

**INVESTIGATION ON CANCER  
CHEMOPREVENTIVE ACTIVITY OF  
FABRICATED TQ ENCAPSULATED PLGA-PF68  
NANOPARTICLES ON SELECTED BREAST  
CANCER CELL LINES**

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CANCER CELL LINES**

by

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## LIST OF SYMBOLS

°C	Degree Celsius
μg	Microgram
μg/mL	Microgram per milliliter
μM	Micromolar
CO <sub>2</sub>	Carbon dioxide
g	Gravity
G0	Gap G0 growth phase
G1	Gap G1 growth phase
G2	Gap G2 growth phase
IC <sub>50</sub>	Inhibitory concentration 50%
L	Liter
M	Molar
mg/mL	Milligram per milliliter
mL	Milliliter
mM	Milimolar
nM	Nanomolar
OD	Optical density
RPM	Revolution per minute
s	singlet
SD	Standard deviation
w/v	Weight per volume

## LIST OF ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
AIC	Anthracycline-induced cardiotoxicity
BSA	Bovine serum albumin
BSC	Breast-conserving surgery
BME	$\beta$ -mercaptoethanol
CYP450	Cytochrome P450
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EE	Encapsulation efficiency
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
FBS	Fetal bovine serum
GCB	Gemcitabine
GIT	Gastrointestinal tract
HCl	Hydrochloric acid
HER2	Human epidermal growth receptor
MDR	Multidrug resistant
MRP	Multidrug resistance protein
Nab	Nanoparticle albumin-bound
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NCCN	National Comprehensive Cancer Network
NSCLC	Non-small cell lung cancer
PBS	Phosphate-buffered saline
PCL	Poly (caprolactone)

PEO	Poly (ethylene oxide)
P-gp	P-glycoprotein
PLA	Poly (lactic acid)
PLGA	Poly lactic-co-glycolic acid
PLGA-PEG	Poly lactic-co-glycolide-block-poly (ethylene glycol)
PPO	Poly (propylene oxide)
PR	Progesterone receptor
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
Rh123	Rhodamine 123
RI	Resistance index
RPMI 1640	Roswell Park Memorial Institute 1640
SDS	Sodium dodecyl sulphate
SERMs	Selective oestrogen receptor modulators
siRNA	Small interfering RNA
SLNs	Solid lipid nanoparticles
TEM	Transmission electron microscope
TFs	Transcription factors
TNBC	Triple negative breast cancer
TQ	Thymoquinone
U.S. FDA	U.S. Food and Drug Administration

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**KAJIAN AKTIVITI PENCEGAHKEMO KANSER BAGI FABRIKASI  
NANOPARTIKEL BERKAPSULKAN TQ DENGAN PF68-PLGA  
TERHADAP TITISAN SEL KANSER PAYUDARA TERPILIH**

**ABSTRAK**

Kerintangan ubat ialah halangan utama dalam terapi kanser payudara. Oleh itu, beberapa kajian telah dijalankan untuk mengatasi masalah ini dengan menggunakan sebatian bioaktif. Thymoquinone (TQ) telah dikaji secara meluas untuk ciri-ciri antikansernya. Dalam keadaan klinikal, potensi terapinya belum diterokai kerana sifatnya yang tidak stabil. Lantaran itu, objektif kajian ini menyasarkan kepada sintesis nanopartikel polimer yang mengandungi TQ untuk mengatasi masalah ini. Tiga jenis sel kanser payudara (MCF 7, UACC 732, MDA-MB 231) yang menunjukkan kerintangan ubat telah dihasilkan menggunakan kaedah *'pulse method'*. Sintesis nanopartikel TQ telah dilakukan dengan menggunakan teknik penyejatan-pelarut emulsi (*emulsion-solvent evaporation*) campuran polimer poly(L-lactide-co-glycolide)-b-poly(ethylene glycol) dan Pluronic F68. Ciri-ciri nanopartikel TQ ini ditentukan menggunakan FTIR, taburan saiz, morfologi, kecekapan enkapsulasi (*encapsulation efficiency*) dan kajian pelepasan (*drug release study*) TQ. Pengesanan indeks rintangan (RI) dilakukan dengan cerakinan MTS, penghasilan P-glycoprotein dan kecerakinan pengaliran keluar ubat. Kesan-kesan anticancer nanopartikel TQ-PLGA-PF68 dikaji menggunakan analisis fungsian, cerakinan TUNEL, qRT-PCR dan kaedah Western blot. Saiz nanopartikel TQ-PLGA-PF68 ialah <100 nm, bulat dengan pengkapsulan jelas TQ dalam teras berdasarkan pemerhatian FTIR and TEM. Ia mempunyai kecekapan pengkapsulan (94%) dan pelepasan TQ >50% dalam keadaan

berasid. RI terhadap tamoksifen bagi sel TamR MCF 7 ialah 2.32 kali ganda dan 1.32 kali ganda pada TamR UACC 732 dan 1.59 kali ganda pada PacR MDA-MB 231 terhadap paclitaxel. Analisis lanjut telah dilakukan dengan TamR MCF 7 memandangkan ia kanser payudara paling tersebar luas dan menunjukkan rintangan lebih tinggi kepada kemoterapi standard. Cerakinan TUNEL menunjukkan bahawa nanopartikel TQ-PLGA-PF68 menggalakkan apoptosis dalam sel TamR MCF 7 ( $P < 0.0001$ ) dan penghentian kitaran sel di fasa S. Penghasilan P-gp adalah 33 kali ganda lebih rendah dalam sel TamR MCF 7 selepas dirawat dengan nanopartikel TQ-PLGA-PF68 ( $P < 0.05$ ). Ini telah disokong oleh cerakinan pengaliran keluar yang menunjukkan pengumpulan intrasel Rhodamine 123 ( $P < 0.05$ ). Analisis sitotoksikan menunjukkan perencatan perkembangbiakan sel (nilai  $IC_{50} = 96.34 \mu\text{g/mL}$ ;  $P < 0.05$ ). Ini disokong oleh kajian fungsian, di mana perencatan pembentukan koloni ( $P < 0.05$ ) dan penghijrahan ( $P < 0.05$ ). Analisis lanjut menunjukkan penurunan dalam ekspresi gen yang terlibat dalam kerintangan ubat, mekanisme kitar sel dan apoptosis (*ABCG2, CYP1A1, CYP2B6, CYPC19, CYP2D6, CYP3A4, CYP3A5, SULT1E1, BRCA1, CDKN1A, CDKN2A, EGFR, NF- $\kappa$ B1, SULT1A1, UGT2B1, ABCC2, CDKN1B*). Analisis KEGG menunjukkan hubungan kepada beberapa jaringan lain yang berhubung kait dengan laluan pengisyaratan rintangan (*resistance signaling pathway*) ( $P < 0.05$ ). Analisis proteomik mengesahkan penurunan ekspresi, P-gp ( $P > 0.05$ ) dan pengisyaratan hilirnya (*downstream signaling*) melibatkan protein NF- $\kappa$ B ( $P > 0.05$ ) and p21 ( $P < 0.05$ ). Penemuan mencadangkan rawatan menggunakan nanopartikel TQ-PLGA-PF68 boleh mengurangkan mekanisme-mekanisme kerintangan ubat oleh sel kanser payudara dari kumpulan reseptor oestrogen positif.

