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Biological Evaluation of Novel Pyrazolotriazolopyrimidine Derivatives as Candidates of EGFR Inhibitors in Different Cancers

التقييم البيولوجي لمثبطات جديدة لمستقبلات عامل النمو الطلائي (EGFR) في انواع مختلفة من السرطان

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إقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Biological Evaluation of Novel Pyrazolotriazolopyrimidine Derivatives as Candidates of EGFR Inhibitors in Different Cancers

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بناءً على موافقة شئون البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحثة/ **مريم جمال محمد غنيم،** لنيل درجة الماجستير في **كلية *العلوم*** قسم **التكنولوجيا الحيوية** وموضوعها:

التقييم البيولوجي لمثبطات جديدة لمستقبلات عامل النمو الطلائي (EGFR) في أنواع مختلفة من السرطان

Biological Evaluation of Novel Pyrazolotriazolopyrimidine Derivatives as Candidates of EGFR Inhibitors in Different Cancers

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واللجنة إذ تمنحها هذه الدرجة فإنها توصيها بتقوى الله ولزوم طاعته وأن يسخر علمها في خدمة دينها ووطنها.

والله ولي التوفيق ،،،

عميد البحث العلمي والدراسات العليا

أ.د. مازن اسماعيل هنية

Abstract

Background:

Cancer is one of the most serious diseases and represents a major threat to human health all over the world. Epidermal growth factor receptors (EGFRs) have been shown to be implicated in tumour initiation and progression. Despite of the important progress in EGFR inhibitors synthesis, many cancers develop resistance to these drugs. Therefore, more effective EGFR tyrosine kinase inhibitors are required for cancer treatment.

Objective:

This study has been conducted to evaluate the biological activities of a series of novel Pyrazolotriazolopyrimidine derivatives (BA623, BA642, BA645) compounds that have been synthesized as potential epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in human breast (MCF7), cervical (HELA) and colorectal (Caco-2) cancer.

Methods:

MTT assay has been employed to evaluate the general toxicity of these compounds in the indicated cancer cells. The anti-cancer effects of these compounds against cancer cells were assessed using trypan blue assay, growth curve and migration assay. To understand the mechanism of action of the most promising Pyrazolotriazolopyrimidine (BA) compound, western blotting analysis and flow cytometry were performed.

Results:

The results show significant toxicity of all three Pyrazolotriazolopyrimidine compounds tested in this study in a time and concentration dependent manner. The toxicity of these compounds includes proliferation inhibition and cell death. Importantly, these compounds showed a significant anti migration effect on HELA cells. Of the tested compounds BA-623 has the most potent cytotoxic effect and we show here that this compound is able to inhibit EGFR/Akt signaling pathway resulting in activating p53 and p21 leading to G1 cell cycle arrest in MDA-MB-231 breast cancer cell line.

Conclusion:

These results suggest that the tested Pyrazolotriazolopyrimidine derivatives especially BA-623 may hold potent anticancer effects.

Keywords: Cancer, EGFR, biological activities, Pyrazolotriazolopyrimidine and tyrosine kinase inhibitors.

الملخص

خلفية البحث:

يعد السرطان واحدا من أكثر الأمراض خطورة، حيث يمثل تهديدا رئيسا لصحة البشر حول العالم، وأظهرت الدراسات أن مستقبلات عامل النمو الطلائي يرتبط بإحداث الورم وتقدمه. بالرغم من التقدم في تصنيع أنواع مختلفة من مثبطات مستقبلات عوامل النمو الطلائي واستخدامها لمعالجة أنواع محددة من السرطان من ضمنها سرطان الرئة والثدي إلا أن العديد من السرطانات أظهرت مقاومة لها مما تسبب ايضا بالعديد من الاثار الجانبية، لذلك نحتاج لمثبطات أكثر فعالية لهذه المستقبلات.

أهداف البحث:

تم اجراء هذا البحث لتقييم النشاطات البيولوجية لسلسلة جديدة من مشتقات Pyrazolotriazolopyrimidine تم تصنيعها كمثبطات محتملة لمستقبلات عوامل النمو الطلائي في سرطان الثدي وسرطان عنق الرحم وسرطان القولون.

منهجية البحث:

تم تطبيق تجربة MTT لتقييم سمية هذه المركبات على انواع السرطان المشار اليها، وتم فحص التأثيرات المضادة للسرطان لهذه المركبات من خلال تجربة trypan blue, growth curve, migration وتم ايضا فحص تأثيرات مشتقات Pyrazolotriazolopyrimidine على دورة الخلية و EGFR باستخدام تجربة western blot and flow cytometer.

نتائج البحث:

تشير النتائج الى سمية واضحة لمركبات Pyrazolotriazolopyrimidin, والسمية تعتمد على التركيز والوقت. بالإضافة الى ان هذه المركبات لها القدرة على تحفيز الموت الخلوي والذي تم اختباره باستخدام تجربة cell viability فلهذه المركبات قدرة على تثبيط قدرة خلايا سرطان عنق الرحم على الهجرة HELA.الاستجابة لمركب 623 مرتبطة بانخفاضEGFR, AKT المفسفر وزيادة في p53, p21 و ايقاف الخلايا في طور G1 وزيادة عدد الخلايا في منطقة ما قبل G1 في خلايا سرطان الثديMDA-MB-231

تشير هذه النتائج الى احتمالية كون هذه المركبات مضادات سرطانية واعدة.

خلاصة البحث:

يمكن لهذه الدراسة أن تكون نقطة بداية فاعلة لتطوير مجموعة جديدة من مثبطات مستقبلات عوامل النمو الطلائي لعلاج سرطان الثدي وعنق الرحم.

الكلمات المفتاحية:

السرطان، مستقبلات عامل النمو الطلائي EGFR، الفعالية البيولوجية، Pyrazolotriazolopyrimidine، مثبطات التيروسين كاينيز.

Dedication

This thesis is dedicated to

The sake of Allah, my Creator and my Master

My great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life

To the Holy Land, my homeland Palestine

My great parents for their endless love, support and encouragement

My beloved brothers and sisters

To all my family, the symbol of love and giving

Anyone who taught me a letter

To those whose in their hearts all the lofty values that brighten our way over time

My friends who encouraged and supported me

My dear students. All love and appreciation to them

To those who struggle the disease.... to cancer patients

Last but not least, to all those who touch my heart and were beside me all the way and those who believe in the richness of learning.

I dedicate this thesis

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List of Abbreviations

APC	**Adenomatous polyposis coli**
AR	**Amphiregulin**
ATP	**Adenosine triphosphate**
BA	**Pyrazolotriazolopyrimidine**
BTC	**Betacellulin**
CBC	**Complete blood count**
CT	**Computed tomograph**
CRC	**Colorectal cancers**
CDKs	**Cyclin-dependent kinase**
DMF	**Dimethylformamide**
DMSO	**Dimethyl sulfoxide**
EGFR	**Epidermal growth factor receptors**
EGF	**Epidermal growth factor**
EPR	**Epiregulin**
ER	**Estrogen receptor**
FAP	**Familial adenomatous polyposis**
FBS	**Fetal bovine serum**
HER2	**Human epidermal growth factor receptor type 2**
HPV	**Human Papillomavirus**
IGF-IR	**Insulin like growth factor I receptor**
MTT	**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**
MRI	**Magnetic resonance imaging**
NTKR	**Non-receptor tyrosine kinase**
NSCLC	**Non-small cell lung cancer**
PTB	**Phosphotyrosine binding**
PI3K	**Phosphatidylinositol 3-kinase**
PDGFR	**Platelet derived growth factor receptors**
P53	**Tumour protein-53**
PBS	**Phosphate buffer saline**
PI	**Propidium iodide**

SH2	**Src homology 2**
SCCs	**Squamous cell carcinomas**
TKI	**Tyrosine kinase inhibitors**
TNBC	**Triple-negative breast cancer**
TGF-α	**transforming growth factor alpha**

Chapter 1
Introduction

Chapter 1

Introduction

Cancer is a disease that occurs due to internal and external factors that lead to uncontrolled growth and existence of abnormal cells, which cause death after uncontrolled distribution. Worldwide cancer is the second leading cause of death and accounts annually for one in seven deaths (American Cancer Society, 2012). According to the international agency for research on cancer, around 14 million cases were diagnosed in 2012 worldwide (World Health organization, 2014). The number of new cases is expected to reach 22 million during the next two decades (American Cancer Society, 2012). In Gaza Strip, the number of new cancer cases during the period from 2009-2014 is 7069 according to reports of the national center for monitoring oncology (Palestinian Ministry of Health, 2015).

Breast cancer is the most common in Gaza Strip where it represents 18.1% of all cancers. It occupies the first kind among female's cancers with a percentage of 31.4% of all malignant diseases among females. In Gaza, the second common cancer is colon cancer where it represents the most prevalent among males with 11.5% of the male cancers (Palestinian Ministry of Health, 2015).

There are many types of cancer treatments, the specific type of treatment that the patients receive depends on the type and stage of the cancer they have. The main types of cancer treatment include Surgery, chemotherapy, radiation therapy, immunotherapy and hormone therapy. Surgery is a procedure in which a surgeon removes tumour from patient's body. It can be used to remove cancer that is found in one area, or to remove part of a tumour, removing part of a tumour can help other treatments work better (National Cancer Institute, 2015). Radiation is an energy based treatment which uses waves or a stream of particles to cause DNA damage and stop cell growth and division. This leads to cell death over time, so radiation can be used to kill cancer cells and shrink tumours (Baskar, Lee, Yeo, & Yeoh, 2012). Hormone therapy slows or stops the growth of cancers that use hormones to grow like prostate and breast cancers. This type of treatment prevents hormones production or blocking receptor binding to hormones (J. Abraham & Staffurth, 2016). Cancer immunotherapy uses certain parts of a person's immune system to attack cancer. This can be done by stimulating immune system to work harder to

attack cancer cells or by giving immune system components, such as man-made immune system proteins to treat cancer. There are many types of cancer immunotherapy that are used to treat cancer such as monoclonal antibodies, immune checkpoint inhibitors, cancer vaccines and non-specific immunotherapies (American Cancer Society, 2015; West, 2015). Chemotherapy is a systemic drug which kills cancer wherever they exist in the body. There are many types of chemotherapeutic drugs which are divided based on several factors such as its nature, chemical structure, mode of action and its relationship with other medicines (American Cancer Society, 2015). Traditional chemotherapeutic agents are nonspecific, cytotoxic, powerful and cause undesirable side-effects, it doesn't kill rapid-growing cancer cells only, but also it kills or slows the growth of healthy cells that normally grow and divide quickly like blood-forming cells in the bone marrow, cells in the mouth, digestive tract, reproductive system and hair follicles. Damaging of healthy cells may cause side effects, such as fatigue, hair loss, easy bruising and bleeding, infection, anemia, nausea, vomiting, appetite changes, diarrhea, mouth sores, nerve and muscle problems (Colagiuri et al., 2013). Due to these side effects for nonspecific traditional chemotherapy, researchers designed new drugs that attack cancer cells more specifically and have less side effects. These drugs called targeted therapies, because it targets certain components in cancer cells that make cancer cells distinguished from normal cells (Padma, 2015).

Recently the strategy of targeting tyrosine kinase enzymes in human cancers is compelling. These enzymes contribute to tumour initiation and progression (Arora & Scholar, 2005). It modulates growth, signaling, differentiation, adhesion, migration and survival of cancer cells. Among these kinases, epidermal growth factor receptors (EGFR), also known as ERBB1 or HER1, which belongs to the HER family of cell-surface receptors that include three other members HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (Takagi, Hayashi, & Ohta, 2009). Ligand binding to EGFR triggers homodimerization or heterodimerization of this receptor leading to phosphorylation of the receptor and activation of downstream effectors such as RAS–RAF–MEK–ERK–MAPK and PI3K–AKT–mTOR resulting in cell proliferation (Chakraborty et al., 2014). EGF, TGF-α, amphiregulin, epigen, betacellulin, heparin-binding EGF and epiregulin are examples of EGFR ligand. EGFR expressed in several types of cancer, including breast, lung, colorectal,

esophageal, head and neck cancers (Manuscript & Syndromes, 2013; Seshacharyulu et al., 2012). EGFR targeted tyrosine kinase inhibitors (TKI) are small molecules able to inhibit EGFR signaling by competing and binding with ATP binding pockets, reversible or irreversible, on the intracellular catalytic kinase domain of receptor. Gefitinib, Erlotinib, Lapatinib, Canertinib are TKI drugs that used to treat different types of cancer (Seshacharyulu et al., 2012).

Whereas EGFR-targeted therapies can significantly improve disease control, multiple mechanisms of acquired resistance to EGFR-targeted inhibitors have been detected in patients. EGFR-T790M mutations, PIK3CA mutations, MET and MAPK1 amplification are responsible for mechanisms of resistance to EGFR TKIs (Ware et al., 2013). In addition to the different types of resistance, targeted cancer therapies can cause fundamental side effects. The most common side effects are skin problems like rash, dry skin, nail changes, hair growth and skin color changes, problems with blood clotting and wound healing, high blood pressure and other side effects (Widakowich, de Castro, de Azambuja, Dinh, & Awada, 2007). Due to these side effects and resistance to the current EGFR TKIs more effective TKIs are required for cancer treatment.

1.1 General Objective

The current study aims to evaluate the anti-cancer properties of a novel Pyrazolotriazolopyrimidine derivatives group against EGFR expressing cancer cells.

1.2 Specific Objectives

1- To study the anti-cancer effects of Pyrazolotriazolopyrimidine compounds against breast, colorectal and cervical cancer cells in vitro which will include:

- Cytotoxic effects
- Anti-proliferative effects
- Anti-migration effects

2- Determination of the mechanism of action by which these compounds work.

1.3 Significance:

- This is the first study in Gaza strip to investigate novel targeted anti-cancer compounds.
- The synthesized compounds are novel derivatives.
- The utility of this scaffold as anticancer agent is novel.
- The first study to test this Pyrazolotriazolopyrimidine compounds against cancer cell lines.
- To our knowledge this is the first study to describe a full mechanism of action for novel compounds in Gaza.

1.4 Limitation:

1- The arrival of the chemical compounds delayed due to the closure of the crossing.

2- The difficulty of availability of cell lines and their importation from outside Palestine.

Chapter 2

Literature Review

Chapter 2

Literature Review

2.1 Introduction

Cancer is one of the most serious diseases and represents a major threat to human health all over the world. It is defined as a set of diseases characterized by unregulated cell division with the ability of these cells to penetrate and migrate to different organs (World Health Organization., 2017). In 2012 there were 14.1 million new cancer cases diagnosed worldwide (American Cancer Society, 2012). In 2015, cancer accounted for 8.8 million of deaths worldwide (World Health Organization., 2017). According to the latest report of cancer registry unit in Gaza strip which were monitored and recorded in the period between 2009 and 2014, 7069 cases have been report (Palestinian Ministry of Health 2015).

Breast cancer is the most common diagnosed cancer in women worldwide. While breast cancer is the first killing cancer between women in developing countries, it is the second leading cause of death by cancer among women in developed countries (American Cancer Society, 2012). In Gaza Strip breast cancer is the most popular type of cancer and accounts for 1283 cases which represent 18% of all cancers with a morbidity rate of 78 per 100.000. Among female's cancers in Gaza, breast cancer occupies the first kind with 31.4% of all malignant diseases (Palestinian Ministry of Health 2015).

The same report showed that colon cancer represents the second common cancer in Gaza strip, it records 709 cases with 10% of all cancers and morbidity rate of 45 per 100.000. In Gazans males, colon cancer represents the most prevalent with 11.5% of all cancers (Palestinian Ministry of Health 2015). However, worldwide colorectal cancer is the third cancer in men and the second in women (American Cancer Society, 2012). Cervical cancer ranks the fourth most common cancer and the fourth killing cancer among women all over the world in 2012 (American Cancer Society, 2012). In Gaza strip cervical cancer represents 4.6% of women cancers with a morbidity rate of 21.9 per 100.000 (Palestinian Ministry of Health 2015). Since this thesis focuses on specific types of cancer, the next part of this chapter focuses on these types.

2.2 Cancer types

2.2.1 Cervical cancers

Cervix is the lower portion of the uterus that connects it the vagina. Cervical cancer occurs by uncontrolled division of abnormal cells in the cervix forming a tumour and these cells lead to congestion and destruction of normal cells when they spread to other parts of the body (World Health organization, 2006). There are two main types of cervical cancer; squamous cell carcinomas (SCCs) which occurs in the squamous cells that cover the ectocervix and adenocarcinoma which arises in the gland cells underlay the endocervical canal. Almost all cases of cervical cancer are caused by continuous infection with the human Papillomavirus (HPV) so that cervical cancer is characterized by virus-induced illness. HPVs are DNA tumour viruses that infect epithelial cells in the skin (cutaneous HPV types) or in the inner lining of tissues (mucosal HPV types) (Burd, 2003).

There are two types of HPV infections; productive infection and proliferative infection. In the productive infection, viral genome is kept away from the host genome and isn't integrated with the genome of the cells in the basal layer of the cervix, and this type of infection leads to form viral particles (González Martín, 2007). In the proliferative infection the viral DNA is integrated with the genome of the cells. Although HPV infection is considered a high risk factor for cervical cancer, but it may be not sufficient to develop this type of cancer. Several factors contribute to the development of cervical cancer in addition to the viral infection such as long-term use of oral contraceptives, cigarette-smoking, alcohol consumption and many growth factor systems contribute to the progression of cervical cancer such as epidermal growth factor receptor (EGFR) and insulin like growth factor I receptor (IGF-IR) (Martín, 2007; Ibeanu, 2017).

Epidermal growth factor receptor (EGFR) expression is upregulated in many cancer types due to overexpression of the receptor or the presence of activating mutations (Yewale, 2013). In cervical cancer EGFR mutation is rarely detected but EGFR is usually overexpressed (Bumrungthai et al., 2015). One known cause of EGFR overexpression and activation is the HPV infection (Soonthornthum et al., 2011). For example, HPV 16 E6 induces hyper activation of EGFR in the presence or absence of growth factors. This is extremely

important as it triggers long-term EGFR and other kinases such as MAPK and PI3K (Soonthornthum et al., 2011; Spangle, 2013). These proteins play a significant role in cervical cancer progression which makes it a potential target for cancer treatment (Soonthornthum et al., 2011).

2.2.2 Colorectal cancer

Colon is an important and critical part of the digestive system which lies in the lower intestinal tract and connects the small intestine to the rectum. It is the place where water, salts and nutrients are absorbed, as well as stored feces and waste. Colon is divided into four regions; ascending colon, transverse colon, descending colon and sigmoid colon. Colon nature makes it exposed to many biological, chemical and physical factors, all of these factors increase the possibility of many diseases, including cancer (Arvelo, 2015).

Colon and rectum (the last piece of the large intestines) are the places where colorectal cancer occurs. Most of colorectal cancers (CRC) are adenocarcinoma that arises in secretory cells in the lining tissue of the internal organs, which release mucus and other fluids in the body (Giglia, 2016). CRC occurs as a result of the gradual accumulation of genomic instability and genetic changes that collaborate to induce the conversion of normal epithelial cells in colon to adenocarcinoma.

Colorectal cancer is divided into three types according to the increased genetic effect and the risk of cancer; sporadic type, family link type and hereditary type (Ivanovich, 1999; Kheirelseid, 2013). Sporadic CRC represents 60% to 85% of all CRC types and it is defined as colorectal cancer that occurs in the absence of certain inherited mutations and family relationship (Jeter, 2006). The majority of this type of CRC occurs in the left side of colon and resulting from the accumulation of mutations in tumour suppressor genes and oncogenes. Mutations in these genes induce cell growth, inhibit cell death and prevent cell cycle arrest (Grady, 2007; Kheirelseid et al., 2013). Family related type represent 10% to 30% of all CRC and it is more likely to happen if there are members of the family with the first kinship with CRC, however there is no clear pattern of inheritance (Jeter, 2006). CRC that occurs due to the presence of specific inherited germline mutation called hereditary type and represent 6% of CRC (Jeter, 2006). Hereditary and familial colorectal cancers are divided into familial adenomatous polyposis

(FAP) and hereditary non-polyposis colorectal cancer (Lynch syndrome). FAP is characterized by the existence of several polyps that may converted to malignance with age up to approximately 40 if these polyps are not clearly removed (Grady, 2007; Kheirelseid et al., 2013). Polyps is defined as benign growths of tissues that arise from the inner layer of colon into the lumen (American Cancer Society, 2017). Lynch syndrome is a malignant type which does not have polyps and able to develop cancers other than gastrointestinal cancers (Grady, 2007; Kheirelseid et al., 2013).

