SYNERGISTIC EFFECTS OF GEFITINIB AND PACLITAXEL IN BREAST CANCER TREATMENT USING COCKLE SHELL-DERIVED NANOPARTICLE

By

SANGARAN CHEMMALAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chair : Professor Md Zuki bin Abu Bakar @ Zakaria, PhD
Institute : Bioscience

Breast cancer is the most common type of cancer in women worldwide, including in Malaysia, ranking second in terms of mortality rate. The problem with conventional chemotherapy and radiotherapy is that they also elicit adverse effects on healthy cells. Nowadays, nanoparticle-based drug carriers and targeted therapies are used as alternative treatments since they demonstrate improved efficacy and safety. Nanoparticle based drug carriers can carry and deliver multiple drugs at the tumor site due to the leaky tumor vasculature. Epidermal growth factor receptor (EGFR) overexpression has been correlated with poor prognosis in many breast cancer patients. Gefitinib (GEF), an EGFR-Tyrosine kinase inhibitor, must be administered orally, and it leads to adverse skin reactions and gastrointestinal problems. Paclitaxel (PTXL), an antimitotic drug, is poorly water-soluble, and needs to be administered with a solvent, which leads to hypersensitivity and neuropathies in some patients. Further, GEF and PTXL have synergistic properties. Calcium carbonate as a nanoparticle has gained popularity due to its inherent properties such as biocompatibility, pH-sensitivity, and slow biodegradability. Hence, this Ph.D. work focuses on developing three types of drug-loaded calcium carbonate nanoparticles ($CSCaCO_3NP$) utilizing the blood cockle shell (*Anadara granosa*) waste, resulting in GEF-$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, and dual drug-loaded GEF-PTXL-$CSCaCO_3NP$. Subsequently, the aim was to determine the physicochemical and biological characteristics of the synthesized nanoparticles in MCF-7 and SK-BR-3 cell lines. The results reveal that the $CSCaCO_3NP$, GEF-$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, and GEF-PTXL-$CSCaCO_3NP$ were almost spherical with a diameter of 63.9 ± 22.3, 83.9 ± 28.2, 78.2 ± 26.4, and 87.2 ± 26.7(nm), respectively, and were negatively charged, aragonite and mesoporous, with a surface area ranging from ~8 to 10(m^2/g). $CSCaCO_3NP$ shows excellent alkalinization properties in plasma simulating conditions and greater solubility in a moderately acidic pH medium (pH 5.6). The nanoparticles

showed zero-order drug kinetics, with a slow and sustained release even up to 100 hours. The biocompatibility study with Human Mammary Epithelial cells (HMEC) revealed that the nanoparticles were nontoxic. The relationship between the drugs was determined using XTT and Resazurin reduction assays and it showed synergism. Colonigenic assay against MCF-7 and SK-BR-3 cell lines resulted in 0% colonies after treatment with PTXL-CSCaCO$_3$NP, and GEF-PTXL-CSCaCO$_3$NP, whereas at IC$_{50}$ concentrations, GEF-CSCaCO$_3$NP treated group had persisting colonies. Electron microscopic examinations showed that the MCF-7 cells had undergone apoptotic changes and showed characteristic changes like autophagosomes and thicker microfilament bundles, respectively, for GEF-CSCaCO$_3$NP and PTXL-CSCaCO$_3$NP treatments, which were concurrent with the beta tubulin stabilization in MCF-7 and SK-BR-3 cells, as detected using immunofluorescence. Final investigations revealed that GEF-PTXL-CSCaCO$_3$NP was very effective in up-regulating the apoptotic markers Caspase-3 and BAX in the MCF-7 cell line. GEF-PTXL-CSCaCO$_3$NP against MCF-7 showed synergistic inhibition of EGFR2 at Y1248, similar to EGFR at Y1173. In contrast, only the pure drug GEF had lower levels of p-Tyr1068 of EGFR, whereas the drug-loaded nanoparticles had lower inhibition of phosphorylation. In conclusion, the properties of GEF-CSCaCO$_3$NP, PTXL-CSCaCO$_3$NP, and GEF-PTXL-CSCaCO$_3$NP prove that they deliver the payload, and GEF-PTXL-CSCaCO$_3$NP has a high potential, possessing synergistic characteristics that will be a boost in certain breast cancer management.

