EFFECTS OF OXIDIZED LOW-DENSITY LIPOPROTEIN BY THYMOQUINONE IN LIPID-LOADED MCF-7 BREAST CANCER CELLS

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DMSO	Dimethyl sulfide
EDTA	Ethylenediamine tetraacetic acid
EGLN1	Egl-9 family hypoxia-inducible factor 1
ER	Estrogen receptor
FASN	Fatty acid synthase
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IC_{50}	Half maximal inhibitory concentration
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPDS	Lipoprotein deficient serum
MTS (4-	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-sulfophenyl)-2H-tetrazolium

NaCl	Sodium chloride
Ox-LDL	Oxidized low-density lipoprotein
PBS	Phosphate-buffered saline
PHD2	Prolyl hydroxylase domain-containing protein 2
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RIPA	Radio immunoprecipitation assay
RPMI	Roswell Park Memorial Institute
TQ	Thymoquinone
TBST	Tris-Buffered Saline and Tween 20
VAMP4	Vesicle-associated membrane protein 4

KESAN LIPOPROTEIN KETUMPATAN RENDAH TEROKSIDA OLEH THYMOQUINONE DALAM SEL BARAH PAYUDARA MCF-7

ABSTRAK

Kanser payudara adalah punca utama kematian di kalangan wanita di seluruh dunia dan kajian baru-baru ini mendedahkan bahawa lipoprotein berketumpatan rendah teroksida (ox-LDL) menyumbang kepada peningkatan risiko kanser payudara. Thymoquinone (TQ) telah dikaji secara meluas dalam patogenesis kanser melalui *in vivo* dan *in vitro*, tetapi terdapat kekurangan laporan mengenai kawal atur lipid dalam sel kanser. Oleh itu, objektif kajian ini adalah untuk menjelaskan kesan ox-LDL kepada pertumbuhan sel kanser payudara serta mekanisma metabolisma lipid dan pertumbuhan sel di peringkat molekul yang dikawal atur oleh TQ. Kesan toksik TQ dianalisis melalui ujian MTS. Sel MCF-7 didedahkan kepada 10 μg/ml ox-LDL, diikuti oleh rawatan dengan 20 μM TQ untuk kajian ke atas kebergantungan kepada masa. Lokasi dan ekspresi protein sasaran dikaji melalui kaedah immunopendarfluor dan pemendapan Western. Ini diikuti oleh analisis *qRT-PCR* ke atas ekspresi *VAMP4* dan *EGLN1*. Nilai IC_{50} untuk tempoh 24, 48 dan 74 jam rawatan TQ adalah masing-masing 20 μM, 24 μM dan 10 μM. Ox-LDL menyebabkan Bcl-2 berada dalam nukleus, manakala NFκB kekal dalam sitoplasma sel selepas rawatan. Ia menunjukkan bahawa pengaktifan NFκB telah dihalang oleh ox-LDL. Analisis lanjut menunjukkan bahawa ox-LDL menggalakkan proliferasi sel MCF-7 melalui peningkatan kawal atur Bcl-2 dan NFκB manakala untuk FASN, ox-LDL telah menurunkan ekspresinya. Selepas rawatan dengan TQ selama 72 jam, LDLR dan

NFκB menunjukkan pengurangan yang lebih tinggi berbanding dua protein lain yang dikaji. Di samping itu, FASN adalah satu-satunya protein yang menunjukkan perencatan yang ketara oleh TQ berbanding ekspresinya dalam sel tanpa rawatan (nilai-p<0.05). Analisis selanjutnya melalui eksperimen *qRT-PCR* mendedahkan bahawa TQ meningkatkan pengawalaturan ekspresi *VAMP4* dengan signifikan selepas 72 jam rawatan (nilai-p<0.05) sebagai tindak balas kepada perencatan FASN dan Bcl-2. Tahap *EGLN1* berkurangan pada 48 jam rawatan TQ tetapi kali ganda perubahannya lebih rendah berbanding *VAMP4*. Secara keseluruhan, kajian ini menyediakan pemahaman ke atas mekanisma pertumbuhan sel disebabkan oleh ox-LDL serta kawal atur TQ di dalam sel yang mengandungi lipid. Fungsi LDLR, FASN dan VAMP4 dalam sel yang mengandungi lipid boleh dikaji dengan lebih mendalam melalui kaedah pengurangan ekspresi genetik menggunakan teknik *siRNA* dan TQ boleh dikaji melalui terapi gabungan dengan ubat kemoterapi bagi kanser positif ER yang telah sedia ada di dalam model sel kultur. Untuk kajian akan datang, adalah disarankan untuk menggunakan mikroskop mengimbas sefokus untuk mengkaji lokasi sesuatu protein dengan lapisan demi lapisan disaring untuk mendapatkan imej pendarfluor tiga dimensi yang jelas disebabkan oleh kesan TQ.

EFFECTS OF OXIDIZED LOW-DENSITY LIPOPROTEIN BY THYMOQUINONE IN LIPID-LOADED MCF-7 BREAST CANCER CELLS

ABSTRACT

Breast cancer is the leading cause of death among women worldwide and recent studies revealed that oxidized low-density lipoprotein (ox-LDL) contributed to increased risk of breast cancer. To date, thymoquinone (TQ) is widely studied both *in vivo* and *in vitro* in cancer pathogenesis, but there is lack of reports on its lipid regulation in cancer cells. Thus, the objectives of this study were to elucidate the effects of ox-LDL on breast cancer cell growth as well as the molecular mechanisms of lipid metabolism and cell proliferation regulated by TQ. Cytotoxicity of TQ was analyzed using MTS assay. MCF-7 cells were exposed to 10 µg/ml of ox-LDL, followed by treatment with 20 µM of TQ for time-dependent study. Localization and expression of target proteins were studied through immunofluorescence and Western blot methods. This was followed by qRT-PCR analysis on genomic expression of *VAMP4* and *EGLN1*. The IC_{50} value for 24, 48 and 74 hours of TQ treatment was 20 µM, 24 µM and 10 µM respectively. Ox-LDL caused localization of Bcl-2 in the nucleus, whereas NFκB remained in the cytoplasm of cells after treatment. This indicated that activation of NFκB was inhibited by ox-LDL. Further analysis showed that ox-LDL induced MCF-7 cell proliferation through up-regulation of Bcl-2 and NFκB expression, whereas in FASN, ox-LDL reduced its expression. After treatment with TQ for 72 hours, LDLR and NFκB showed more reduction in their expression compared to the other two proteins studied. In addition, FASN was the only protein that indicated significant inhibition by TQ compared to its expression in native cells