**INVESTIGATION ON CANCER CHEMOPREVENTIVE ACTIVITY OF  
FABRICATED TQ ENCAPSULATED PLGA-PF68 NANOPARTICLES ON  
SELECTED BREAST CANCER CELL LINES**

**ABSTRACT**

Drug resistance is remains as a major obstacle in breast cancer therapy. This has attracted considerable interest to overcome drug resistance with bioactive compounds. Free thymoquinone (TQ) has been extensively studied for its anticancer properties. In the clinical setting, its therapeutic potential has not been explored due to its volatile nature. Therefore, the study aimed to develop TQ polymeric nanoparticles and evaluate its effects on drug resistant breast cancer cells. Development of drug resistant breast cancer subtype (MCF 7, UACC 732, MDA-MB 231) were done using pulse method. Synthesis of nanoparticles was done by encapsulating TQ with polymeric poly(L-lactide-co-glycolide)-b-poly(ethylene glycol) and Pluronic F68 using emulsion-solvent evaporation technique and characterised using FTIR spectroscopic study, size distributions, morphology, entrapment efficiency, and drug release study. Confirmation of resistance index (RI) was performed with MTS assay, P-glycoprotein expression and efflux assay. Anticancer effects were studied using functional analysis, TUNEL assay, qRT-PCR array and Western blot method. Size of TQ-PLGA-PF68 nanoparticles was <100 nm, spherical with confirmed encapsulation of TQ within core based on FTIR and TEM observation. It had high encapsulation (94%) and drug release of >50% at acidic conditions. RI of TamR MCF 7 cells was 2.32-fold and 1.32-fold in TamR UACC 732 to tamoxifen and 1.59-fold in PacR MDA-MB 231 to paclitaxel. Further analysis was done with TamR MCF 7 as it is the most prevalent breast cancer and exhibited higher resistance to standard

chemotherapy. TUNEL assay indicated that TQ-PLGA-PF68 nanoparticles induced apoptosis in TamR MCF 7 cells was time dependant ( $P<0.0001$ ) and arrest of cells at S-phase. P-gp expression was 33-fold lower in TamR MCF 7 cells after treatment with TQ-PLGA-PF68 nanoparticles ( $P<0.05$ ). This was supported by efflux assay which exhibited intracellular accumulation of Rhodamine 123 ( $P<0.05$ ). Cytotoxicity analysis exhibited significant inhibition of cell proliferation ( $IC_{50}$  value=96.34  $\mu$ g/mL;  $P<0.05$ ). This is supported by functional study, where inhibition of colony formation ( $P<0.05$ ) and migration ( $P<0.05$ ) were noted. Further analysis on relative gene expression provides evidence of downregulation of genes involved in drug resistance, cell cycle and apoptosis mechanisms (*ABCG2*, *CYP1A1*, *CYP2B6*, *CYPC19*, *CYP2D6*, *CYP3A4*, *CYP3A5*, *SULT1E1*, *BRCA1*, *CDKN1A*, *CDKN2A*, *EGFR*, *NF- $\kappa$ B1*, *SULT1A1*, *UGT2B1*, *ABCC2*, *CDKN1B*). KEGG analysis showed link to several resistance signaling pathways ( $P<0.05$ ). Finally, proteomic analysis confirmed the downregulation of P-gp ( $P>0.05$ ) and its downstream signaling involving NF- $\kappa$ B ( $P>0.05$ ) and p21 proteins ( $P<0.05$ ). Findings suggest TQ-PLGA-PF68 nanoparticles treatment could mitigate drug resistant mechanisms of oestrogen receptor positive breast cancer cells.

# CHAPTER 1

## INTRODUCTION

### 1.1 Breast Cancer: An Overview

Cancer is defined as the unregulated proliferation of cancer cells by dividing continuously and have the capability of invading other tissues or organs (Pugazhendhi *et al.*, 2018). Cancer can arise almost anywhere in the body. It is characterised as carcinoma (originates from the epithelial cells i.e. breast cancer), sarcoma (arise from connective tissue i.e. osteosarcoma), lymphoma (originates from the lymph nodes) and leukaemia (immature blood cells that grow in the bone marrow) (Zhou *et al.*, 2012).

Globally, breast cancer is one of the most common cancer and is the highest incidence of cancer-related death among women (Siegel *et al.*, 2018). Breast cancer affects more than 2 million women annually. In 2018, it was reported that 627,000 (15%) women died from breast cancer (WHO, 2018). In Malaysia, breast cancer is the most commonly diagnosed cancer among Malaysian women regardless of their backgrounds and it is also the main cause of cancer death in Malaysia women (Bray *et al.*, 2018).

Breast cancer subtypes are classified based on pathological biomarkers. There are four breast cancer subtypes and defined by the expression of oestrogen receptor (ER), progesterone receptor (PR) and overexpression of human epidermal growth receptor (HER2) as shown in Table 1.1. They are classified into luminal A subtype (ER+/PR+/HER2-) that makes up approximately 50-60% of breast cancers which are slow-growing and less aggressive (Perou & Borresen-Dale, 2011; Yersal & Barutca,

2014; Dai *et al.*, 2017). The luminal B subtype (ER+/PR+/HER2+) represents 15-20% of breast cancers and exhibit more aggressive phenotype compared to luminal A subtype (Perou & Borresen-Dale, 2011; Yersal & Barutca, 2014; Dai *et al.*, 2017). Meanwhile, HER2 positive subtype (ER-/PR-/HER2+) accounts for 15-20% of breast cancer subtypes, which are highly invasive and aggressive in terms of biological and clinical behaviour (Yersal & Barutca, 2014; Dai *et al.*, 2017; Vernieri *et al.*, 2019). While, 10-20% of breast cancer is basal-like, which is referred as triple negative breast cancer (TNBC) that ER-, PR-, and does not overexpress HER2 (Perou & Borresen-Dale, 2011; Dai *et al.*, 2017). Generally, it has the worse prognosis of all sub-types (Sprouse & Herbert, 2014). Meanwhile, the Ki-67 is a cell proliferation marker and present only during the active phases of the cell cycle. Proliferation is a key feature of the progression of tumours (Urruticoechea *et al.*, 2005). High levels of Ki-67 indicate cells that are actively proliferating, thus are prone to mutation initiation and expansion. In breast cancer, a result of Ki-67 less than 10% is considered low, 10-20% borderline, and high if more than 20%.

In United States, it was reported that between 2012-2016, the breast cancer luminal A subtype is the common subtype with the prevalence of 85 new cases per 100,000 women (Fallahpour *et al.*, 2017). Meanwhile, an increasing trend is seen in Malaysia. The luminal A subtype rate increased by 2% in every five-year cohort, where it increased from 54.5% in 1994–1998, to 56.4% in 1999–2003 and 58.4% in 2004–2008 (Yip *et al.*, 2011). Unlike, the rate of HER2 positive breast cancer which remained constant.

