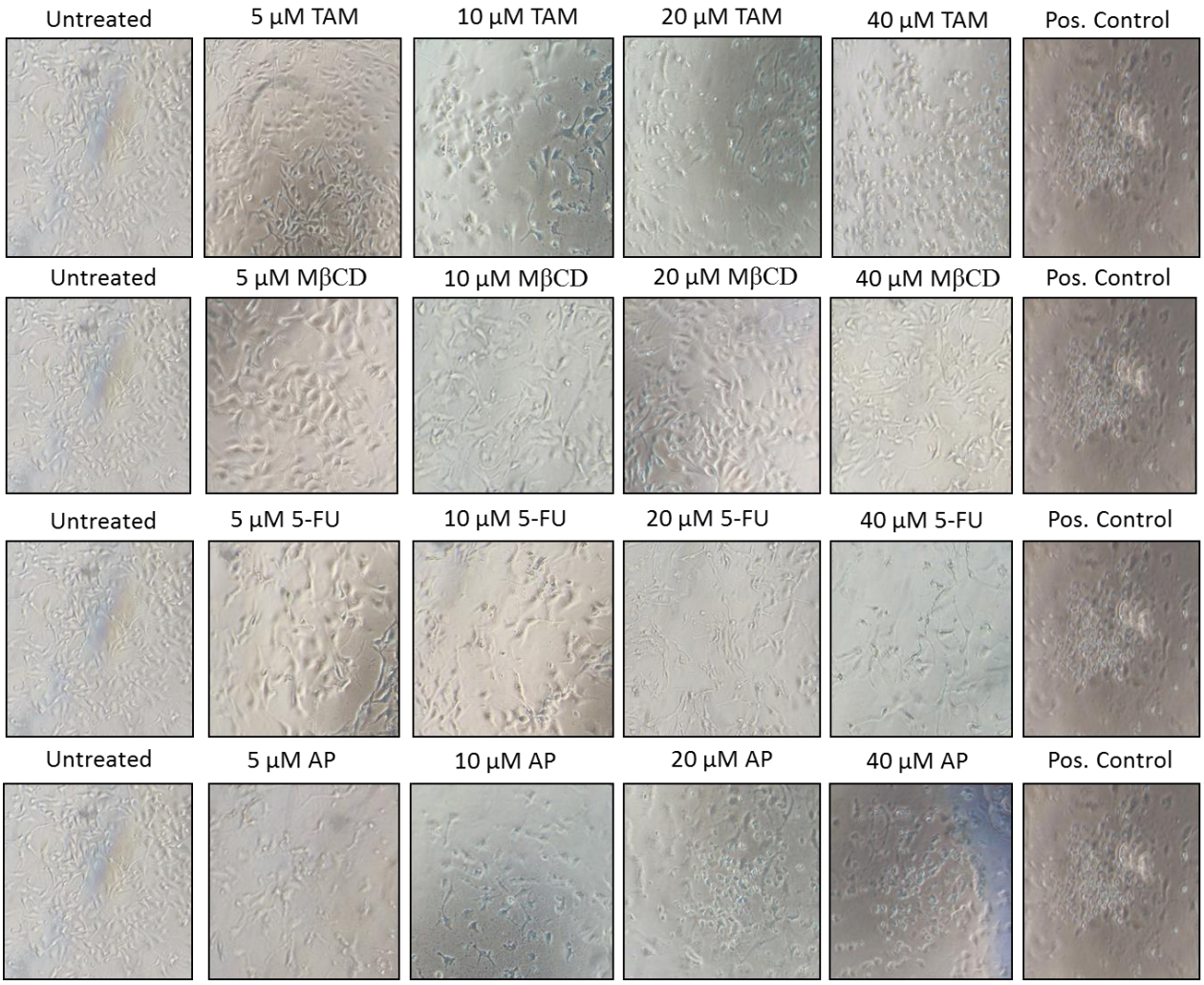


Figure 4.4: Visual representation of non-transfected MCF-cells when treated with various concentrations of drugs for 24 hrs

The images Fig. 4.4 are a visual representation of the effects of various drug concentration treatments in non-transfected MCF-7 cells compared to the untreated MCF-7 cells presented in Fig. 4.3.

It can be observed that with increasing concentrations of TAM, 5-FU and AP, MCF-7 cells appear to be progressively more stressed and more spherical in shape. In contrast, MβCD did not seem to have much effect on growth inhibition and cells appear to be healthy as compared to the positive control (pos. control) - 40 µM PL. Thus, these images confirm the results shown in Fig. 4.3.

Previously, Esau et al, (2016) showed that *CETP* was found to assist ER+ BC (such as MCF-7 cells) in cell proliferation, cholesterol uptake and thus been identified as a cell survival gene (Esau et al., 2016). Therefore, we show that in the absence of *CETP*, the treatment of various

drugs impeded cancer cell growth and indicate a lowered cell survival rate as well as drug resistance (Fig. 4.3) with exception at 40 µM. However, this could suggest that high concentrations of TAM could possibly increase the expression of ABCA1 that assists the export of cytotoxic compounds. The increase in growth inhibition observed in Fig. 4.3a-d suggested that knock-down of *CETP* does indeed increase drug efficacy, especially at lower concentrations. This is significant as this would result in a reduced IC_{50}, which is the concentration that induces 50% of cell growth inhibition or cell death (Miyoshi et al., 2003). As previously mentioned, TAM, is the most commonly used drug in the treatment of hormone responsive BC cells. TAM has been shown to induce a dose-dependent cytotoxicity, where it caused a 50 - 60% reduction in cancer cell survival at 10 µM for 24 hrs (Perry et al., 1995; Zhang et al., 1999a). However, in our results, we only observed approximately 10% cancer cell growth inhibition in non-transfected MCF-7 cells at 10 µM for 24 hrs and a four-fold increase in growth inhibition in transfected MCF-7 cells. This is possibly an indication that our MCF-7 cell lines are more resistant, yet still causing a 40% increase in growth inhibition in the absence of *CETP*.

Interestingly, *CETP* knock-down resulted in a significant 8–10% increase in growth inhibition at 20 and 40 µM of MβCD. Although this increase seems low, MβCD is a known cholesterol-depleting agent that is relatively low in cytotoxicity and several studies have shown that it is only effective in concentrations that are greater than 2 mM (Grosse et al., 1998; Li et al., 2006; Onodera et al., 2013). Therefore, we show that the absence of *CETP* increases the efficacy of MβCD, even at low concentrations (20 and 40 µM). This increase in efficacy in transfected MCF-7 cells may indicate that by knocking-down *CETP* with the use of a cholesterol-depleting agent, such as MβCD, increases cholesterol depletion and possibly induces membrane instability thus sensitizes cells to foreign compounds (Krause and Regen, 2014; Yeagle, 1985). This membrane instability from knocking-down *CETP* may also explain the enhanced cell growth inhibition when treated with 5, 20 and 40 µM 5-FU. However, when cells were treated with 10 µM, there was a slight decrease in growth inhibition. This could possibly suggest that there was uneven loading of cell numbers in the wells. Therefore, the overall increase in growth inhibition when treated with a variety of drugs/compounds may suggest that *CETP* knock-down possibly reduced MDR by decreasing P-gp activity due to membrane fluidity or decrease in membrane cholesterol (Reungpatthanaphong et al., 2004).

AP is a cholesterol-depleting agent derived from PL and has been found to be less cytotoxic to normal cells while still effective in cancer cells (Sagar et al., 2014). In addition, AP treated cancer cells have been observed to reduce *CETP* mRNA expression by 35% (Esau et al., 2016). In this study, we observed a decrease in cell growth inhibition in transfected MCF-7 cells when compared to non-transfected MCF-7 cells at lower concentrations. There is no biological explanation available for this observation and warrants further investigations. In this study, there was an increase in growth inhibition in transfected MCF-7 cells at 40 µM. However, this concentration becomes too cytotoxic to cells therefore it was expected to decrease cell viability.

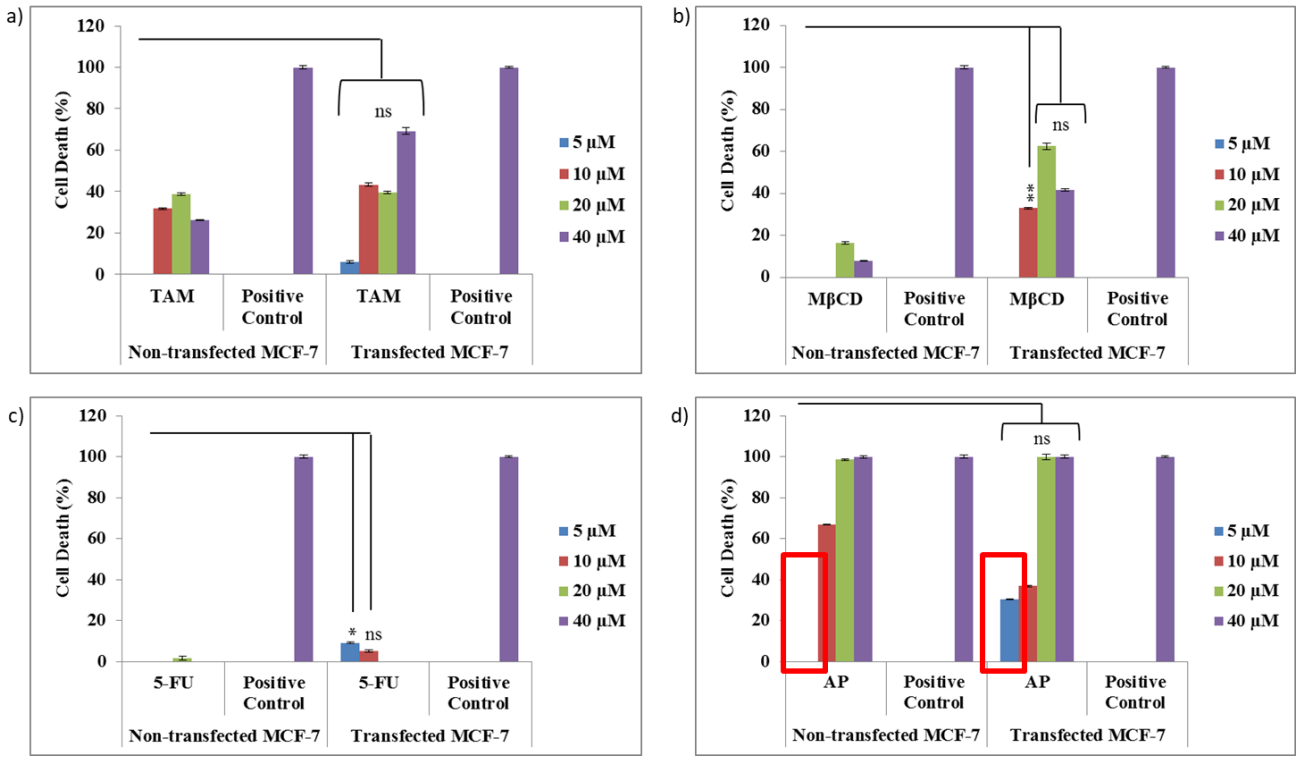


Figure 4.5: *CETP* knock-down increased apoptosis in transfected MCF-7

*Fig. 4.5 depicts the comparison of cell death, through the analysis of APO% assay. The transfected MCF-7 cells to non-transfected MCF-7 cells after treatment with various concentrations (5, 10, 20 and 40 µM) of a) TAM, b) MβCD, c) 5-FU and d) AP. 40 µM PL was used as a technical positive control. Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z- factor > 0.5.*

Subsequently, APO% assay was performed to observe the amount of apoptosis caused by the various drugs in non-transfected and transfected MCF-7 cell line.Although not significant, knock-down of *CETP* resulted in an increase in apoptosis when treated with a) TAM (by 10-40% at different concentrations), b) MβCD (by 30-60%) and c) 5-FU (by 5-10%). This correlates with the results obtained from MTT (Fig. 4.3) as an increase of growth inhibition was observed in transfected MCF-7 cells compared to the non-transfected MCF-7 cells. The same was observed for AP in Fig. 4.5d, where no significant change in apoptosis percentage was noted except at 5 µM (shown in red box). Although there was no statistical significance, there is a 30% biological significance in the analysis. However, this could possibly due to the small absorbance values as the smaller the number the greater the chance for error.