(*p*-value < 0.05). Further analysis through qRT-PCR experiments revealed that TQ significantly up-regulated *VAMP4* expression at 72 hours TQ treatment (*p*-value < 0.05) in response to the inhibition of FASN and Bcl-2. *EGLN1* level was reduced at 48 hours TQ treatment but the fold change was lower compared to *VAMP4*. Taken together, this study provides insight into molecular mechanism of ox-LDL-induced cell growth as well as regulation of TQ in lipid-loaded cells. The roles of LDLR, FASN and VAMP4 in lipid-loaded cells could be further studied through their gene knockdown using siRNA technique and TQ may also be examined in combined therapy with the existing ER-positive chemotherapeutic agent in cell culture model. For future studies, it is recommended to use confocal scanning microscope to study the localization of a protein by scanning many thin sections through a sample to obtain a clear three-dimensional fluorescent image due to TQ effect.

CHAPTER 1

INTRODUCTION

1.1 Cancer

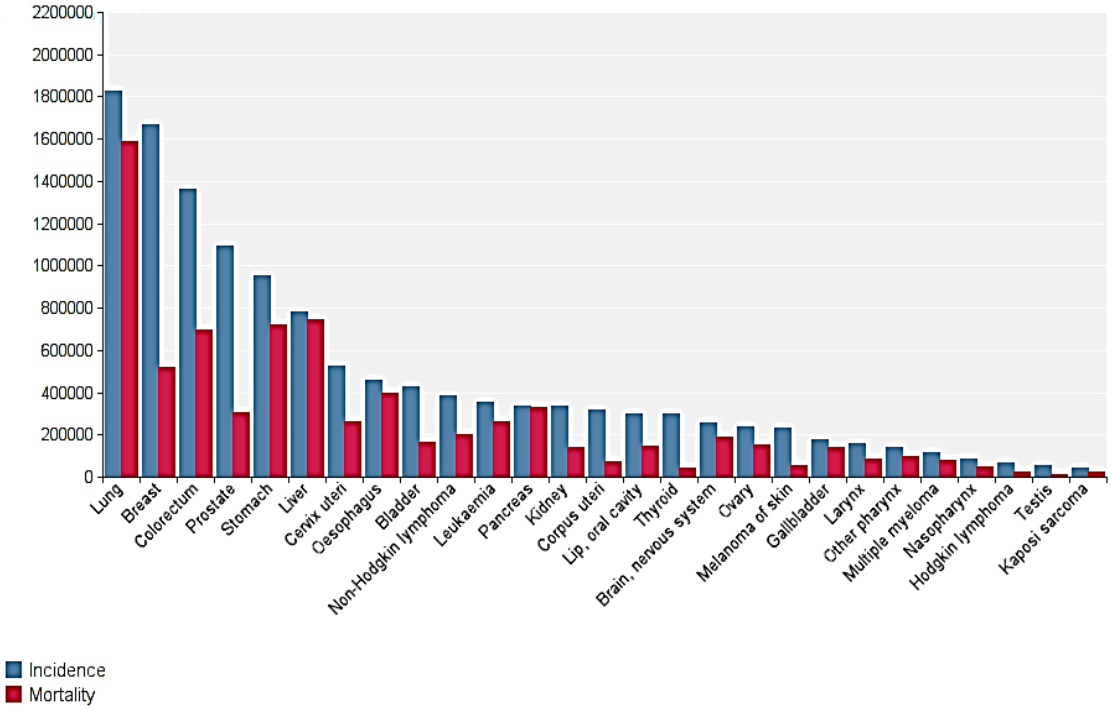
Cancer is among the leading causes of death worldwide. According to the World Health Organization (WHO), approximately 8.2 million deaths were due to cancer and 14.1 million new cancer cases were reported in 2012 across the world. It is also a major health problem in Malaysia, accounting for 11.28 % of total death in 2007 and the third cause of death among Malaysian population (Omar and Ibrahim, 2011). Cancer is a group of diseases characterized by rapid and uncontrolled proliferation of abnormal cells. It can start almost anywhere in the human body due to the genetic changes or external factors such as chemicals and radiation (National Cancer Institute, 2015).

There are more than 100 types of cancers (National Cancer Institute, 2015). Carcinomas are cancers that arise in the epithelium and it can be divided into adenocarcinomas and squamous cell carcinomas. Adenocarcinomas refer to cancers that develop in an organ or a gland, while squamous cell carcinomas are cancers that originate in the skin. Melanomas also develop in the skin, usually in the pigment cells called melanocytes. Sarcomas are cancers of the supporting tissues of the body such as blood vessels, bone and muscle. Gliomas are cancers of the nerve tissue. Cancers of the blood are known as leukemias while cancers of the lymph glands are known as lymphomas (Cancer Research UK, 2014).

Most cancers form a lump called a tumour, but not all lumps are cancer. Tumours can be benign tumours which do not invade other tissues, whereas malignant tumours have the ability to invade and metastasize from their primary site to other parts of the body through bloodstream or lymph vessels (American Cancer Society, 2015). Statistics from the International Agency for Research on Cancer (IARC) in 2012 showed that the four leading types of cancer in the world are lung, breast, colorectum and prostate cancer (Figure 1.1).