**Table 1.1: 2013 St. Gallen - Breast cancer subtypes classification.**

Subtype	Characteristics		Medical therapy
Luminal A	ER PR HER2 Low Ki-67 (<14%)	++ + - 	Endocrine therapy
Luminal B	ER PR HER2  High Ki-67 (>40%)	+ +/- +/-  	Chemotherapy; Endocrine therapy; Anti-HER2 targeted therapy
HER2-positive	ER PR  HER2 High Ki-67	- -  ++	Chemotherapy; Anti-HER2 targeted therapy
Triple negative	ER PR HER2 High Ki-67	- - - 	Chemotherapy

(Nielsen *et al.*, 2004; Goldhirsch *et al.*, 2011)

Several factors may lead to the initiation of breast cancer, such as genetic abnormalities that affected the gene expression and mutation of the tumour suppressor genes. The oncogenes that are frequently deregulated in breast cancer includes *ErbB2*, *PI3KCA*, *MYC*, and *CCND1* (encodes cyclin D1) (Lee & Muller, 2012). Occurrence of mutations in the tumour suppressor genes *BRCA1*, *BRCA2*, and *p53* are also responsible for development of breast cancer (Lee & Muller, 2012). Mutation in oncogenes and in tumour suppressor genes can generate a clonal cell population with proliferative characteristics that leads to the induction of cancer (Basu, 2018).

Hormonal factor has an important role in breast cancer and is strongly linked with risk of ER positive PR positive breast cancer (Cotterchio *et al.*, 2003; Yue *et al.*, 2010). Women with increased exposure to oestrogen because of early menarche, late

menopause, long-term menopausal oestrogen therapy, and oestradiol ( $E_2$ ) levels have higher risk of breast cancer.

## 1.2 Breast Cancer Treatment

A mammary tumour develops when the cells that make up the breast tissue replicate abnormally. This caused the complexity of breast cancer mechanisms that demands for comprehensive therapies. For patients, treatments rely on the stage and subtype. Surgical removal of the tumour is the first choice in treatment of breast cancer. Due to this the treatment is done by surgery or radiotherapy. Subsequently, the treatment involves chemotherapy and endocrine therapies. This is done to lower the risk of relapse and improve overall survival. Meanwhile, the higher classification and inoperable tumours are normally treated with chemotherapy. It is addressed as neoadjuvant treatment, which aims to reduce the size of the tumours. This will be followed by surgical treatment. In breast cancer treatment, type of treatment will be based on the status of the axillary nodes, subtypes and menopausal state (Wesolowski & Ramaswamy, 2011). However, majority of the breast cancer patients would need multiple therapies because of acquired resistance towards chemotherapy, anti-HER2 therapy and endocrine therapy (Cree & Charlton, 2017). Around 75% of breast cancer patients are reported to express oestrogen alpha ( $ER\alpha$ ) (Piva *et al.*, 2014). Therefore, this has led to the strategy of inhibiting oestrogen signalling as a therapeutic approach. Despite development of targeted anti-oestrogen therapies for ER positive, approximately 30-50% of patients still encounter relapse (Szostakowska *et al.*, 2019). Studies have shown that almost 15% of them with early stage had developed signs of recurrence within five years on adjuvant setting with endocrine therapy (Dowsett *et al.*, 2012). By 15 years, the recurrence rate was as high as 30% (Dixon, 2014).

However, approximately 40% of patients presenting with metastatic ER-positive state would eventually develop resistance after 2 years of endocrine therapy (Dixon, 2014).

The molecular targeting of tamoxifen is directed towards oestrogen receptor and is the standard treatment for patients expressing ER-positive (Manna & Holz, 2016). Interestingly, about 10% of patients with ER-negative breast cancers have responded to tamoxifen treatment (Manna & Holz, 2016). Numerous reports have found a significant fraction of women with ER-negative benefited from tamoxifen treatment (Radin & Patel, 2016).

For HER2 positive subtype, it has responded well to anti-HER2 therapies including trastuzumab and pertuzumab, lapatinib and the antibody-drug conjugate trastuzumab emtansine (Rinnerthaler *et al.*, 2019). Despite all these therapies, relapse is still reported to occur. This is because the HER2 metastatic ability in distant sites can counter the anti-HER2 targeting (Goel *et al.*, 2017; Vernieri *et al.*, 2019). Thus, resistance develops eventually, with primary or acquired resistance to anti-HER2 therapies is contributing most to the treatment failure (Wang & Dang, 2016; Vernieri *et al.*, 2019).

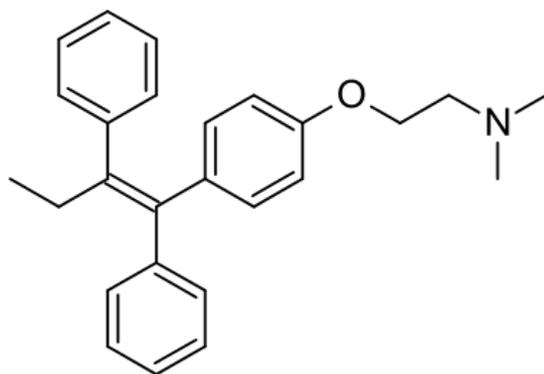
On the other hand, triple negative breast cancer (TNBC) is characterised by poor survival in comparison to the other breast cancer subtypes mainly due to absence of ER, PR and HER2 where targeted therapies towards ER and HER2 are ineffective in TNBCs. This subtype shows a higher tendency of relapse particularly in distant metastasis (Yuan *et al.*, 2014; Wahba & El-Hadaad, 2015). Moreover, resistance to chemotherapy is a major concern as treatment for TNBCs rely entirely on chemotherapy as standard of care (Sprouse, 2014; Nedeljković & Damjanović, 2019) and the respond of TNBCs towards chemotherapy is poor (Liedtke *et al.*, 2008).

Consequently, TNBCs patients often develop resistance (Nedeljković & Damjanović, 2019).

Furthermore, for many women with early breast cancer, breast-conserving surgery (BSC) is currently considered the best treatment option, followed by breast irradiation as recommended by National Comprehensive Cancer Network (NCCN) guidelines. This to destroy cancer cells that may not have been removed in the conserved breast tissue to prevent local recurrence and distant metastasis. Studies show that women who have BCS followed by radiation therapy have similar long-term survival rates as women who have a mastectomy. The likelihood of local relapse after breast conservations is low, at roughly 2-3% after 5 years (Nijenhuis & Rutgers, 2013). These techniques allow women with different forms of breast cancer to conserve their breasts.