Genetic alterations lead to the stimulation of oncogenes and inactivation of tumour suppressor genes are critical for initiation and progression of CRC. The Adenomatous polyposis coli (APC) genes encodes large protein with multiple functional domains, these domains mediate oligomerization and enable it to bind with a different intracellular proteins. This protein is a member of the Wingless/Wnt signaling pathway (Grady, 2007). In this pathway APC binds with different proteins such as ß-catenin and glycogen synthase kinase (GSK)-3ß where it enables GSK to phosphorylate ß-catenin targeting it to ubiquitin-mediated proteasomal degradation. Germline mutation in this gene consider as a fundamental cause for FAP syndrome and also APC gene is mutated in 70% of sporadic CRC (Grady, 2007; Jeter, 2006). Most CRC initiate by mutations in APC genes, so that these mutations are seen in the earliest stages of CRC formation. Mutations in this gene affect Wingless/Wnt signaling pathway by prevent APC to bind with ß- catenin which lead to the accumulation of ß-catenin and over activation of Wingless/Wnt signaling pathway. ß-catenin causes overexpression of c-MYC (Grady, 2007; Kheirelseid et al., 2013). C-MYC is a proto-oncogene that regulates many cellular processes such as trigger of cell proliferation by up-regulation of cyclins (cyclins act as a regulators of CDKs Cyclin-dependent kinase(CDKs) (Leu et al., 2016). C-MYC also down regulate one of the main cell cycle inhibitors p21, inducing cell growth and inhibition of apoptosis through down-regulation of the major anti-apoptotic protein Bcl-2 (Grady, 2007; Kheirelseid et al., 2013).

Tumour protein-53 (P53) is a tumour suppressor transcription factor that is named as “guardian of the genome” due to its role in maintaining the stability of genome via recognizing of DNA damage and trigger cell cycle arrest, DNA repair

and apoptosis (Zilfou, 2009). P53 is mutated in more than 50% of human cancers. In colorectal cancer, p53 mutations appear in the late stages and lead to convert adenoma to carcinoma (Grady, 2007; Kheirelseid et al., 2013).

Epidermal growth factor receptor (EGFR) is overexpressed in 25% to 82% of CRC where it plays an important role development and progression of colorectal cancer (Spano et al., 2005). In addition to the role of EGFR in CRC, up-regulation of some EGFR downstream effectors due to mutations in these effectors contribute in CRC development and progression (Krasinskas, 2011). Mutations in KRas, BRAF, and p-AKT are the most common EGFR downstream effectors mutation (Krasinskas, 2011).

2.2.3 Breast cancer

To understand breast cancer development it is important to know the structure of the normal breast. The female breast consists mostly of fatty and fibrous connective tissues and is divided into about 20 sections called lobes. Each lobe is further subdivided into a group of lobules which are the structures that contain milk-producing glands. Once milk has been produced, a complex system of tiny ducts carries it through the breast to the collecting chamber located below the nipple (Allred, 2001). Breast cancers mostly originate from either the ducts or the lobules in which case they are referred to as ductal carcinomas or lobular carcinomas, respectively. When confined to the site of origin, they are further classified as non-invasive (or in situ breast cancer) but when they spread beyond the basement membrane and invade the underlying connective tissue they are classified as invasive breast cancer (Weigelt, 2010). There are also less common breast cancers such as medullary, papillary and mucinous carcinoma.

While the transition from non-invasive to invasive breast cancer has been reported, breast cancer progression remains a point of debate and two prevailing models (linear and parallel) have been proposed. The linear model proposes that cells in the primary (non-invasive) tumour accumulate progressive mutations in genes regulating cell division and growth (C. a Klein, 2009; G. Klein, 1998). The most common mutations identified in the primary tumour include BRCA1, BRCA2, p53 and RB and amplification of the HER-2 receptor (Baker et al., 2010; Bosco, 2007; Ross, 1998; Slamon et al., 1987). Some of these cells eventually

acquire the ability to proliferate autonomously; they expand clonally and leave the primary site to travel the vascular systems to a distant organ where they develop into a secondary metastatic growth. This model also suggests that metastasized cells in the secondary site should also be able to leave that site to set up at a tertiary site (C. a Klein, 2009; Polyak, 2008). The parallel progression model proposes that cancer cells may leave the primary tumour site at a very early stage and may be subsequently genetically modified in the metastatic niche where they later settle (C. Klein, 2009). However, these tumour cells may also differ genetically from cells that eventually develop into a metastasis in the same patient which could reflect the requirements for these cells to adapt and be able to successfully grow in the new microenvironment (Stoecklein, 2010).

Breast cancer is divided according to the presence of estrogen receptor (ER) to ER positive breast cancer and ER negative breast cancer. More than 70% of human breast cancers (BCs) are ER-positive which means that these cells are hormone-dependent and approximately 15% are hormone-receptor-negative which includes BCs lacking estrogen receptor expression (Massarweh, 2006). Triple negative breast cancers (TNBCs) are called such because they lack receptors for estrogen, progesterone and Her2. TNBCs are highly aggressive and resistant to conventional chemotherapy (Fumagalli, 2012; Hima, 2011; Khokhar, 2012; Reis-Filho, 2008). It is important to note that more than 70% of TNBCs overexpress genes implicated in metastasis and invasion as well as genes involved in proliferation and resistance to apoptosis including AKT, PI3K, RAS and NF-κB (Sørlie et al., 2001; Vogelstein, 2004; Wilhelmsen, 2006). More importantly, mutations in p53 is reported to be one of the most common features of TNBCs and several studies indicate that these mutant p53 proteins enhance tumourigenesis and treatment resistance (Brosh, 2009; Holstege et al., 2009; Selivanova, 2007). EGFR is overexpressed in all breast cancer subtypes, however it is more frequently overexpressed (approximately 80%) in triple-negative breast cancer (TNBC) (Lehmann et al., 2011; Simon, 2016).

2.3 Cancer treatments

Cancer is multifactorial in nature and it is unlikely to be cured by a single therapeutic approach**.** There are many types of cancer treatments, the specific type of treatment that the patients receive depends on the type and stage of the cancer they have. The main types of cancer treatments include surgery, chemotherapy, radiation therapy, immunotherapy and hormone therapy. The next section of this chapter will provide a general overview of the key cancer treatment types.

Surgery is a procedure in which a surgeon removes tumour from patient's body. It can be used to removes cancer that is found in one area, or removes part of a cancer tumour, removing part of a tumour can help other treatments work better (National Cancer Institute, 2015). Surgery is very effective in the treatment of cancer, especially at the early stages of the diseases and sometimes common problems do occur, like pain, infection. Other risks of surgery include bleeding, harm to adjacent tissues, drug reactions and dysfunction in some body functions (American Cancer Society, 2016d).

Radiation is an energy that's carried by waves or a stream of particles, which cause DNA damage and stop cell growth and division. This leads to cell death over time (American Cancer Society, 2014). Radiation can be used alone to cure some cancers such as skin, prostate, Lymphomas, cervix, head, neck and non-small lung cancers. It is also used with other cancer treatments such as surgery, chemotherapy or immunotherapy to treat breast cancer, rectal and anal carcinomas, advanced head and neck carcinomas, advanced lung carcinomas, advanced lymphomas, advanced cervix carcinomas, endometrial carcinomas, CNS tumours, soft tissue sarcomas and pediatric tumours (Delaney, 2005). Although radiation kills or slows the growth of cancer cells, it has several side effects, such as killing of nearby healthy cells and causes fatigue, skin irritation, fever or chills, sore mouth, nausea and diarrhea (American Cancer Society, 2016d).

Hormone therapy slows or stops the growth of cancers that depend on hormones to grow like breast and prostate cancers. This type of treatment prevents hormones production or blocking receptor binding with hormone. Tamoxifen is an example on hormone therapy that binds to estrogen receptor preventing estrogen binding to its receptor. It is used to cure ER-positive breast cancer (J. Abraham,

2016; Erickson, 2013). Immunohistochemical studies showed that breast cancers that express the estrogen receptor, progesterone receptor or both respond well to hormone therapy. For example, five years of adjuvant tamoxifen safely reduces 15-year risks of breast cancer recurrence and death (Davies et al., 2011). It causes unwanted side effects such as hot flashes, night sweat, vaginal discharge and increase the risk for endometrial cancer (J. Abraham, 2016).

Immunotherapy depends on the use of certain parts of the immune system to fight cancer. This can be done by stimulating immune system to work harder to attack cancer cells or by giving immune system components, such as man-made immune system proteins to treat cancer. There are many types of cancer immunotherapy that are used to cure cancer such as monoclonal antibodies, checkpoints inhibitors, vaccines and non-specific immunotherapies. Monoclonal antibodies are man-made versions of immune system proteins. It is constructed to attack a certain part of a cancer cells. Cetuximab is a monoclonal antibody attacks EGFR protein which is common in normal epithelial cells and many types of cancer cells (American Cancer Society, 2015). Checkpoints are specific locations on T cells surface that called receptors. These receptors need to be activated or inactivated to begin an immune response. Cancer cells use these checkpoints to avoid attack them by the immune system through binding to activated T cells receptors and turn them off. Checkpoint inhibitors are drugs that prevent T cells from turning off by cancer cells, which allow infiltration of T cells into the tumour stopping its growth (West, 2015). Pembrolizumab (Keytruda) is another example on immune checkpoint inhibitors that target PD-1a checkpoint protein on T cells that mainly used to treat advanced melanoma and non-small cell lung cancer (American Cancer Society, 2015; McDermott, 2015). Cancer vaccines are (parts of tumour cells- proteins or antigen) used to prevent and treat cancer. Sipuleucel-T (Provenge®) is an example on vaccines that treat advanced prostate cancer (Enock & Ndefo, 2011). Non-specific cancer immunotherapies target cancer cells randomly, they activate the immune system in general. Cancer immunotherapeutic cytokines such as interleukins, interferons that are usually synthesized by certain types of immune system cells are necessary for control of the growth and activity of other immune system cells and blood cells (American Cancer Society, 2015).

Chemotherapy, anti-neoplastic therapy and cytotoxic therapy are three medical terms used to describe chemical agents used in cancer therapy but the most commonly used is chemotherapy. Unlike surgery and radiation, chemotherapy is used as a systemic approach to treat cancer and is especially important for patients with advanced stages of cancer. Currently, more than 100 chemotherapeutic agents are used either as single treatments or in combination with other treatments and divided based on several factors such as the nature of its work, chemical structure and its relationship with other medicines (American Cancer Society, 2016b).

The most common types of chemotherapeutics are: (1) Alkylating agents such as nitrogen mustard (mechlorethamine) that binds to DNA and causes DNA damage. Platinum drugs such as cisplatin are often classified in this group because they kill cells by the same way, Cisplatin is used to treat many cancer types such as sarcomas, cancers of soft tissue, bones, muscles, and blood vessels (Cheung-Ong, 2013; Dasari, 2014a); (2) Anti-metabolite drugs such as 6-mercaptopurine (6-MP) which is a chemotherapy that damage the cells in S phase by interfering with genetic material. It is used to treat leukemia, breast, ovary, and the intestinal tract cancers; (3) Anti-tumour antibiotic is a chemotherapy that causes alteration in the DNA inside the cells. Anthracyclines are types of anti-tumour antibiotics, they interfere with DNA replication enzymes and act during all cell phases like doxorubicin. Actinomycin-D and bleomycin are examples of not anthracyclines anti-tumour antibiotics (American Cancer Society, 2015; Minotti, 2004); (4) Topoisomerase inhibitors which inhibit a class of enzymes responsible for separating the strands of DNA during DNA replication. The topoisomerase inhibitors work by maintaining the DNA-enzyme intermediate as a complex, preventing re-ligation of the break between DNA strands and thus inhibiting the replication process. Several studies carried out on the early topoisomerase inhibitors, etoposide and teniposide, showed that these compounds are unable to bind purified DNA. Further investigations revealed that these inhibitors bind to the topoisomerase-DNA complexes causing double strand breaks (Chen et al., 1984; Dow, 1983); (5) Mitotic inhibitors are natural products such as plant alkaloids. It can stopping mitosis in the M phase and it can work in all cell cycle phases by preventing the enzymes from producing the proteins necessary for cell

reproduction. The common limitations of using these drugs lie in their potential ability to cause peripheral nerve damage and to induce chemoresistance in various cancer types. However, members of this chemotherapeutic class such as paclitaxel and ixabepilone are still used to treat patients with breast cancer, lung cancer and leukaemia (American Cancer Society, 2015; Priyadarshini 2012).

Most of the current drugs have limited efficiency against cancers, partly due to, tumour cells acquiring resistance to chemotherapy (Sawicka, 2004). In many cases, cancer cells modify the expression of cell surface receptors to reduce the drug uptake and to increase the drug efflux. In the case of enzyme-inhibiting chemotherapies, drug resistance can arise from genetic mutation or by gene amplification of the targeted enzymes (Gottesman, 2002). Tumour cell resistance to DNA damaging agents are usually associated with modifications in the DNA damage response to increase the DNA repair capacity (D. Wang, 2005). Specific combination treatments can be used to improve the effect of a certain chemotherapy and to overcome drug resistance (Cheung-Ong et al., 2013). However traditional chemotherapeutic agents are nonspecific, cytotoxic, powerful and cause undesirable side-effects, it doesn't only kill rapid-growing cancer cells, but also kills or slows the growth of healthy cells that normally grow and divide quickly like blood-generating cells in the bone marrow, cells in the mouth, digestive tract, reproductive system and hair follicles (Minotti et al., 2004). For example, use of doxorubicin and other anthracyclines have been linked to cardio-toxicity, including cardiomyopathy and congestive heart failure. Damaging of healthy cells may cause side effects, such as fatigue, hair loss, easy bruising and bleeding, infection, anemia, nausea, vomiting, appetite changes, diarrhea, mouth sores, nerve and muscle problems, skin and nail changes such as dry skin and color change, urine and bladder changes and kidney problems, weight changes and fertility problems (American Cancer Society, 2016c; Minotti et al., 2004).

Due to these side effects for nonspecific traditional chemotherapy, researchers designed new drugs that attack cancer cells more specifically than traditional chemotherapy and have less side effects. These drugs called targeted therapies.

Targeted therapy targets certain characteristics in cancer cells that make cancer cells distinguished from normal cells (Vijaya, 2015). Cancer cells make some changes on their own genomes which enabling them to continuously divide forming tumours, invade neighboring tissues and migrate to different parts of the human body. These genetic changes that occur in cancer cells appear as overexpression or down regulation of certain molecules or signals that responsible for the maintenance of the carcinogenesis process (American Cancer Society, 2013b). Targeted drug designed to target proteins play a major role in the maintenance of cancer cells from death, also these drugs block the tumour ability to form blood vessels, thus cutting off the blood supply from tumour cells and to inhibit and stopping of signals that have a role in the development and spreading of cancer (American Cancer Society, 2013b; Vijaya, 2015).

Targeted therapy are divided based on the nature of their work and place of work directed to him. It includes monoclonal antibodies and small molecule inhibitors (Vijaya, 2015). Drugs that target enzymes which have a role in cancer growth and development called enzyme inhibitors like tyrosine kinase inhibitors (TKI), growth factor inhibitors, mTOR inhibitors, signal transduction inhibitors and proteasome inhibitors (American Cancer Society, 2013b). Sunitinib is an example of TKI that considered as a multi-targeted kinase inhibitors because it can inhibit the ability of cancer cells to form blood vessels and inhibit tyrosine kinase enzyme (Tourneau, 2007). This drug is currently used to treat patients with advanced renal cell carcinoma (RCC) and gastrointestinal stromal tumours (GIST) (Le Tourneau, Raymond, 2007). Proteasome is a group of enzymes that break down proteins that are not needed by the cell, but some of these proteins have a role in controlling and regulating growth and division of the cell. Bortezomib is proteasome targeted drug prevent proteasome from degrading these proteins and is used to cure patients with multiple (Adams, 2004; Field-Smith, 2006).

2.4 Tyrosine kinases enzymes

Tyrosine kinases are groups of enzymes which are categorized into receptor tyrosine kinase (TKR) enzymes and non-receptor tyrosine kinase (NTKR) enzymes. The receptor tyrosine kinases have the ability to influence the control of cellular functions and the transmission of extracellular signals from outside the cells to the inside. Structurally these enzymes consist of three parts;

the first one is the extracellular N-terminal which dedicated to ligand binding, the hydrophobic transmembrane rejoin and an intracellular tyrosine kinase domain. Non receptor tyrosine kinase enzymes in turn activated depend on internal signal in the cells. The function of tyrosine kinase enzymes is to transfer the phosphate group from adenosine triphosphate ATP to certain proteins which leads to either activation or inhibition of these proteins. When ligand binds to extracellular domain of tyrosine kinase receptors, it triggers the dimerization process of these receptors leading to autophosphorylation of the catalytic domain of receptor the tyrosine kinase enzyme. Activated tyrosine kinase receptor work as activator for cytosolic mediator proteins through which signals are transmitted to the nucleus and caused changing in the DNA synthesis and then in the cell division and many characteristics of the cell as migration, differentiation and cell death (Arora, 2005; Paul, 2004).

Cancer cells have active forms of these receptors which occurs due to autocrine-paracrine loops or the presence of a mutation in these receptors that make them constitutively active and lead to carcinogenesis. Hyper activation of TKRs takes place due to the presence of high concentration of ligands thus resulting in overexpression of the receptors or vice versa overexpressed receptors lead to ligands overexpression, these mechanism of activation for TKRs called autocrine-Paracrine loops (Paul et al, 2004). This type of activation was detected in different types of cancer in different types of receptors like epidermal growth factor receptors (EGFR), platelet derived growth factor receptors (PDGFR) and insulin growth factor receptors (IGFR) and ligands that related of these receptors EGF and TGFα for EGFR, PDGF-A and PDGF-B for PDGFR and IGF I and IGF II for IGFR. Different mutations in TKRS are responsible for the hyper activation of the function of these receptor, it might occur in the extracellular or intracellular domain of the receptor. These mutation may be point mutation or translocation (Paul, 2004).

Since tyrosine kinase receptors have a critical role in cancer initiation and development, they become the focus of scientists' attention. Scientists and researcher have developed inhibitors to combat and reduce their activity. There are many targeted therapies which designed to inhibit tyrosine kinase receptors

(Corkery, 2009; Lockhart et al., 2006; Yamasaki et al., 2008). These drugs target either the extracellular part of these receptors or the intracellular part of them. Targeted therapy that binds to the extracellular domain and prevents ligand binding to the binding sites called monoclonal antibody (Paul , 2004). Herceptin is the first approved monoclonal antibody that designed to target HER-2 (the second type of EGFR family) receptor. Herceptin binds to HER-2 receptor and inhibits its activity leading to cell cycle suppression (Molina et al., 2001). Tyrosine kinase inhibitors (TKIs) are targeted drugs that inhibit TKRs by competing on the ATP binding site in the intracellular (the tyrosine kinase enzyme) domain of the receptor. These drugs are designed to mimic ATP and have at least two aromatic rings. Gefitinib is a common approved TKI that selectively inhibit the tyrosine kinase domain of EGFR in non-small cell lung cancer and squamous cell carcinoma (Paul, 2004; Yewale et al., 2013).

2.5 EGFR targeted drug

Epidermal growth factor receptor (EGFR) was discovered by Stanly Cohen who won the Nobel Prize in Physiology/Medicine in 1986 (Seshacharyulu et al., 2012). EGFR is also called ErbB1/ HER1 and belongs to the ErbB family. This family of cell-surface receptors consist of three other members HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) whose molecular weight range between 170 to 185 KDa **Figure 2.1** (Seshacharyulu et al., 2012; Yewale et al., 2013).

These members are characterized by having the same structure, they consist of three parts; extracellular N-terminal part that are wealthy with cysteine and it is dedicated to the correlation of ligand, hydrophobic transmembrane and intracellular C-terminal tyrosine kinase part which is extremely conserved and contain several tyrosine residues. The extracellular part of EGFR consists of I, II, III and IV domains. While the interaction of I and III facilitates ligand binding and leads to the active open conformation, II and IV interaction counteract ligand binding and lead to closed inactive conformation (Seshacharyulu et al., 2012).

Phosphorylation of tyrosine residues in the C-terminal tail followed by receptors homodimeriazation or heterodimerization. Homodimeriazation occurs as a result of dimerization between EGFR receptors, but heterodimerization results from the interaction between EGFR and other ErbB receptors. Dimerization

process is induced by ligand binding and this process followed by stimulation and then binding of adaptor and effector proteins like Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain in the phosphorylated tyrosine residues that found in the cytosolic part of the receptor which lead to activation of several cellular pathways **(Figure 2.1)**. KRAS-BRAF- mitogen-activated protein kinase pathway ERK1/2 (extracellular signal-regulated kinase) are signaling proteins that induce cell division and cell growth (Dhillon, 2007). Active phosphatidylinositol 3-kinase (PI3K)/Akt pathway activates the transcription activator (STAT) which are responsible for cell survival and regulate cell death (Beck, 2014).