1.2 The epidemiology of breast cancer

In Malaysia, breast cancer is the most frequent type of cancer reported in 2007 (Omar and Ibrahim, 2011) and the second most frequent cancer worldwide in 2012 (IARC, 2012). As shown in Table 1.1, breast cancer constitutes 18.1 % of all cancer cases reported among Malaysian population in 2007, with high incidence among women aged 35 years old and above. Among the different ethnic groups in Malaysia, Chinese has the highest incidence rate (38.1 per 100,000) followed by the Indians (33.7 per 100,000) and Malays (25.4 per 100,000) for age-standardized rate (ASR) in 2007. Out of 3,242 female breast cancer cases diagnosed in 2007, 42 % of them were at stage 3 and 4 (Omar and Ibrahim, 2011). Malay women have a poorer survival compared to the other two main ethnics. Even though the cause is unclear, it was thought that pharmacogenomics, lifestyle factors (weight-gain, diet, and exercise) and psychosocial factors (acceptance of second or third line chemotherapy) may be responsible for the difference in survival. Nulliparity, family history, not breastfeeding and use of oral contraceptives are among the factors that are believed to increase risk of breast cancer among Malaysian women (Yip et al., 2014).



Source: IARC (2012)

Figure 1.1: Estimated cancer incidence and mortality worldwide in 2012 among men and women in all age groups.

Table 1.1: The ten leading cancers among the Malaysian population in 2007.

Type of cancer	**Percentage (%)**
Breast	18.1
Colorectal	12.3
Trachea, Bronchus, Lung	10.2
Nasopharynx	5.2
Cervix uteri	4.6
Lymphoma	4.3
Leukaemia	4.1
Ovary	3.6
Stomach	3.5
Liver	3.3

Source: Omar and Ibrahim (2011)

1.3 Breast cancer subtypes

Breast cancer can be classified into different categories based on its histopathological type, stage of the tumour, grade of the tumour and expression of hormone receptors. Majority of breast cancers are derived from the epithelium lining the ducts or lobules and are classified as mammary ductal carcinoma. Carcinoma *in situ* (CIS) is proliferation of cancer cells within the epithelial tissue without invasion of the surrounding tissue, whereas invasive carcinoma invades the surrounding tissue (Tripathi et al., 2013).

The Tumour-Nodes-Metastasis (TNM) is one of the most widely used cancer staging systems (WHO, 2015, Edge and Compton, 2010). The system is based on three important criteria which include the primary tumour size (T), the absence or presence and extent of regional lymph node metastasis (N) as well as the absence or presence of distant metastases (M). There are five stages of breast cancer (0, I, II, III, and IV) with stage 0 being in situ, stage I being the early stage invasive cancer, and stage IV being the most advanced cancer (WHO, 2015, Edge and Compton, 2010, Tavassoli and Devilee, 2003).

In addition, three histological grades I, II, and III (also known as Scarff Bloom-Richardson grade, Nottingham grade, or Elston-Ellis grade) are used to assess the degree of tumour differentiation. It is based on the tubule formation, nuclear pleomorphism or how closely they resemble normal breast cells and mitotic rate (Edge et al., 2010, Dixon, 2013,). Grade I (well differentiated) cancers have relatively normal-looking cells that do not appear to be growing rapidly and are arranged in small tubules, whereas grade II (moderately differentiated) cancers have moderate features. Grade III (poorly differentiated) cancers, which is the highest

grade, are lack of normal features and tend to grow more rapidly and aggressively (WHO, 2015).

Estrogen and progesterone are the hormones that often fuel the growth of breast cancer cells. Cancer cells may have one, both or neither of these receptors. Breast cancers that have estrogen receptors are often referred to as ER-positive cancers, while those containing progesterone receptors are known as PR-positive cancers. Both ER-positive and PR-positive cancers are also classified as hormone receptor-positive cancer. They are more common in women after menopause. In addition, tumours with increased levels of *HER2/neu* gene are referred to as HER2-positive. This type of cancer often has high number of copies of the *HER2/neu* gene, resulting in greater than normal amounts of the growth-promoting protein. Breast cancer cells which do not have estrogen or progesterone receptors and do not have too much HER2 expression are called triple-negative. This cancer is more common in younger women and among African-American or Hispanic/Latina women. Triple-negative breast cancers tend to grow and spread more quickly than other types of breast cancer (American Cancer Society, 2015).

In Malaysia, out of 94 cases of invasive breast ductal carcinoma studied between 2006 and 2010, 53.2 % was ER-positive while 24.5 % was HER2-positive breast cancer. Triple negative breast cancer constituted of 22.3 % cases (Ch'ng et al., 2012). Another study on breast cancer cases in Sarawak also found that ER-positive breast cancer had the highest percentage (57 %), followed by triple negative breast cancer (29 %), and HER2-positive breast cancer (23 %) (Devi et al., 2012).

1.4 Breast cancer risk factors

Breast cancer is 100 times more common among women than men (American Cancer Society, 2015). According to Dahlui et al. (2011), a woman in Malaysia had a 1 in 20 chance of developing breast cancer in her lifetime. The risk increases as a person gets older. About 1 out of 8 invasive breast cancers are found in women at age below 45 whereas in women age 55 or older, 2 of 3 invasive breast cancers are found (American Cancer Society, 2015).

Besides gender and age, hormonal factors are likewise implicated. Post-menopausal women with highest levels of estrogen and testosterone were found to have 2-3 times the risk compared to women with the lowest levels (Key et al., 2002). Early menarche, nulliparity or first birth after the age of 35 and late menopause may increase the risk of breast cancer. Combined hormonal replacement therapy for menopausal symptoms increases the risk of breast cancer especially after prolonged use (McPherson et al., 2000, Chlebowski et al., 2003). On the other hand, continuous breastfeeding up to one and a half or two years may lower the risk, possibly because breastfeeding reduces a woman's total number of lifetime menstrual cycles, which is similar to the effects of starting menstrual periods at a later age or going through early menopause (American Cancer Society, 2015).