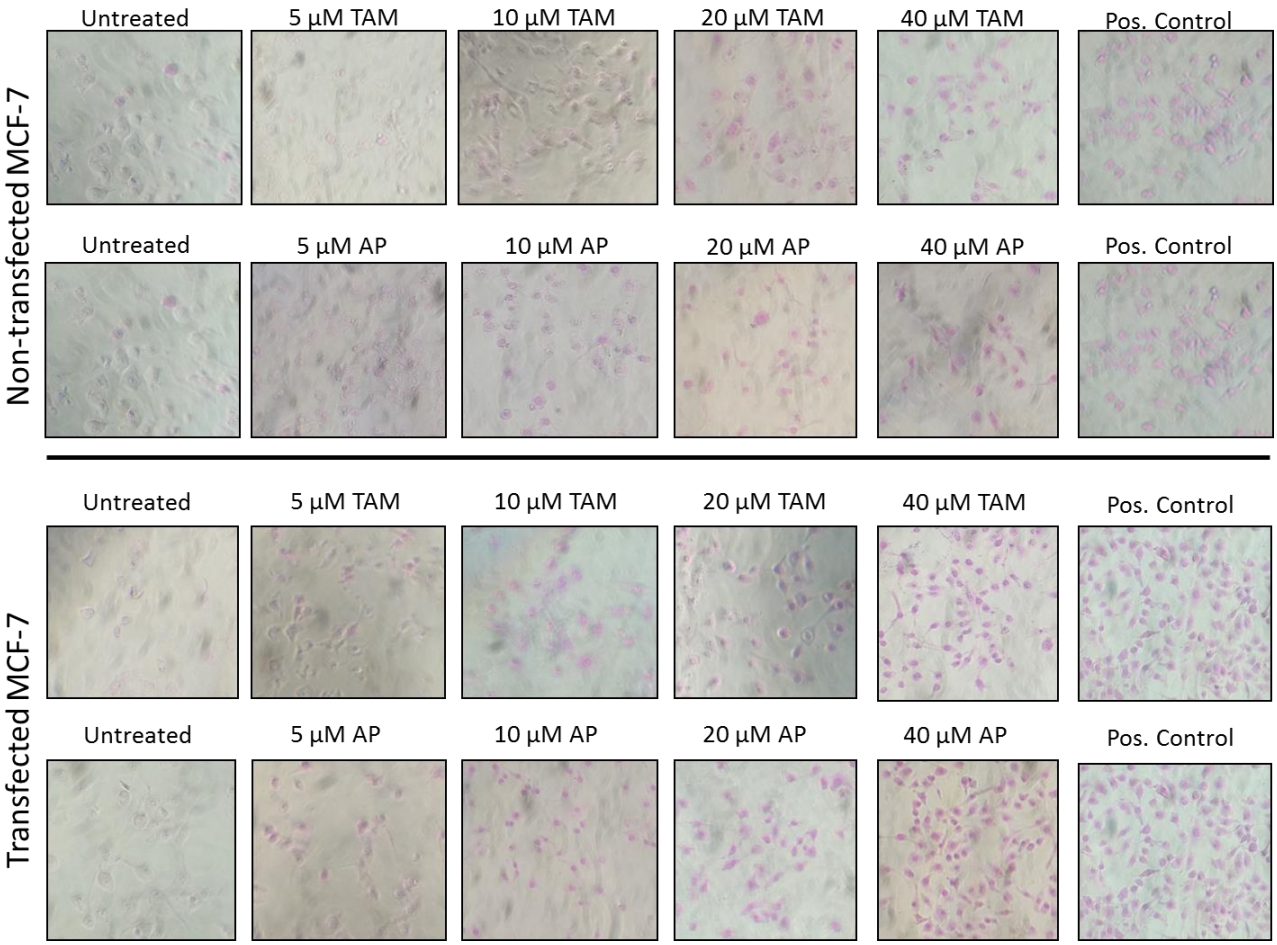


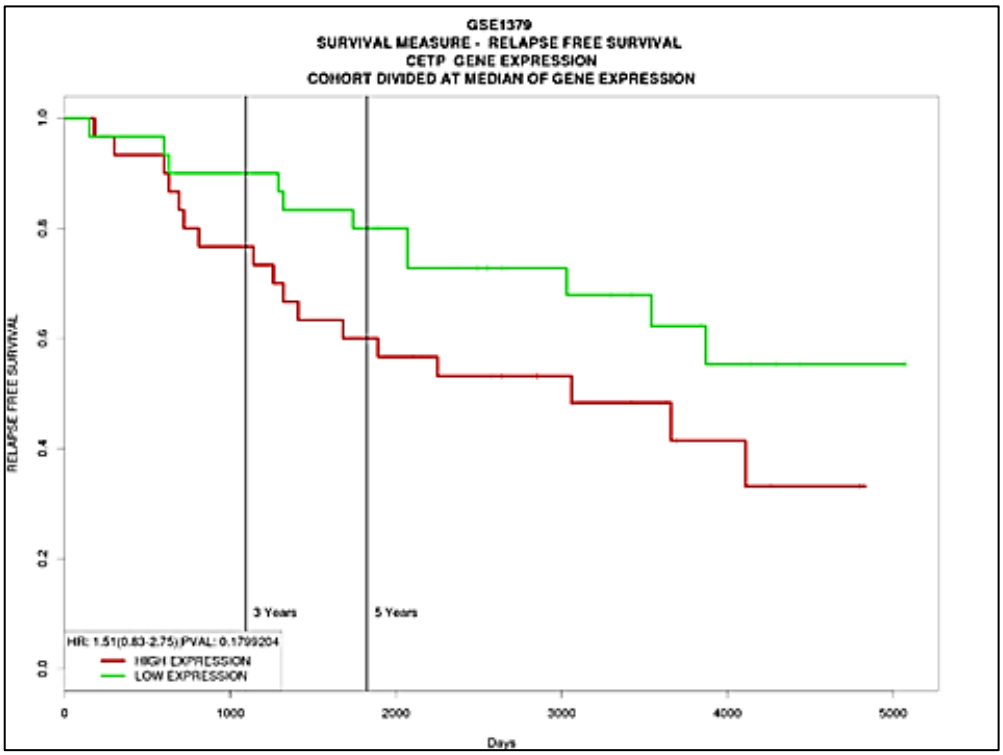
Figure 4.6: Visual representation of APO% assay between transfected and non-transfected MCF-7 cells treated with TAM and AP

The images in Fig. 4.6 are a visual representation of the effects of various drug concentration treatments in non-transfected MCF-7 cells compared to the untreated MCF-7 cells presented in Fig. 4.5.

As observed above, increasing drug concentrations lead to a progressive increase in APO% dye intake and thus the cells appear more pink when compared to the untreated cells. Furthermore, transfected cells appear to have retained more dye as compared to the non-transfected MCF-7cells with a direct relation to drug concentration. Therefore, confirms the resultes depicted in Fig. 4.5.

APO% assay that the knock-down of *CETP* enhanced apoptotic activities when cells were treated with TAM, MβCD and 5-FU. Interestingly, at 5 µM AP however, there was about a 30% increase in apoptosis in transfected MCF-7 cells when compared to non-transfected cells. This correlates to the results obtained in (Esau et al., 2016), where *CETP* knock-down showed an increase in apoptosis. This is favourable as it was mentioned previously, lowered effective concentration would suggest a smaller IC_{50} value. This could possibly reduce adverse side effects that are usually associated with various drugs. Moreover, the increase in apoptosis is further supported by the Kaplan-Meier plots in Fig. 4.7 displaying a link between *CETP* expression and BC patient survival from four different databases (Esau et al., 2016). These databases depicted that a low *CETP* expression is associated with longer relapse-free survival as well as overall survival. In addition, ER+ BC was revealed to express more *CETP* compared to ER- BC and are less likely of long term survival (Esau et al., 2016; Sukkasem et al., 2016).

a)



b)

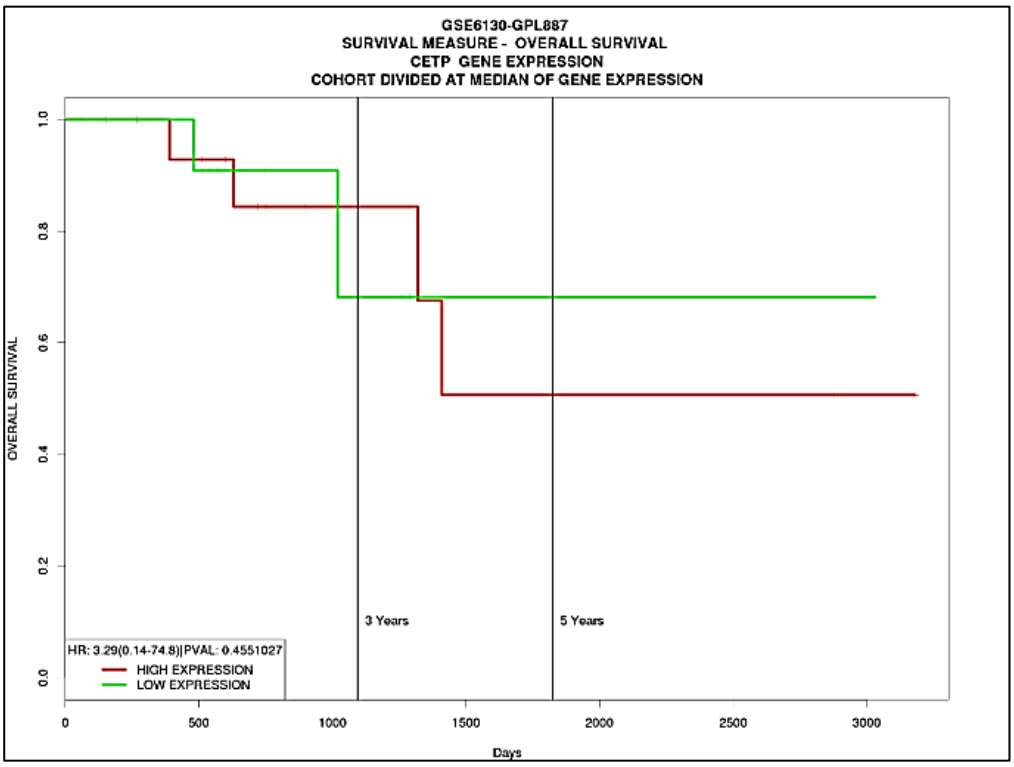


Figure 4.7: Kaplan-Meier Plot on relapse-free survival and overall survival

Fig. 4.7a and b are Kaplan-Meier plots of (a) relapse-free survival and (b) overall survival of BC patients stratified by median CETP expression in the PROGgene database (Esau et al., 2016; Goswami and Nakshatri, 2013). The red line represents high CETP expression and green line represents low CETP expression.