There are many different ligands that bind and activate EGFR these includes epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin (AR), betacellulin (BTC) and epiregulin (EPR) (Ono, 2006; Seshacharyulu et al., 2012). Certain cancers such as breast, cervical, colorectal, head and neck, gastric, lung, esophageal, prostate, bladder, renal, pancreatic, ovarian and liver cancers usually express high level of epidermal growth factor receptors (EGFR) (Arteaga, 2002). Several therapeutic approaches have been developed to be EGFR targeted drugs. Monoclonal antibodies and small molecule tyrosine kinase (TK) inhibitors are the main therapeutic approaches that target EGFR in different malignancies. Monoclonal antibodies target extracellular domains (ligand binding domain) of EGFR, however TK inhibitors bind into the intracellular domain (TK domain) of EGFR. Gefitinib, Erlotinib, Lapatinib, Osimertinib and Afatinib are the most commonly used EGFR tyrosine kinase inhibitors (Seshacharyulu et al., 2012; Yewale et al., 2013).

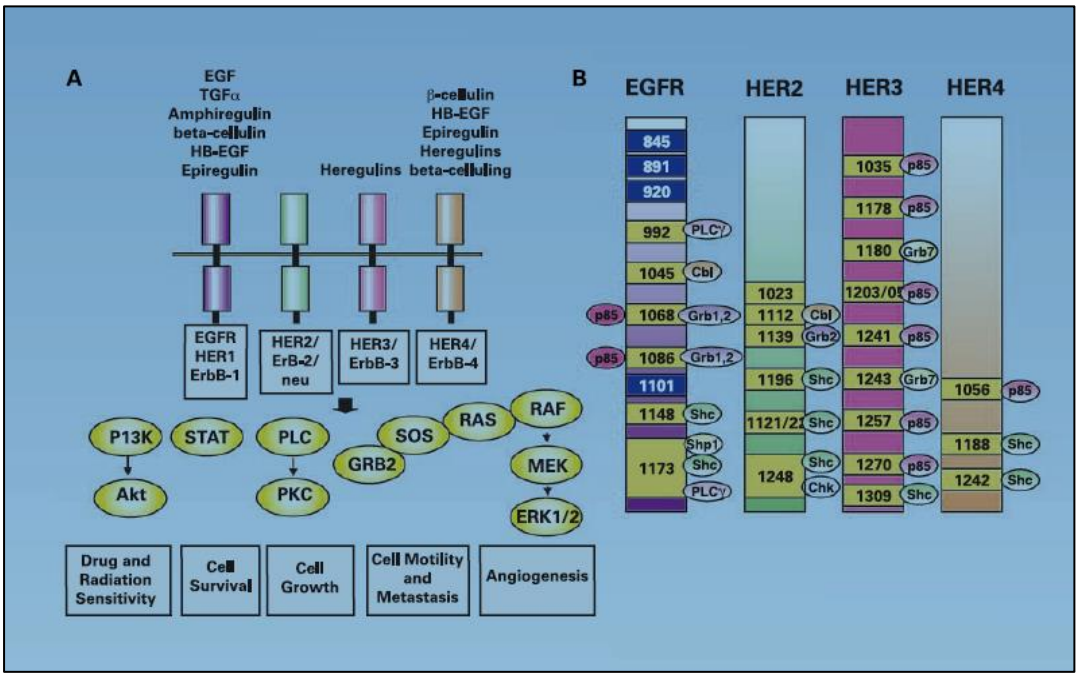

Figure (2.1): The EGFR family and its downstream signaling molecules as targets for cancer therapy. EGFR family proteins and their ligands (A). EGFR family proteins, their specific phosphorylation sites, and binding sites of signaling molecules to the tyrosine kinase domains (Ono, 2006).

2.6 EGFR tyrosine kinase inhibitors

2.6.1 Gefitinib

Gefitinib (Iressais) is an anilinoquinazoline derivative compound which was firstly characterized in 1996 and was approved by the FDA for the treatment of advanced non-small cell lung carcinoma in 2003 (Cohen, Williams, Sridhara, Chen, & Pazdur, 2003; Seshacharyulu et al., 2012). Structural similarity of this compound to adenosine triphosphate (ATP) enables it to bind to the ATP binding site of the catalytic tyrosine kinase domain of the EGFR thereby preventing its autophosphorylation and activation (Segovia-Mendoza, 2015).

Corkery et al, studied the effect of gefitinib on triple-negative breast cancer cells (TNBCs) as a potential targeted therapy (Corkery et al., 2009). Enzyme-linked immunosorbent assay (ELISA) was used to measure phosphorylated EGFR in different cancer cell lines. The results revealed that all TNBC cell lines express high levels of EGFR and very low level of HER-2. Proliferation assays are used to assess the effects of gefitinib on the tested cell lines, the results showed that TNBC cell lines which overexpressed EGFR are less sensitive to gefitinib than HER-2 overexpressing cell lines. Western blot analysis

results showed that gefitinib inhibits MAPK and Akt phosphorylation in HER2 overexpressing cell lines. Furthermore, G1 cell cycle arrest was induced in HER2 overexpressing cell lines. In contrast, almost there was no change in cell cycle analysis profile of TNBC. This study also tested the inhibitory effects of gefitinib in combination with chemotherapeutic drugs such as carboplatin and docetaxel on TNBC cell lines and the results showed that this combination synergistically improved the response in TNBC cell lines.

Another study investigated the effect of gefitinib, and PI3K/AKT inhibitors (PI-103) as combined treatment on triple-negative breast cancer cells where EGFR is frequently overexpressed (Yi, 2013). Cell proliferation was evaluated by MTT assay and results showed a synergistic anti-proliferative effect. The effect of gefitinib/PI-103 combination also examined by western blot analysis and demonstrated that the level of both phospho-AKT and phospho-ERK significantly reduced in two TNBC cell lines. The combination of gefitinib/PI-103 markedly increased caspase-3/7 activity and synergistically induced the level of PARP cleavage. In addition, levels of three anti-apoptotic proteins, XIAP (X-linked inhibitor of apoptosis protein), Bcl-2 and Mcl-1(myeloid cell leukemia 1), were profoundly reduced in cells treated with gefitinib/PI-103 combination than cells treated with either drug alone. Apoptotic cell death was further confirmed by annexin V/PI staining followed by flow cytometric analysis in MDA-MB-468 cells treated with combination of both drugs. The results showed that cells were treated with combination of gefitinib/PI-103 showed, early apoptotic cell death with a 5-fold increase compared with the vehicle-treated control. Combination treatment also increased the late apoptotic/necrotic cell death about 2-fold over the control treatment.

Clinical studies showed that gefitinib is more effective in treating lung cancer patients than other chemotherapies such as cisplatin due to its ability to delay cancer recurrence. In a phase III trial, lung cancer patients who are treated with gefitinib have median progression-free survival time of 10.4-10.8 months (Maemondo et al., 2010; Yang et al., 2017). Comparing with other chemotherapies, gefitinib is more effective as it has longer median progression-free survival time (10.8 months) than carboplatin–paclitaxel chemotherapeutic treatments (5.4 months) (Maemondo et al., 2010).

2.6.2 Lapatinib

Lapatinib is a dual tyrosine kinase inhibitors that target both epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor type 2 (HER2) which belongs to 4-anilinoquinazolines derivative compounds. It was approved by FDA in 2007 for breast and lung cancers treatment in combination with chemotherapy. It has a similar basic structure and function to gefitinib (Dai et al., 2008; Segovia-Mendoza et al., 2015).

Early study investigated the effects of lapatinib on breast cancer cell lines and explored its therapeutic potential (Konecny et al., 2006). The effects of this compound on human breast cancer cells were tested using a group of 22 breast cancer cell lines that express different levels of both EGFR and HER2. Quantitative ELISA used to evaluate the level of both receptors in these cell lines. Results showed that nine of them amplify HER2 gene and overexpress HER2 protein more than EGFR with range from 108 ng/mg HER2 protein in MDA-MB-453 to 1,161 ng/mg HER2 protein in SUM225. MDA-MB-468, MDA-MB-231, and BT20 cells overexpress EGFR with range from 58 ng/mg EGFR protein in MDA-MB-231 to 908 ng/mg EGFR protein in MDA- MB-468. However ten of the established breast cancer cell lines showed lower level of both HER-2 and EGFR. After this, researchers examined the ability of Lapatinib to inhibit the proliferation of all investigated cell lines in a concentration-dependent manner and results showed that lapatinib displayed different levels of toxicity with IC_{50}s (up to 1,000-fold) between the tested cell lines with range from 0.010 μM in UACC-812 breast cancer cells that express high level of HER-2 to 18.6 μM in MDA-MB-231 breast cancer cells which mainly express EGFR. These results might show that the sensitivity of breast cancer cell lines to lapatinib depend on HER2 expression more than on EGFR expression. For the mechanism of action, western blot analysis results showed that lapatinib reduces EGFR, HER-2, AKT, and ERK phosphorylation in a time and dose dependent manner in HER2-overexpressing breast cancer cell lines SK-BR-3 and BT474. To confirm the inhibitory effect of lapatinib on HER2-overexpressing breast cancer cells, cell cycle analysis was performed and the results showed increasing levels of sub G1 phase which was accompanied by a reduction of cells in G0/G1 phase which means that lapatinib mainly induces apoptosis in tested breast cancer cell lines. The same study

showed that lapatinib treatment completely inhibited the growth of HER-2-overexpressing human breast cancer cells in mice. Long-term in vivo treatment over 77 days showed that extended dosing of lapatinib results in significant and persistent reduction of tumour growth in mice comparing with mice that treated with vehicle control. Furthermore, this study evaluated the inhibitory effect of lapatinib in combination with trastuzumab (a monoclonal antibody targeted drug that is used to treat HER2-positive cancers mainly breast cancer) in HER2 overexpressing breast cancer cell lines. In vitro results showed that the two agents displayed a strong synergistic interaction against tested cell lines. From these finding, this tested compound can be used alone or in combination to treat HER2 overexpressing breast cancer.

Clinically, lapatinib is an effective treatment for relapsed or refractory HER2 positive inflammatory breast cancer. Phase II trial showed that lapatinib delays cancer recurrence in HER2 positive breast cancer patients with median progression-free survival time of 3.65 months (Kaufman et al., 2009).

2.6.3 Erlotinib

Erlotinib HCl (Tarceva) is another anilinoquinazoline derivative compound has a similar mode of action (García-Claver et al., 2013). It was approved by FDA for treatment advanced non-small cell lung cancer (NSCLC) patients after failure chemotherapeutic drugs (Cohen, 2005).

Yamasaki et al, examined the effect of erlotinib on breast cancer cells and role of cyclin-dependent kinase 2 (CDK2) on the sensitivity to this drug (Yamasaki et al., 2008). The cytotoxicity of erlotinib on 10 breast cancer cell lines which express different levels of EGFR was assessed using MTT. The results showed that four of the tested cell lines A-431, SK-BR-3, BT-474 and T-47D were sensitive to erlotinib with IC_{50}s ranged between 1.53µM and 9.80µM and the remaining cell lines were resistant to erlotinib with IC_{50}s more than 20 µM. The ability of this drug to inhibit EGFR phosphorylation was tested by immunoprecipitation and western blot analysis in the A-431, SK-BR-3 (erlotinib sensitive) cell lines and MDA-MB-468 (erlotinib resistant) cell line. The results revealed that erlotinib able to block EGFR phosphorylation in both erlotinib-sensitive and erlotinib-resistant cell lines. The study further tested the inhibitory

effects of erlotinib on EGFR downstream signaling pathways. After 72 hours of erlotinib treatment the level of p-ERK1/ERK2 was down regulated and the cell cycle regulator p27 was up-regulated in all erlotinib sensitive cell lines and in some erlotinib resistant cell lines. From these data, there is no correlation between the sensitivity to erlotinib and ERK/p-ERK, or p27 expression in these cell lines. However, cell cycle analysis results indicated that erlotinib exhibited G1 cell cycle arrest and apoptosis indicated by increased proportion of cells in sub-G1 in the erlotinib sensitive cell lines only. S-phase reduction has been shown in all erlotinib sensitive cell lines after 72 hours of the treatment using BrdUrd assay. Researchers also examined the effects of erlotinib on CDKs (important cell cycle regulators) to confirm cell cycle analysis results and data revealed that CDK2 activity obviously decreased in all erlotinib sensitive cell lines after treated with10μM erlotinib. Importantly, restoration of CDK2 activity partially restored proliferation and induced erlotinib resistance in erlotinib-sensitive cell lines, indicating that sensitivity to erlotinib in these breast cancer cells depends, at least in part, on CDK2 activity.

Erlotinib ability to delay cancer recurrence isn't more efficient than gefitinib when it used to treat lung cancer patients (Yang et al., 2017). Phase III trial shows that erlotinib has median progression-free survival time of 13 months which means that no significant differences between erlotinib and gefitinib (10.4 months) (Yang et al., 2017). Phase II trial of erlotinib concluded that it delays the recurrence of squamous cell cancer of head and neck in Patients with median progression-free survival time of 2.4 months (Soulieres et al., 2004). While erlotinib treatment results in long median progression-free survival time (13months) for lung cancer patients, gemcitabine plus carboplatin chemotherapeutic treatments provide (4.6 monthes) (Zhou et al., 2011).

2.7 Current EGFR TKIs side effects

Although targeted cancer therapies are less toxic but it can cause fundamental side effects. The most common side effects are skin problems like rash, dry skin, nail changes, hair growth and skin color changes, problems with blood clotting and wound healing, high blood pressure and other side effects (American Cancer Society, 2013a). In addition to these side effects many cancers developed mechanisms of resistance to EGFR-targeted therapies. Multiple mechanisms of intrinsic (primary) and acquired (secondary) resistant to EGFR-targeted inhibitors have been detected in patients. EGFR-(T790M, C797S) secondary mutations, amplification of alternative signaling pathways such as MAPK1, MET, HGF, AXL, Hh and IGF-1R, loss of PTEN and inactivating mutations of pro-apoptotic protein BCL2-like 11(BIM) are known mechanisms behind the resistance to EGFR TKIs (Morgillo, 2016; Ware et al., 2013).

Due to these side effects and resistance to the current EGFR TKIs, more effective TKIs are required for cancer treatment. Many scientist and researcher designed and synthesized pyrazolo and pyrazolo-pyrimidine derivatives compounds and evaluated their ability to be anti-cancer agents. From these studies some of researcher are directing these derivatives compounds to target and inhibit an epidermal growth factor receptor tyrosine kinase (EGFR-TK) (Sebastian et al., 2016) (Li Chen, 2017). In this regard, the current study aims to biological evaluation of novel pyrazolotriazolopyrimidine based (BA) compounds as an EGFR TKIs in different types of cancer.

Chapter 3

Material and methods

Chapter 3
Material and methods

3.1 Cell culture

The MCF7 human breast adenocarcinoma (estrogen receptor positive) cells and Caco-2 (human colorectal adenocarcinoma) kind gifts of Dr. Mazen ALzaharna, faculty of health sciences, Islamic University of Gaza and HELA cells from Dr Johnny Stiban, Birzeit University of Palestine. MCF7 and Caco-2 were maintained in DMEM medium and HELA (Cervical cancer cells) were maintained in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37°C in a 5% CO2 and 95% air-humidified incubator. Media was replaced every 2-3 days (Aliwaini et al., 2015; H. C. Wang et al., 2016).

3.2 Treatments

A panel of compounds designed to be EGFR targeted agents was synthesized by Prof. Bassam Abu Thaher (Ph.D. Organic Chemistry- Islamic university of Gaza) and his colleagues which includes (BA623, BA642 and BA645) **(Figure 3.1)**. The compound BA642 dissolved in DMF (at 100 °C) to give 10mM. The compounds BA623, and BA645 dissolved in heated DMSO (at 100 °C) to give a final concentration of 10 mM. All compounds stored at room temperature for no more than 7 days. At use and in order to get the final concentration, subsequent dilutions in the appropriate media for each cell line were prepared as shown in **Figure 3.2**. Vehicle treated cells were incubated in normal media with DMF or DMSO (the vehicle in which BA-compounds was dissolved in).

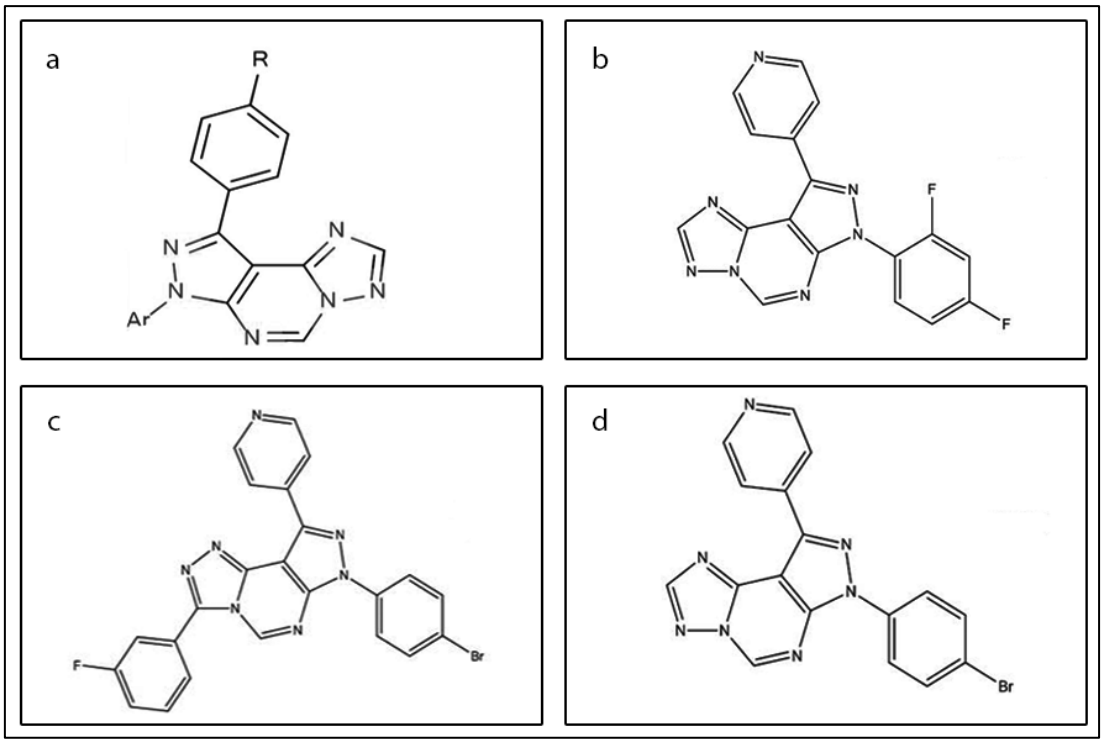

Figure (3.1): Structural formulas of the Pyrazolotriazolopyrimidine derivatives (BA) compounds. General structural formula of BA- compounds 7-Aryl-9-(pyridin-4-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (a), structural formula for 7-(2,4-difluorophenyl)-9-(pyridine-4-yl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*] pyrimidine (BA642) compound (b), structural formula for 7-(4-bromophenyl)-3-(3-fluorophenyl)-9-(pyridine-4-yl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine (BA623) compound (c) and structural formula for 7-(4-bromophenyl)-9-(pyridine-4-yl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine (BA645) compound (d).

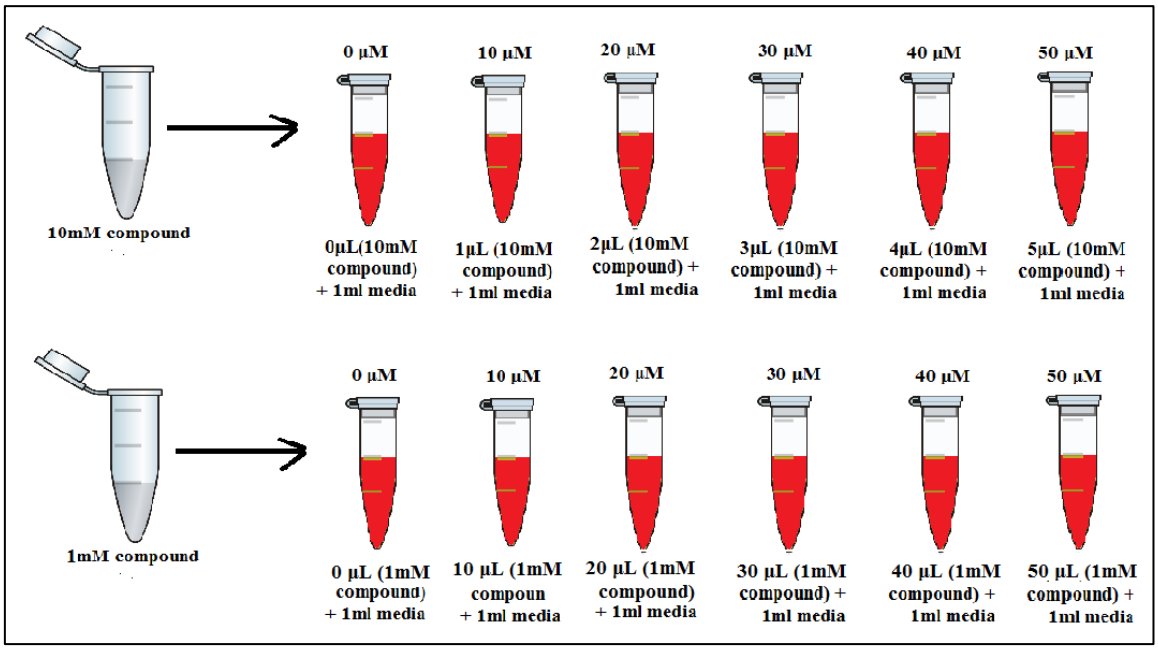

Figure (3.2): Dilutions of the Pyrazolotriazolopyrimidine derivatives (BA) compounds in the appropriate media to get the final concentration.