Moreover, genetic predisposition is estimated to contribute to 5-10 % of all breast cancer cases (Palma et al., 2006). Susceptibility to breast cancer is generally inherited as an autosomal dominant with limited penetrance. Inherited mutations in *BRCA1* and *BRCA2* increase the risk of breast cancer in addition to ovarian cancers (McPherson et al., 2000). The *BRCA1* and *BRCA2* are genes that code for BRCA1 and BRCA2 tumour suppressor proteins. They are located on the long arms of

chromosomes 17 and 13 respectively, functioning to repair damaged dsDNA and mutation or alteration in either of these genes can lead to cancer (Foulkes and Shuen, 2013). Meta-analysis shows that breast cancer risks for mutation carriers are approximately 57 % for *BRCA1* and approximately 49 % for *BRCA2* (Chen and Parmigiani, 2007). Other high penetrance alleles identified as part of inherited breast cancer syndromes include *TP53, PTEN, STK11, CDH1* mutations (Mavaddat et al., 2010).

High consumption of meat, dairy products, fat and alcohol were reported to increase the risk, whereas healthy diet with high amount of fibre, fruits, vegetables, and anti-oxidants may lower the risk of breast cancer. Other well-known risk factors are race and ethnicity, a first degree-relative with breast cancer, overweight and obesity (especially postmenopausal obesity), oral contraceptives, exposure to tobacco products, alcohol intake and radiation (McPherson et al., 2000, Kushi et al., 2012, Assi et al., 2013). Before menopause, being overweight or obese modestly decreases breast cancer risk, whereas after menopause, being overweight or obese increases breast cancer risk (Reeves et al., 2007, Nelson et al., 2012). Barnard et al. (2015) conducted a review on current knowledge about the associations between established breast cancer risk factors and risk of specific tumour subtypes. Summary of risk factor with regard to breast cancer subtype (Luminal A, Luminal B, HER-2 overexpressing, and triple negative) are shown in Table 1.2.

1.5 Treatment for breast cancer

There are several treatment options for women diagnosed with breast cancer which include surgery, radiation therapy, chemotherapy, hormonal therapy and

Table 1.2: Summary of breast cancer risk factor according to subtype.

Risk factor	Luminal A	Luminal B	HER2-overexpressing	Triple negative
Younger age at menarche	+ +	+	unk	+ + +
Greater parity	− − −	unk	unk	+ +
Older age at first birth	+ +	unk	unk	unk
Breastfeeding	− −	− −	unk	− − −
Older age at menopause	+ +	unk	unk	+
Greater BMI (premenopausal)	−	unk	unk	+
Greater BMI (postmenopausal)	unk	unk	unk	unk
Family history	+ + +	+	+ + +	+ + +
Alcohol use	+	unk	+	unk
Use of oral contraceptives	−	unk	unk	+
Menopausal hormonal therapy use	+ +	unk	unk	unk

Source: Barnard et al. (2015)

+++: indicates consistent evidence of a positive association between the risk factor and the subtype.

− − −: indicates consistent evidence of a negative association between the risk factor and the subtype.

++: indicates a probable positive association between the risk factor and the subtype.

− −: indicates a probable negative association between the risk factor and the subtype.

+: indicates a possible positive association between the risk factor and the subtype.

−: indicates a possible negative association between the risk factor and the subtype.

unk: indicates insufficient or inconsistent evidence.

targeted therapies. The most appropriate treatment depends on stage and type of breast cancer, characteristics of the cancer cells, menopausal status, and the patient's state of health (Peepliwal and Tandale, 2013). There are two types of breast cancer surgery which include breast-conserving surgery and mastectomy (American Cancer Society, 2015). Radiation therapy is often used after breast-conserving surgery to lower the chance of cancer recurrence by destroying residual breast cancer or shrinking the tumour before surgery by damaging the double strand DNA of tumour cells via radiation. Chemotherapy involves treatment with cancer killing drugs which are generally given by injection into a vein to reduce the chance of cancer recurrence after surgery or to shrink the tumour before the surgery. In most cases, especially adjuvant and neoadjuvant treatment, chemotherapy is most effective when combinations of more than one drug are used and the combinations are based upon size of tumour, lymph node metastasis, and presence of ER receptor and HER2 receptor (Peepliwal and Tandale, 2013, Tripathi et al., 2013).

Hormone therapy is beneficial for hormone receptor-positive breast cancer through lowering estrogen levels or inhibiting estrogen from acting on the breast cancer. Tamoxifen is among the most common drugs used to block estrogen receptors in breast cancer cells and it is often given to patients with early localized breast cancer that can be removed by surgery and those with metastatic breast cancer (American Cancer Society, 2015). Targeted therapy uses drugs or other substances to attack specific cancer cells without harming normal cells. Monoclonal antibodies, tyrosine kinase inhibitors, and cyclin-dependent kinase inhibitors are types of targeted therapies used in the treatment of breast cancer (National Cancer Institute, 2015).

1.6 Lipid metabolism and cancer

Lipid is a diverse group of hydrophobic molecules. It can be obtained either from dietary sources or from carbohydrate-derived fatty acids synthesized in the liver or in adipocytes. Fatty acids are molecules with long hydrocarbon chains topped with a carboxyl group which can be used to generate many types of lipids including triacylglycerides, sterols, sphingolipids, phosphoinositides and eicosanoids (Baenke et al., 2013). Cholesterol is a type of animal sterols which is essential for structural component of animal cell membranes in maintaining membrane integrity and fluidity (Hu et al., 2010). The formation of long chain fatty acids is catalyzed by fatty acid synthase (FASN) through condensation of acetyl-CoA and malonyl-CoA to produce palmitate (Rossi et al., 2003). In normal cells, FASN expression and activity are usually low and it is strictly regulated by hormones, diet and growth factors, except for liver and adipose tissue (Menendez and Lupu, 2007). In contrast, fatty acids in the rapidly proliferating cancer cells can be synthesized *de novo* to sustain the high demand of lipids required for formation of new cell membrane and energy production (Menendez and Lupu, 2007, Flavin et al., 2010, Santos and Schulze, 2012). As such, FASN is often present at high levels in many human cancer cells (Kuhajda, 2006).