### **1.3 Tamoxifen**

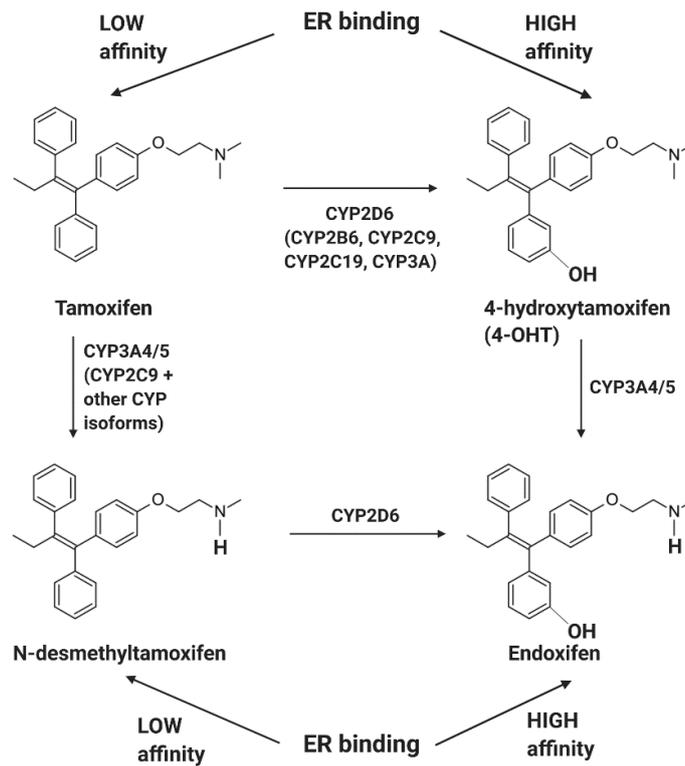
Tamoxifen (Figure 1.1), a selective oestrogen receptor modulators (SERMs) is currently prescribed for ER positive breast cancer (An, 2016). It was approved by the U.S. Food and Drug Administration (FDA) for the treatment of premenopausal women suffering from advanced breast cancer (Macgregor & Jordan, 1998; Hultsch, 2018). It is also used for treating man with breast cancer (Eggemann *et al.*, 2019). For the past three decades, tamoxifen has been used and significantly improved disease-free survival among breast cancer patients (Early Breast Cancer Trials Collaborative Group, 1992). Therefore, it has become the main hormonal therapy to prevent relapse among patient with ER-positive.



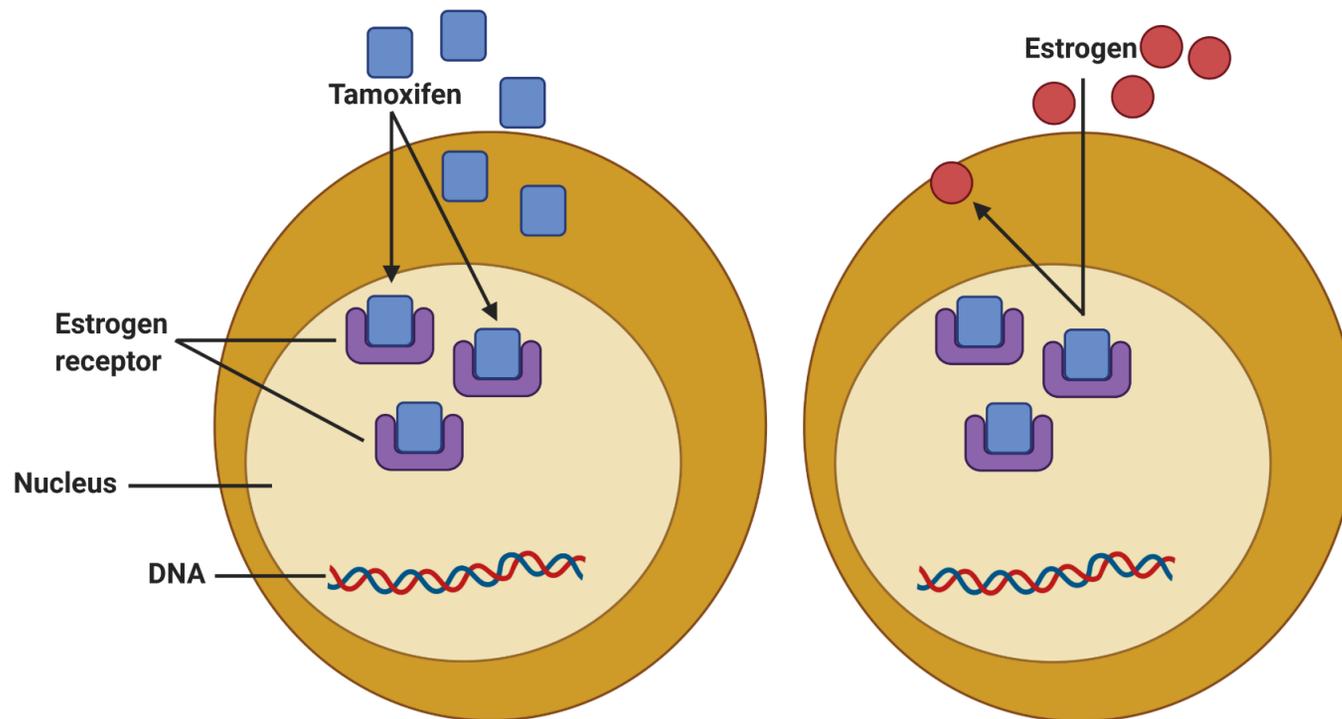
**Figure 1.1: Chemical structure of tamoxifen. PubChem CID=2733526. <https://pubchem.ncbi.nlm.nih.gov/compound/Tamoxifen>. Created with BioRender.com.**

### **1.3.1 Tamoxifen Metabolism**

Tamoxifen is a nonsteroidal triphenylethylene derivative that is metabolised by cytochrome P450 (CYP450) to form active metabolites such as 4-hydroxytamoxifen (4-OHT) and endoxifen (Figure 1.2). Principally, tamoxifen blocks oestrogen signalling in breast cancer cells and thus, inhibit ER activity associated with tumour cell growth (Dean, 2012) (Figure 1.3). The 4-OHT and endoxifen exhibit a 10-fold higher affinity to ER. They have 30 to 100-fold potency to inhibit cell proliferation of estrogen-dependent cells compared to tamoxifen. In fact, level of endoxifen in serum is more than 6-fold higher compared to 4-OHT (Higgins & Stearns, 2010). Thus, this provide evidence that endoxifen plays an important role for the activity of tamoxifen (Higgins & Stearns, 2010).