Keywords: Breast cancer, Cockle shell derived nanoparticles, Gefitinib, Paclitaxel, dual drug-loaded nanoparticles

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KESAN SINERGISTIK GEFITINIB DAN PACLITAXEL DALAM RAWATAN KANSER PAYUDARA MENGGUNAKAN NANOPARTIKEL YANG DIHASILKAN DARIPADA TEMPURUNG COCKLE

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Kanser payudara merupakan sejenis kanser yang paling kerap berlaku di kalangan wanita di seluruh dunia, termasuk Malaysia, yang menduduki tempat kedua dalam hal kadar kematian. Masalah dengan kemoterapi konvensional dan radioterapi, ialah mereka juga menimbulkan kesan buruk pada sel-sel sihat. Hari ini, penggunaan pembawa dadah berasaskan nanopartikel dan terapi sasaran digunakan sebagai rawatan secara alternatif kerana mereka menunjukkan kecekapan dan keselamatan yang lebih baik. Pembawa dadah berasaskan nanopartikel boleh membawa dan menghantar pelbagai dadah di kawasan tumor kerana vaskulat tumor yang bocor. Hiperekspresi reseptor faktor pertumbuhan epidermal (EGFR) telah dikaitkan dengan prognosis yang buruk pada ramai pesakit kanser payudara. Gefitinib (GEF), penghalang EGFR-Tyrosine kinase mesti diberikan secara oral dan ia membawa kepada reaksi kulit yang tidak baik dan masalah gastrointestinal. Paclitaxel (PTXL), merupakan dadah antimitotik yang kurang larut dalam air, dan perlu diberikan dengan pelarut yang boleh menyebabkan hipersensitiviti dan neuropati pada sesetengah pesakit. Selanjutnya GEF dan PTXL mempunyai sifat sinergistik. Karbonat kalsium sebagai nanopartikel telah mencapai popularity kerana sifat-sifatnya seperti biokompatibiliti, sensitiviti pH, dan biopenguraian yang perlahan. Oleh itu projek kajian Ph.D. ini memberi tumpuan kepada pembangunan tiga jenis pemuat dadah nanopartikel kalsium karbonat ($CSCaCO_3NP$) menggunakan sisa kulit kerang darah (*Anadara granosa*), iaitu GEF-$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, dan dwi dadah GEF-PTXL-$CSCaCO_3NP$. Seterusnya, matlamat kajian ini adalah untuk menentukan ciri-ciri fizikal-kimia dan biologi nanopartikel yang disintesis dalam MCF-7 dan SK-BR-3 baris sel. Hasil kajian mendedahkan bahawa $CSCaCO_3NP$, GEF-$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, dan GEF-PTXL-$CSCaCO_3NP$ adalah hampir sferik dengan diameter 63.9 ± 22.3, 83.8 ± 28.2, 78.2 ± 26.4 dan 87.2 ± 26.7(nm), masing-masing. Penyebaran cahaya dinamik (DLS) dan eksperimen adsorpsi-desorpsi N_2 mendedahkan bahawa nanopartikel yang disintesis mempunyai beban negative, argonite dan