Apart from that, lipids also play important roles as signalling molecules in cancer. Examples of lipid-derived signalling molecules include phosphoinositides, lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerides (DAG), ceramide and sphingosine. Phosphoinositides, LPA, PA, and DAG are important second messengers, whereas ceramide and sphingosine are generated in response to pro-apoptotic signals such as chemotherapy and UV radiation (Baenke et al., 2013). It was reported that the LPA could promote cancer cell proliferation, migration and

survival through binding to G-protein-coupled receptors (Mills and Moolenaar, 2003). In response to innate immunity and inflammation, the cytokine signaling pathways induces phospholipases (A2, C, and D), sphingomyelinases and the enzymes that regulate cholesterol metabolism (McLaren et al., 2011). According to Huang and Freter (2015), most of the mitogenic signaling in mammalian cells is carried out by growth factors which regulates cell growth and proliferation as well as involves in the activation of many lipid metabolism-related enzymes.

Furthermore, cholesterol is the source for biosynthesis of steroid hormones such as testosterone, estradiol, progesterone, cortisol/corticosterone and aldosterone (Hu et al., 2010). One of the mechanisms by which steroid producing cells acquire cholesterol is from plasma low-density lipoprotein (LDL) or other apolipoprotein B-(apoB) or apoE-containing lipoproteins through the LDL receptor-mediated endocytic pathway (Goldstein and Brown, 2009). Another important derivatives of cholesterol are bile salts and vitamin D. Formation of bile salts in the liver is the main route for removal of cholesterol circulating in the blood (Papachristodoulou et al., 2014), while vitamin D plays a crucial role in the control of calcium and phosphorus metabolism (Berg et al., 2002). Numerous studies have suggested that high dietary fat intake promotes the growth of colorectal, liver, breast, pancreatic, gastrointestinal and prostate cancers (Fleshner et al., 2004, Kwan et al., 2015). Reactivation of lipid biosynthesis in cancer cells could lead to enhanced membrane lipid saturation, which potentially increases cancer cells resistance to oxidative stress and cell death induced by chemotherapeutic agents. It was also reported that tumour-associated fatty acid synthesis could alter the mobility of membrane components and therefore affects the uptake of common chemotherapeutics into the cells (Rysman et al., 2010).

In human, excess lipids are stored in lipid droplets in the form of triacylglycerides and cholesteryl esters (Beloribi-Djefaflia et al., 2016). Lipid droplets are essential for storage of metabolic energy and also provide reservoirs of lipids for membrane synthesis (Thiam et al., 2013). In mammary gland, the formation of milk globules begins with the formation of ADRP- and TIP47-containing lipid droplets in the endoplasmic reticulum. They increase in size through fusion between lipid droplets, which is catalysed by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein domain including SNAP23, syntaxin-5 and vesicle-associated membrane protein 4 (VAMP4) (Olofsson et al., 2009). In addition, knockdown of the genes for SNAP23, syntaxin-5 or VAMP4 in heart muscle cells showed reduction in the rate of fusion and the size of the lipid droplets, suggesting that the SNARE system is important in lipid droplet fusion (Bostrom et al., 2007). Fatty acids mobilized from lipid stores can be degraded in the mitochondria through β-oxidation to provide energy when required by cells (Santos and Schulze, 2012). Previous studies have reported that diverting fatty acids to storage and reducing fatty acid release from storage can control proliferation of cancer cells (Currie et al.,2013). Although the exact role of lipid droplets in promoting survival of cancer cell remains unclear, it has been suggested that lipid droplets could be beneficial during the state of low oxygen availability. After re-oxygenation, they could be used as a readily available source of energy to sustain cancer cells growth (Santos and Schulze, 2012). In other words, previous studies demonstrated that both FASN and VAMP4 are the important regulators of cellular fatty acid metabolism which can promote cancer cell survival.

1.7 Hypoxia in cancer

Solid tumours require the right amount of nutrients and oxygen to sustain their metabolic needs. Many tumours proliferate very quickly that they exceed their own vascular supply, resulting in low oxygen availability or hypoxia. Hypoxia can increase cellular reactive oxygen species (ROS) and cause endoplasmic reticulum stress (Ackerman and Simon, 2014). This leads to an adaptive response that is transcriptionally regulated by the hypoxia-inducible factors (HIF) (Ameln et al., 2011). Prolyl hydroxylase domain-containing protein 2 (PHD2) is an enzyme encoded by the *EGLN1* gene (Semenza, 2014) and it is a direct HIF target gene (Metzen et al., 2005). HIF activation causes a metabolic switch to anaerobic energy production through expression of the glucose transporter 1 (GLUT1) and several glycolytic enzymes (Denko, 2008). HIF also prevents the entry of pyruvate into the tricarboxylic acid cycle (TCA) cycle by inducing the expression of pyruvate dehydrogenase kinase 1.

In addition, it has been suggested that PHD2 has the potential as a target for monitoring cancer progression and that inactivation of PHD2 could eventually lead to overexpression of FASN in hypoxic cancerous cells. This is because during hypoxia, HIF-1α does not undergo proteosomal degradation and translocates into the nucleus whereby it targets the glycolytic pathway and fatty acid synthesis to fulfill the requirement of energy (Singh et al., 2016). Besides, it has been reported that hypoxia can activate NFκB activity which is mediated through elevated expression of IκB kinases, resulting in increased levels of proinflammatory cytokines. *In vitro* studies on HeLa cells also showed that prolyl hydroxylases (PHDs) suppressed NFκB expression and hypoxia increases activity of cellular IKKβ, a critical regulator of

NFκB (Cummins et al., 2006). Based on these reports, it is clear that PHD2, FASN and NFκB are among the key regulators in cancer cells during hypoxia which potentially contribute towards cancer cells survival. Due to its role as a lipid metabolic gene and it is important for cellular transformation (Hirsch et al., 2010), this study aimed to analyse the link between *EGLN1* gene and other lipid metabolism genes particularly in breast cancer cells.