3.3 Cell morphology

Cells were plated at suitable numbers in order to obtain 60-70% confluency on the day of treatment. After treating the cells with the BA-compounds the morphological changes were monitored and photographed using an inverted light microscope (Olympus 1X71, USA) and camera (Zeiss AxioCam, Germany) respectively. Any morphological changes were photographed using a light microscope.

3.4 Growth curves

Two methods were utilized to determine short term cell growth, namely: cell viability as a measure of cell growth was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Kit (Roche, Germany) and counting of cells at specific time points using a haemocytometer.

3.4.1 Cytotoxicity assays (MTT)

To determine the cytotoxic effect of the indicated compound HELA, MCF7, Caco-2 cells were seeded respectively (3000-6000 HELA cells/well), (7000-9000 MCF7 cells/well), (10,000-12,000 Caco-2 cells/well) in quadruplicate in a 96-well plate and treated after 24 hours with a range of the indicated concentrations of specific compounds or vehicles for 48 or 72 hours. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay according to the manufacturer‘s instructions (Roche, USA). Briefly, 10µL of MTT solution was added to each well and incubated for 4 hours at 37°C. This was followed by the addition of 100µL solubilization buffer (10% SDS in 0.01 M HCl) and incubated overnight at 37°C. Absorbance (550 nm) was measured with ELISA reader for each well and the mean cell viability was calculated as a percentage of the control. Two separate experiments were performed to determine the concentration of BA compounds required to kill 50% of the cells (IC_{50}). The IC_{50} values were calculated from linear equation from Microsoft (Behray et al., 2016; Deshpande, 2016; Martinho et al., 2017).

3.4.2 Viability assay /trypan blue

To determine the ability of BA-compounds to induce cell death in cancer cells, HELA and MCF7 cells were seeded respectively (200,000- 300,000 HELA cells/well), (7000- 9000 MCF7 cells/well) in duplicate in a 6-well plate and treated in a second day with the indicated concentrations of specific compounds or vehicles. Cell viability was assessed by the trypan blue assay after 24, 48 and 72 hours of treatment and results were analyzed by Excel Microsoft office 2010. Total cells and viable cells were counted by trypan blue exclusion as follows. Floating and adherent cells were collected at each time point, centrifuged, and resuspended in medium. The cells were then diluted at a 1:9 ratio of 0.4% trypan blue (Sigma) and scored under a light microscope. Viable (unstained) and nonviable (blue-stained) cells were counted, and the total numbers of living and dead cells were calculated (Yamasaki et al., 2008).

The % cell viability was calculated using the following equation:

% cell viability = total viable cells (unstained) X 100 / total cells (stained + unstained)

3.4.3 Growth curve assay

To determine the effects of the BA-compounds, HELA and MCF7 cells were seeded respectively (200,000- 300,000 HELA cells/well) and (7000- 9000 MCF7 cells/well) in duplicate in a 6-well plate and treated in a second day with the indicated concentrations of specific compounds or vehicles. Cell numbers were assessed by counted on a haemocytometer after 24, 48 and 72 hours of treatment. Briefly, the cell were collected by trypsinization and after centrifugation, 1ml of phosphate buffer saline (PBS) was added to the pellet. Then 17µl of cell suspension was taken and counted on a haemocytometer at 24, 48 72 hours. The data represents pooled results of tow experiments performed in twice replicate (Konecny et al., 2006). Furthermore, growth curves were drown using Excel Microsoft office 2010 and the population doubling time (PDT), or the time required for a culture to double in number, was calculated by the following formula:

$$\mathbf{Y=mXb}$$

Y= number of cells,

X= time (hours),

m= the slope of the line,

b= the point at which the line crosses the y-axis (y-intercept).

3.5 In vitro cell migration assay

In vitro scratch motility assay: Cells were grown to confluence in 6cm tissue culture dishes. A linear wound was made by scratching through the monolayer using a sterile 1000 µl pipette tip. To remove cell debris, the growth medium was replaced and several markings were made along the edges of the scratch line which were used as reference points and the wound widths measured at the time of the scratching (0 hour) and thereafter at 24, 48 and 72 hours intervals. Pictures were taken using an inverted light microscope (Olympus 1X71, USA) and camera (Zeiss AxioCam, Germany) respectively. Migration distances were measured using Axiovert software (Zeiss, Germany). The difference in width represents the distance migrated in µm (Martinho et al., 2017).

3.6 Cell cycle analysis

Cells were collected by trypsinisation, washed twice with 1XPBS, resuspended in 2 ml of cold 1XPBS and counted on a haemocytometer to determine

the volume of propidium iodide (PI) solution that was added. Cells were fixed in 8 ml of 70% cold ethanol for at least 30 min at -20°C. Fixed cells were collected by centrifugation at 1500 rpm for 5 min at room temperature, washed twice with 1XPBS and centrifuged at 6000 rpm for 1 min at room temperature. Before flow cytometry analyses, the samples were treated with RNase A (50 μg/ml) for 15 min at 37°C and immediately stained for 30 min at room temperature with PI solution (see appendix, section 6.3), to a final concentration of 1 X 106 cells/ml. A minimum of 50 000 cells/sample were subjected to analysis using a Beckman Coulter FACSCalibur flow cytometer (Beckman Coulter, USA) (Aliwaini, Swarts, Blanckenberg, Mapolie, & Prince, 2013).

3.7 Western blot analysis

For SDS-PAGE and immunoblotting, cells were plated at 6×10^5 in 6 cm dishes and treated with 10μM BA623 for 24, 48 and 72 hours. Cells were analyzed in whole cell lysis buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% b-mercaptoethanol and 0.02% bromophenol blue) and samples boiled for 10 min. Proteins were resolved by SDS/PAGE (8–15% gels) as required and transferred to Hybond ECL membranes (Amersham Biosciences). The membranes were incubated with primary antibodies against phosphor-EGFR, phospho-AKT (no. 9271), p53 (sc-126), p21 (sc-756) and a-Tubulin (sc-8035), (Santa Cruz, CA, USA). After the primary antibody incubation, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (1:5000) (Biorad) and antibody-reactive proteins were visualized using the chemiluminescence reaction (ECL) detection system (Thermo Scientific, Hudson, NH, USA) (Aliwaini et al., 2013; Yamasaki et al., 2008)**.**

3.8 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of appropriate replicates. Statistical significance was assessed between the groups using the Student's t-test.

Chapter 4

Results

Chapter 4

Results

4.1. Introduction

Cancer is a major health problem worldwide and it is considered the second leading cause of death. It accounts annually for one in seven deaths worldwide and the number of new cases is expected to reach 22 million during the next two decades (American Cancer Society, 2012). Cancer is defined as a heterogeneous illness because it occurs in different parts of the human body. However, the most frequent cancer types in males are lung, prostate, colorectal, stomach and liver cancers while breast, colorectal, lung, cervix and stomach cancers are the most common among women (World Health Organization. Fact sheet, 2011). There are many types of cancer treatments, the specific type of treatment that the patients receive depends on the type and stage of the cancer they have. The most common types of cancer treatments include surgery, chemotherapy, radiation therapy, immunotherapy and hormone therapy (American Cancer Society, 2016a).

Traditional chemotherapeutic agents are powerful, cytotoxic agents however, they are nonspecific and cause serious side-effects, it doesn't kill rapid-growing cancer cells only, but also kills or slows the growth of healthy cells that normally grow and divide quickly. Efforts to overcome these side-effects have focused on identifying novel targeted therapeutic agents and understanding the mechanisms by which they function.

Recently EGFR targeted tyrosine kinase inhibitors (TKIs) have attracted a lot of interest as targeted therapeutic agents because they have been shown to exert a significant cytotoxic effect on cancer cells. TKIs normally exert its cytotoxic effect by competing with ATP for binding to the tyrosine kinase domain of EGFR leading to ablation of both phosphorylation of the receptor and downstream signaling (Seshacharyulu et al., 2012). While early EGFR TKIs significantly showed anti-tumour activity, there are multiple mechanisms of acquired resistance to EGFR-targeted inhibitors have been detected in patients and can cause fundamental side effects (Ware et al., 2013).

This chapter of the study investigates the possible anti-cancer activity of a group of pyrazolotriazolopyrimidine based (BA) compounds in different types of cancer cells and evaluates their inhibitory effect against EGFR in these cell lines.

4.2. Screening of a group of pyrazolotriazolopyrimidine based (BA) compounds in breast, cervical and colorectal cancer cell lines

A panel of novel compounds designed to be EGFR targeted agents was synthesized by Prof. Bassam Abu Thaher and his colleagues which includes (BA623, BA642 and BA645). Cytotoxic effects of these compounds were first screened on HELA cells using a range (0 to 50 µM) of the compounds dissolved in DMSO at room temperature for 48 hours. Cell viability was determined by MTT assay.

Figure (4.1) shows the cytotoxic effect of these compounds and data exhibited that only one of the tested compounds (BA642) has a cytotoxic effect at moderate and high concentrations. However, the concentrations that inhibit cell growth by 50% (IC_{50}) weren't achieved by this experiment.

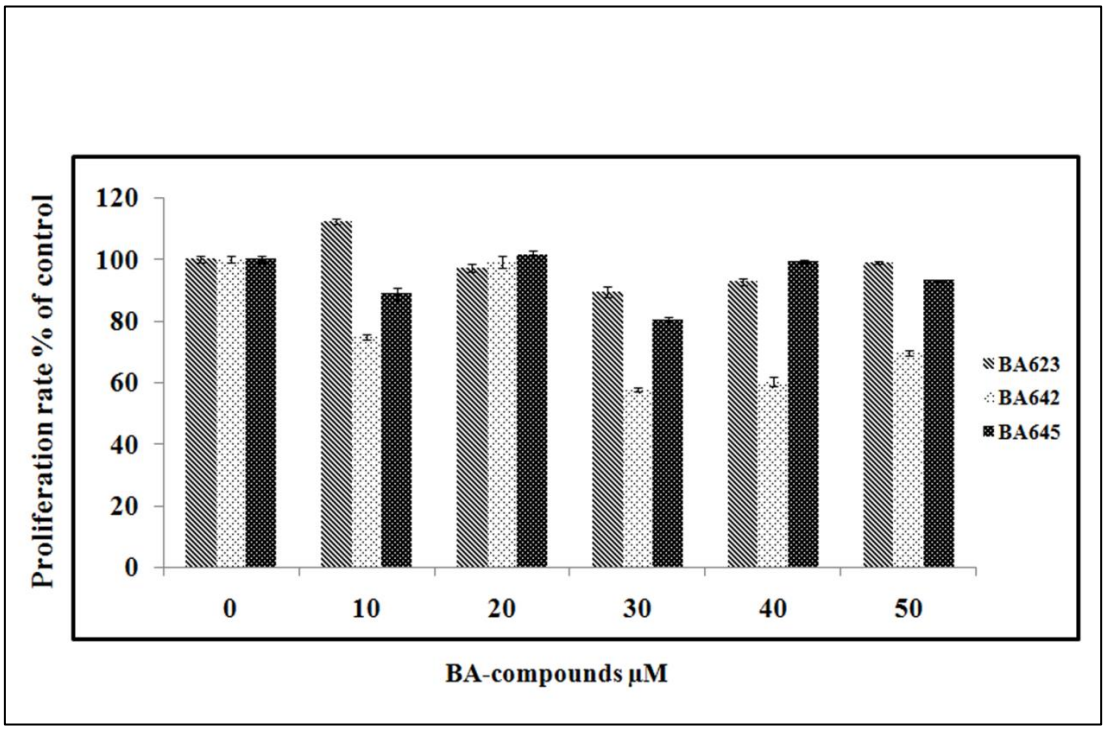

Figure (4.1): The cytotoxic effects of a panel of synthesized compounds on cervical cancer cell line. HELA cells were plated in 96-well plates and after 24 hours cells were treated with increasing concentrations of the indicated compounds (0-50 µM). Cell viability

was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment. Results represent the mean percentage ± SE.

Importantly, under microscope we observed that these compounds were not completely dissolved by this method and therefore different solubilizing techniques were used to enhance its solubility. Of these methods; DMSO (Dimethyl sulfoxide) at room temperature, DMSO with heating (100°C), DMF (Dimethylformamide) with heating (100°C). The best solubilizing method was DMSO with heating for BA623 and BA645 and heated DMF for BA642 and these methods were used for the next experiments.

4.2.1 BA642 has a cytotoxic effect on human breast and cervical cancer cells

The cytotoxic effects of BA642 on HELA cervical cancer cell line **(Figure 4.2)**, MCF7 breast cancer cell line (**Figure 4.3**) and Caco-2 colorectal cancer cell line **(Figure 4.4)** were examined using the MTT assay. After 72 hours of BA642 treatment results show strong dose dependent inhibition in both cervical and breast cancer cell lines with IC_{50} of 33.2 µM and 78.5µM respectively **(Figure 4.2a and 4.3a)**. While BA642 kills around 75% of HELA cells at 40 µM it kills only 30% of MCF7 cells at the same concentration. Furthermore, morphological sings of apoptosis such as cell shrinkage and apoptotic membrane appeared after 72h of the treatment **(Figure 4.2c and 4.3c)**. In contrast it has a little or no effect on colorectal cancer cell line **(Figure 4.4)**. However, it also exerts an effect on the morphology of HELA and MCF7 cells like cell shrinkage **(Figure 4.4c)**. These results show that BA642 displays potent cytotoxicity against HELA and MCF7 cells and that HELA cells are more sensitive to this compound than MCF7cells.

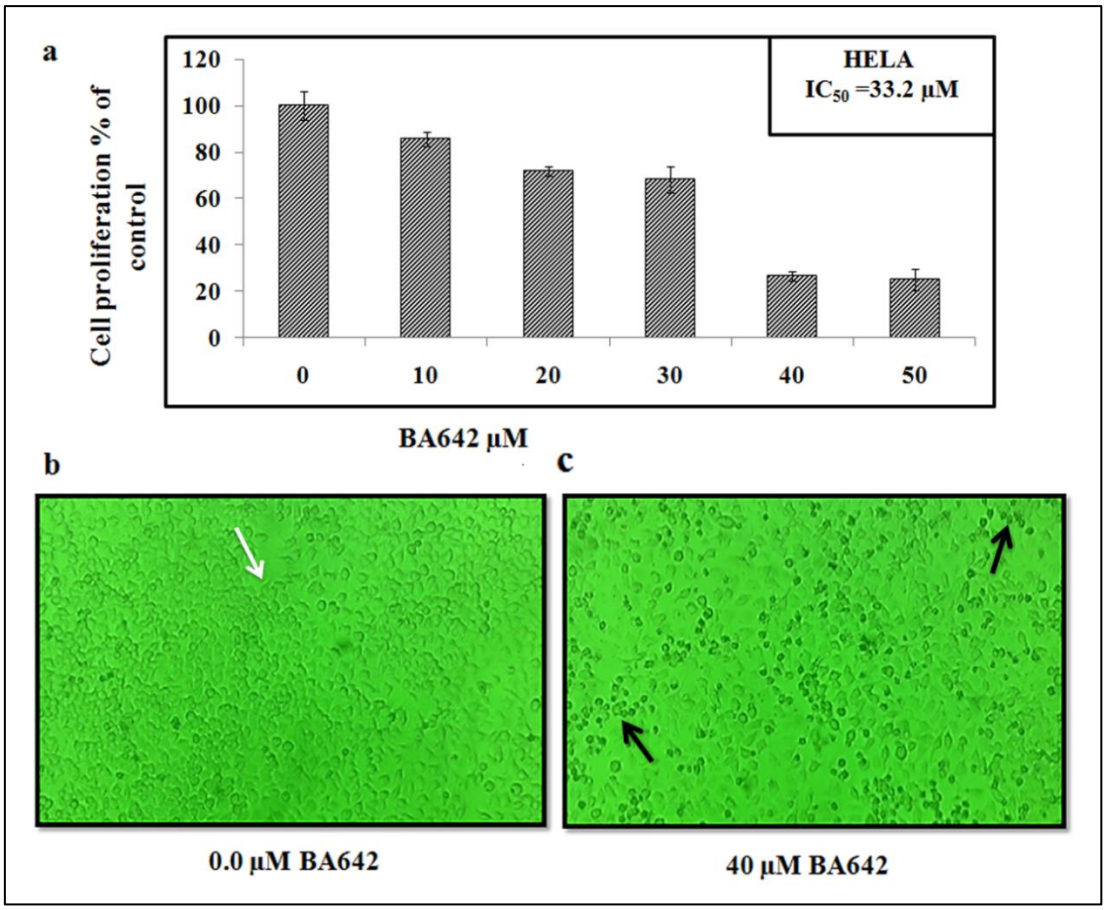

Figure (4.2): The cytotoxicity of BA642 on cervical cancer cell line. HELA cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 72 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate (a). Morphology of HELA cells (at magnification 10X) treated either with vehicle (b) or BA642 (c) for 72 hours. The white arrow refers to healthy cell and the black ones refer to stressed cells, fragmented cells and dead cells.

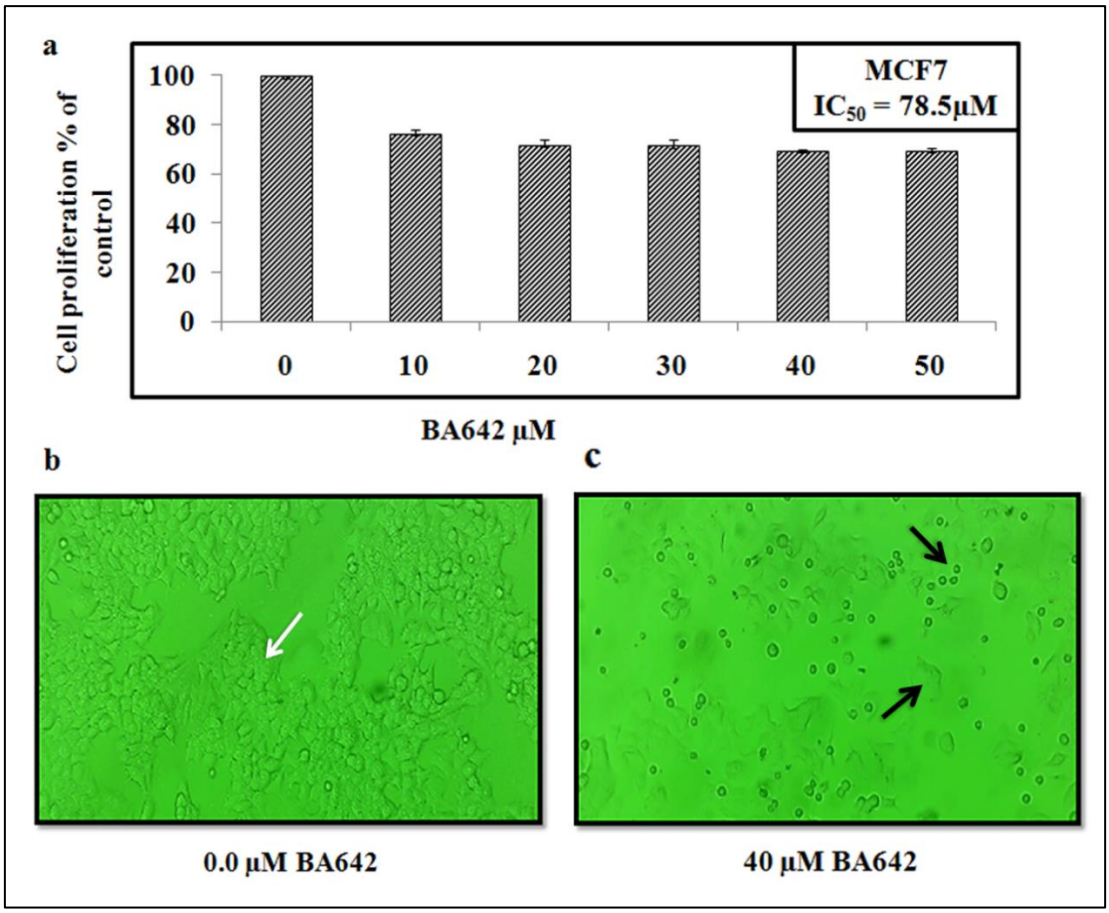

Figure (4.3): Cytotoxicity of BA642 on breast cancer cell line. MCF7 cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 72 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate (a). Morphology of MCF7 cells treated either with vehicle (b) or BA642 (c) after 72 hours at magnification 10X. The white arrow refers to healthy cells and the black ones refer to shrunk and dead cells.