mesoporous, dengan kawasan permukaan berkisar antara 8 hingga 10(m^2/g). $CSCaCO_3NP$ menunjukkan sifat-sifat alkalizasi yang sangat baik dalam keadaan simulasi plasma dan penyelesaian yang lebih besar dalam persekitaran pH moderat asid (pH 5.6). Nanopartikel menunjukkan kinetik ubat urutan nol dengan pelepasan yang perlahan dan berterusan sehingga 100 jam. Hubungan antara dadah-dadah, ditentukan menggunakan ujian XTT dan ujian pengurangan Resazurin ia menunjukkan sinergi. Ujian kolonigenik terhadap MCF-7 dan SK-BR-3 talian sel menghasilkan 0% koloni selepas rawatan dengan PTXL-$CSCaCO_3NP$, dan GEF-PTXL-$CSCaCO_3NP$ manakala, pada kepekatan IC_{50} koloni GEF-$CSCaCO_3NP$ masih berterusan. Pemeriksaan mikroskop elektron menunjukkan bahawa sel-sel MCF-7 telah mengalami perubahan apoptotik dan menunjukkan perubahan ciri-ciri seperti autophagosomes dan bundles mikrofilament yang lebih tebal, masing-masing untuk GEF-$CSCaCO_3NP$, dan rawatan PTXL-$CSCaCO_3NP$, yang bersamaan dengan stabilisasi beta tubulin dalam sel MCF-7 dan SK-BR-3, seperti yang ditemui dalam dapatan kajian imunofluorescence. Penyelidikan akhir mendedahkan bahawa GEF-PTXL-$CSCaCO_3NP$ sangat berkesan dalam 'up'-regulasi penanda apoptotik Caspase-3 dan BAX dalam talian sel MCF-7. GEF-PTXL-$CSCaCO_3NP$ berbanding MCF-7 menunjukkan penghalang sinergistik EGFR2 pada Y1248 serupa dengan EGFR Y1173. Sebaliknya, hanya dadah asli GEF mempunyai tahap EGFR p-Tyr1068 yang lebih rendah, manakala nanopartikel yang dimuat dadah mempunyai penindasan fosforilasi yang berkurangan. Kesimpulannya, sifat-sifat GEF-$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, dan GEF-PTXL-$CSCaCO_3$ NP membuktikan bahawa mereka membekalkan beban berguna dan GEF-PTXL-$CSCaCO_3NP$ mempunyai potensi yang tinggi mempunyai ciri-ciri sinergistik yang akan menjadi penambahbaik pengurusan kanser payudara tertentu.

Kata kunci: Kanser payudara, nanozarah terbitan cengkerang kerang, Gefitinib, Paclitaxel, nanozarah dua muatan dadah

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4-Oct	Octamer- binding transcription factor 4
µg/mL	Microgram per Millilitre
µL	Microlitre
µm	Micrometre
µM	Micromolar
mL	Milliliter
ABC	ATP-binding cassette
4T1luc-GFP	4T1 mouse mammary gland adenocarcinoma with red-shifted luciferase gene fused to Green fluorescent protein
AAG	Alpha 1-acid glycoprotein
ADME	Absorption, distribution, metabolism, and excretion
AIDS	Acquired Immuno Deficiency Syndrome
Akt	Protein kinase B, serine/threonine kinase
AR	Amphiregulin
ATP	Adenosine triphosphate
AVO	Acidic vesicular organelles
BAK	Bcl-2 homologous antagonist/ killer
BAX	Bcl-2 like protein 4
BCL 2	B- cell lymphoma family proteins
BH3	Bcl-2 homology 3
BL 1	basal- like 1
BL2	basal- like 2
BRCA	BReast CAncer
BT-474	Human ductal breast carcinoma

BTC	Betacellulin
$CaCO_3$	Calcium carbonate
CAL-51	Cellosaurus cell line- triple negative breast cancer cell line
CaP	Calcium Phosphate
Caspases	Cysteine- dependent aspartate specific proteases
CDK	Cyclin dependent kinase
CDR	Cumulative drug release
Ce6(Mn)	Mn^{2+}-chelated chlorine6
CHMm	Canine mammary tumor cell lines
CHOP	C/EBP Homologous Protein
CI	Combination Index
$CSCaCO_3NP$	Cockle shell-derived calcium carbonate nanoparticle
CYP2D6	Cytochrome P450 2D6
DAMP	(N-(3-[2,4-dinitrophenyl amino] propyl)-N-(3-amino-propyl)methylamine)
DCIS	Ductal carcinoma in situ
DIW	Deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
DOX	Doxorubicin hydrochloride
DW	Distilled water
DUAL	GEF+PTXL
DUAL-CS	GEF-PTXL-$CSCaCO_3NP$
dual-GEF	Concentration of GEF in GEF+PTXL
dual-PTXL	Concentration of PTXL in GEF+PTXL