1.8 Oxidized low-density lipoprotein (ox-LDL)

A lipoprotein particle is a complex of lipids and proteins. Lipids are naturally water-insoluble and they are transported in the form of lipid/protein complexes. The five major groups of lipoproteins are high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), intermediate-density lipoproteins (IDL), and chylomicrons. The main difference between these lipoprotein types are their size, density and protein composition (Jain et al., 2013). Description and properties of the different types of lipoproteins are summarized in Table 1.3. This study will focus on one of the lipoproteins, LDL, which functions mainly to transport cholesterol from liver to other parts of the body. The core of an LDL particle is hydrophobic and consists of esterified cholesterol and triacylglycerol, while its outer surface layer of phospholipids is surrounded by a single apo B-100 protein (Harisa & Alanazi, 2013). Uptake of cholesterol by a cell occurs through receptor-mediated endocytosis whereby LDL will bind to LDL receptor (LDLR) and form a complex which will be internalized into the cells. LDL dissociates from the receptor when the pH drops. The receptor is then recycled to the surface of the cell,

Table 1.3: Subtypes of lipoproteins and their properties.

Properties	Chylomicron	Very low density lipoprotein	Low density lipoprotein	High density lipoprotein
Abbreviations	–	VLDL	LDL	HDL
Diameter (Å)	6,000–2,000	300–900	200–250	80–120
Density (g/ml)	<0.94	0.95–1.006	1.019–1.063	1.063–1.210
Electrophoretic Mobility	Does not exhibit electrophoretic mobility	Pre-β-mobility	β-Mobility (β-lipoprotein)	α-Mobility (α-lipoprotein)
Protein content (%dry weight)	1–2	8–10	20–25	50–60
Type of apolipoprotein	Apo B-48	Apo B-100, Apo C-1, Apo E	Apo B-100	Apo A-1, Apo A-2
%Triacyglycerols	80–85	45–53	5–9	2–3
%Cholesterol	2–4	17–27	43–50	12–25
%Phospholipids	7–9	17–19	19–21	17–24
Functions	Transport dietary lipids from the intestinal epithelial cells to the liver or other locations around the body	Distribute triacylglycerols to peripheral tissues	Major vehicle for transport of cholesterol to peripheral tissues	Involved in transport of cholesterol from peripheral tissues to liver for excretion

Apo B-48 Apolipoprotein B 48, *Apo B* Apolipoprotein B, *Apo B-100* Apolipoprotein B 100, *Apo C-1* Apolipoprotein C 1, *Apo E* Apolipoprotein E, *Apo A-1* Apolipoprotein A 1, *Apo A-2* Apolipoprotein A 2

Source: Jain et al., (2013)

while LDL is transferred into the lysosomes for degradation to monomers and released into the cytoplasm (Harisa & Alanazi, 2013).

Production of LDLR is subject to feedback regulation. When cells accumulate excess cholesterol, they reduce their receptors whereas when cells are deprived of cholesterol, more LDLR is being synthesized. This mechanism of feedback regulation of LDLR synthesis was discovered by Goldstein and Brown (1975), who studied the LDLR activity in cultured human fibroblasts. It was also reported that LDLR makes one round trip into and out of the cell every 10 minutes for a total of several hundred trips within its 20 hours of lifespan (Brown et al., 1983). Mechanism of LDL uptake through receptor-mediated endocytosis is illustrated in Figure 1.2. LDLR is regulated by a pair of sterol-regulated membrane-bound transcription factors called SREBPs. It is down-regulated when LDL concentrations are high, and fewer receptors appear on the cell surface. When the complex of LDLR and LDL are endocytosed, the receptors are recycled back to the cell surface and LDL is hydrolysed in lysosomes to release cholesterol, cholesterol esters, phospholipids, and amino acids (Papachristodoulou et al., 2014).

LDL is often linked to atherosclerosis and the main target of therapy for the prevention of coronary heart disease (Carmena et al., 2004). It is susceptible to oxidative modification (Levitan et al., 2010) and oxidation of LDL is assumed to occur in two main stages (Yoshida and Kisugi, 2010). During the initial stages of LDL oxidation *in vitro*, oxidative modifications of LDL result in little change in apolipoprotein B100 and such modified LDL is called minimally oxidized LDL (Yoshida and Kisugi, 2010). In the second stage, LDL is further oxidized along with modification in the protein portion of LDL, resulting in loss of recognition by the LDL receptor and a shift to recognition by the scavenger receptors. This can give rise