**Figure 1.2: Major metabolic pathways for tamoxifen and the main cytochrome P450 (CYP) enzymes involved with the capacity of ER binding. Created with BioRender.com.**



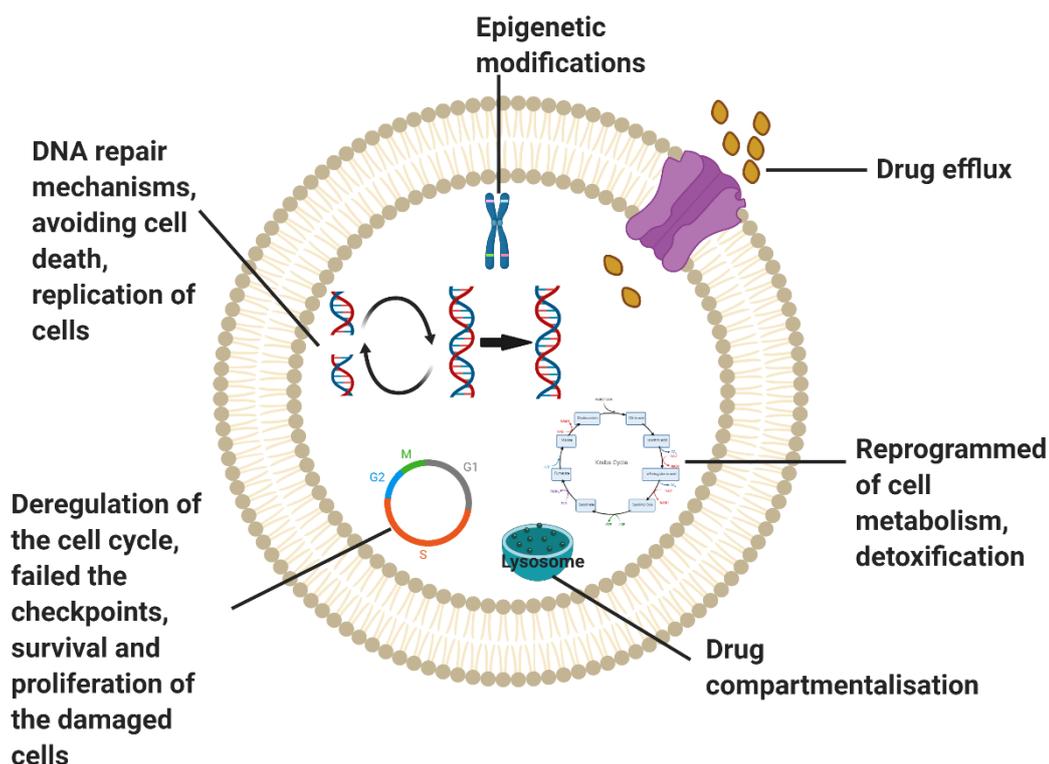
**Figure 1.3: Schematic diagram shows tamoxifen binding to oestrogen receptor and prevention of oestrogen to its receptor (adapted from <https://www.riverpharmacy.ca>). Created with BioRender.com.**

## 1.4 Chemoresistance

Resistance of cancer cell limits the effectiveness of chemotherapy. Cancer may be intrinsically drug-resistant or acquired-resistance to chemotherapy during course of treatment (Longley & Johnston, 2005; Holohan *et al.*, 2013; Clarke *et al.*, 2015). Intrinsic resistance is contributed by pre-existing intracellular resistance-mediating factors (Holohan *et al.*, 2013). In contrast, development of acquired drug resistance happens during treatment of tumours (Holohan *et al.*, 2013). Additionally, resistance can also be acquired through the selection pressure of a resistant population of cells. By such pressure it will cause the development of resistant cell population that has the ability to cause recurrence with larger evolvability characteristic (Foo & Michor, 2014; Sprouse & Herbert, 2014). Therefore, to reduce the incidence of breast cancer resistance, new therapy is needed to overcome this challenge.

In general, both pharmacokinetic factors and pharmacodynamic properties of the drugs have been associated with the development of drug resistance (Asaduzzaman, 2016). Cancer cells may use multiple mechanisms to escape drug treatment (Figure 1.4). This includes inadequate access of the drug to the tumour cells due to the inhibition of drug uptake (Asaduzzaman, 2016), or more commonly through increase of drug efflux (Sprouse, 2014). Proteins that are involved in drug efflux is controlled by the overexpression of multidrug efflux pumps such as P-glycoprotein (encoded by *MDR1*), MRP1 (encoded by *ABCC1*) and ABCG2 (encoded by *BCRP*) (Januchowski *et al.*, 2016; Spitzwieser *et al.*, 2016). Another mechanism to decrease the drug uptake is by compartmentalising the drug into intracellular vesicles, thus the drug would not be able to reach the target (Lu, 2015). Nevertheless, effect of the drug can be modulated by the capability of the cancer cell to repair DNA damage and enhance DNA repair mechanism (Zhu *et al.*, 2018). Deregulation of the cancer cell

cycle or ability to escape the apoptosis pathway enables the limitless cell division (Thu *et al.*, 2018). Recently, alteration of local drug metabolism (reprogrammed metabolism) (Hultsch *et al.*, 2018) and detoxification (Cree & Charlton, 2017) were reported as characteristics of cancer resistance.



**Figure 1.4: Multiple mechanisms that are involved in the development of resistance to therapeutic agents. Created with BioRender.com.**

Although endocrine resistance is widely used, between 25-35% of the treated patients would develop recurrence. It can be contributed by either intrinsic or acquired resistance towards endocrine therapies (Haque & Desai, 2019). Potential mechanisms that have been associated with endocrine resistance involve alterations of cell survival and cell proliferation (Osborne & Schiff, 2011). Moreover, the acquisition of endocrine resistance is manifested by morphological changes that show epithelial-mesenchymal transition (EMT) transition along with increased migratory rate in both

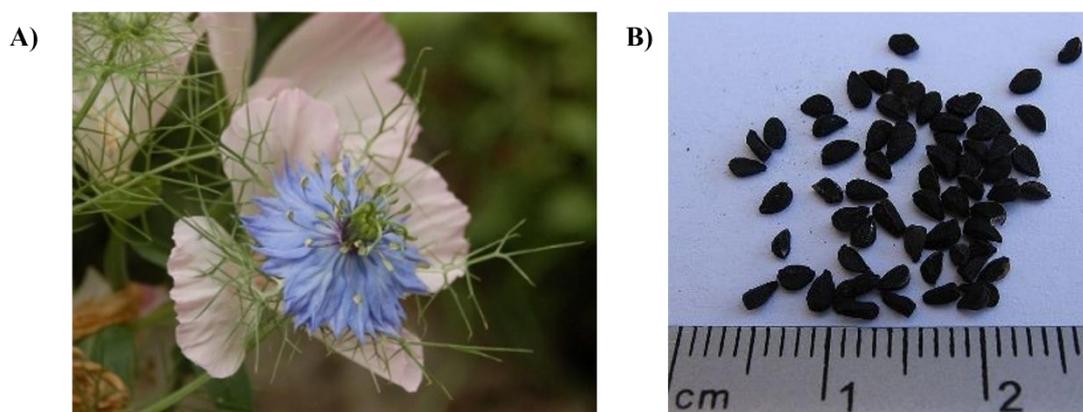
anti-oestrogen resistant cells (Piggott *et al.*, 2018; Zhu *et al.*, 2018). Besides that, resistance to endocrine therapy is exhibited by tumour-inducing cells which is stem-like protrusions. This features could contribute to disease progression (Brien *et al.*, 2011; Luqmani & Alam-Eldin, 2016). Endocrine resistance exhibited higher expression of *cyclin-D1* gene that is involved in cell growth. Additionally it causes the activation of *CDK4* and *CDK6* genes in tandem with high expression of Bcl2 proteins, the anti-apoptotic protein (Rudas *et al.*, 2008). Endocrine resistance also modify the expression of proteins that are involve in cell cycle such as C-MYC, RB1, p21 and P27KIP1 (Thangavel *et al.*, 2011; Dixon, 2014). Besides cell cycle, the other mechanism involves ligand binding to growth receptors such EGFR, HER2 and IGF-1. Activation of the receptors would cause downstream signaling that involved NF- $\kappa$ B and PI3K/Akt/mTOR pathway.