EDX	Energy Diffraction X-ray
EE	Entrapment efficiency
EGF	Epidermal growth factor
EGFR/EGFR1/ErbB1/ HER1	Epidermal growth factor receptor 1
EGFR2/ErbB2/ HER2	Epidermal growth factor receptor 2
EGFR3/ErbB3/ HER3	Epidermal growth factor receptor 3
EGFR4/ErbB4/ HER4	Epidermal growth factor receptor 4
EGFR TKI	Epidermal growth factor receptor tyrosine kinase inhibitor
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
EMT	Epithelial mesenchymal transition
EPR	Enhanced permeability and retention
EREG	Epiregulin
ER	Estrogen receptor
ERK-2	Extracellular signal- regulated kinase
FA	Folic acid
FA conjugated Gnb/ Cap polymeric (PLGA -PEG) NPs	Folic acid (FA)-conjugated Gefitinib/Capsaicin polymeric (PLGA-PEG) nanoparticles
Gefitinib- entrapped FA-BSA-CM-β-CD NPs	Gefitinib- entrapped Folic acid (FA) decorated bovine serum albumin (BSA) conjugated carboxymethyl-β-cyclodextrin (CM-β-CD) nanoparticles
FDA	Food and Drug Administration
FESEM	Field emission scanning electron microscopy
FITC	Fluorescein isothiocyanate isomer
FTIR	Fourier- transformed infrared

FTY720	Fingolimod
GEF	Gefitinib
GEF- $CSCaCO_3NP$	Gefitinib-loaded cockleshell-derived calcium carbonate nanoparticle
GEF-PTXL-$CSCaCO_3NP$	Gefitinib and Paclitaxel loaded Cockleshell-derived calcium carbonate nanoparticle
GPCR	G-protein coupled receptor
h	hours
H_2	Histamine
H-AFFt	Heavy chain of apoferritin
HAS	Human serum albumin
HB-EGF	heparin-binding EGF-like growth factor
HCC1806	Cell line isolated from the mammary gland having acantholytic squamous cell carcinoma, stage IIB, grade 2
hFOB 1.19	Human fetal osteoblastic cells
HMEC	Primary mammary epithelial cells; normal, Human
IUPAC	International Union of Pure and Applied Chemistry
IC_{50}	Half-maximal inhibitory concentration
IGF	Insulin like growth factor
IHC	Immunohistochemistry
IM	Immunomodulatory
kg	Kilogram
L	Liter
LAR	Luminal androgen receptor
M523595	Desmethyl-gefitinib
M537194	Metabolite of Gefitinib
MAC	Micron-size aragonite calcium carbonate particles

MAPK	Mitogen -activated protein kinase
MCF-7	Human breast cancer cell line acronym for Michigan Cancer Foundation-7
MDA-MB-468	Cell line isolated form pleural effusion of female with metastatic adenocarcinoma of breast
MDR	multidrug resistance
MEK-1	Mitogen- activated protein kinase
MEN	menadione
MET	mesenchymal epithelial transition factor receptor
MMP	Matrix metalloproteins
MSL	mesenchymal stem-like
MTT	3-[4, 5- dimethylthiazol-2-yl]-3, 5-diphenyl tetrazolium
MWCO	Molecular weight cut-off
NA	Not available
nm	Nanometre
NP	Nanoparticle
NRG	Neuregulin
NSCLC	Non-small-cell lung cancer
p21(CIP1/WAF1)	Cyclin dependent kinase inhibitor 1
p53	Tumor protein 53
p70s6k	Ribosomal protein S6 kinase beta-1, p70S6
p85s6k	Represent isomer of p70s6k derived by differential splicing from a common gene
PBS	Phosphate Buffer Saline
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PI(3)K	Phosphatidylinositol-3-OH kinase