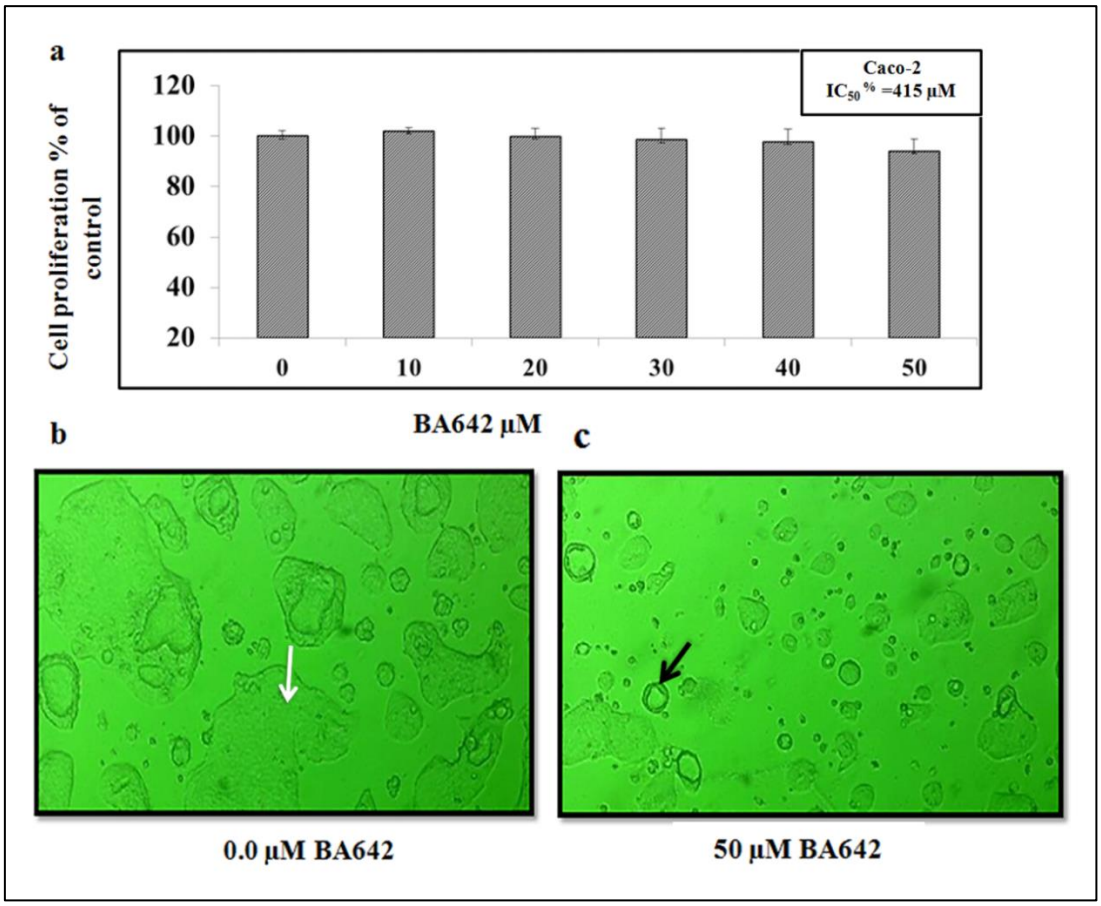

Figure (4.4): The cytotoxicity of BA642 on colorectal cancer cell line. Caco-2 cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 72 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate (a). Morphology of Caco-2 cells treated either with vehicle (b) or BA642 (c) after 72 hours at magnification 10X. The white arrows refer to healthy cells and the black refer to stressed cells.

4.2.2 BA623 has a cytotoxic effect on different cancer cells

Cytotoxic effects of BA623 on HELA cervical cancer cell line **(Figure 4.5)**, MCF7 breast cancer cell line **(Figure 4.6)** and Caco-2 colorectal cancer cell line **(Figure 4.7)** were examined using the MTT assay. Data showed that BA623 has cytotoxic effects on both HELA cells with IC_{50} (equal 53.67 µM) **(Figure 4.5a)** and Caco-2 cells with IC_{50} (equal 87.6 µM) **(Figure 4.7a)**, it has no or little effect on MCF7 cells with IC_{50} (equal 197.8 µM) **(Figure 4.6a)** after 48hr of treatment. The same experiment was repeated on HELA and MCF7 cells at 72hr. Results show

significance decrease of the IC_{50}% of the compound on the indicated cell lines. The IC_{50} of BA623 on HELA became 28.87µM **(Figure 4.5b)** and 44.56 µM on MCF7 **(Figure 4.6b)** cells. Furthermore, morphological sings of apoptosis such as cell shrinkage and apoptotic membrane appeared after 72h of the treatment **(Figure 4.5b, 4.6b and 4.7b)**. These results show that BA623 displays potent cytotoxicity in time dependent manner. It has a cytotoxic effect against all cell lines and that HELA cells are more sensitive to this compound than other cell lines.

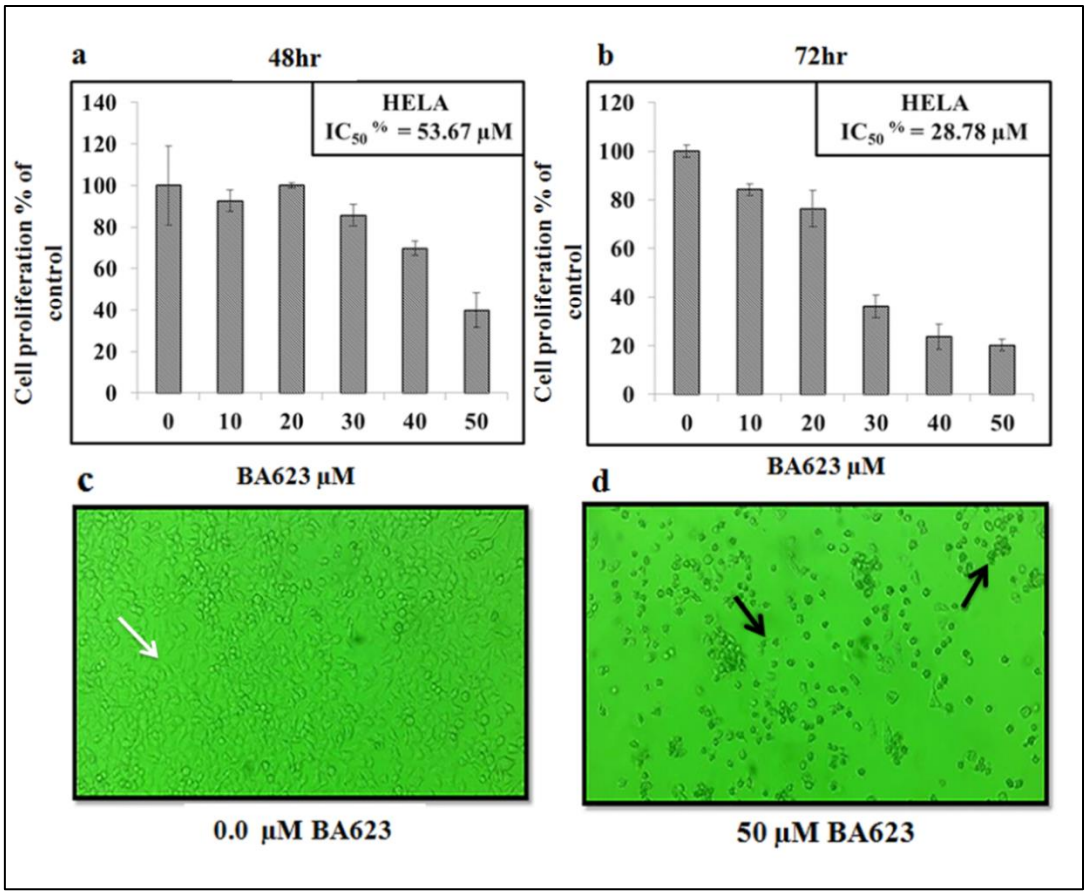

Figure (4.5): The cytotoxicity of BA623 on cervical cancer cell line. HELA cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment (a) and 72 hours (b). Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate. Morphology of HELA cells were treated either with vehicle (b) or BA623 (c) after 72 hours at magnification 10X. The white arrows refer to healthy cells and the black refer to stressed cells, fragmented cells and dead cells.

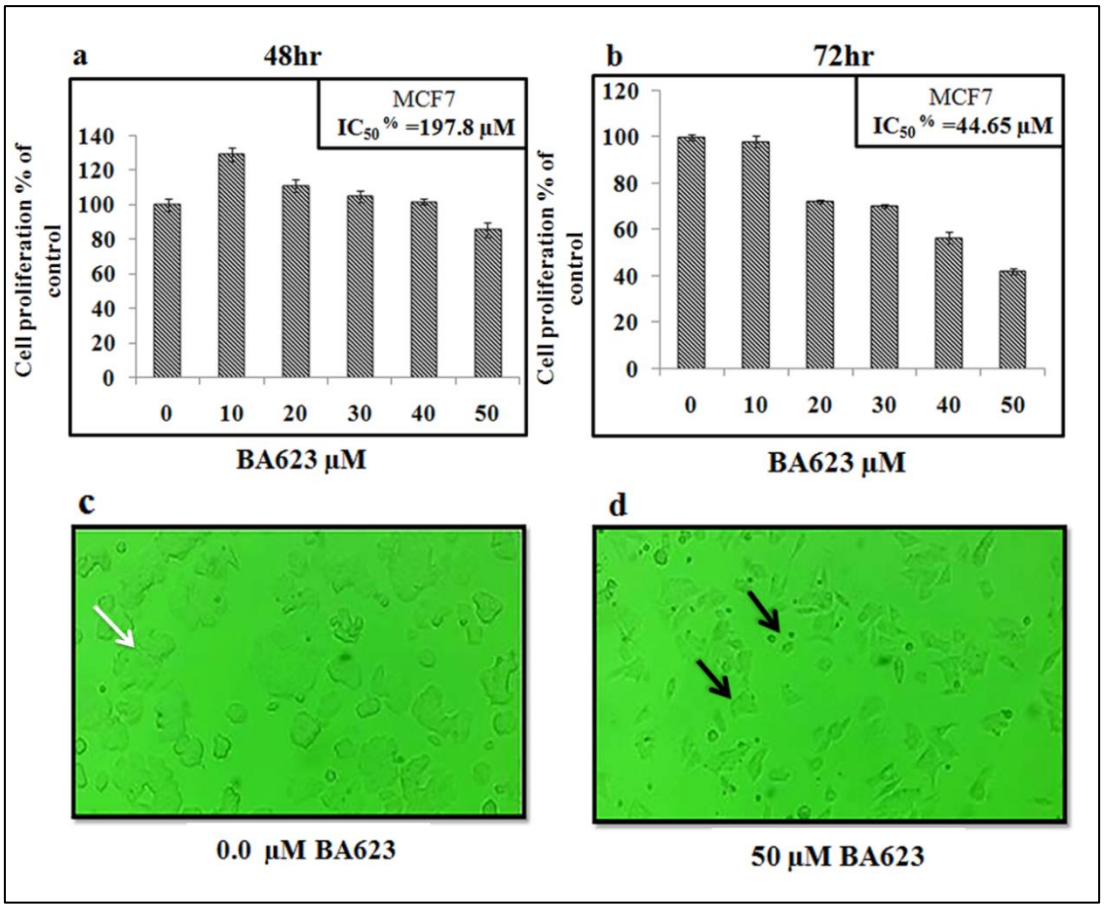

Figure (4.6): The cytotoxicity of BA623 on breast cancer cell line. MCF7 cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment (a) and 72 hours (b). Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate. Morphology of MCF7 cells were treated either with vehicle (b) or BA623 (c) after 72 hours at magnification 10X. The white arrows refer to healthy cells and the black refer to shrunk of cells.

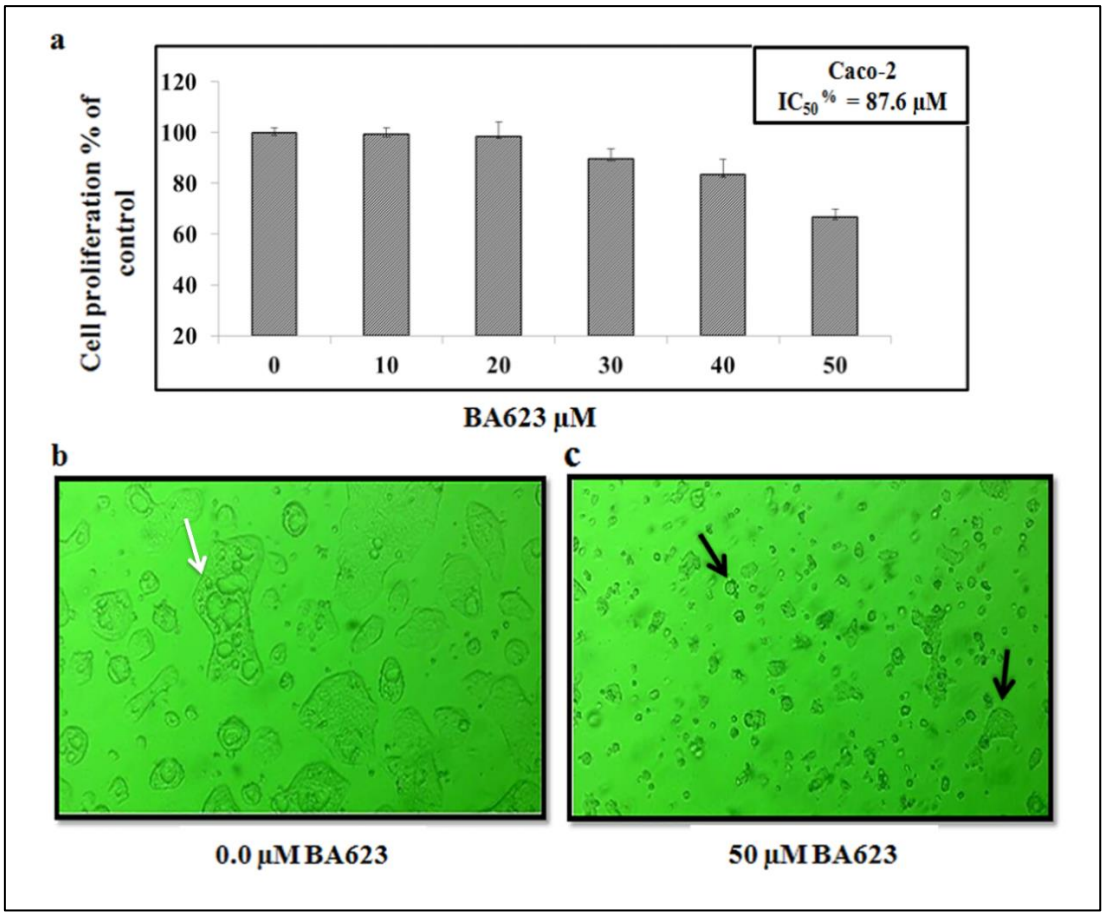

Figure (4.7): The cytotoxicity of BA623 on colorectal cancer cell line. Caco-2 cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment (a). Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate. Morphology of Caco-2 cells were treated either with vehicle (b) or BA623 (c) after 72 hours at magnification 10X. The white arrow refers to healthy cells and the black ones refer to shrunk and dead cells.

4.2.3 BA645 has a cytotoxic effect on different cancer

The cytotoxic effect of BA645 on MCF7, HELA and Caco-2 was examined as in above. Results exhibit that BA645 compound exerts a cytotoxic effects on both HELA with IC_{50} (equal 47 µM) **(Figure 4.8a)** and Caco-2 with IC_{50} (equal 79.77 µM) **(Figure 4.10a)** but it has little effect on MCF7 cells with IC_{50} (equal 145.9 µM) **(Figure 4.9a)**. The same experiment was repeated on HELA and MCF7 cells at 72hr. The results showed little differences in the IC_{50}s of the compound on the indicated cell lines. The IC_{50} of BA645 on HELA became 41µM **(Figure 4.8b)** and 107.8µM

on MCF7 cells **(Figure 4.9b)**. Furthermore, morphological sings of apoptosis such as cell shrinkage and apoptotic membrane appeared after 72h of the treatment **(Figure 4.8b and 4.10b)**. These results show that BA645 displays a potent cytotoxicity in time dependent manner significantly on HELA cells. It also has a cytotoxic effects against Caco-2 cells and that MCF7 cells are less sensitive to this compound than other cell lines.

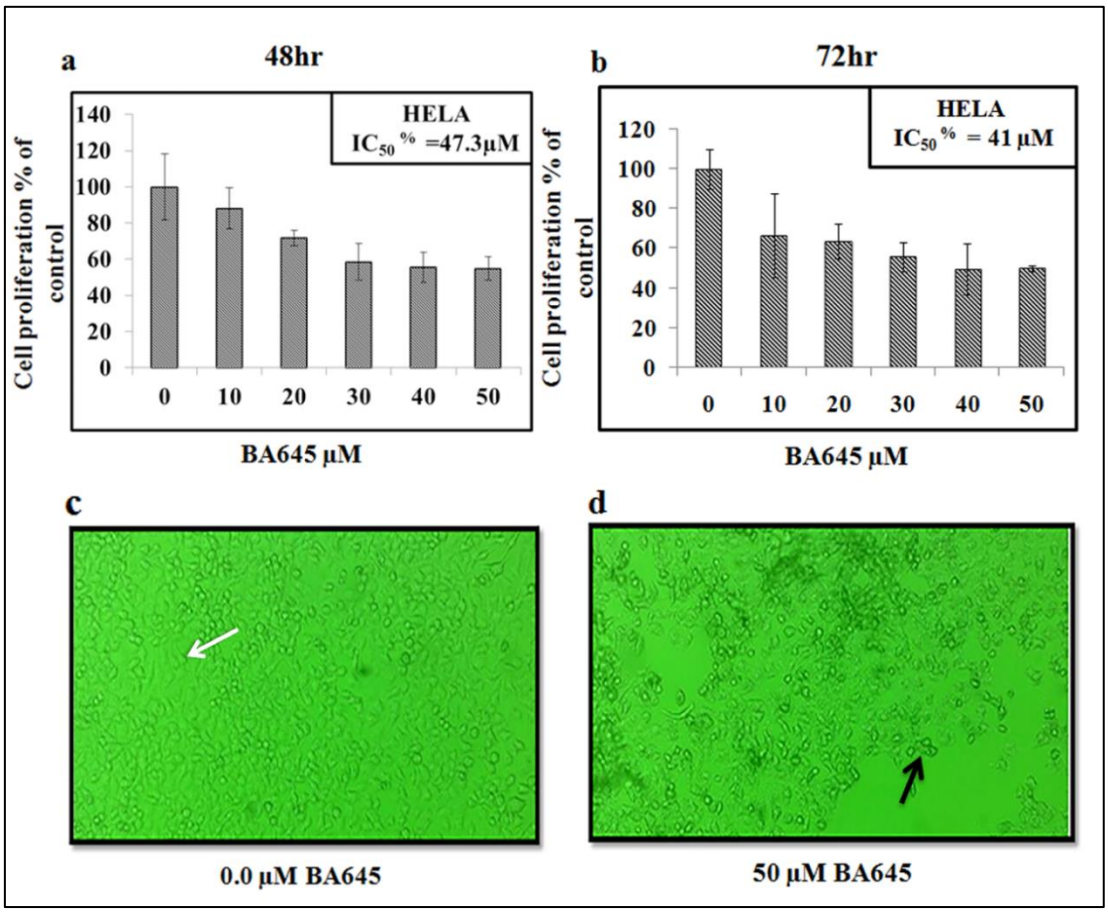

Figure (4.8): The cytotoxicity of BA645 on cervical cancer cell line. HELA cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours (a) and 72 hours (b) of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate. Morphology of HELA cells were treated either with vehicle (b) or BA645 (c) after 72 hours at magnification 10X. The white arrow refers to healthy cells and the black ones refer to stressed cells and dead cells.