This indicates that, resistant cells have the capability to survive even with existing mode of therapies. Therefore, to mitigate resistance to anti-cancer drug, there is a pressing need to explore into new drug as an alternative to the current therapies. Ongoing clinical trials are venturing new agents to be given along with endocrine therapy (Leary *et al.*, 2007; Alfakeeh & Brezden-Masley, 2018). Another possibility to overcome drug resistance is by materializing the herbal medicines that confer the anti-cancer properties. In addition, the plant-derived compounds have been the source of many chemotherapeutic agents (Mokashi, 2004; Wang *et al.*, 2015). Natural compounds like quercetin and curcumin have been used for treatment in clinical trials to overcome cancer drug resistance (Sotiropoulou *et al.*, 2014). Some studies have reported that alkaloids, flavonoids and other plant compounds could inhibit P-gp when given along with chemotherapeutic drugs (Lee *et al.*, 2018). One of the extensively studied plant is *Nigella sativa*, has long been regarded as the most treasured nutrient-

rich herbs in history around the world and numerous scientific studies are in progress to validate the traditionally claimed uses of small seed of this species (Mariod *et al.*, 2017; Ahmad *et al.*, 2019).

### 1.5 *Nigella sativa*

*Nigella sativa* L. (*N. sativa*) is a small shrub (20-90 cm tall) from the family *Ranunculaceae*. It is native plant to Southern Europe, North Africa and Southeast Asia. *N. sativa* has tapering green leaves and rosaceous white, yellow, pink, pale blue or purplish flowers with 5-10 petals. The ripe fruit (capsule: 3-7 united follicles) contains numerous tiny seeds, dark black in color and possess a severe pungent smell, contains considerable amount of oil (Figure 1.5). *N. sativa* was first botanically described and characterized by Linnaeus in 1753 (Table 1.2).



**Figure 1.5:** *Nigella sativa* flower (A) and black seeds (B). (Adapted from <http://bioweb.uwlax.edu/bio203/>)

**Table 1.2: Taxonomic group of *Nigella sativa* (NCBI: txid555479)**

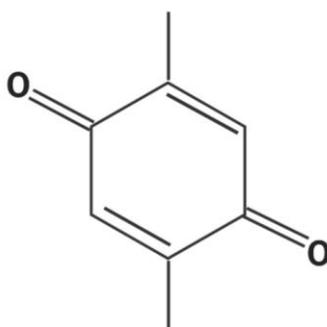
<b>Kingdom</b>	<b>Plantae</b>
Order	Ranunculales
Family	Ranunculaceae
Genus	<i>Nigella</i>
Species	<i>N. sativa</i>

The black seeds of *N. sativa* (Ranunculaceae) have been mentioned in many historical and religious references. The black seed contains several components of the volatile oil including TQ (2-Isopropyl-5-methylbenzo- 1,4-quinone, 30-48%), thymohydroquinone, dithymoquinone, p-cymene (7-15%), carvacrol (6-12%), 4-terpineol (2-7%), t-anethol (1-4%), sesquiterpene longifolene (1-8%),  $\alpha$ -pinene and thymol (Ali & Blunden, 2003; Islam, 2018). TQ has been reported to exhibit anti-oxidant (Dur *et al.*, 2016; Imran *et al.*, 2018), anti-inflammatory (Woo *et al.*, 2013; Ballout *et al.*, 2018) and chemo-sensitization effects (Mostofa *et al.*, 2017). The antiproliferative effects of TQ have been reviewed by Almajali *et al.* (2021) in many types of cancer, such as breast cancer, lung cancer, gastric cancer, colon cancer, prostate cancer, skin cancer, ovarian cancer, liver, cervical cancer and blood cancer. Additionally, TQ protects healthy cells from oxidative damage, making it an efficient chemotherapeutic agent (Ecevit *et al.*, 2017; Mariod *et al.*, 2017).

## **1.6 Thymoquinone: A Promising Drug for Circumventing Multidrug Resistance (MDR)**

TQ, discovered as a substantial component of the volatile oil (hydrophobic chemical compounds from plants that can easily evaporate at normal temperatures), is the most bioactive chemical and demonstrates a wide range of therapeutic advantages among the several active ingredients reported thus far (Mariod *et al.*, 2017). TQ shows

potential pharmacological properties such as anti-oxidant, anti-inflammatory, anti-cancer and other important biological activities (Khan *et al.*, 2011). These pharmacological effects of TQ are due to quinone constituent (Figure 1.6). It inhibited cancer cell growth and progression based on *in vitro* and *in vivo* study models (Majdalawieh *et al.*, 2017; Ballout *et al.*, 2018). Moreover, TQ has also been found to be cytotoxic in several types of parental and multi-drug resistant human tumour cell lines (<https://patents.justia.com/patent/6218434>, Worthen *et al.*, 1998; Ali & Blunden, 2003).



**Figure 1.6: Chemical structure of TQ. PubChem SID=386221103. (<https://pubchem.ncbi.nlm.nih.gov/substance/386221103>). Created with BioRender.com**

For instance, TQ treatment showed promising results in doxorubicin-resistant human breast cancer cells (Arafa *et al.*, 2011). There was an extensive decrease of the cell survival regulators, phosphorylated Akt and Bcl2 along with an increased expression of PTEN and apoptotic markers such as Bax, cleaved caspases, and cleaved PARP after doxorubicin-resistant MCF-7/DOX cells were exposed to TQ. TQ also produced an augmented expression of p53 and p21 proteins with a concomitant G2/M arrest in the same cell line.

Meanwhile, study by Bashmail *et al.* (2018) had assessed the chemomodulatory potential of TQ to gemcitabine (GCB) against human breast

adenocarcinoma (MCF-7) and ductal carcinoma (T47D) cells. TQ induced apoptosis, necrosis, and autophagy in MCF-7 and T47D breast cancer cells, demonstrating cytotoxic effects and promising chemo-modulatory effects to GCB against these breast cancer cells, in addition to depleting tumour associated resistant stem cell fraction (Bashmail *et al.*, 2018). Although the use of GCB was clinically approved for the treatment of metastatic breast cancer since 2004, it suffers from many drawbacks such as lack of selectivity, exaggerated normal tissue toxicity and the emergence of tumour resistance (Barton-Burke, 1999; Burstein, 2000) and appear in the form of tumour relapse and/or recurrence and remote organ metastasis (Jia & Xie, 2015). Therefore, TQ might be able to overcome resistance to GCB and would be a potential successful therapy for breast cancer.