pKa	Acid dissociation constant
PLGA	Poly lactic glycolic acid
PMS	Phenazine methosulfate
PR	Progesterone receptor
PTXL	Paclitaxel
PTXL-CSCaCO$_3$NP	Paclitaxel -loaded Cockle shell-derived calcium carbonate nanoparticle
QGY cell line	Hepatocellular carcinoma cell line
QTc	QT corrected for heart rate
Raf	Rapidly accelerated fibrosarcoma kinases
Ras	“Rat sarcoma virus” protein
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Rosewell Park Memorial Institute medium
RSD	Relative standard deviation
S/SER	Serine
SD	Standard deviation
SEAFDEC	Southeast Asian Fisheries Development Center
SEM	Scanning electron microscopy
SHP-1	Src homology 2 domain-containing protein tyrosine phosphatase 1
SK-BR-3	Human breast cancer cell line isolated by the Memorial Sloan- Kettering Cancer Center
Src	Proto-oncogene tyrosine-protein kinase Src
T/ TYR	Amino acid Tyrosine
t1/2	Half-life of drug
T80	Tween 80

TEM	Transmission Electron microscopy
TGF-α	Transforming growth factor-α
TNBC	Triple negative breast cancer
TPGS	Vitamin E-tocopheryl polyethylene glycol succinate
TS-CS-PEG-FA	Tocopherol succinate-chitosan- polyethylene glycol-folic acid
TQR	Drug resistance inhibitor -tariquidar
UMR-106	Rat osteogenic sarcoma cells
VEGF	Vascular endothelial growth factor
XRD	X-ray diffraction
XTT	2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide
Y	Tyrosine amino acid

CHAPTER 1

INTRODUCTION

1.1 Study background

The field of nanomedicine is multidisciplinary and encompasses the application of nanotechnology for the diagnosis, treatment, monitoring, and control of biological systems (Moghimi et al., 2005). Nanoparticles are colloidal systems composed of organic or inorganic materials which are usually less than 200nm or lesser in size (Morton et al., 2014), but are commonly 5-350nm in diameter. The various types of inorganic nanoparticles are usually synthesized from gold (Alkilany et al., 2013), silica (Zhang et al., 2010), silicon, iron (Calero et al., 2015), carbon (Albert et al., 2018), calcium carbonate ($CaCO_3$) (Wenliang et al., 2018; Kamba et al., 2014a; Ghaji et al., 2018), carbon (Kokalari et al., 2021), calcium phosphate ($Ca(H_2PO_4)_2$), hydroxy apatite ($Ca_5(PO_4)_3OH$) (Fan et al., 2021) and many more. Nanomaterials have been used in the treatment of cancer. The primary principle behind nanomedicine for cancer treatment is that tumors have leaky vasculature due to sustained tumor angiogenesis called the enhanced permeability and retention (EPR) effect (Matsumura & Maeda, 1986; Maeda, 2021). The tumor contains underdeveloped lymphatic drainage, facilitating the passive targeting of nanoparticles to the tumor with subsequently enhanced permeability retention (Danhier et al., 2010). Hence the nanoparticles can accumulate at a higher concentration in the tumor tissues and elicit the required therapeutic effect.

In the past three decades, nanotechnology and branches of nanobiotechnology have gained a lot of attention and popularity. It is due to the high economic impact in various fields, including cosmetics, electronics, food, molecular diagnostics, and even personalized medicines. Since the approval of liposomal doxorubicin to the lipid-based nanoparticle vaccines for COVID-19, the area of nanotechnology has revolutionized the field of medicine (Vahedifard & Chakravarthy, 2021).

In the year 2020, the incidence of breast cancer was 11.7% (2262419) with a mortality of 6.9% (684996) in both sexes in the world (WHO, 2021a). Breast cancer is the most common type of cancer in women in Malaysia, ranking second for causing death following lung cancer (WHO, 2021b). In the field of oncology, nanomedicines have gained recognition for their use when conventional medicines failed. The most fundamental characteristic of tumor cells is their propensity for chronic proliferation, which is not the status quo in healthy cells. Tumor cells have deregulated cellular signaling mechanisms, and they are relayed mostly by growth factors that bind to the cell surface, typically comprising intracellular tyrosine kinase domains. The tyrosine kinase domains regulate the cell cycle and cellular growth, these signals influence cell survival and energy metabolism. Cancer cells can show sustainable growth by evading the growth

suppressing mechanisms, more so by producing the growth factor ligands on their own, more so due to mitogenic signaling and they respond to its growth factors via similar receptors, hence resulting in the autocrine proliferative stimulus (Lemmon & Schlessinger, 2010).