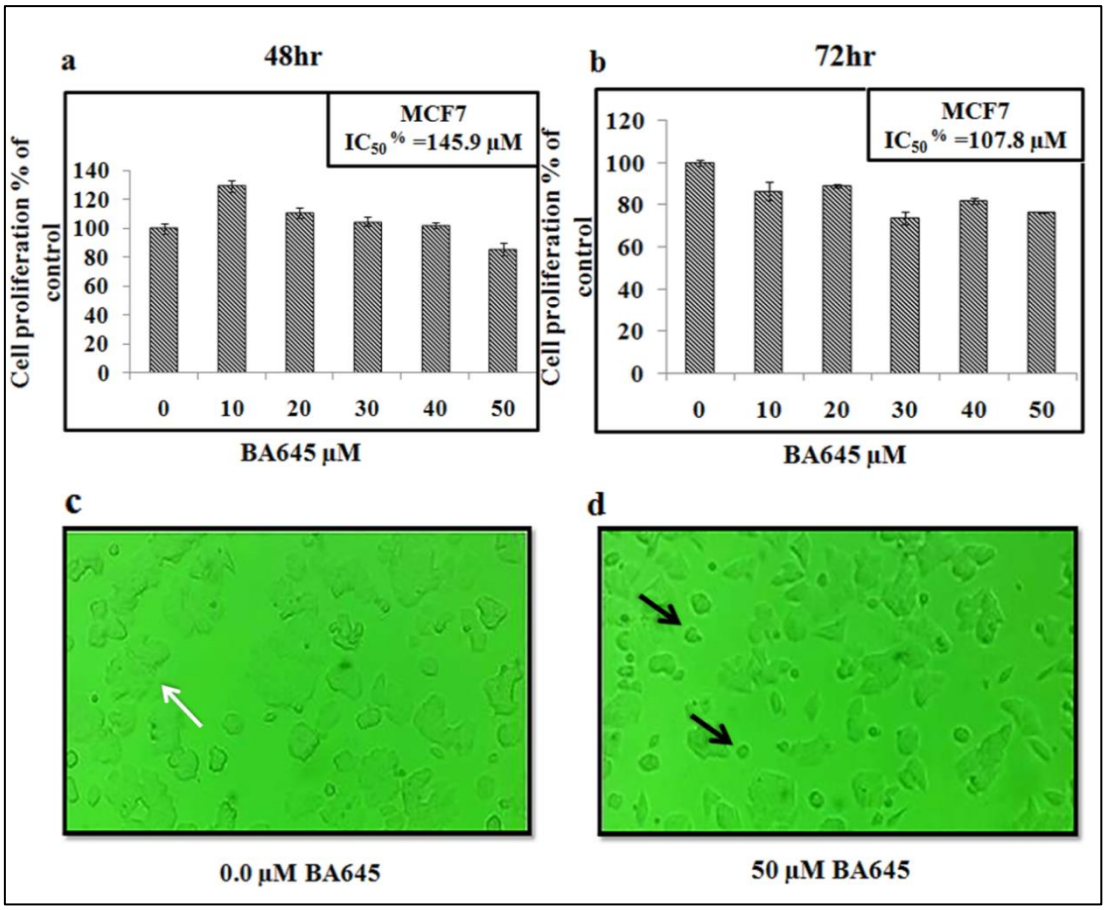

Figure (4.9): The cytotoxicity of BA645 on breast cancer cell line. MCF7 cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment (a) and 72 hours (b) Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate. Morphology of MCF7 cells were treated either with vehicle (b) or BA645 (c) after 72 hours at magnification 10X. The white arrow refers to healthy cells and the black ones refer to shrunk and dead cells.

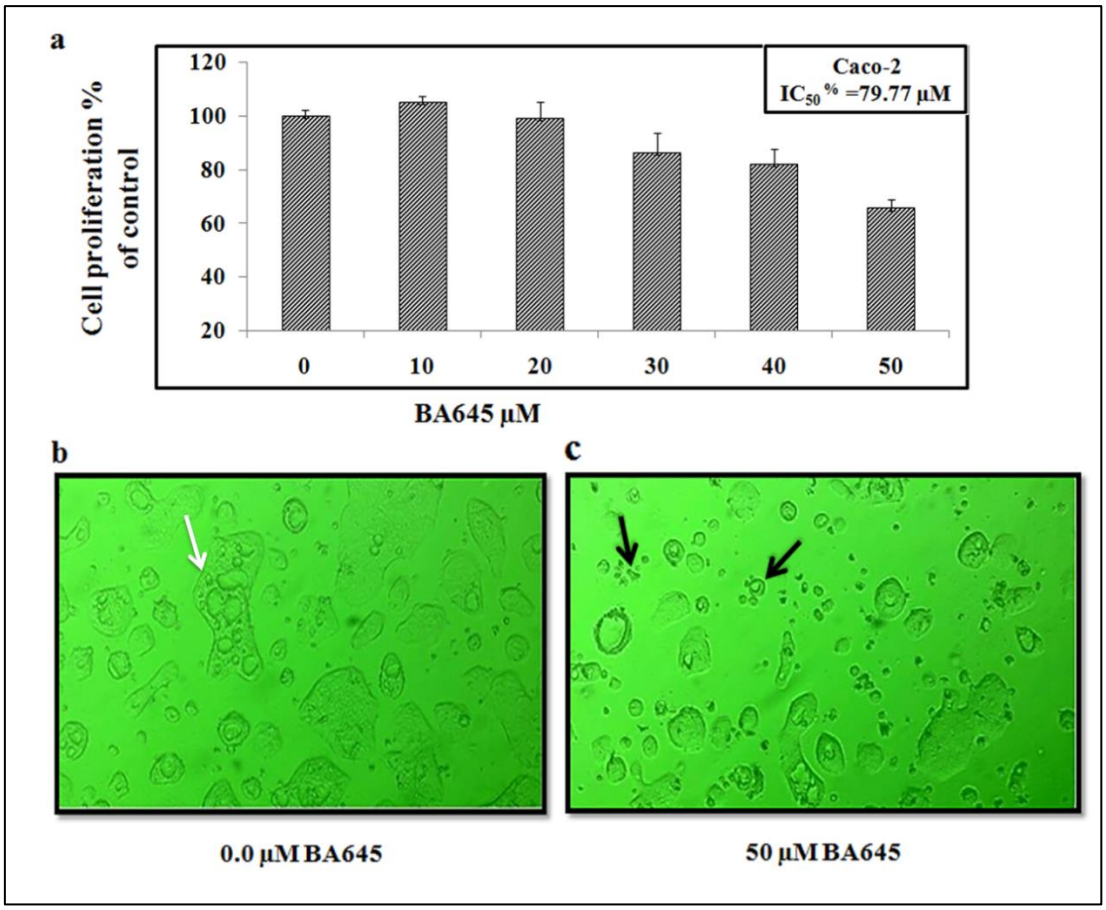

Figure (4.10): The cytotoxicity of BA645 on colorectal cancer cell line. Caco-2 cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-50 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment (a). Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate. Morphology of Caco-2 cells were treated either with vehicle (b) or BA645 (c) after 72 hours at magnification 10X. The white arrow refers to healthy cells and the black ones refer to shrinkage of cells and dead cells.

4.2.4 BA-compounds have little cytotoxic effects on normal cells

The cytotoxic effects of BA-compounds on DNB normal skin fibroblast cell line were examined as in above. Results show that BA645 compound exerts the least cytotoxic effect on DNB cells with IC_{50} of (839 µM) **(Figure 4.13).** BA623 also has a little cytotoxicity on DNB cells with IC_{50} (238.28 µM) **(Figure 4.12)** but BA642 has a strong cytotoxic effect on DNB cells with IC_{50} (56.38 µM) **(Figure 4.11)**.

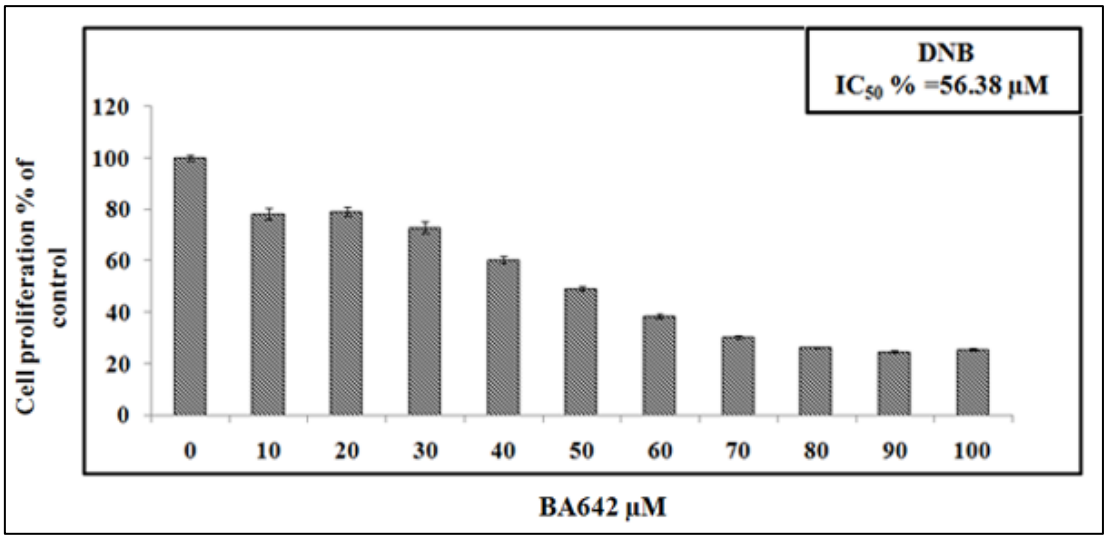

Figure (4.11): The cytotoxicity of BA642 on skin fibroblast cell line. DNB cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-100 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 72 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate.

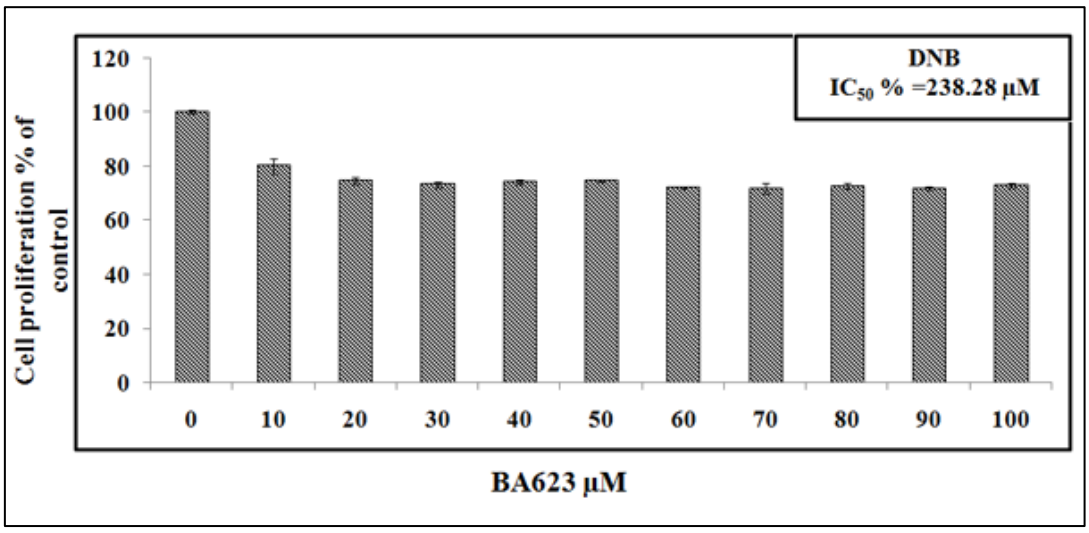

Figure (4.12): The cytotoxicity of BA623 on skin fibroblast cell line. DNB cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-100 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 72 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate**.**

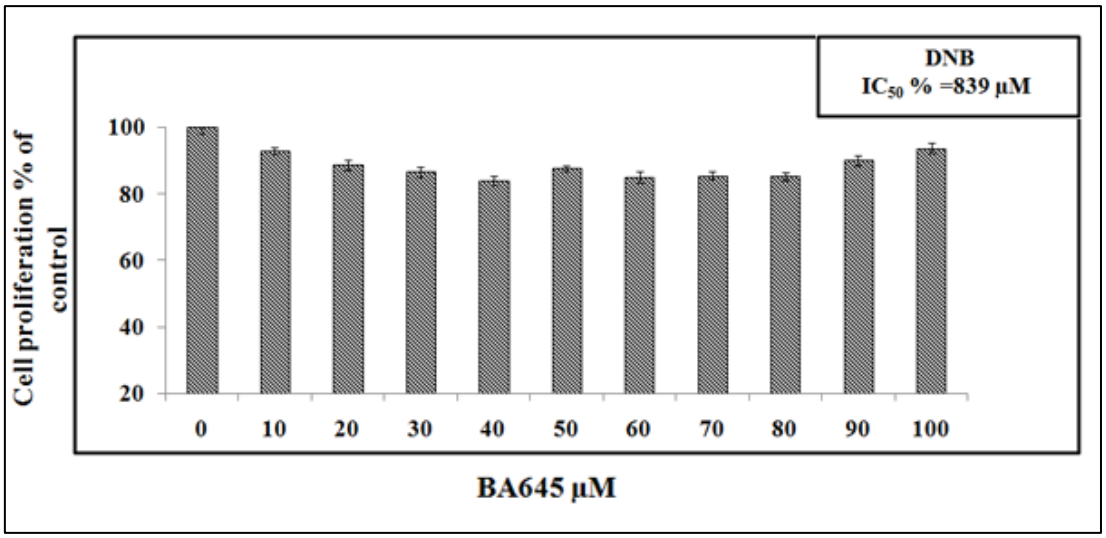

Figure (4.13): The cytotoxicity of BA645 on skin fibroblast cell line. DNB cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-100 µM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 72 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate.

All together we show her that all three novel compounds exert different levels of toxicity against the tested cell lines **(Table 4.1).** However it is obvious that HELA is the most sensitive to these compounds than other cell lines. While BA645 has no cytotoxic effect on normal cells, BA623 is the most effective compound against all three types of cancer and it is toxicity on normal cells seems very little. Therefore in the next parts of this thesis we tested the effect of BA-compounds on the MCF7 breast cancer cells and HELA the cervical cancer cells and investigated the molecular mechanism of their action.

Table (4.1): The IC_{50}s of BA-compounds on different cell lines

Compounds	**IC_{50} on HELA**	**IC_{50} on MCF7**	**IC_{50} on DNB**
BA623	28.87 µM	44.56 µM	238.28 µM
BA642	33.2 µM	78.5 µM	56.38 µM
BA645	41 µM	107.8 µM	839 µM

4.3 BA-compounds are able to induce cell death in cancer cells

Trypan blue assay was used to measure the effect of BA 642, BA 623, and BA 645 on the cell viability of MCF7 and HELA cells. Caco2 was excluded as it looks resistant to BA-compounds.

4.3.1 BA642 induces cell death in both HELA and MCF7 cells

The effect of BA642 on the cell viability of MCF7 and HELA cells was measured by trypan blue assay. Results show that the cell viability of both cancer clines was decreased in a time-dependent manner. For example, BA642 kills 22% of HELA cells at 24 hours, 40% at 48 hours and 55% at 72 hours. It also kills 5% of MCF7 cells at 24hours and about 50% at both time points 48 and 72 hours as shown in **Figure 4.14a and c.**

Taken together these findings show that BA642 kills HELA and MCF7 at similar levels (about 50 %) after 72 hours of the treatment.

Although trypan blue assay fundamentally measures cell death rate and cell proliferation rate by cell counting can be also evaluated by this assay. In support of MTT data, trypan blue results show that BA642 exerts anti-proliferation effects on HELA and MCF7 cells by killing cancer cells and decreasing its number **(Figure 4.14b and d).**

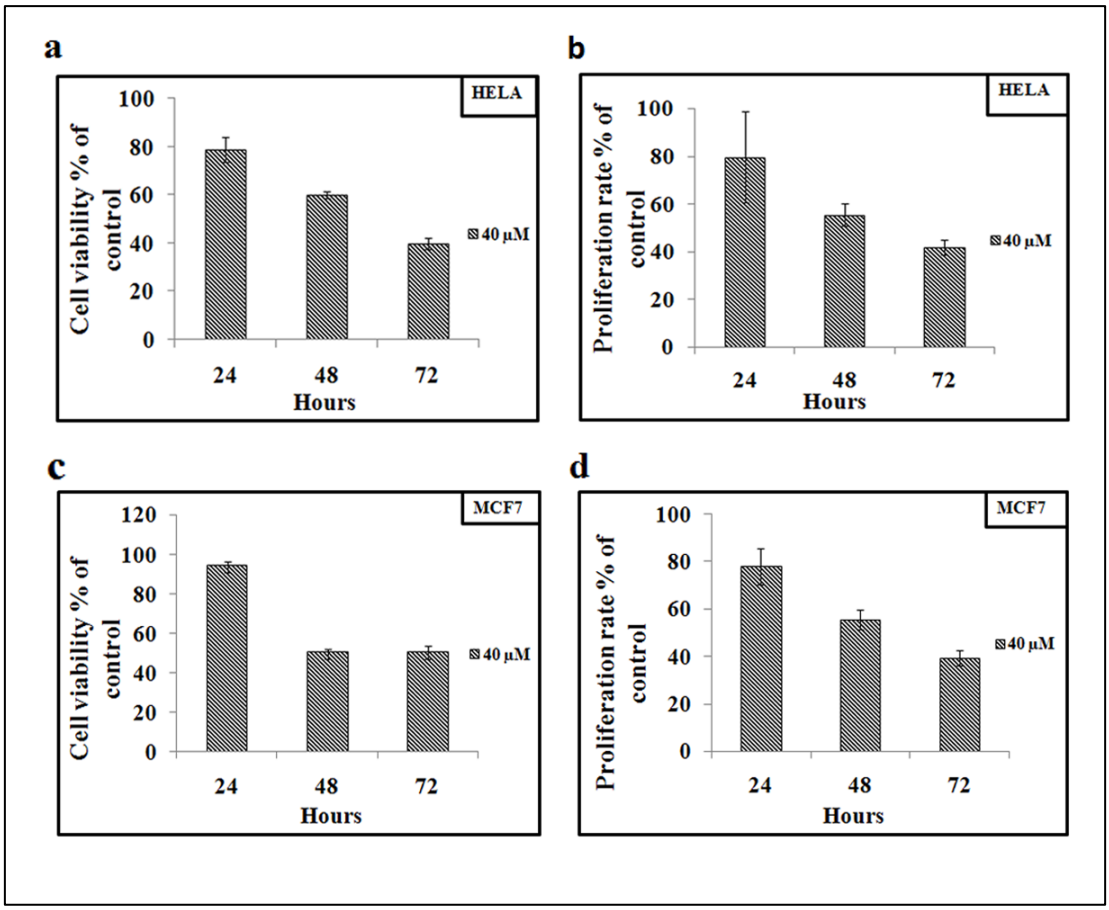

Figure (4.14): BA642 inhibits cancer cell viability and proliferation. Effect of BA642 on cell viability of cervical (a) and breast (c) cancer cells. Cancer cell lines were plated in 6-well plates and after 24 hours, cells were treated with 40 µM of BA642 or vehicle. Cell viability was assessed by the trypan blue assay after 24, 48 and 72 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate. Right graphs of the figure show the anti-proliferation effect of BA642 on HELA (b) and MCF7 (d) cancer cells.

4.3.2 BA623 induces cell death in both HELA and MCF7 cells

The effect of BA623 on the cell viability of MCF7 and HELA cells was measured by trypan blue assay. Results show that the cell viability of both cancer clines was decreased in a time-dependent manner. For example, BA623 kills 22.5% of HELA cells at 24 hours, 50% at 48 hours and 95% at 72 hours. It also kills 25% of MCF7 cells at 72hr as shown in **Figure 4.15a and c.** In conclusion, HELA cells are more sensitive to BA623 treatment than MCF7 cells.

Although trypan blue assay basically estimates cell death rate, cell proliferation rate by cell counting cane be also evaluated by this assay. In agreement

of MTT data, trypan blue results show that BA623 exerts anti-proliferation effects on HELA and MCF7 cells by killing cancer cells and decreasing its number **(Figure 4.15b and d).**

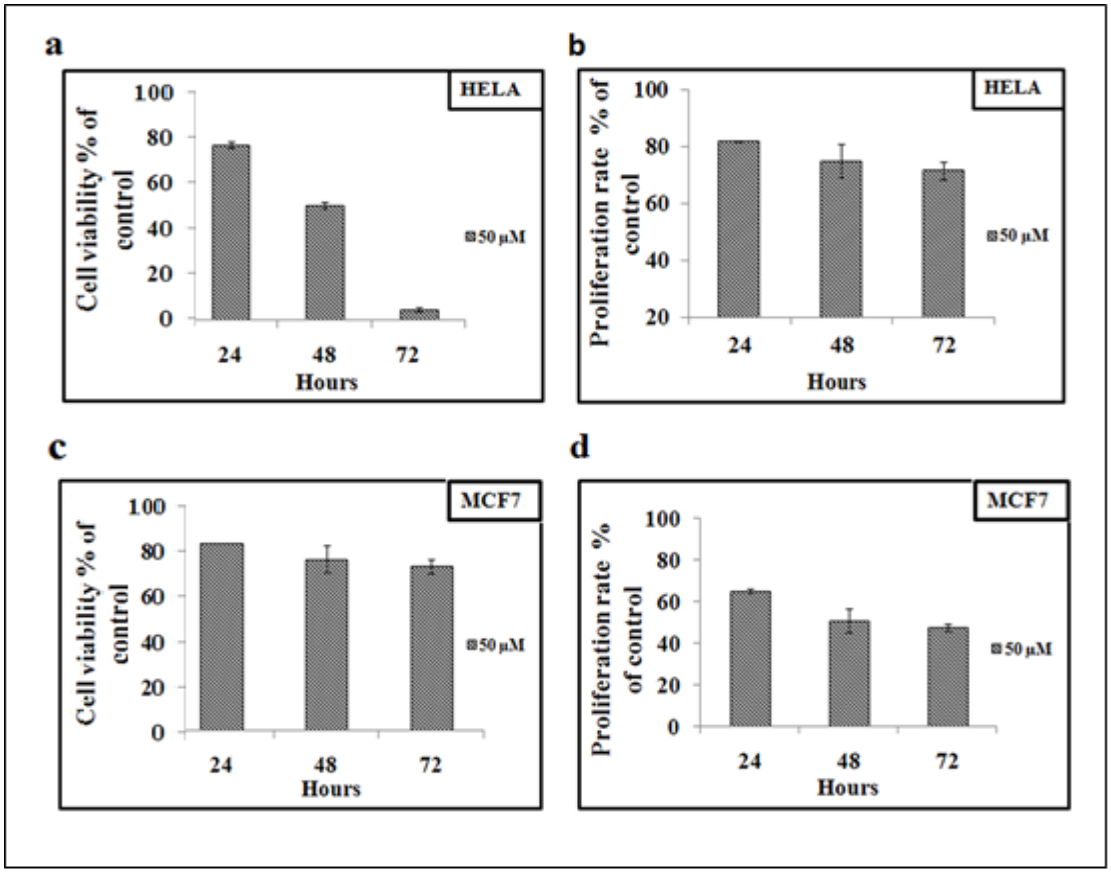

Figure (4.15): BA623 inhibits cancer cell viability and proliferation. Effect of BA623 on cell viability of cervical (a) and breast (c) cancer cells. Cancer cell lines were plated in 6-well plates and after 24 hours, cells were treated with 50 µM of BA623 or vehicle. Cell viability was assessed by the trypan blue assay after 24, 48 and 72 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate. Right graphs of the figure show the anti-proliferation effect of BA623 on HELA (b) and MCF7 (d) cancer cells.