4.3 Measurement of cholesterol levels in transfected and non-transfected MCF-7 cells

Cholesterol assay was then performed to measure CE levels in non-transfected and transfected MCF-7 cells (objective 2).

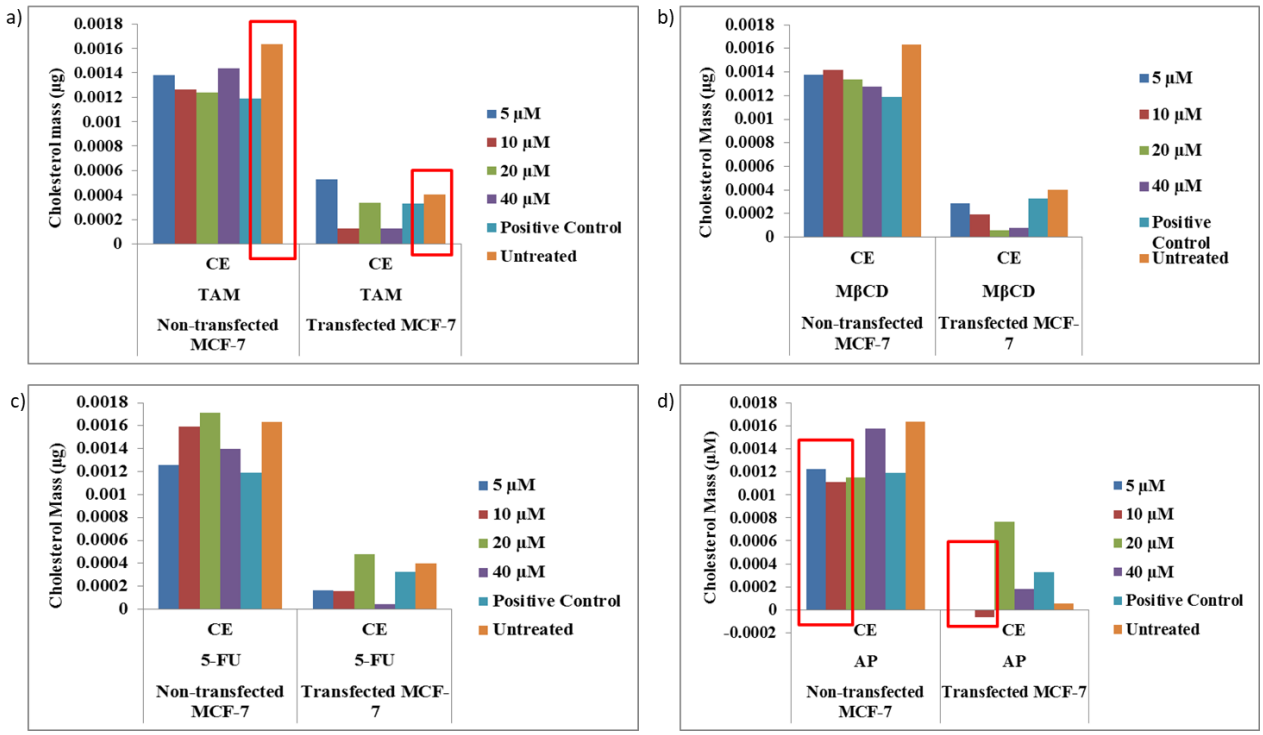


Figure 4.8: Representation of CE levels in non-transfected and transfected MCF-7 cells

Fig. 4.8 illustrates the CE level, through the analysis of cholesterol assay. The transfected MCF-7 cells were compared to non-transfected MCF-7 cells before and after treatment with various concentrations of a) TAM, b) MβCD, c) 5-FU and d) AP. 40 µM PL was used as a technical positive control.

We observed a 70-90% decrease in CEs across the various drug/compound treatment in transfected MCF-7 cells compared to the non-transfected cells. Interestingly, apart from a decrease in CEs in drug/compound treated cells, there was also a decrease in CEs in the untreated transfected MCF-7 cells (indicated in the red boxes in Fig. 4.8a). Therefore, this suggests that by knocking down *CETP*, intracellular CE levels also reduced. Samples were performed in duplicates due to financial constraints. In addition, CE levels decreased drastically in AP-treated transfected MCF-7 cells at 5 and 10 µM. This could possibly explain the increase in cell death observed in transfected MDA-MB 231 cells (Fig. 4.5).

As aforementioned, cancer cells are rich in cholesterol and thus have an altered lipid metabolism maintained by SREBP and ACAT (involved in CE synthesis) (Peck and Schulze, 2014). It has been found that an increased activity of SREBP have been associated with several cancers and SREBP inhibition halted cancer cell growth and proliferation (Peck and Schulze, 2014). Therefore, this decrease in CE level that we observed in transfected MCF-7 cells could possibly suggest a reduced SREBP activity. In addition, the accumulation of intracellular CEs result in an escalated PI3K-dependent SREBP activity and thus fueling

cancer aggressiveness (Peck and Schulze, 2014; Yue et al., 2014). Thus, we speculate that the surge in cancer cell growth inhibition and apoptosis in transfected MCF-7 cells treated with various drugs and compounds could be due to a reduction in CEs thereby managing cancer aggressiveness and increasing response to toxic drugs or compounds. Furthermore, high levels of intracellular CEs have been found to be associated with drug resistance (Peck and Schulze, 2014). This is due to the fact that in normal cells, the excess cholesterol stops the SREBP and ACAT activity. However, in cancer cells, ACAT and SREBP are continuously producing CEs for storage and thus reducing intracellular toxicity (Peck and Schulze, 2014). Additionally, in (Reungpatthanaphong et al., 2004), it was found that P-gp activity (involved in MDR) is directly correlated to cholesterol levels and a decrease in cholesterol level resulted in a reduced P-gp activity thus reducing drug resistance. For these reasons, the increased cell death in transfected MCF-7 cells that we observed could have been due to the drop in intracellular CE levels thus redcuing drug resistance.

4.4 Analysis of growth inhibition and apoptosis in transfected and non-transfected MCF-7 cells before and after the combination treatment of TAM and AP

In addition, MCF-7 cells were treated with various concentrations of TAM together with different concentrations of AP to observe the effect on growth inhibition and apoptosis. The combination treatment was performed to observe whether the efficacy of TAM can be increased, especially at low concentrations, while reducing TAM resistance.

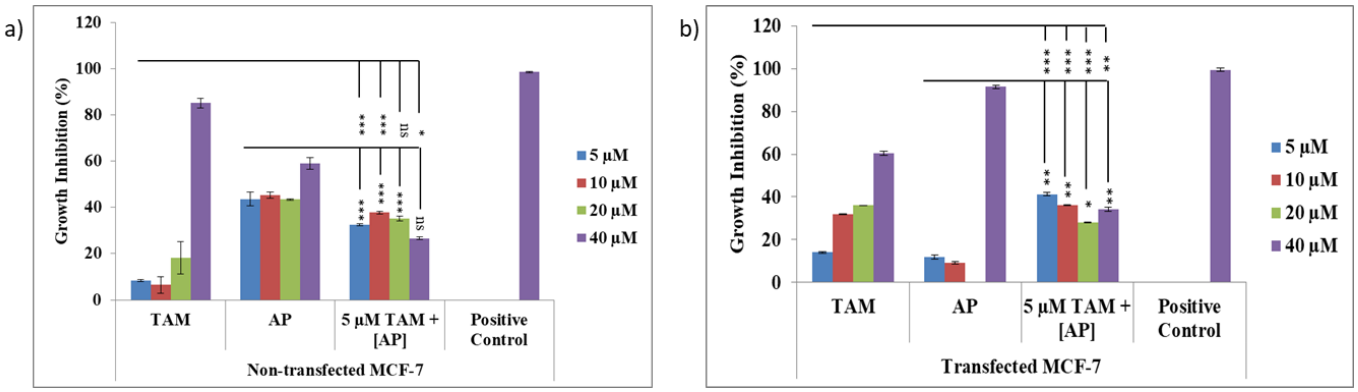


Figure 4.9: Representative of combination treatments (5 µM TAM with various AP concentrations) in comparison to single drug treatment in non-transfected MCF-7 cells and transfected MCF-7 cells

Fig. 4.9 illustrates the effects of combination treatment on growth inhibition, through the analysis of MTT assay. The transfected MCF-7 cells (a) were compared to the non-transfected MCF-7 cells (b). The concentration of TAM is constant (5 µM) whereas the

*concentration of AP is varied (5, 10, 20 and 40 µM). 40 µM PL was used as a technical positive control. Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z- factor > 0.5.*

Although, in the non-transfected MCF-7 cells (Fig. 4.9a), the combination treatment appear to have increased growth inhibition when compared to TAM single treatment, there was actually a decrease in growth inhibition when compared to AP single treatment. In the transfected MCF-7 cells (b) however, we observed an increase in growth inhibition about 30% when compared to both single treatments. Therefore, the increase in the combination treatment observed in transfected MCF-7 cells is possibly due to an increased efficacy of TAM. Supporting data (other concentrations of TAM and AP) are in the appendix (Fig 8.1 and 8.2).

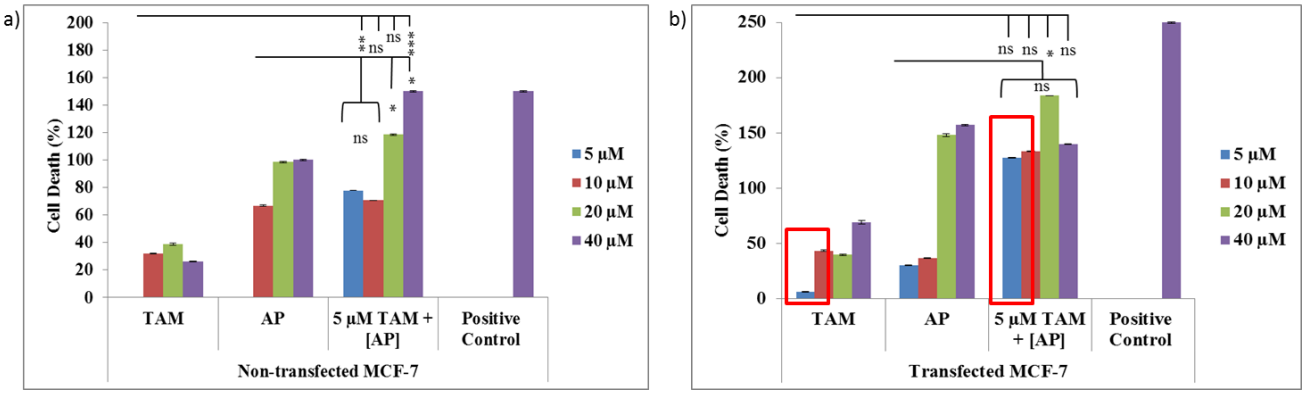


Figure 4.10: Representative of the combination treatment (5 µM TAM with various AP concentrations) in non-transfected and transfected MCF-7 cells

*Fig. 4.10 illustrates the effects of combination treatments on apoptosis, through the analysis of APO% assay. The combination treatments were compared to single drug treatment (TAM or AP alone) in non-transfected MCF-7 cells. Similarly, the combination treatment was compared to the single treatment in transfected MCF-7 cells. The concentration of the combination treatment is composed of 5 µM TAM and varying concentrations of AP (5, 10, 20 and 40 µM). 40 µM PL was used as a technical positive control. Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z- factor > 0.5.*

Similar results were observed in APO% assay where in (Fig. 4.10a) non-transfected MCF-7 cells, even though apoptosis appear to have increased in the combination treatment compared to TAM single treatment, when compared to AP single treatment not much difference was observed at lower concentrations. However, (b) in transfected MCF-7 cells, the combination treatment caused a 15-fold increase in apoptosis compared to both single treatments (shown

in red box). Therefore, the increase in the combination treatment observed in transfected MCF-7 cells is possibly due to an increased efficacy of TAM. Supporting data (other concentrations of TAM and AP) are in the appendix (Fig. 8.3 and 8.4).