A separate study have demonstrated anti-proliferative and pro-apoptotic activities of TQ in both a non-small cell lung cancer (NSCLC) and a small cell lung cancer SCLC cell lines (Jafri *et al.*, 2010). It also appears that there may be synergism between TQ and cisplatin. This combination was active *in vivo* as demonstrated by the mouse xenograft study. By suppressing NF- $\kappa$ B, TQ may be able to overcome ciplastin resistance and enhance its efficacy. Thus, TQ or likely synthetic analogues of TQ should be developed for possible future human use not only in lung cancer but in possibly other tumour types as well.

TQ anti-cancer activities have been studied in numerous experimental animal models (Khan *et al.*, 2011; Goyal *et al.*, 2017). Although it has been extensively studied, its application in clinical setting is still limited due to its poor bioavailability and hydrophobicity (Odeh *et al.*, 2012; Ballout *et al.*, 2018) for considering it as the primary therapeutic agent. In order to overcome TQ solubility and biodistribution, TQ

encapsulation may improve its delivery to the target sites (Ballout *et al.*, 2018) (Figure 1.7)

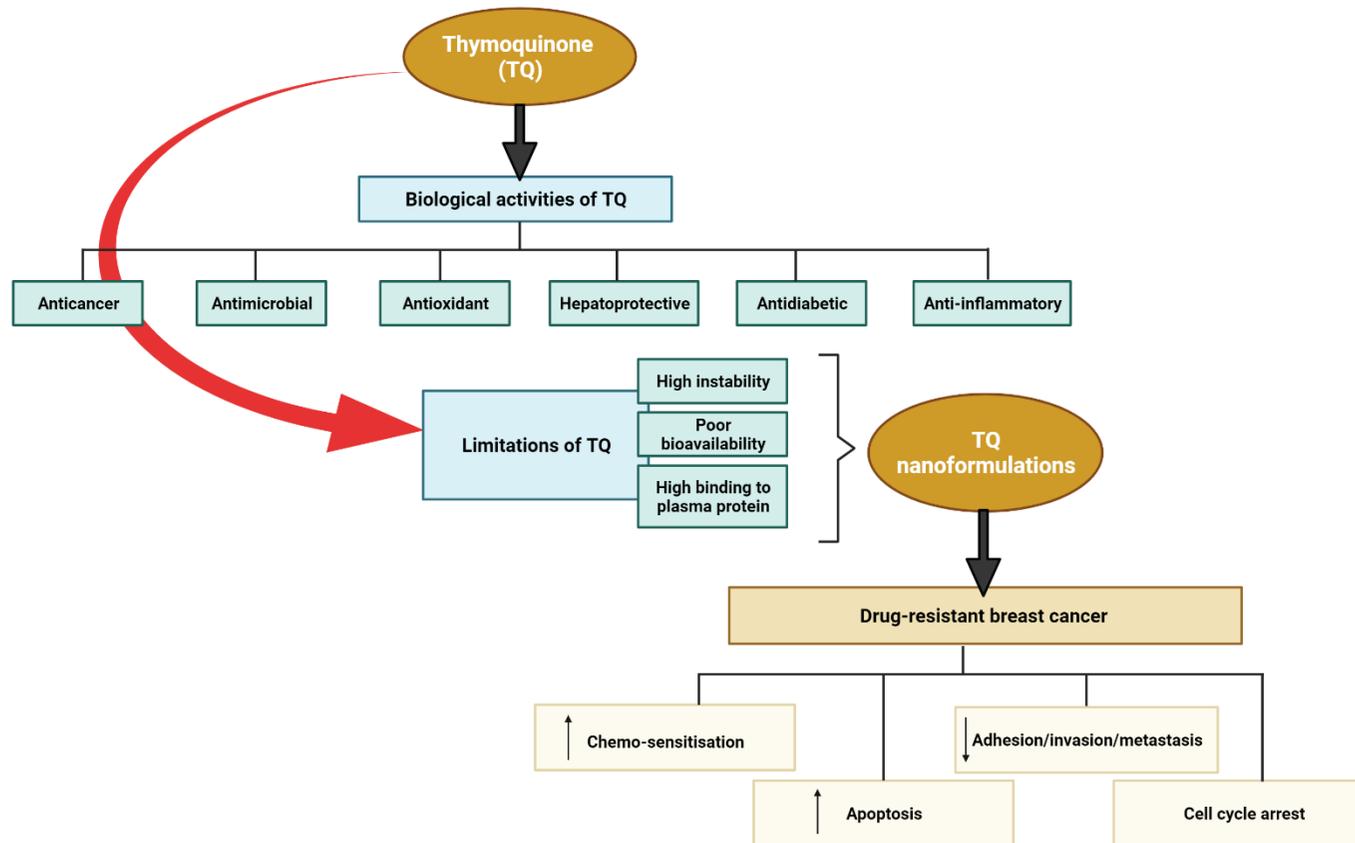


Figure 1.7: Multitargeted protective effects of TQ mechanism against drug-resistant breast cancer. Created with BioRender.com.

## 1.7 Nanoparticulates to Treat Breast Cancer

Nanoparticulates have been gaining research interest over the recent years. Their application in cancer therapy has given promising findings over existing breast cancer therapy that includes surgery, radiotherapy, chemotherapy and hormonal therapy (Hortobagyi, 1998). These therapies demonstrate low specificity and side effects towards the patients (Adair *et al.*, 2010; Tang *et al.*, 2017). For instance, doxorubicin from the family of anthracyclines promotes cardiotoxicity in patients with breast cancer (Smith *et al.*, 2010). This is due to the fact that their ability to treat cancer is hampered by cumulative dose-dependent cardiotoxicity, which can result in irreparable heart failure (Volkova & Russell, 2012; Cai *et al.*, 2019). Studies in cells and animals reveal that the mechanism of anthracycline-induced cardiotoxicity (AIC) is multifaceted, with free radical generation inducing numerous types of cellular harm (Geisberg & Sawyer, 2010; Cai *et al.*, 2019). In addition, anthracyclines alter nucleic acid biology by intercalation into DNA and modulate intracellular signaling, leading to cell death and the disruption of homeostatic processes such as sarcomere maintenance (Rawat *et al.*, 2021). Meanwhile, paclitaxel and docetaxel from the family of taxanes caused bone marrow suppression (Nurgalieva *et al.*, 2011) and hypersensitivity reactions (Lee *et al.*, 2009) in the breast cancer patients. Compared with conventional cancer therapies, nanomedicine has many benefits, as they are less prone to drug degradation while being transported. The other advantage includes improve biocompatibility and increase delivery of drug to tissues. The ideal characteristics of a nanoparticle should comprise various aspects such as non-toxic, biocompatible, nano-scale size, encapsulation efficiency, stability, drug release and targeting sites (Table 1.3). Nanomedicine also exhibits great potential to effectively target and eliminate breast cancer stem cells, which are involved in resistance. List of

formulations for breast cancer therapeutics with approval of Food and Drug Administration (FDA) and ongoing clinical trials are provided in Table 1.4. The liposomes, polymeric nanoparticles and nanoparticle albumin-bound (Nab™)-paclitaxel are the most common nanoparticles designed to treat breast cancer (Figure 1.8).