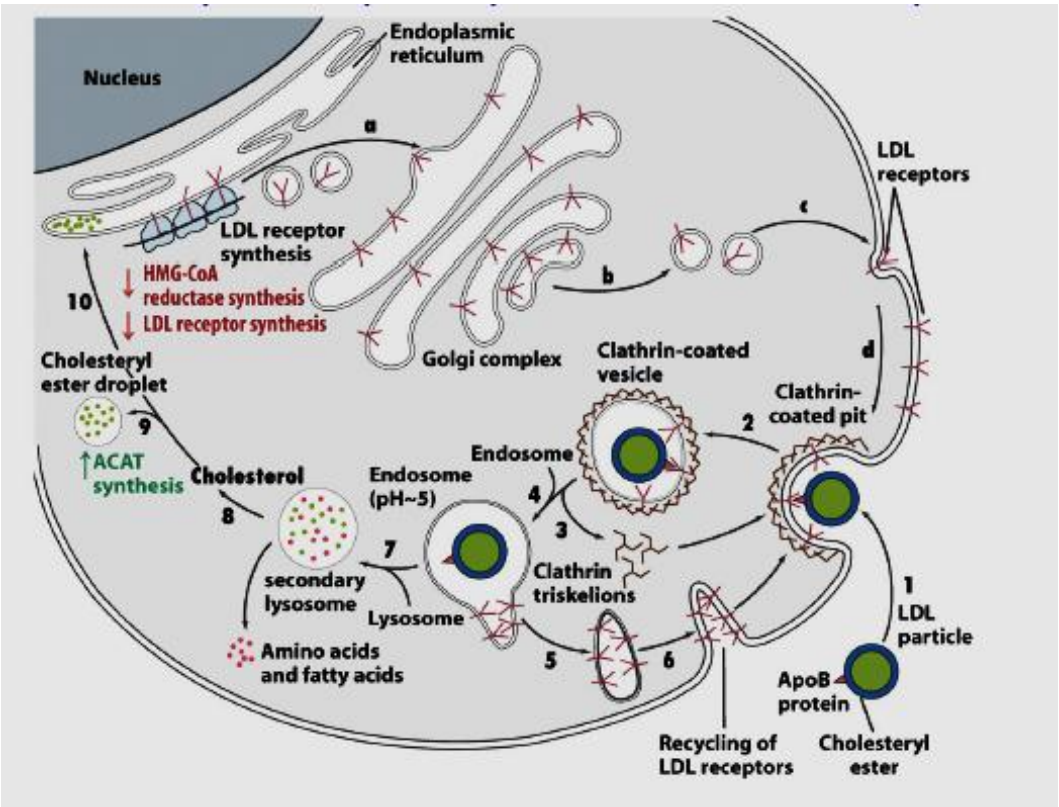


Figure 1.2: Mechanism of LDL uptake through receptor-mediated endocytosis. Adapted from http://www.unifr.ch/biology/assets/files/schneiter/lectures/voet_chap_20_new.pdf

to foam cells and causes ox-LDL to be more atherogenic than native LDL (Steinberg, 1997). Levitan et al. (2010) summarized the various bioactive compounds generated from LDL oxidation which include phospholipid products, sphingolipid products, free fatty acids, oxysterols, cholesteryl ester products, hydroxynonenal and malondialdehyde besides modification in functional groups of the Apo-B.

Apart from its atherogenic property, recent studies also reported that ox-LDL stimulated the proliferation of prostate cancer cells (Wan et al., 2015) and human mammary epithelial cells MCF-10A (Khaidakov and Mehta, 2012). Delimaris et al. (2007) found increased ox-LDL serum levels in stage I and II breast cancer patients, and they suggested that serum ox-LDL levels predict an increased risk of breast cancer. In contrast, study by Zabirnyk et al. (2010) had demonstrated that ox-LDL can initiate cell death of various cancer cell lines including HT29, HeLa, OVCAR3, OVCAR5, MCF7, A549 and PC3. However, the cytotoxicity of ox-LDL on these cancer cell lines was dose-dependent. Many studies have reported on overexpression of LDLR in various types of cancer including adrenal adenoma, colon, pancreatic, lung, brain, and prostate cancer (Chen and Hughes-Fulford, 2001). In human breast carcinoma cells, studies also showed that treatment with LDL cholesterol or overexpression of LDLR could induce tumour cell proliferation (Liu et al., 2013).

1.9 Thymoquinone and its anti-cancer effects

Nigella sativa is an annual flowering plant of the *Ranunculaceae* family that is abundant in countries bordering the Mediterranean Sea, Pakistan and India (Bassim Atta, 2003). Other common names for *Nigella sativa* include Black Cumin, Fennel Flower, and Habbatus Sauda. It has been used in traditional medicine for

treatment of various diseases such as asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness, and influenza (Ali and Blunden, 2003, Entok et al., 2014). In fact, Prophet Muhammad (PBUH) once stated that this plant has the source of healing for every disease except death. The black seeds of the plant are the most extensively studied since 1880. The chemical composition of the seeds include fixed oil (32 - 40 %), volatile oil (0.4 – 0.45 %), proteins (16 – 19.9 %), minerals (1.79 – 3.74 %), alkaloids, coumarins and saponins (Hussein El-Tahir and Bakeet, 2006). The volatile oil of black seeds contains a bioactive constituent called thymoquinone (TQ).

The chemical structure of TQ is 2-isopropyl-5-methyl-benzoquinon (AbuKhader, 2013). Studies on TQ revealed that the compound is capable to induce cell death of several human cancer cell lines derived from lung, colon, liver, melanoma, and breast tumours (Attoub et al., 2013). In addition, TQ was also found to be cytotoxic to squamous carcinoma cells (Chu et al., 2014), ovarian adenocarcinoma (Shoieb et al., 2003), human pancreatic adenocarcinoma and uterine sarcoma (Worthen et al., 1998), as well as colorectal cancer (Hala Gali-Muhtasib, 2004). In HCT-116 human colon cancer cells, the pro-apoptotic effects of TQ are dependent on p53 (Hala Gali-Muhtasib, 2004), whereas in myeloblastic leukemia HL-60 cells, induction of apoptosis by TQ occurs through the activation of caspase 3, 8, and 9 (El-Mahdy et al., 2005). Reports have shown that TQ treatment of HCT-116 cells demonstrated a marked increase in p53 and p21 protein levels and a significant inhibition of anti-apoptotic protein Bcl-2 (Hala Gali-Muhtasib, 2004).