CETP knock-down along with the use of a cholesterol-depleting agent, AP, and treating the cells with TAM did indeed increase the apoptosis compared to single treatment by approximately 15-fold even at low concentrations (5 and 10 µM). This is possibly due to a decrease in CEs from knocking-down *CETP* together with the use of a cholesterol-depleting agent resulting in increased vulnerability of cells to TAM. Therefore, this suggests that when the three treatments are combined, the efficacy of TAM increases. Moreover, it appears that cells are less resistant to TAM when *CETP* was knocked-down along with the use of AP. Therefore, *CETP* knock-down along with the use of a cholesterol-depleting agent could possibly reduce resistance and increase efficacy of TAM and potentially other chemotherapeutic drugs. This increase in drug efficacy would mean that the dose of highly potent drugs could be reduced without lowering its effects and ultimately reduce possible side effects.

4.5 CETP protein knock-down was confirmed using Western blotting in MDA-MB 231 cells

These assays proved to be highly effective in hormone responsive or ER+ BC cells. However, the effects of knocking-down *CETP* together with the use of AP have never been studied before in a triple negative BC cell line (MDA-MB-231). Due to time constraints, only certain assays were performed on MDA-MB-231 cells, however further experiments will be performed during my PhD study.

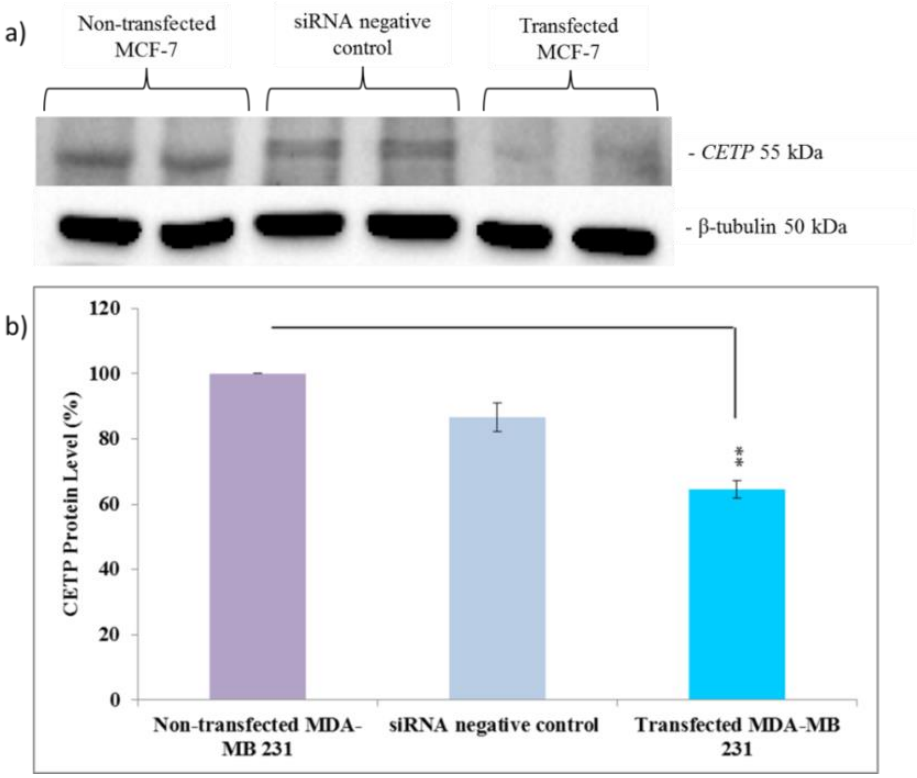


Figure 4.11: CETP protein was significantly knocked-down in MDA-MB 231 cells

*Fig. 4.11a illustrating protein bands from Western blotting using Chemidoc and b) depicts the densitometry analysis in MDA-MB 231 cells. Data represents the mean ± standard deviation S.D. (n=3), ** P < 0.001.*

Densitometry analysis revealed a 63% decrease in CETP mRNA expression in MDA-MB 231 cells.

4.6 Growth Inhibition and Apoptosis analysis in transfected and non-transfected MDA-MB 231 cells

Transfected and non-transfected MDA-MB 231 cells were then treated with the various drug concentrations and the effects of the drugs on growth inhibition and apoptosis was analysed.

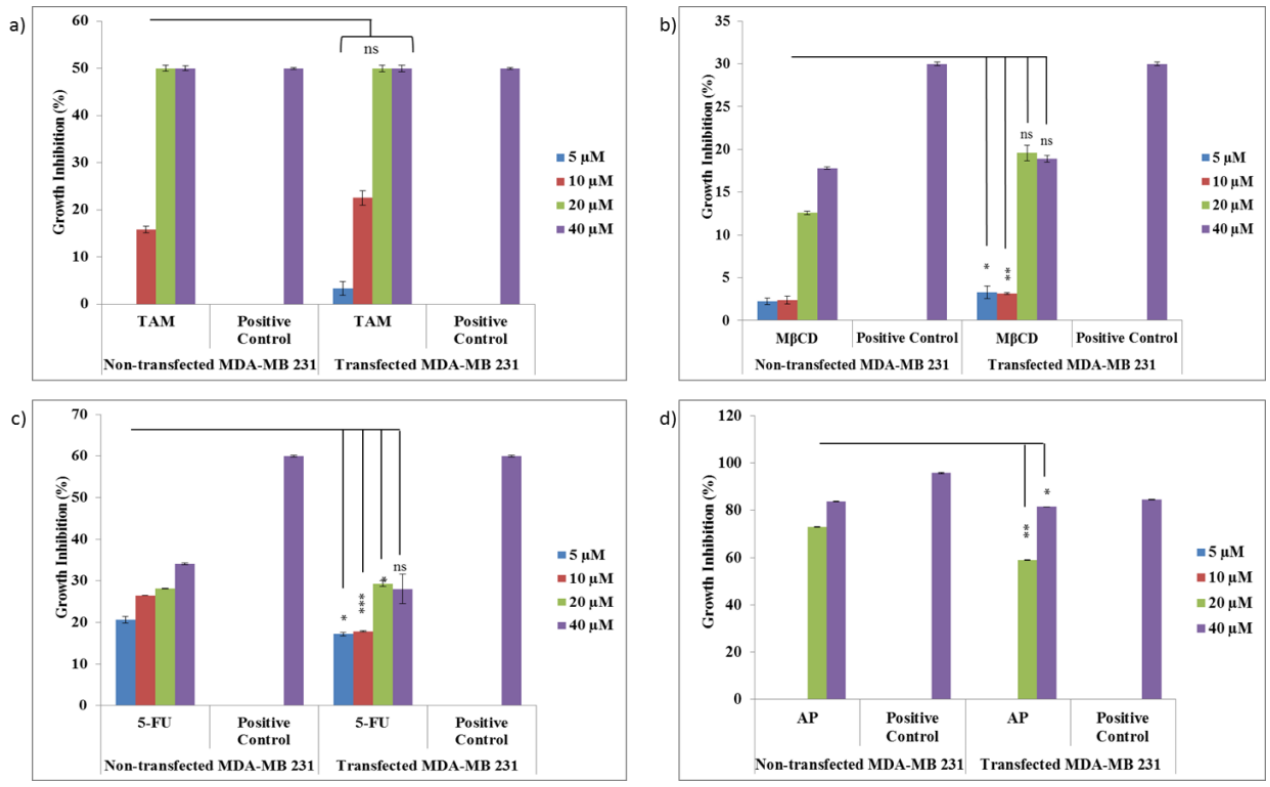


Figure 4.12: Growth inhibition was not significantly affected by the knock-down of CETP in MDA-MB 231 cells

*Figure 4.12 shows comparative growth inhibition, through the analysis of MTT assay. The transfected MDA-MB 231 cells were compared to non-transfected MDA-MB 231 cells treated with a) TAM, b) MβCD, c) 5-FU and d) AP. 40 µM PL was used as a technical positive control. Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z- factor > 0.5.*

A small increase in growth inhibition was observed in transfected MDA-MB 231 cells treated with (a) lower concentrations of TAM (5 and 10 µM). In addition, the same was observed in transfected MDA-MB 231 cells treated with (b) MβCD at all concentrations. However, no significant difference was observed for 5-FU and AP treated transfected MDA-MB 231 cells. 40 µM PL was used as positive control. Positive control was cut off at 50% cell death (Fig. 4.12a), 30% (Fig. 4.12b), 60% (Fig. 4.12c) and Fig. 4.12d did not change. The positive control was cut off at various percentages due to the large difference between the test compounds and positive control.

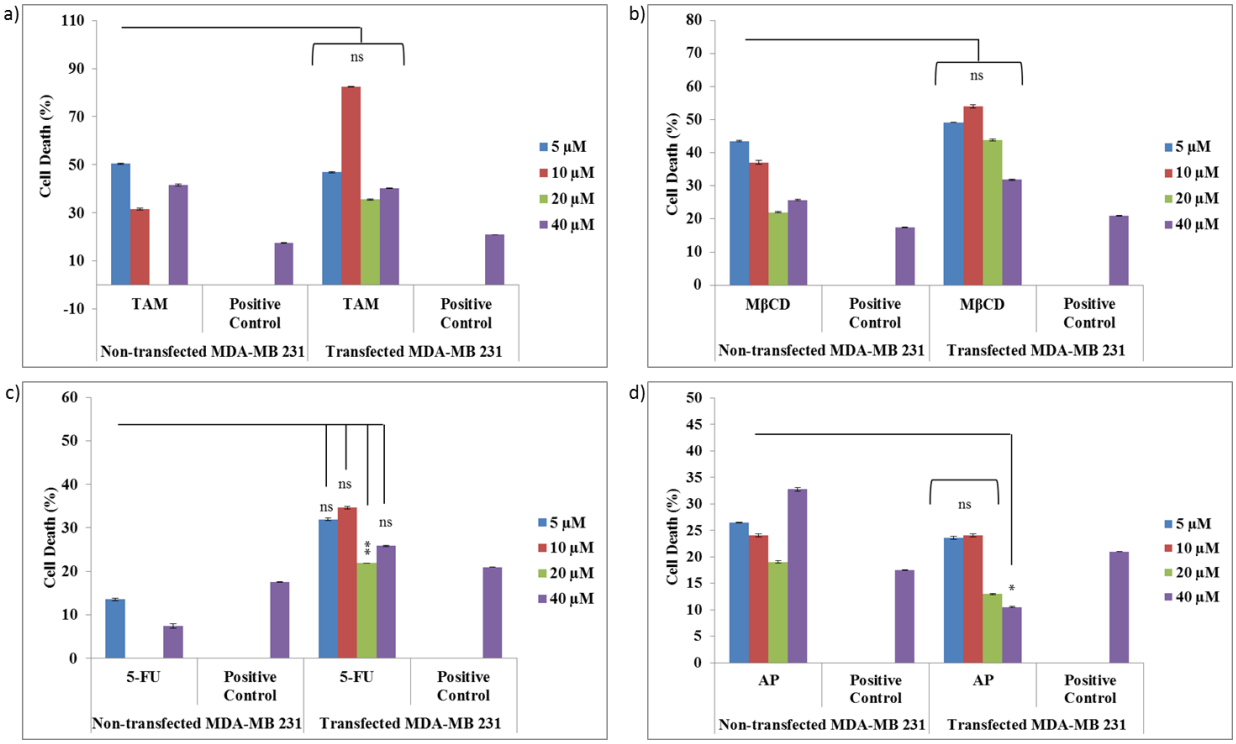


Figure 4.13: CETP knock-down resulted in an increase in apoptosis in transfected MDA-MB 231 cells when treated with tamoxifen, MβCD and 5-FU when compared to non-transfected cells

*Figure 4.13 shows comparative analysis of level of apoptosis, through APO% assay. The transfected MDA-MB 231 cells were compared to non-transfected MDA-MB 231 cells treated with a) TAM, b) MβCD, c) 5-FU and d) AP. 40 µM PL was used as a technical positive control. Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z- factor > 0.5.*

Again, a small increase in apoptosis was observed in transfected MDA-MB 231 treated with 10 and 20 µM TAM and MβCD when compared to non-transfected cells. Interestingly, the efficacy of 5-FU appeared to have increased when *CETP* was knocked-down compared to non-transfected MDA-MB 231 cells. However, when transfected MDA-MB 231 cells were treated with AP not much difference in apoptotic activity was observed when compared to the non-transfected cells. In addition, there was actually a reduction in apoptotic activity in higher concentrations of AP in transfected cells compared to non-transfected cells.