**Table 1.3: Selection criteria for a nanoparticulate-based therapeutic platform.**

Desired characteristics	Comments
Inherently nontoxic and biocompatible materials	The initial material selections should be based on nontoxic and biocompatible materials especially with an aim toward human healthcare.
Small size (10-200 nm)	This is a proven range of effective particle diameter for a wide variety of delivery system. However, there is no particular size that seems most efficacious, especially for <i>in vivo</i> studies.
Encapsulation of active agent	The active agents/drugs must be encapsulated within the nanoparticles to be protected from unwanted degradation or clearance during blood circulation. To achieve the therapeutic dosage, the nanoparticles must encapsulate a high percentage of the active agents/drugs (>50%).
Colloidally stable in physiological conditions/environments	The nanoparticles and surface functionalisation should be resistant to agglomeration caused by solution Ph values, ionic strength, macromolecular interactions, and temperature encountered in the physiological environment.
Targeting to cell or tissue of choice	Targeting ensures the greatest uptake concentration of chemotherapeutics within the desired lesions and the least side effects with healthy tissues.
Biologically or extrinsically controlled release of active agents	There should be a trigger mechanism such as the acidic Ph within the tumour or during endosome maturation to

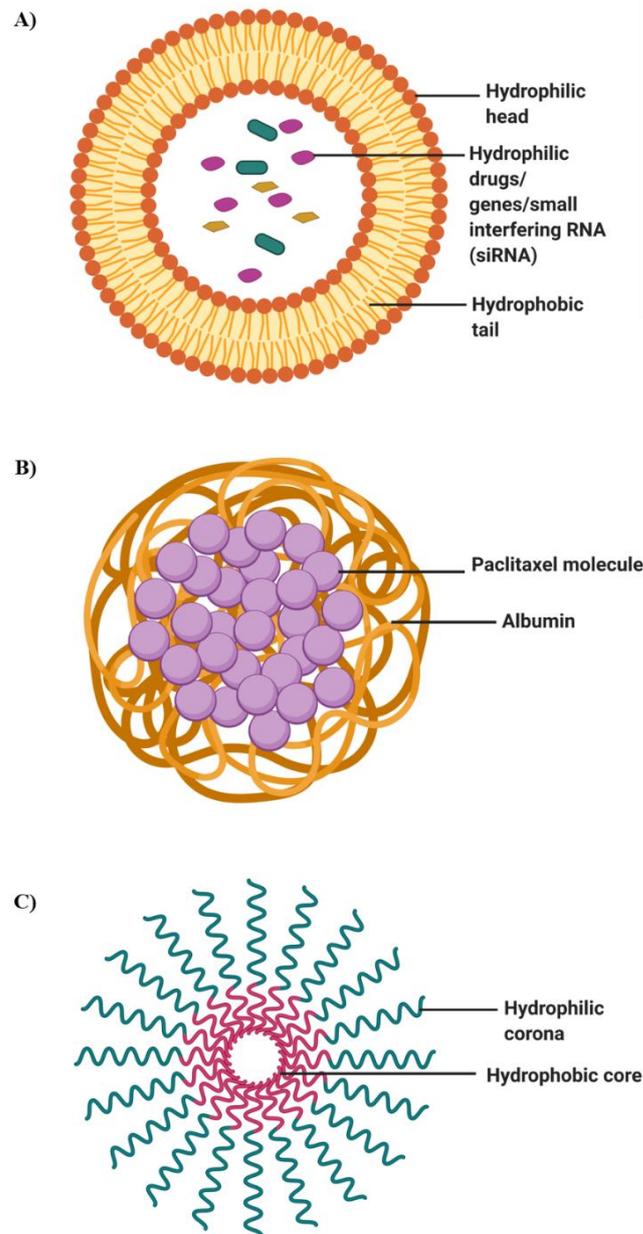
	ensure the release of the encapsulated drug into the targeted tissues
Reasonable circulation time	<p>Resistance to agglomeration that remove the nanoparticle-encapsulated drug from the patient must be avoided to promote long circulation times in the circulatory system for as many of the nanoparticles to find and sequester in the cancer cells as possible.</p> <p>There must be clearance mechanisms for the nanoparticle vehicle after completing its task, to avoid the cumulative and/or systemic side effects such as interference with biological functions.</p>

Adapted with permission from Adair *et al.* (2010).

**Table 1.4: List of nanomedicines as breast cancer therapeutics approved by the U.S. FDA and those in clinical trials.**

<b>Product</b>	<b>Nanoplatfrom/agent</b>	<b>Manufacturer (in USA)</b>	<b>Status in USA</b>	<b>Reference/Clinical trial identifier</b>
Doxil®	PEGylated liposomal/doxorubicin hydrochloride	Janssen Products	Approved in 1995	Barenholz, 2012
Abraxane®	Nanoparticle albumin-bound paclitaxel	Abraxis Bioscience	Approved in 2005	Hawkins <i>et al.</i> , 2008
Myocet®	Non-PEGylated liposomal/doxorubicin	Sopherion Therapeutics	Phase III	NCT00294996
NK-105	PEG-polyaspartate/paclitaxel	Nippon Kayaku	Phase III	NCT01644890
Genexol® -PM	PEG-poly (D, L-lactide)/paclitaxel	Samyang Biopharmaceuticals	Phase III	NCT00876486
NK-012	PEG-polyglutamic acid/SN-38	Nippon Kayaku	Phase II	NCT00951054
Xyotax®	Paclitaxel poliglumex	Dana-Farber Cancer Institute	Phase II	NCT00148707
ThermoDox®	Heat-activated liposomal/doxorubicin	Celsion	Phase I/II	NCT00826085
Liposomal annamycin	Liposome/semi-synthetic doxorubicin analogue annamycin	New York University, School of Medicine	Phase I/II	NCT00012129

Rexin-G	Targeting protein tagged phospholipid/microRNA-122	Epeius Biotechnologies	Phase I/II	NCT00505271
SPI-077	Stealth liposomal cisplatin	LiPlasome Pharma	Phase I	NCT01861496
S-CKD602	PEGylated liposomal/CKD602	Alza	Phase I	NCT00177281
Nanoxel®	PEG-poly (D, L-lactide)/docetaxel PEG-Polylactic-co-glycolic	Fresenius Kabi Oncology	Phase I	NCT00915369
BIND-014	PEG-Polylactic-co-glycolic acid/docetaxel	BIND	Phase I	NCT01300533



**Figure 1.8: Nanoparticulate-based chemotherapeutic delivery platforms approved by the US FDA for cancer treatment. (A) Liposomes are spherical vesicle composed of an aqueous core and a membranous lipid bilayer, preferable for encapsulation of hydrophilic drugs; (B) Nanoparticle albumin-bound paclitaxel (Nab-paclitaxel) is a colloid suspension of paclitaxel; (C) Polymeric nanoparticles are self-assembled from amphiphilic and biodegradable polymers in aqueous solution and are effective to encapsulate hydrophobic drugs. Created with BioRender.com.**