4.3.3 BA645 induce cell death in both HELA and MCF7 cells

The effect of BA645 on the cell viability of MCF7 and HELA cells was measured by trypan blue assay. Results show that the cell viability of both cancer clines was decreased in a time-dependent manner. For example, BA645 kills 18% of HELA cells at 24 hours, 30% at 48 hours and 80% at 72 hours. It also kills 5% of MCF7 cells at 72hr as shown in **Figure 4.16a and c.**

From the above results we conclude that BA645 has a significant effect on HELA cells viability and proliferation rate, but again it has a little effect on MCF7 cells.

Although trypan blue assay mainly measures cell death rate, cell proliferation rate by cell counting cane be also evaluated by this assay. In support of MTT data, trypan blue results show that BA645 exerts anti-proliferation effects on HELA cells by killing HELA cells and decreasing its number. However, it exerts a little effect on MCF7 cells proliferation rate **(Figure 16b and d).**

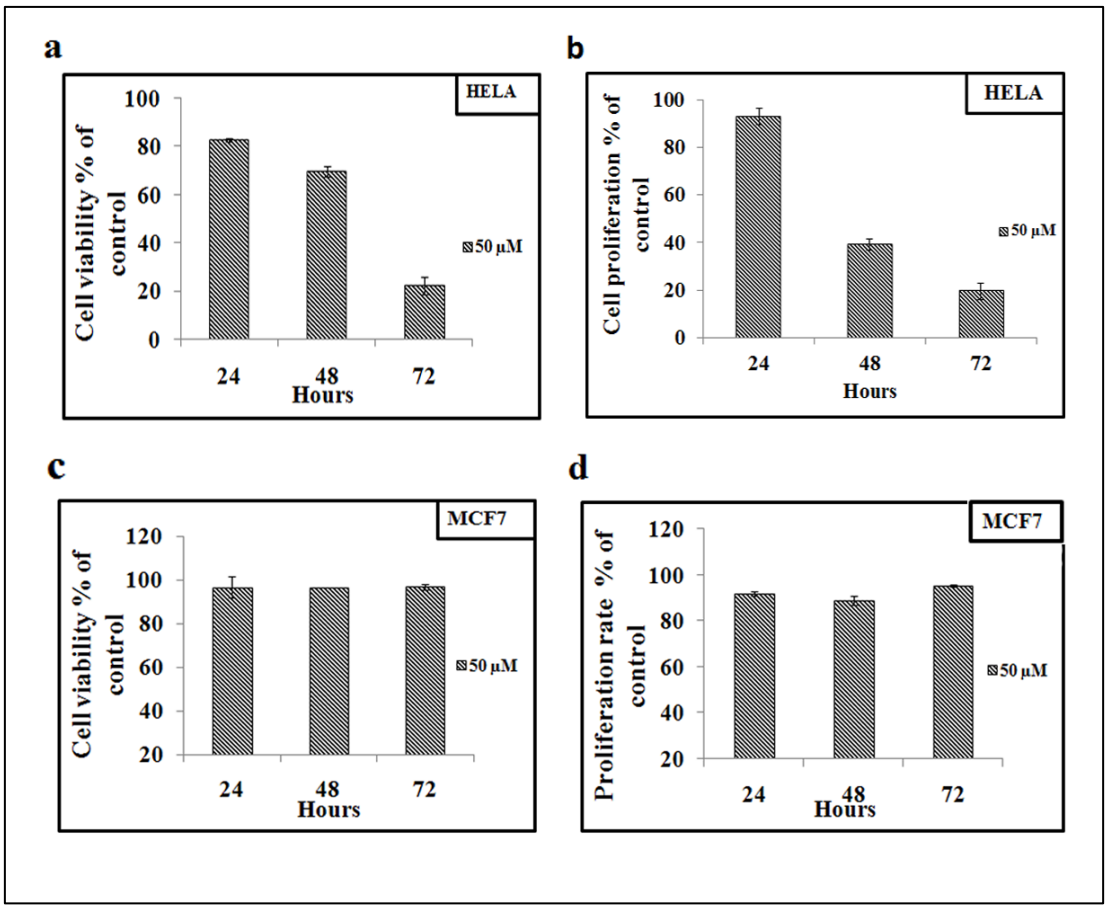

Figure (4.16): BA645 inhibits cancer cell viability and proliferation. Effect of BA645 on cell viability of cervical (a) and breast (c) cancer cells. Cancer cell lines were plated in 6-well plates and after 24 hours, cells were treated with 50 µM of BA645 or vehicle. Cell viability was assessed by the trypan blue assay after 24, 48 and 72 hours of the treatment. Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate. Right graphs of the figure show the anti-proliferation effect of BA645 on HELA (b) and MCF7 (d) cancer cells.

Finally we show her that all three novel compounds induce different levels of cell death and anti-proliferative effect in the tested cell lines after 72 hours of the treatment **(Table 4.2 and 4.3).** However it is obvious that HELA cells are the most sensitive almost for all novel BA-compounds. Therefore in the next parts of this thesis we present the effect of BA-compounds on the proliferation rate (as growth curve) of MCF7 and HELA cancer cells. Furthermore, we show the molecular mechanism behind this effect.

Table (4.2): Percentage of cell death induced by BA-compounds in MCF7 and HELA cells at 72 hours.

Compounds	**% of HELA cell death**	**% of MCF7 cell death**
BA623	95%	25%
BA642	55%	52%
BA645	80%	5%

Table (4.3): Anti-proliferative effect of BA-compounds in MCF7 and HELA cells (percent of control) at 72 hours.

Compounds	**%of HELA proliferation rate reduction**	**%of MCF7 proliferation rate reduction**
BA623	30%	53%
BA642	59%	63%
BA645	80%	5%

4.4 BA-compounds inhibit growth of cancer cells

Trypan blue results revealed that BA-compounds had an effects on the proliferation of cancer cells. Growth curve assays and their characterization were used to confirm these results.

4.4.1 BA642 inhibits growth of human breast and cervical cancer cells

The effect of BA642 on the growth of MCF7 breast cancer cell line, HELA cervical cancer cell line was studied using growth curve assay. Furthermore, population doubling time (PDT) was calculated from the linear equation (Excel Microsoft office 2010). Results show that 10µM of BA642 inhibits the growth of HELA cells around 35% at 72 hours and increases population doubling time (PDT) from 24.96 hours for control cells to 40.32 hours for treated cells **(Figure 4.17a)**. The same concentration of BA642 also inhibits the growth of MCF7 cells around 35% at 72 hours and increases population doubling time (PDT) from 36.86 hours for control cells to 62.4 hours for treated cells **(Figure 17b)**.

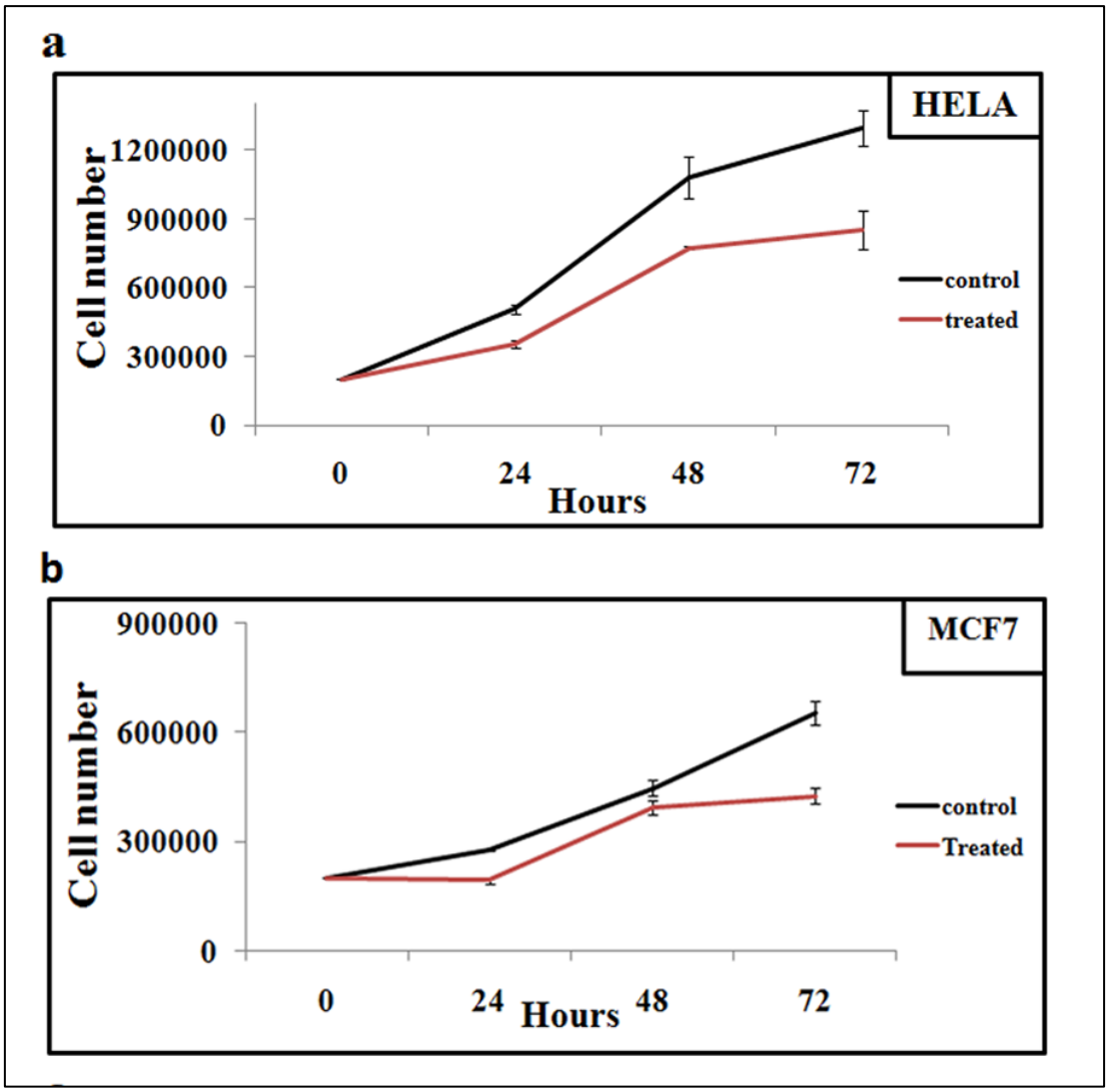

Figure (4.17): The effect of BA642 on the growth of cervical and breast cancer cell lines. Cells were plated in 6-well plates and treated with 10µM of BA642. Growth curve for HELA cells with and without BA642 (a); growth curve for MCF7 with and without BA642 (b). Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate.

4.4.2 BA623 inhibits growth of human cervical and breast cancer cells

The effect of BA623 on the growth of HELA cervical cancer cell line and MCF7 breast cancer cell line was studied using growth curve assay. Furthermore, population doubling time (PDT) was calculated from the linear equation (Excel Microsoft office 2010). Results Turn out that 50µM of BA623 inhibits the growth of HELA cells around 50% at 72 hours and increases PDT from 19.4 hours in control to 40.94 hours in treated cells **(Figure 4.18a)**. The same concentration of BA623

reduces the growth of MCF7 cells about 70% at 72 hours and increases PDT from 68.88 hours in control to 201.6 hours in treated cells **(Figure 4.18b).**

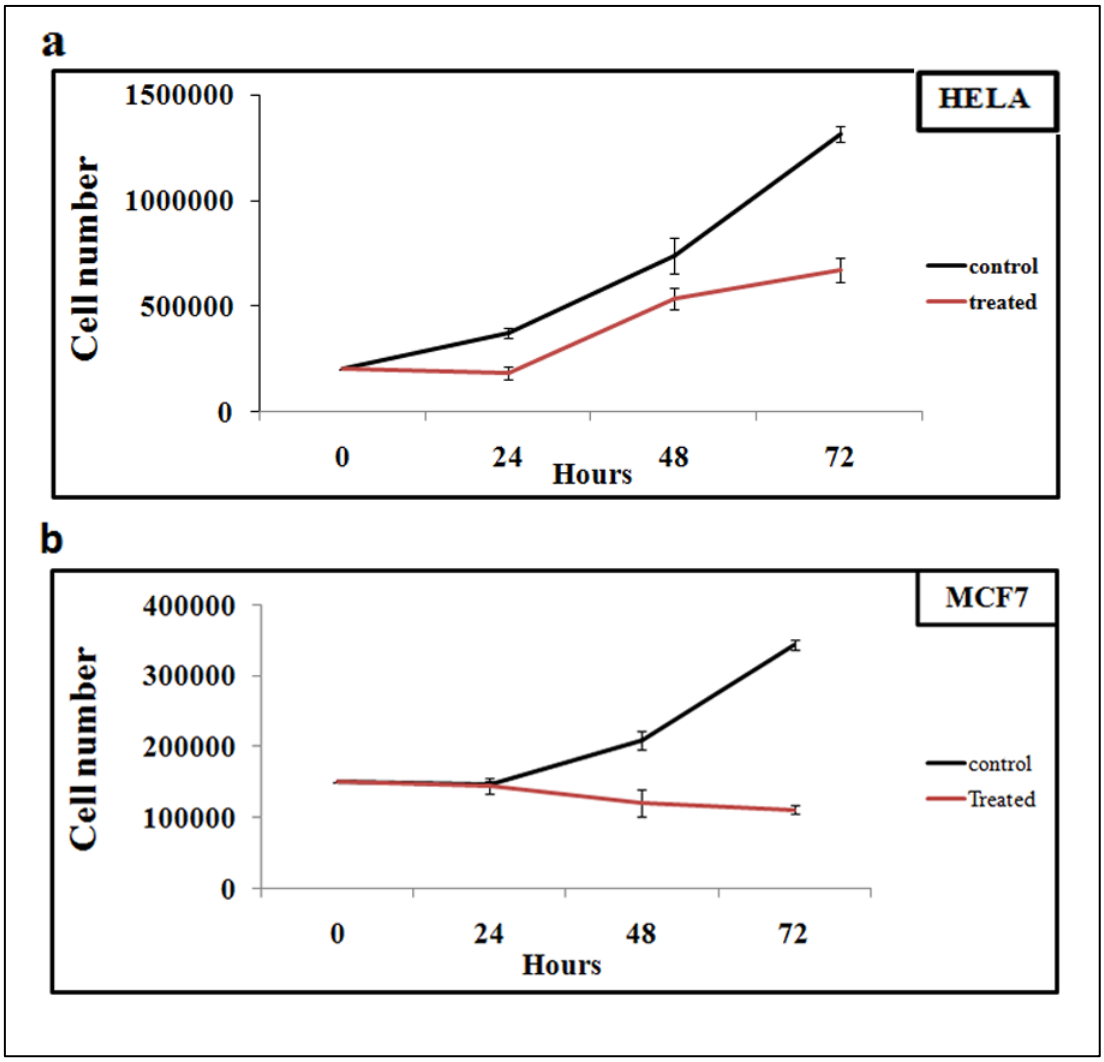

Figure (4.18): The effect of BA623 on the growth of cervical and breast cancer cell lines. Cells were plated in 6-well plates and treated with 50µM of BA623. Growth curve for HELA cells with and without BA623 (a); growth curve for MCF7 with and without BA623 (b). Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate.

4.4.3 BA645 inhibits growth of human cervical and breast cancer cell lines

The effect of BA645 on the growth of HELA cervical cancer cell line and MCF7 breast cancer cell line was studied using growth curve assay and population doubling time (PDT) was calculated from linear equation by Excel Microsoft office 2010. Results reveal that 50µM of BA645 inhibits the growth of HELA cells around 75% at 72 hours and increases PDT from 7.2 hours in control to 28.5 hours in treated cells **(Figure 4.19a)**. The same concentration of BA 645 also reduces the growth of MCF7 cells about 20% at 72hours and increases PDT from 68.88 hours in control to 108.96 hours in treated cells **(Figure 4.19b)**.

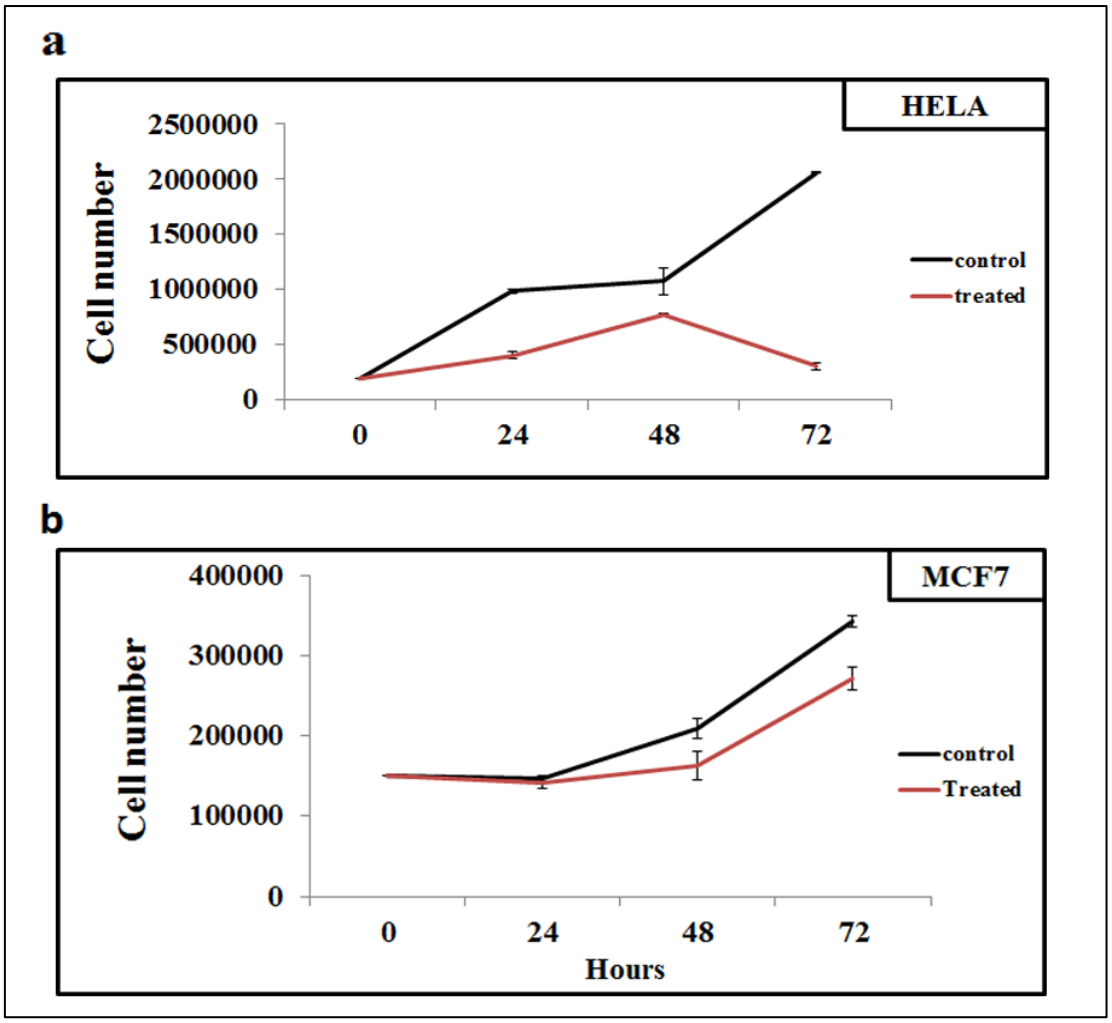

Figure (4.19): The effect of BA645 on the growth of cervical and breast cancer cell lines. Cells were plated in 6-well plates and treated with 50µM of the indicated compound. Growth curve for HELA cells with and without BA645 (a); growth curve for MCF7 with and without BA645 (b). Results represent the mean percentage ± SEM of control of at least two experiments performed in twice replicate.