Hormone responsive BCs, such as MCF-7, are rich in cholesterol. However MDA-MB 231 cells are triple negative cells that lack the hormone receptors as well as the HER2 receptor for cell growth and proliferation. In addition, it was found in our lab that the cholesterol levels in

MDA-MB 231 cells are several times lower compared to MCF-7 cells. Previously, in (Esau et al., 2016), *CETP* knock-down was performed in BT20 cell line (another triple negative BC cell line) to study the role of CETP in triple negative cell line, however, it has never been performed in MDA-MB 231 cell line. Therefore, these results serve as baseline data for further investigations. From the cell viability assay, we observed a small increase in growth inhibition at low concentrations of TAM but not significant increase was observed at higher concentrations. Additionally, no significant increase in growth inhibition and cell death was observed in transfected MDA-MB 231 cells when treated with MβCD, 5-FU and AP. TAM, as discussed previously, plays an antagonistic role to estrogen therefore binds to ER to prevent cell proliferation and survival (Hodges et al., 2003; Mourits et al., 2001). Furthermore, the effects of TAM has been found to be ER dependent at low concentrations, while at higher concentrations the effects are independent of ER (Perry et al., 1995). This is evident in this study, where TAM is more effective in MCF-7 cells however, in MDA-MB 231 cells TAM is only effective at higher concentrations (Fig. 4.12 and 4.13).

In addition, no significant change was observed in growth inhibition and cell death in transfected MDA-MB 231 cells when treated with MβCD (Fig. 4.12 and 4.13). This is probably due to the fact that MDA-MB 231 cells are already low in cholesterol and thus the depletion of cholesterol does not affect the survival of MDA-MB 231 cells. No significant increase in growth inhibition was observed in transfected MDA-MB 231 cells when treated with 5-FU (Fig. 4.12). However, this was opposite to what was observed for cell death as there was an increase in cell death in transfected MDA-MB 231 cells. However, the results obtained from MTT assay may not always correlate to the results obtained from apoptosis assays as MTT assay measures metabolic activities and not apoptosis (Gerlier and Thomasset, 1986). The increase in apoptosis may indicate a possibility that *CETP* knock-down resulted in a decrease in cholesterol and thus may have weakened the cell membrane. This could then possibly allow 5-FU to enter the cells more easily (as 5-FU targets the DNA replication process as mentioned previously) and thus causing an increased cell death in transfected MDA-MB 231 cells (Fig. 4.13). In addition, it has been found that 5-FU treated MDA-MB 231 cells increases Fas/*FasL* expression levels (Chhipa and Bhat, 2007). Fas is a type I transmembrane protein and it is part of the tumour necrosis factor-R (TNF-R) family involved in the induction of apoptosis (Huang et al., 1999). Therefore, this could possibly be a more effective strategy in treating ER- cancer cells.

AP-treated transfected MDA-MB 231 cells also did not show a significant difference in both growth inhibition as well as apoptosis. This was also evident in (Sagar et al., 2014) where AP did not induce apoptosis in BT20 cells. In addition, it was also found that AP disrupted lipid rafts in MCF-7 cells (35% reduction in cholesterol) however in BT20 cells, lipid rafts appear to be intact and could possibly suggest AP's selectivity to ER+ BC cells (Esau et al., 2016).

Although single treatment appears to be less effective against ER- BC cells, a combination treatment was performed to investigate whether TAM together with AP would increase growth inhibition and cell death.

4.7 Analysis of growth inhibition and apoptosis in transfected and non-transfected MDA-MB 231 cells before and after the combination treatment of TAM and AP

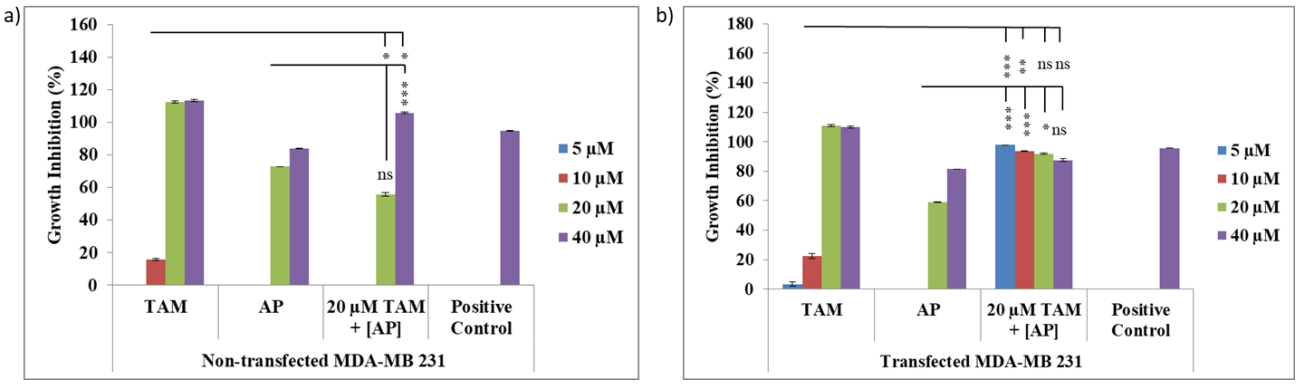


Figure 4.14: Representative of combination treatment in transfected and non-transfected MDA-MB 231 cells

*Fig. 4.14 illustrates growth inhibition analysis, through the MTT assay. The combination treatments (20 µM TAM and various concentrations of AP; 5, 10, 20 and 40 µM) were compared to the single treatments (TAM and AP alone at 5, 10, 20 and 40 µM) in non-transfected MDA-MB 231 cells. Similarly, the combination treatments were compared to the single treatments in transfected MDA-MB 231 cells. 40 µM PL was used as a technical positive control. Supporting data are represented in appendix (Fig. 8.5 and 8.6). Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z- factor > 0.5.*

The results suggest that *CETP* knock-down does not increase the efficacy of chemotherapeutic drugs, hormone targeted chemotherapeutic drug or compounds. This could

possibly be due to the lower cholesterol levels in MDA-MB 231 cells however, this will be further investigated. Interestingly enough, combination treatment increased the efficacy of 20 µM TAM in transfected MDA-MB 231 cells when treated along with varying concentrations of AP (5, 10, 20 and 40 µM)

Subsequently, the cytotoxicity effects of various drugs and compounds were tested in HEK 293 cell line (normal cell line).

4.8 Comparison of growth inhibition in non-transfected MCF-7 cells and HEK 293 cells after various drug treatments

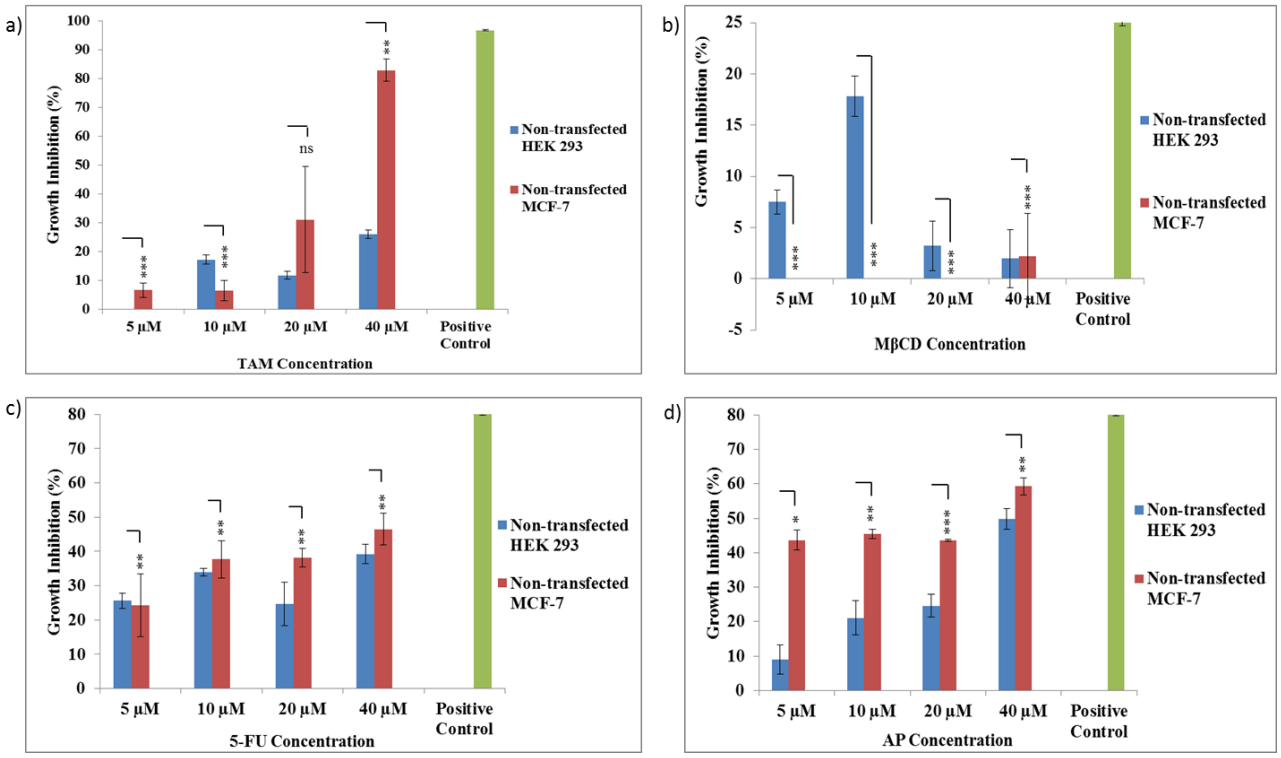


Figure 4.15: Comparison in growth inhibition between non-transfected MCF-7 and HEK 293 cells when treated with various drugs and compounds

*Fig. 4.15 represents growth inhibition, through the analysis of MTT assay, between non-transfected MCF-7 and non-transfected HEK 293 cells treated with a) TAM (at 5, 10, 20 and 40 µM), b) MβCD (at 5, 10, 20 and 40 µM), c) 5-FU (at 5, 10, 20 and 40 µM) and d) AP (at 5, 10, 20 and 40 µM). 40 µM PL was used as a technical positive control. Data represents the mean ± S.D. (n=3), where * P < 0.05, ** P < 0.001, *** P < 0.0001 and ns = not significant. All plates had a Z- factor > 0.5.*

The effects of the drugs and compounds were then tested in HEK 293 cells to observe the cytotoxicity of each drug and compounds in normal cells. In TAM treated non-transfected HEK 293, we observed a much lower toxicity when compared to MCF-7 cells (Fig. 4.16a). This suggest that TAM is relatively non-cytotoxic even at 40 µM. It can also be observed that when HEK 293 cells were treated with AP (Fig. 4.16d), only higher concentrations of AP were quite toxic in both normal and cancer cells. However, as explained previously, we are targetting low concentrations that are just as effective as higher concentrations in cancer cells and not normal cells to reduce drug resistance. In contrast to TAM and AP, 5-FU appear to be cytotoxic in both normal as well as cancer cells (Fig. 4.16c). This is possibly due to the non-specific, DNA replication inhibitory properties of 5-FU. MβCD, as discussed, is a known cholesterol depleter however it was observed that MβCD is more cytotoxic in normal cells and not effective in cancer cells at low concentrations compared to AP (Fig. 4.16b and d). This could imply that AP could possibly be a better cholesterol depleting agent than MβCD.