Breast cancer cells are relatively autonomous of exogenous growth factors as compared to healthy breast tissue. This is due to the innate capacity of the tumor to synthesize increased quantities of growth factors and receptors for these growth factors (Salomon et al.,1995). According to Goldhirsch et al. (2011), breast cancer is no longer considered a single disease due to its various subtypes. Hence, breast cancer cases should be managed and treated according to their distinct subtypes: luminal (ER/PR-positive and HER2-negative), HER2 (HER2-positive regardless of ER/PR status), and triple-negative (TN; ER-, PR-, and HER2-negative) breast cancer subtypes. The first line of treatment is usually the removal of tumors through surgery, and to reduce recurrence, the patients are given adjuvant therapies, including hormonal and HER2 targeted therapies.

Since overexpression of epidermal growth factor receptor (EGFR) has been correlated with poor prognosis in patients, multiple agents have been developed to act against EGFR. Gefitinib (GEF) is an orally active, selective epidermal growth factor receptor-tyrosine kinase inhibitor that results in blocking the signal transduction pathways responsible for the proliferation and maintenance of cancer cells (Baselga & Averbuch, 2000). Paclitaxel (PTXL) is a taxane which promotes abnormal microtubule assemble hence, stabilizes microtubules after they are formed. PTXL blocks mitotic spindle formation during cell division resulting in bundling of microtubules (Kuhn, 1994), hence bringing mitotic arrest and cell death. PTXL has been used widely used for breast cancer cases and is administered weekly (Kataja & Castiglione, 2008). Myelosuppression, with leukopenia and/or neutropenia, was the dose-limiting toxicity of Paclitaxel (Walker, 1993). Other symptoms include alopecia, transient myalgia, arthralgia, fatigue, and headaches, which have been observed in patients (Rowinsky et al., 1990). Because of the toxicity of PTXL, researchers developed albumin-bound paclitaxel (nab-PTXL, Abraxane®, Abraxis Bioscience) which has been approved by the European Medicines Agency (EMA) for patients with metastatic breast cancer who have failed combination therapy or replacement therapy within 6 months of adjuvant therapy where prior therapy included an anthracycline (EMA, 2015). This medication does not need toxic solvents and it has produced more responses when compared to conventional PTXL-containing medicines (EMA, 2015).

The focus of the current study is to use the newly synthesized cockle shell-derived calcium carbonate ($CaCO_3$) aragonite nanoparticles ($CSCaCO_3NP$) as a nanocarrier for GEF and PTXL. The use of GEF-loaded $CSCaCO_3NP$ would be justified for the following two reasons. Firstly, co-expression of increased quantities of EGFR and their respective ligands results in a transformed cellular phenotype (Woodburn, 1999; Bi et al., 2016), in this context, cancer. Secondly, though EGFR is observed in typical epithelial cells, it is found to be excessively

expressed in many epithelial tumors, including breast cancer (Bi et al., 2016; Baselga & Averbuch, 2000), and this has been related to worse clinical output in several patients (Woodburn, 1999; Bi et al., 2016). Research carried out on ZR-75-1 and MCF-10A ras cell lines indicate that GEF, coupled with two taxanes (Docetaxel and PTXL), shows a dose-dependent supra-additive rise in inhibition of growth (Ciardiello et al., 2000). When combined with cytotoxic agents like PTXL and other agents, GEF displays enhanced cell growth inhibition, induction of apoptosis, and increased tumor destruction in a dose-dependent manner *in vitro* and *in vivo* in various cancer cell lines (Takabatake et al., 2006). Different cross-talking pathways targets have been identified in various breast cancer cell lines. It was found that GEF produces anticancer effects, which redeems the counteractive EGFR-hypoxia crosstalk in resisting PTXL's pro-apoptotic property (Jia et al., 2009). Hence, embracing the possibility of synthesizing calcium carbonate nanoparticles with their distinguishing characteristic features and loading them with GEF and PTXL would be the first step in discovering nanomedicines as a targeted therapy against breast cancer.