Regulation of apoptosis by the Bcl-2 family of proteins can be characterized into pro-apoptotic and anti-apoptotic members. The pro-apoptotic proteins are such as Bax and Bak, whereas the anti-apoptotic proteins include Bcl-2 and Bcl-XL

(Portier and Taglialatela, 2006). Previous studies have shown that *Bcl-2* is among the genes that are regulated by a transcription factor, nuclear factor-*kappa* B (NFκB) besides *Bcl-XL, cIAP, suvivin, TRAF, COX-2, MMP-9* and *iNOS* (Luqman and Pezzuto, 2010). It was reported that activation of NFκB is common in carcinomas, which are mainly driven by inflammatory cytokines surrounding the tumour microenvironment (DiDonato et al., 2012). NFκB plays important roles in inflammation, immunity, cell proliferation and apoptosis. It is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, ox-LDL and microbial antigens (Luqman and Pezzuto, 2010). To date, numerous studies and clinical trials on TQ have been conducted to measure its safety, tolerability and toxicity at higher doses. Some researchers also proposed the use of combination of TQ with other chemotherapeutic agents such as doxorubicin, as well as TQ-loaded nanoparticles for treatment of cancer.

1.10 Problem statement

Breast cancer is the most common type of cancer in women and it is a major life threatening illness in the world. Numerous anti-cancer modalities have been developed and chemotherapy is one of the important treatments for breast cancer. Nevertheless, drug resistance is a major challenge in breast cancer chemotherapy and frequently accounts for the failure of the treatment. Recurrence of breast cancer was reported in almost 30 % of all patients with early stage of metastatic breast cancer (Chang, 2012). On top of that, elevated levels of serum lipid was detected in stage I and II of breast cancer patients (Delimaris et al., 2007), thus suggesting its role as an important factor that leads to the breast cancer development in these patients. There

is limited information on the exact molecular mechanism which relates ox-LDL to cancer, therefore it requires further investigation by studying the potential of loading breast cancer cells with ox-LDL. This will provide insight into the mechanism by which lipid metabolism is regulated in cancer cells. In addition, TQ is widely known for its anti-cancer properties and many other medicinal benefits, yet there is lack of knowledge on its regulation particularly in lipid-loaded breast cancer cells. Further understanding on the targeting sites of TQ may offer therapeutic strategy for cancer treatment, since there is increasing evidence that lipids are associated with cancer progression. There is a need to discover the molecular mechanism and potential of new drugs, which could be used to complement the existing chemotherapeutic treatment and overcome the complication of drug-resistance among breast cancer patients.

1.11 Objectives of study

This study sought to elucidate the effects of ox-LDL on breast cancer cell growth and the molecular mechanisms of TQ regulation in lipid-accumulated MCF-7 cells. To accomplish this goal, the following specific objectives were developed:

1. To determine the optimum concentration of TQ for treatment on MCF-7 cell line based on its half maximal inhibitory concentration (IC_{50}) value.

2. To assess the roles of ox-LDL in breast cancer cells based on comparison between native MCF-7 cells and lipid-loaded MCF-7 cells.

3. To compare the effects of TQ on localization and expression of the proteins involved in lipid metabolism and cell proliferation using proteomic analysis.

4. To study the roles of TQ in regulating lipid metabolism and cancer specific genes via qRT-PCR method.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

The human mammary breast cancer epithelial cell line, MCF-7, was obtained from American Type Culture Collection (ATCC). The adherent cell line was cultured in RPMI-1640 medium with L-glutamine (ATCC, USA), supplemented with 10 % heat-inactivated fetal bovine serum (JR Scientific Inc., USA) and 1 % antibiotics (10,000 U/ml of penicilin, 10,000 µg/ml of streptomycin) (Gibco, USA). The MCF-7 cells were seeded in 25 cm^2 culture flasks (Corning, USA) containing 5 ml complete growth medium. All culture flasks were incubated in 37 °C humidified incubator (Thermo Fisher Scientific, USA) with 5 % carbon dioxide. Medium renewal was done 2 to 3 times per week.

2.2 Subculturing cells

The MCF-7 cells were subcultured when they reached 80 - 90 % confluent. Medium was removed from the flasks and the cell layer was rinsed twice with phosphate buffered saline (PBS) to remove all traces of serum or trypsin inhibitor. Then, 3 ml of 0.05 % trypsin-EDTA solution (Gibco, USA) was added to detach the cells and culture flasks were incubated in a humidified CO_2 incubator for 10 minutes. About 4 ml of culture medium was added into the flasks to stop trypsin-EDTA reaction on the cells. The detached MCF-7 cells were transferred into centrifuge tubes and centrifuged at 125 x g for 7 minutes. After the supernatant was removed,

cell pellet was re-suspended with 1 ml fresh culture medium and approximately $7x10^4$ cells were seeded each new culture flask. The cells were incubated in 37 °C incubator.

2.3 Cryopreservation of cell line

Cryopreservation was done to preserve cell stock of MCF-7 cell line. After cells were collected and pelleted by centrifugation at125 x g for 7 minutes, the cell pellet was re-suspended in 1 ml fresh growth medium. Dimethyl sulfoxide (DMSO) was used as a cryoprotective agent to prevent formation of ice crystals and fragmentation of the cell membrane (Langdon, 2003). DMSO was added to the cell suspension at a final concentration of 10 % (Corsini, 2004). Finally, the cell stock was transferred into cryotubes and stored at -80 °C.

2.4 Determination of cell number

To count the total cell number, medium was first aspirated and transferred into 15 ml centrifuge tube. Culture flasks were rinsed with 4 ml phosphate-buffered saline (PBS) and 3 ml of 0.05 % Trypsin-EDTA (Gibco, USA) was added into the flasks. They were incubated at 37 °C for 10 minutes and observed under the microscope to ensure that the cells have completely detached from the flask surface. Then, 4 ml of complete growth medium was added to stop the reaction of trypsin on the cells. Cells were pipetted into the tube and centrifuged at 125 x g for 7 minutes. After the supernatant was removed, 1 ml of growth medium was added to the cell pellet and mixed well. Approximately 10 µl of the cell suspension was diluted with