5. Summary

As mentioned previously, that this study was performed to generate baseline data for future experiments and therefore the key findings in this seminal study will be highlighted here. It was found that *CETP* knock-down resulted in a decrease in CE levels and thus possibly reducing drug resistance, as CE levels have been previously associated with the aggressiveness of cancers. In addition, it was also found that lower concentration of TAM (5 µM) combined with AP and *CETP* knock-down enhances drug efficacy by possibly reducing drug resistance in ER+ BC. However, *CETP* knock-down still increases TAM efficacy at higher concentrations (20 µM) in the combination treatment in ER- BC cells. Interestingly, 5-FU appeared to be more effective in ER- BC cells when *CETP* was knocked-down when compared to ER+ BC cells. Therefore, 5-FU in combination with AP could possibly be a promising strategy in treating ER- BC cells. Thus this strategy should be exploited further as AP has been proven, in this study as well as in one other study by (Sagar et al., 2014), that it is safe and low in cytotoxicity in normal cells. Nevertheless, the effects of the drugs and compounds in both single and combination treatments were more demarcated in MCF-7 cells compared to MDA-MB 231 cells. MCF-7 cells are ER+ BC cells therefore, it would be interesting to check the expression level of estrogen receptor gene (*ESR1*) in future studies. Prof Kaur in her previous work found that AP decreased expression of *ESR1* in microarray experiment (unpublished result). Furthermore, the biological explanations for most of the

observations made during the study are not yet available at the moment as this is a totally new concept and background literature are limited to compare the results of this investigation. Future studies will be focused on understanding the effects of *CETP* knock-down on cholesterol pathway associated proteins such as; SREBP, ACAT1, LDLR, ESR1 and LXR. Additionally, apoptotic assays such as caspase assays, mitochondrial outer membrane potential assays and Western blotting on various apoptotic proteins will be performed to elucidate molecular mechanisms involved and thus provide a possible explanation for the effects we observed especially for AP treatment. Moreover, *CETP* knock-down should be performed in HEK 293 cell line for the combination treatment to observe cytotoxicity effects on normal cells as well as checking for CE levels. The CE levels will also be investigated in MDA-MB 231 (ER-) cell line after transfections. In this study, cholesterol-depleting agents in combination with TAM have shown to be a promising strategy in treating BC however, other cholesterol-depleting agents (such as cyclodextrins) along with TAM could also be investigated. Subsequently, an *in-vivo* study will be performed to test the effect of *CETP* knock-down on tumour growth and response to various drugs or compounds. In contrast to knocking-down *CETP*, an over-expression study should be performed to observe what the effects would be if *CETP* is expressed abundantly in cancer cells and normal cells.

6. Conclusions

BC is amongst the top five lethal cancers in South Africa with increasing reported deaths yearly. Treatment for BC is limited as patients often suffer from relapses, especially from long term use of TAM, as it is the most common and effective treatment for BC. Therefore strategies in enhancing drug efficacy, while reducing drug resistance may be a beneficial approach. In this study, we showed that *CETP* knock-down increased cell death in MCF-7 cells possibly due to a reduction in CE levels. In addition, *CETP* knock-down along with combination treatment involving a cholesterol-depleting agent such as AP increased TAM efficacy even at low concentrations (5 µM). Therefore, CETP could thus serve as a potential drug-resistance marker in cancer cells, more specifically BC. Furthermore, strategies targeting CEs could possibly be used as a potential combination treatment for cholesterol-related diseases as well as reduce drug resistance and dosage

7. Trouble-shooting

All experiments were standardised in non-transfected MCF-7 cells and assays were performed in various ways for optimal results. These are as follows:

WESTERN BLOTTING

Western blotting was standardised by loading various concentrations of sample lysates; 5, 10 and 20 mg/ml. In addition, 40% and 30% bis-acrylamide solutions were tested in a gel to determine which pore size was more suitable. In addition, various concentrations of the antibodies were also tested and 2 blocking solutions (3 and 5% blocking solutions) were tested.

RNA EXTRACTION

RNA was initially extracted from the protocol using trizol (Esau et al., 2016) however, the purity of the RNA was poor and thus the Direct-zol™ RNA MiniPrep kit (Zymo Research, Inqaba, SA) was used to extract good quality RNA.

RT-qPCPR

RT-qPCR to quantify *CETP* mRNA expression was first performed using a probe-based assay – PrimePCR™ Assay kit (Bio-Rad, USA). However, expression levels were not able to be detected. This could possibly be due to the fact that probes are light sensitivity and thus light could have reduced probe activity during preparation of the assay. SYBR green was thus used as an alternative method.

MTT ASSAY

MTT assay was standardised with different concentrations of H_2O_2 (100 µM, 200 µM, 500 µM, 1 mM, 5mM and 10 mM) and PL (5 µM, 10 µM, 20 µM and 40 µM). In addition, MTT solution was added in drug treated and non-treated cells and incubated at 37°C for varying times: 2, 3 and 4 hrs). Furthermore, different MTT solubilisation solutions (between 10% SDS, 10 mM HCl solution and isopropanol) were used to dissolve formazan crystals to determine which solubilising solution was the most effective. 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 (as isopropanol evaporated during overnight incubation).

APO% ASSAY

APO% was initially performed as per protocol in (Esau et al., 2016), where cells were detached from the plate with 15 mM and 17mM EDTA (previously found to be an effective chemical in lifting cells when added to media but cytotoxic if added directly to cells – the mechanism of this is still unknown). In addition, the same concentrations of H_2O_2 and PL as

per MTT assay were tested to pick out the optimal positive control. Furthermore, the cells were treated for various times (2, 3, 4, 6, and 24 hrs) and different volumes of APO% dye (provided in the APO% assay kit) 0.5, 1, 1.5, 2 and 2.5 µl were tested against the cells as the APO% dye becomes toxic at high concentrations. However, background stain was high therefore, several changes to the parameters for the assay were made. Due to high background reading, we then followed the protocol provided by the manufacturer (APO% assay kit, Biocolor, UK), where media and dye were removed after incubation and cells were washed with 1 X PBS between each step (no EDTA step). Thereafter, APO% assay was performed with and without 'dye release (provided in the APO% kit)' and the contents of the wells were mixed using a plate shaker at 500rpm for 30s to 1 min. However, with the shaking of the plate on the plate shaker, we observed cross contamination between wells even at low rpm. Therefore, mixing was performed using a pipette but the absorbance values were not consistent between samples. Subsequently, different concentrations of trypan blue (0.4%, 0.04%, 0.004% and 0.0004%) were added after APO% dye incubation without lifting the cells. This was done as trypan blue is a fluorescence quencher, thereby it was used to quench background noise however, it appeared that trypan blue also masked intracellular APO% dye. It was then decided that cells will be incubated with APO% dye without detaching the cells for the stipulated time, then discarded from each well and washed with 1 X PBS. Thereafter, cells were stored in PBS until measurement. This last method was used as it was found to produce the best and most consistent results.

CHOLESTEROL ASSAY

Cholesterol assay was standardised by a colleague in the laboratory. This was done by testing different volumes of various components in the assay for optimal results. These included; Ampliflu red (Sigma Aldrich, UK), cholesterol esterase and cholesterol oxidase. In addition, the assay was performed with and without catalase and it was found that with catalase, the background noise was reduced.

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9. Appendix

The following results represents the combination treatments in MCF-7 cells and MDA-MB 231 cells with variable TAM and AP concentrations.

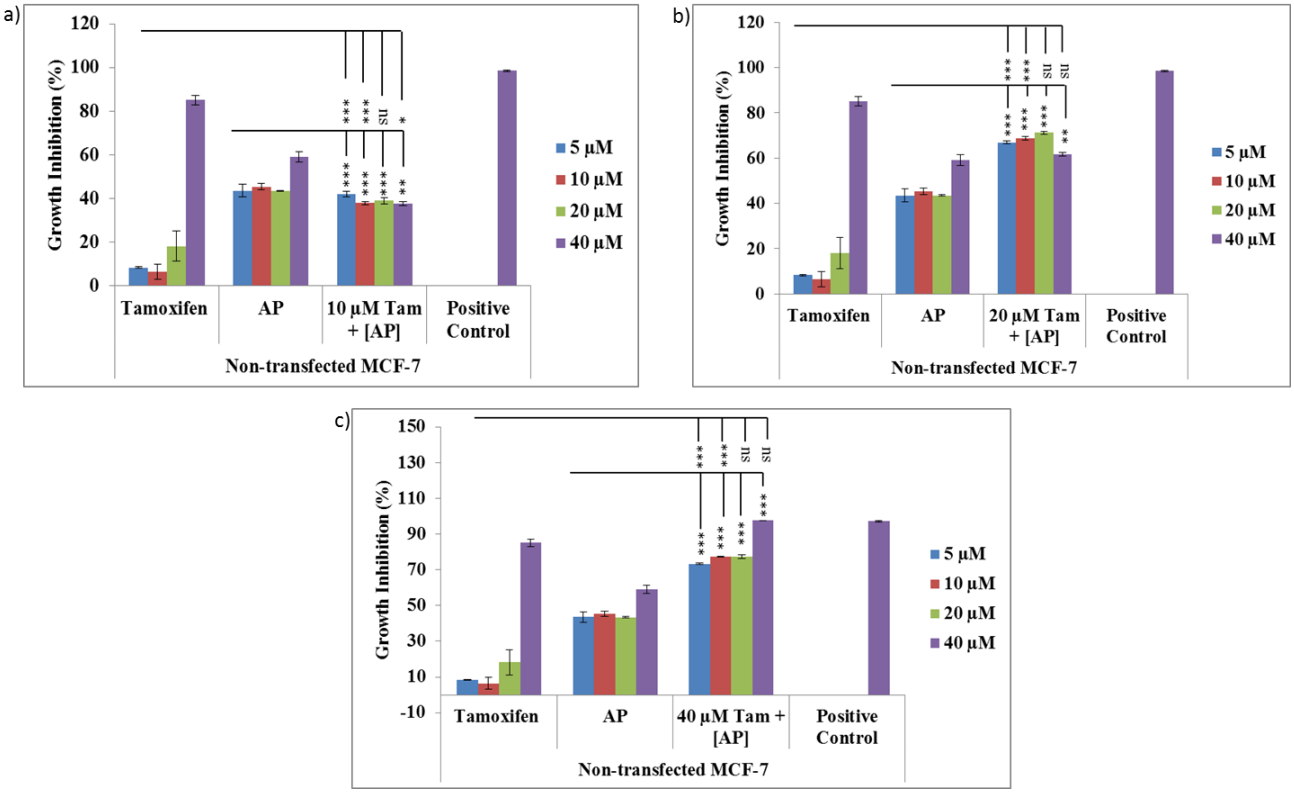

Figure 9.1: Representative of combination treatment on growth inhibition in non-transfected MCF-7 cells

*Fig. 8.1 represents growth inhibition analysis, through MTT assay. The combination treatments were compared to the single treatments in non-transfected MCF-7 cells. The combination treatments were composed of a) 10 µM TAM and various AP concentrations (5, 10, 20 and 40 µM), b) 20 µM TAM and various AP concentrations and c) 40 µM TAM and various AP concentrations. 40 µM PL was used as a technical positive control. Data represents the mean ± standard deviation S.D. (n=3), where * P< 0.05, ** P<0.001, *** P<0. 0001and ns = not significant with a Z-factor > 0.5.*

Although an increase in growth inhibition was observed in the various combination treatments when compared to TAM single treatment, there was no significant change when compared to AP single treatment. Therefore, the effect observed from the combination treatment is more likely to be from AP.