1.2 Problem statements

Although with the modern healthcare system, a 5-year survival rate of 80% is reported in the USA, many survivors experience long-term side effects due to the cancer treatment (Yip et al., 2014). Cancer chemotherapy exerts its actions on cancer cells more intensely, but the normal cells are also exposed to the adverse actions of the drugs or radiation administered. In addition to the fact that these above-stated treatments can result in resistance to certain medications or even multi-drug resistance (MDR) through various mechanisms, especially due to the overexpression of the ATP-binding cassette (ABC) transporters in the tumor cells (Liu et al., 2016). PTXL, a conventional chemotherapeutic drug is poorly water-soluble, and it is always administered with ethanol and Cremophor EL, which leads to hypersensitivity in some patients (Rowinsky et al., 1991) which is a major problem with PTXL in addition to drug resistance. GEF is a targeted drug which needs to be administered everyday orally and has adverse skin reactions and gastrointestinal problems (Bernsdorf et al., 2011).

Inadequate drug release and poor drug penetration at the cancer site are also problems in conventional medicine (Maleki Dizaj et al., 2019). In addition, conventional chemotherapy has poor specificity, high toxicity, and induces drug resistance (Din et al., 2017). Hence, the situation calls for other alternative modalities like the combination therapy with cytotoxic agents or other co-targeting drugs or radiotherapy. Combination therapy came into use in chemotherapy because of various factors that include overcoming drug resistance and multitargeted treatments for disturbing multiple nodes of pathways of interest for better outcomes (Bulusu et al., 2016). When combined with cytotoxic agents like PTXL and other agents, GEF displays enhanced cell growth inhibition, induction of apoptosis, and increased tumor destruction in a dose-dependent manner in various cancer cell lines (Takabatake et al., 2006).

Targeted drug delivery to a specific site is a sensible option to solve these issues faced in the terms of conventional chemotherapy. So, nanotechnology paves the way in solving all these critical issues by providing targeted therapy along with controlled drug release by delivering the payload to the targeted sites, hence reducing the toxicity (Santos et al., 2015; Patra et al., 2018). Nanotechnology can be used to improve the delivery of poorly water-soluble drugs, co-delivery of two or more drugs for combination therapy, and transcytosis of drugs across tight epithelial and endothelial barriers (Farokhzad & Langer, 2009).

Inorganic nanoparticles are famous for the controlled release of drugs due to their longer biodegradability. The biogenic inorganic calcium carbonate nanoparticles derived from the Blood cockle shells have shown promising results in delivering chemotherapeutic drugs (Kamba et al., 2014b; Danmaigoro et al., 2017; Ibiyeye et al., 2019). To overcome the problems with PTXL and GEF, in this study, PTXL is loaded onto the calcium carbonate nanoparticle along with GEF. So, by combining these two drugs in a single drug delivery system with the pH dependent calcium carbonate nanoparticles we aim to overcome the problems faced with conventional chemotherapy and benefit with the single drug delivery system.

1.3 Significance of the study

Combined therapies with targeted drugs loaded onto nanoparticles have shown promising results due to the targeted release and synergistic effects of the drugs. Literature indicates that the drugs PTXL and GEF elicit toxicities when administered to cancer patients. The approach to tackle this problem with the cockle shell derived calcium carbonate nanoparticles loaded with GEF and PTXL would be beneficial in terms of drug administration and toxic profile due to lowered dosage of each drug.

In addition, pH dependent solubility of the calcium carbonate nanoparticles would release the drugs in the tumor site which has lowered pH. Hence, there is a need to synthesize and characterize the GEF and PTXL drug loaded calcium carbonate nanoparticles and evaluate the *in vitro* efficacy in comparison to the pure drugs in breast cancer cell lines. Finally, the outcome of this research is expected to improve the current knowledge on the inorganic nano-drug carriers and the anti-cancer efficacy in MCF-7 and SK-BR-3 cell lines.