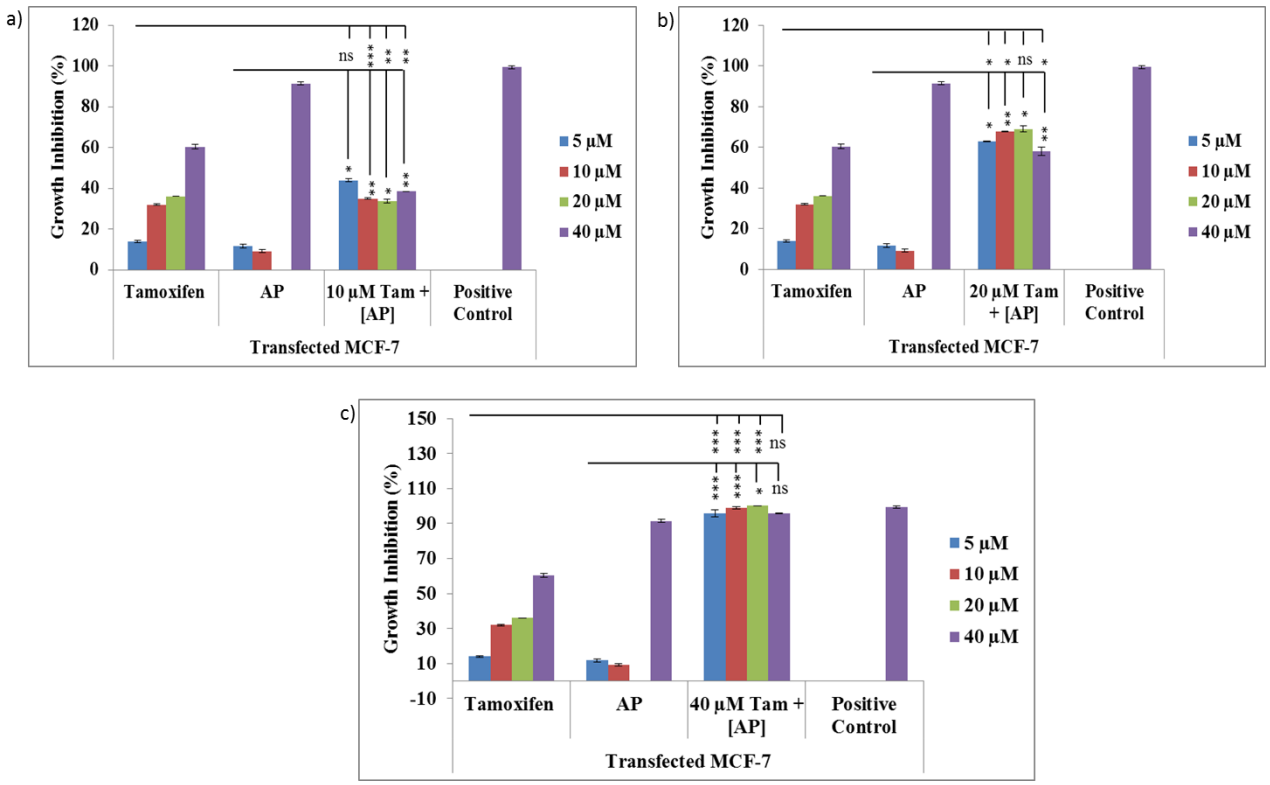

Figure 9.2: Representative of combination treatment on growth inhibition in transfected MCF-7 cells

*Fig. 8.2 represents growth inhibition analysis, through MTT assay. The combination treatments were compared to the single treatments in transfected MCF-7 cells. The combination treatments were composed of a) 10 µM TAM and various AP concentrations (5, 10, 20 and 40 µM), b) 20 µM TAM and various AP concentrations and c) 40 µM TAM and various AP concentrations. 40 µM PL was used as a technical positive control. Data represents the mean ± standard deviation S.D. (n=3), where * P< 0.05, ** P<0.001, *** P<0. 0001and ns = not significant with a Z-factor > 0.5.*

In transfected MCF-7 cells however, we observed a significant increase in growth inhibition in the combination treatments compared to both single treatments especially at low concentrations. Therefore, the effects observed here could possibly suggest that CETP knock-down may have increased TAM efficacy.

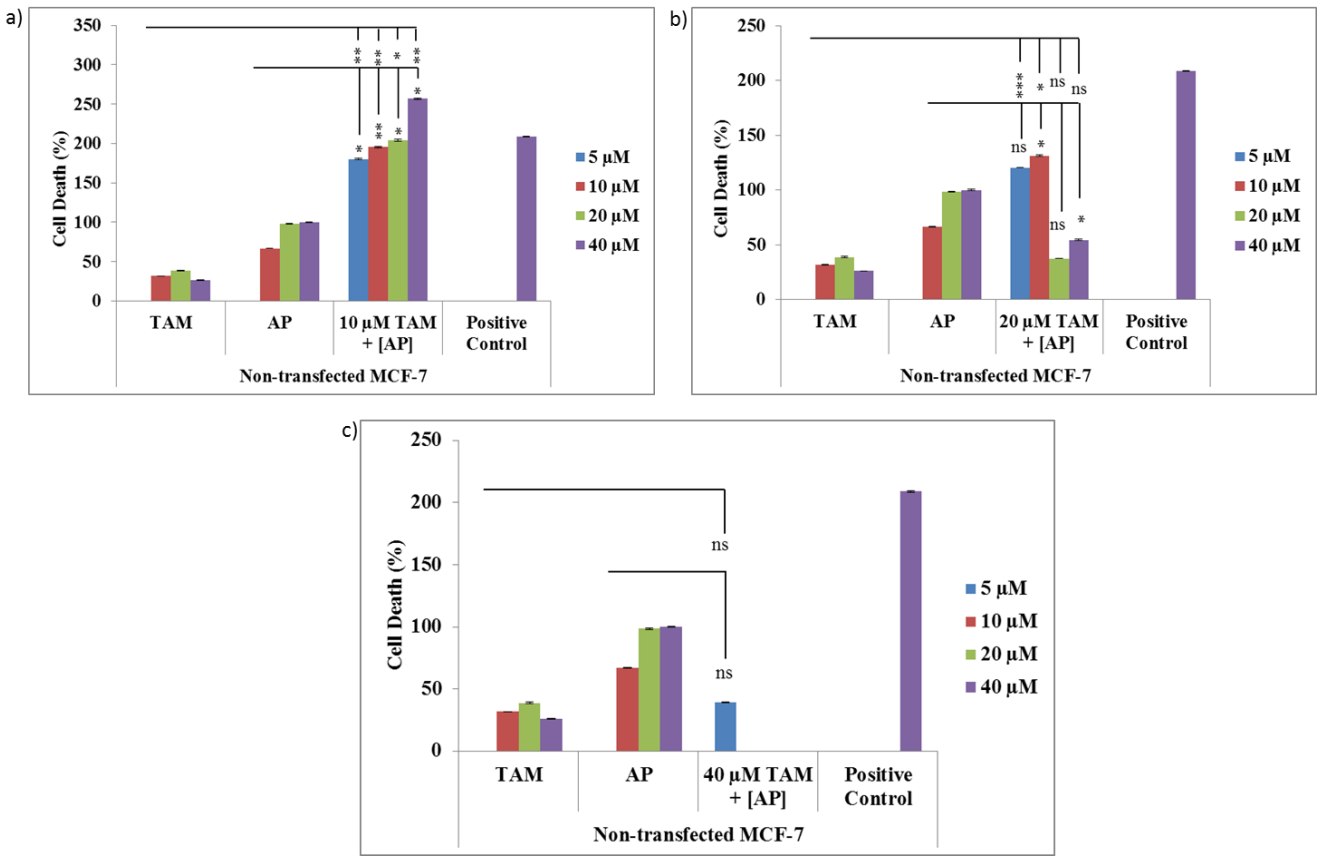

Figure 9.3: Representative of combination treatment on cell death in non-transfected MCF-7 cells

*Fig. 8.3 represents apoptosis analysis, through APO% assay. The combination treatments were compared to the single treatments in non-transfected MCF-7 cells. The combination treatments were composed of a) 10 µM TAM and various AP concentrations (5, 10, 20 and 40 µM), b) 20 µM TAM and various AP concentrations and c) 40 µM TAM and various AP concentrations. 40 µM PL was used as a technical positive control. Data represents the mean ± standard deviation S.D. (n=3), where * P< 0.05, ** P<0.001, *** P<0. 0001and ns = not significant with a Z-factor > 0.5.*

Once again no significant increase in cell death was observed in the combination treatment compared to AP single treatment in non-transfected MCF-7 cells except at 5 µM TAM (which was shown in Fig. 4.10) with various AP concentrations. However, this could possibly due to uneven cell number as APO% assay will be standardised further.

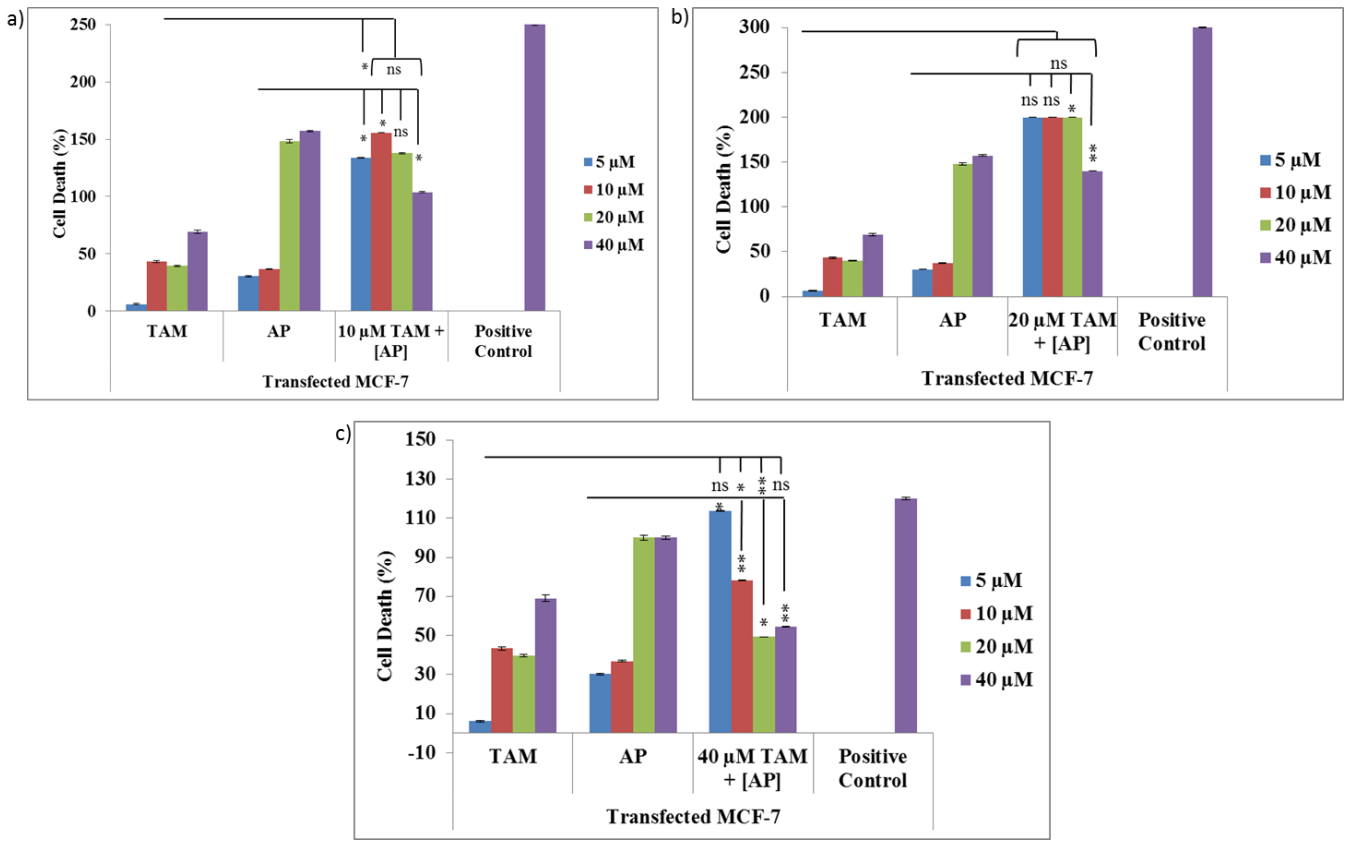

Figure 9.4: Representative of combination treatment on cell death in transfected MCF-7 cells

*Fig. 8.4 represents apoptosis analysis, through APO% assay. The combination treatments were compared to the single treatments in transfected MCF-7 cells. The combination treatments were composed of a) 10 µM TAM and various AP concentrations (5, 10, 20 and 40 µM), b) 20 µM TAM and various AP concentrations and c) 40 µM TAM and various AP concentrations. 40 µM PL was used as a technical positive control. Data represents the mean ± standard deviation S.D. (n=3), where * P< 0.05, ** P<0.001, *** P<0. 0001and ns = not significant with a Z-factor > 0.5.*

CETP knock-down, resulted in an increased cells death in the combination treatments compared to both single treatments. This suggests that *CETP* knock-down increased TAM efficacy even at low concentrations.

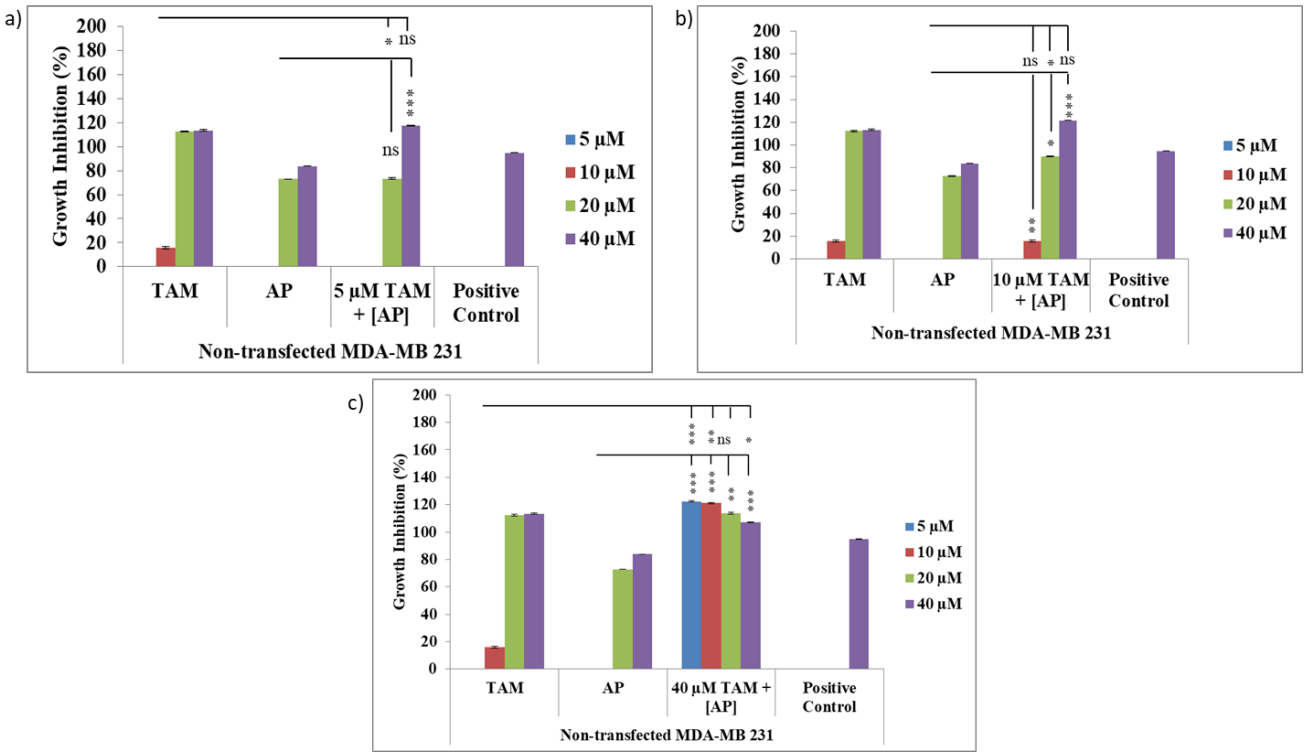

Figure 9.5: Representative of combination treatments in non-transfected MDA-MB 231 cells

*Fig. 8.5 represents growth inhibition analysis, through MTT assay. The combination treatments were compared to the single treatments in non-transfected MDA-MB 231 cells. The combination treatments were composed of a) 5 µM TAM and various AP concentrations (5, 10, 20 and 40 µM), b) 10 µM TAM and various AP concentrations and c) 40 µM TAM and various AP concentrations. 40 µM PL was used as a technical positive control. Data represents the mean ± standard deviation S.D. (n=3), where * P< 0.05, ** P<0.001, *** P<0. 0001and ns = not significant with a z-factor > 0.5.*

No significant difference in growth inhibition was observed in the combination treatment when compared to the single treatment in non-transfected MDA-MB 231 cells (Fig. 8.5a and b) except at higher concentrations (Fig. 4.14b; 20 µM TAM with varying concentrations of AP). In addition, an enhanced activity was observed at 40 µM TAM along with AP however, it is a very high concentration and therefore too cytotoxic.

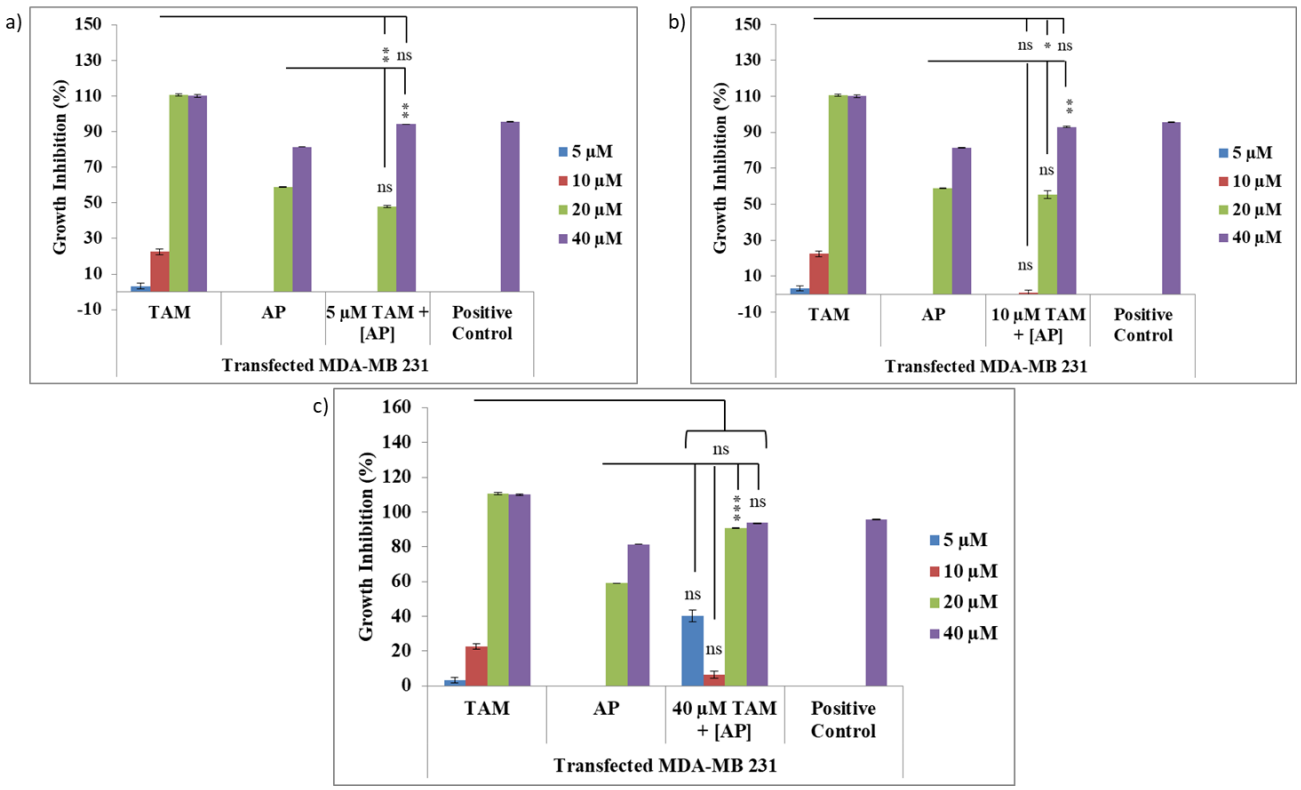

Figure 9.6: Representative of combination treatments in transfected MDA-MB 231 cells

*Fig. 8.6 represents growth inhibition analysis, through MTT assay. The combination treatments were compared to the single treatments in transfected MDA-MB 231 cells. The combination treatments were composed of a) 5 µM TAM and various AP concentrations (5, 10, 20 and 40 µM), b) 10 µM TAM and various AP concentrations and c) 40 µM TAM and various AP concentrations. 40 µM PL was used as a technical positive control. Data represents the mean ± standard deviation S.D. (n=3), where * P< 0.05, ** P<0.001, *** P<0. 0001and ns = not significant with a z-factor > 0.5.*

Similarly, in transfected MDA-MB 231 cell line, no significant increase was observed in the combination treatment when compared to single treatment (Fig. 8.6a, b and c). Once again, growth inhibition was observed at high concentrations in transfected MDA-MB 231 cell line (Fig. 4.14b).

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