1.4 Hypothesis

In MCF-7 and SK-BR-3 breast cancer cell lines, the anti-neoplastic effects of dual agents (GEF-PTXL-CSCaCO$_3$NP) are potentiated as compared to the anti-neoplastic effects of single agents (PTXL-CSCaCO$_3$NP and GEF-CSCaCO$_3$NP).

1.5 Research questions

i. Could a nanoparticulate drug delivery system loaded with Gefitinib and Paclitaxel be produced with the acceptable characteristics required for a nanovector with an adequate loading capacity and entrapment efficiency from cockle shells?
ii. To what extent will the dual (GEF-PTXL-CSCaCO$_3$NP) or the mono drug be loaded and will GEF-CSCaCO$_3$NP and PTXL-CSCaCO$_3$NP act synergistically in bringing about a dose-dependent anti-proliferative effect in the MCF-7 and SK-BR-3 breast cancer cell lines?
iii. What are the biological effects of CSCaCO$_3$NP, GEF-CSCaCO$_3$NP, PTXL-CSCaCO$_3$NP and GEF-PTXL-CSCaCO$_3$NP in MCF-7 and SK-BR-3 breast cancer cell lines?
iv. What effect does the dual (GEF-PTXL-CSCaCO$_3$NP) or the mono drug loaded (GEF-CSCaCO$_3$NP and PTXL-CSCaCO$_3$NP) have on the production of pro-apoptotic factor-like BAX and blocking the phosphorylation of the Tyrosine kinases in MCF-7 cell line?

1.6 Objectives of the study

1.6.1 General Objectives

The main objective is to evaluate the synergistic effects (anti-neoplastic) of mono drug-loaded GEF-CSCaCO$_3$NP and PTXL-CSCaCO$_3$NP and dual-drug loaded (GEF-PTXL-CSCaCO$_3$NP) on MCF-7 and SK-BR-3 cell lines.

1.6.2 Specific Objectives

i. To synthesize and evaluate the physicochemical characteristics of CSCaCO$_3$NP from cockle shells, mono drug-loaded GEF-CSCaCO$_3$NP and PTXL-CSCaCO$_3$NP and dual drug-loaded Gefitinib and Paclitaxel calcium carbonate nanoparticle (GEF-PTXL-CSCaCO$_3$NP).
ii. To assess the *in vitro* drug release kinetics profile of mono drug-loaded GEF-CSCaCO$_3$NP and PTXL-CSCaCO$_3$NP, as well as dual drug-loaded Gefitinib and Paclitaxel loaded calcium carbonate (GEF-PTXL-CSCaCO$_3$NP).
iii. To determine and compare the inhibitory concentration (IC_{50}) of CSCaCO$_3$NP, GEF-CSCaCO$_3$NP, PTXL-CSCaCO$_3$NP, and GEF-PTXL-CSCaCO$_3$NP and compare the level of synergism between the drugs in the MCF-7 and SK-BR-3 cell lines using both XTT and Resazurin assay.
iv. To determine the IC_{50} and biocompatibility of blank and drug-loaded CSCaCO$_3$NP in Human Mammary Epithelial cells (HMEC).
v. To evaluate and compare the anti-colonigenic ability, anti-migration capacity, and beta tubulin stabilization of CSCaCO$_3$NP, GEF-

$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, and GEF-PTXL-$CSCaCO_3NP$ on MCF-7 and SK-BR-3 cell lines.

vi. To study the light microscopic, fluorescent microscopic and ultramicroscopic cytopathological changes after treatment with $CSCaCO_3NP$, GEF-$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, and GEF-PTXL-$CSCaCO_3NP$ on MCF-7 cell line.

vii. To determine the effects of $CSCaCO_3NP$, GEF-$CSCaCO_3NP$, PTXL-$CSCaCO_3NP$, and GEF-PTXL-$CSCaCO_3NP$ on apoptotic markers: Caspase-3 and BAX, and to estimate the level of inhibition of phosphorylation of EGFR and EGFR2 in MCF-7 cell line.

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