All together we show her that all three novel compounds increase population doubling times in the tested cell lines **(Table 4.4).**

Table (4.4): The population doubling times (hours).

Compounds	**PDT in HELA control** (hours)	**PDT in treated HELA** (hours)	**PDT in MCF7 control** (hours)	**PDT in treated MCF7** (hours)
BA623	19.4	40.94	68.88	201.6
BA642	24.96	40.32	36.86	62.4
BA645	7.2	28.5	68.88	108.96

4.5 BA-compounds inhibit cell migration of human cervical cancer cells

To further explore the anti-tumour activity of BA-compounds, a scratch motility assay was performed and a significant reduction in cell migration was observed for HELA cell line exposed to 10 μM of all BA-compounds after 24, 48 and 72 hours of the treatment.

4.5.1 BA642 inhibits cell migration of human cervical cancer cells

To further explore the anti-tumour activity of BA642, a scratch motility assay was performed and a significant reduction in cell migration was observed for HELA cells exposed to 10μM BA642 for 24, 48 and 72 hours **(Figure 4.20)**. Importantly, the anti-migration effect was more obvious at 24 and 72 hours of the treatment time point.

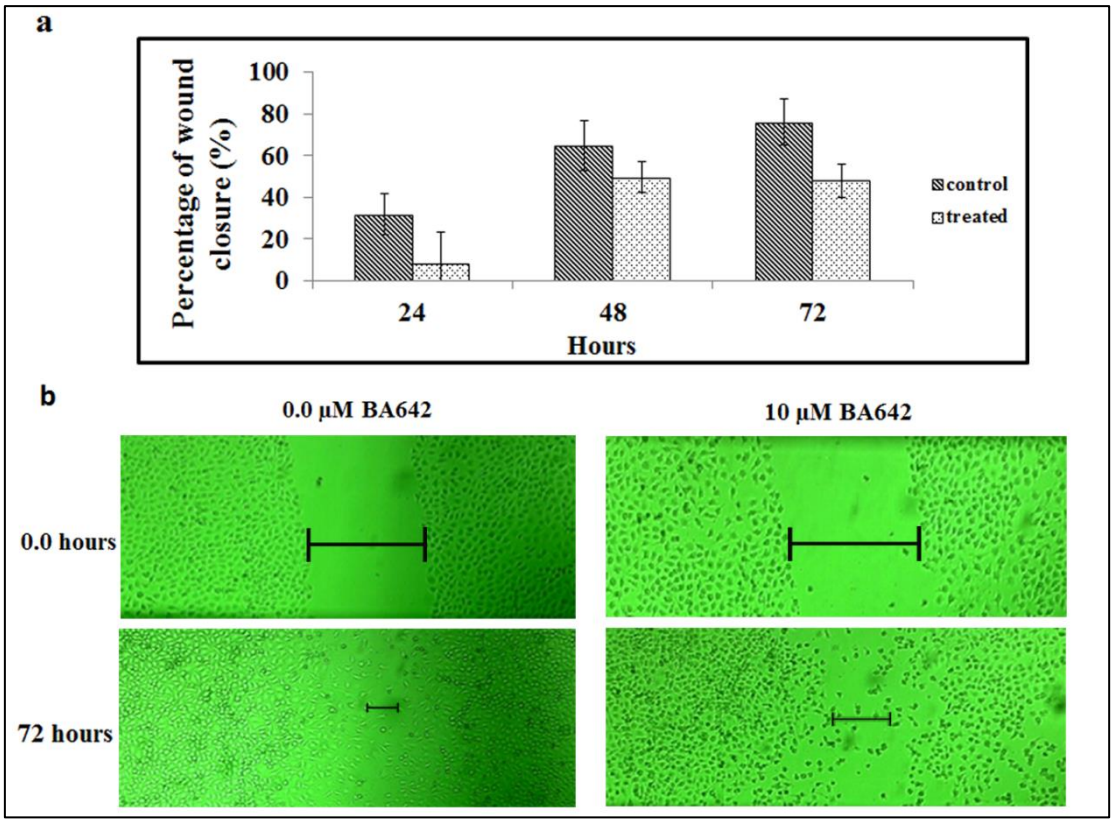

Figure (4.20): BA642 inhibits migration of cervical cancer cells. BA642 inhibits the migration ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 90-100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or BA642 (10µM) for 72 hours. At specified time points (x-axis) cells were photographed using (4x; Olympus 1X71) and the area migrated was measured and expressed relative to zero time (y-axis). Assays were done in duplicate and two independent experiments were performed.

4.5.2 BA623 inhibits cell migration of human cervical cancer cells

To further explore the anti-tumour activity of BA623, a scratch motility assay was performed and a significant reduction in cell migration was observed for HELA cell line exposed to 10µM BA623 for 24, 48 and 72 hours **(Figure 4.21)**. BA623 inhibits the migration ability of cervical cancer cells around 21% at 72 hours. These results indicate that the most obvious effect was at 72 hours of treatment.

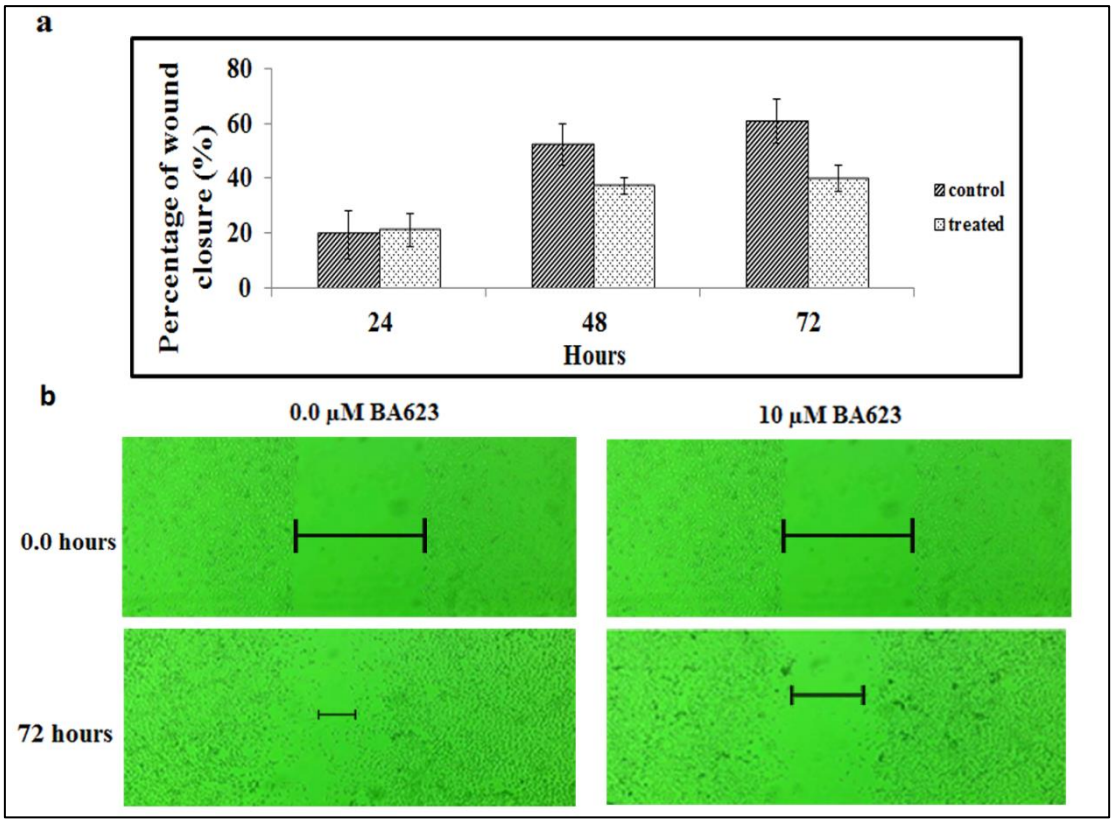

Figure (4.21): BA623 inhibits migration of cervical cancer cells. BA623 inhibits the migration ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 90-100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or BA623 (10µM) for 72 hours. At specified time points (x-axis) cells were photographed using (4x; Olympus 1X71) and the area migrated was measured and expressed relative to zero time (y-axis). Assays were done in duplicate and two independent experiments were performed.

4.5.3 BA645 inhibits cell migration of human cervical cancer cells

To further explore the anti-tumour activity of BA645, a scratch motility assay was performed and a significant reduction in cell migration was observed for HELA cell line exposed to 10µM BA645 for 24, 48 and 72 hours **(Figure 4.22)**. Importantly, the anti-migration effect was clear at 72 hours of the treatment time point.

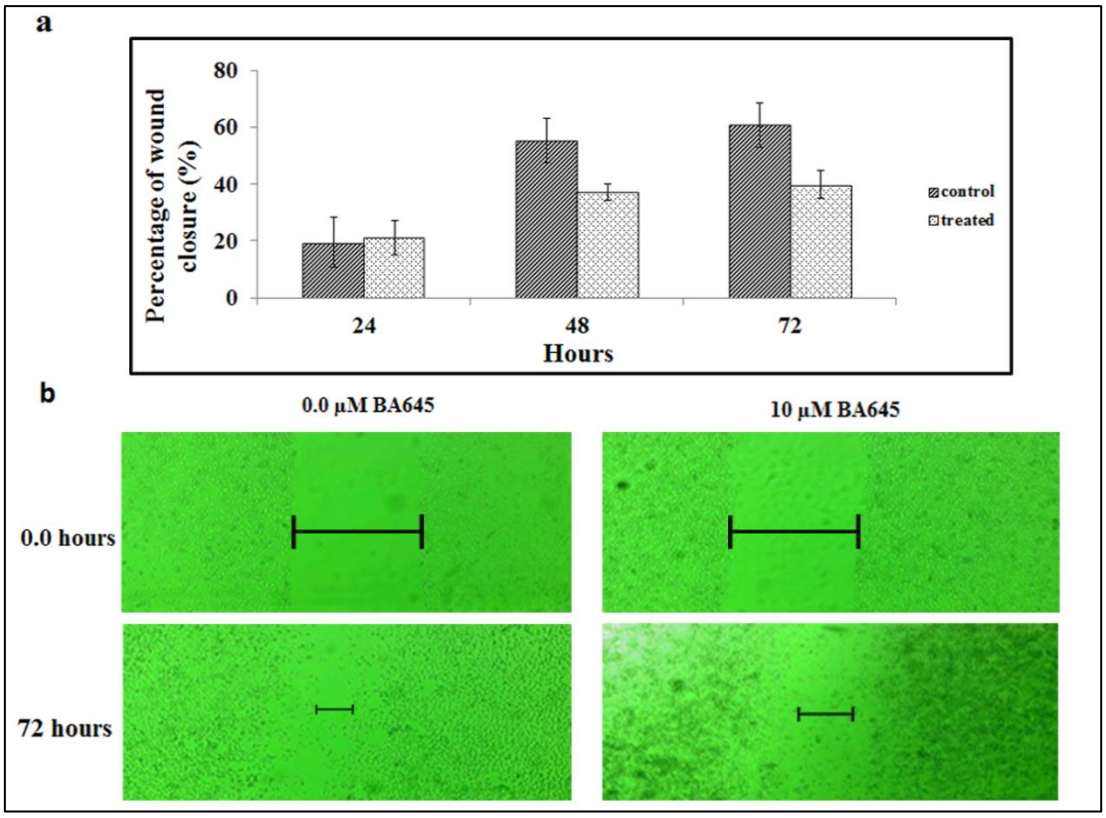

Figure (4.22): BA645 inhibits migration of cervical cancer cells. BA645 inhibits the migration ability of cervical cancer cells in an in vitro scratch assay. Cells were grown to 90-100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or BA645 (10µM) for 72 hours. At specified time points (x-axis) cells were photographed using (4x; Olympus 1X71) and the area migrated was measured and expressed relative to zero time (y-axis). Assays were done in duplicate and two independent experiments were performed.

4.6 BA623 inhibits EGFR activation and induces cell cycle arrest

Triple negative breast cancers (TNBCs) lack receptors for estrogen, progesterone and Her2 hence this subtype is highly aggressive and resistant to conventional chemotherapy (Crown, O'Shaughnessy, & Gullo, 2012). However, TNBCs usually express high level of EGFR, and its level is significantly associated with a negative prognosis in TNBC (Changavi, Shashikala, & Ramji, 2015). Several targeted therapeutics for TNBCs have entered clinical trials, but unfortunately, none of them have yet been approved for TNBC. Our results in **Figure 4.23** showed that MDA-MB-231 (TNBCs) are sensitive to BA623 with an IC50 (10.88μM) and therefore this section of the study investigates the effect of BA623 and the mechanism of action behind this effect.

Activation of EGFR upon the binding of its ligand to the extracellular domain of results in the phosphorylation of a group of tyrosine residues (Y992, Y1068, Y1086, Y1148, and Y1173) in the carboxy-terminal domain of EGFR (Nyati, Morgan, Feng, & Lawrence, 2006). Therefore, inhibition of phosphorylation of specific tyrosine residues abrogates multiple growth-promoting pathways. To determine whether BA623 play a role as EGFR targeted agent, we performed western blot analysis with MDA-MB-231 breast cancer cell line. **Figure 4.24** shows that untreated MDA-MB-231 expresses a high level of phosphorylated-EGFR. In comparison to total EGFR protein level, pEGFR was reduced in a time dependent manner in treated MDA-MB-231 cells. Of the tested forms we show here that BA623 inhibits EGFR phosphorylation at (Y1068) site. Inhibition of EGFR abrogates its downstream signaling pathways, such as phosphatidylinositol 3 kinase pathway PI3K/AKT/mTOR. This pathway is one the main signaling pathway that regulated by EGFR and play critical role in cancer development. Phosphorylation of EGFR at (Y1068) site cause activation of AKT at (s473) site (Sette et al., 2015). Our data shows that BA623 inhibits this pathway as evident by the inhibition of AKT phosphorylation at (s473) site in a time dependent manner **(Figure 4.24)**. Activating mutations of AKT indirectly down regulates p53 (tumour suppressor protein) levels by enhancing MDM2 (murine double minute 2) protein which mediates p53 degradation. Inhibition of phosphorylated AKT leads to stabilize p53 from degradation (A. G. Abraham & O'Neill, 2014). **Figure 4.24** shows that BA623 increases p53 level and its target p21 (main cell cycle regulator protein).

Activation of p21 is normally considered a sign of cell cycle arrest (Aliwaini et al., 2013). Therefore, we then tested the effect of BA623 on the cell cycle profile of MDA-MB-231. So we analyzed the cell cycle distribution pattern of MDA-MB-231 cells following treatment with BA623 (10μM) for 72 hours using flow cytometry. Interestingly, BA623 inhibited cell cycle progression thereby leading to a decreased cell proliferation. Cell cycle analysis exhibited that BA623 treatment increases MDA-MB-231 cells in G0/G1phase by 20.18 % which was accompanied by a reduction of cells in G2-M phase mainly **(Figure 4.25)**. Results also show a gradual accumulation of cells in the subG1 phase which means that BA623 also induce apoptosis **(Figure 4.25)**. Altogether these findings show that BA623 might represent potential EGFR targeted agent which induces G1 cell cycle arrest and apoptosis.

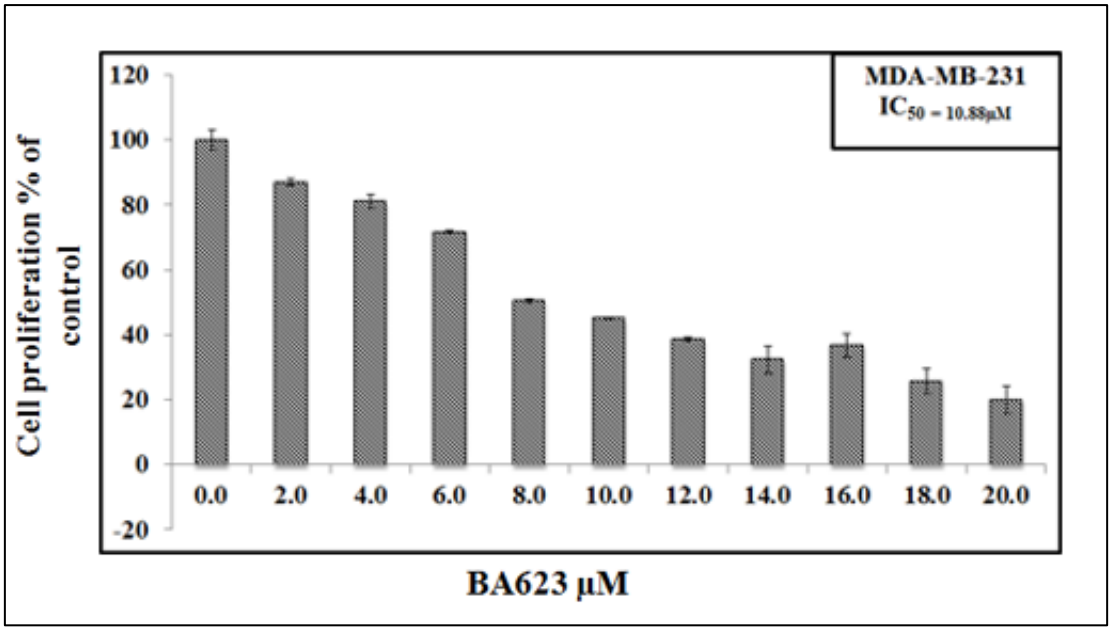

Figure (4.23): The cytotoxicity of BA623 on breast cancer cell line. MDA-MB-231 cells were plated in 96-well plates and after 24 hours the cells were treated with increasing concentrations of the indicated compound (0-20μM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 72 hours of treatment. Results represent the mean percentage ± SEM of control of at least three experiments performed in quadruplicate.

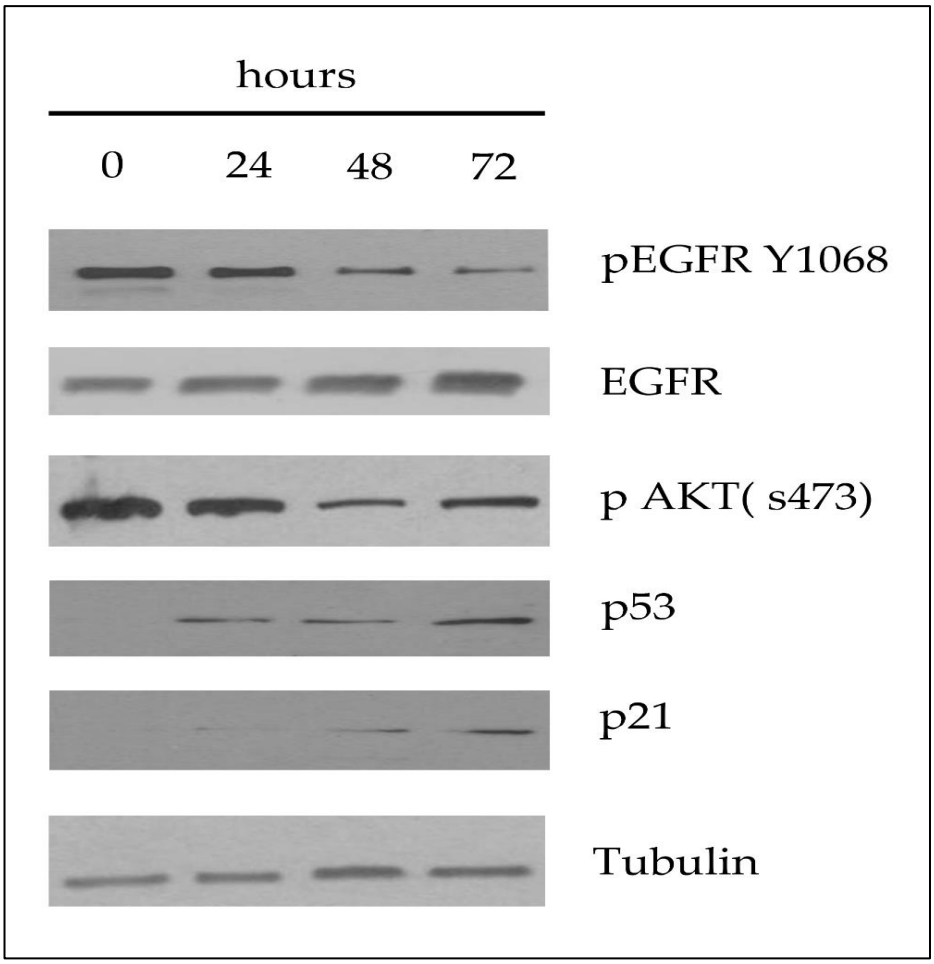

Figure (4.24): Activity of BA623 on EGFR, AKT, p53 and p21 in MDAMB-231 cells. Cells were treated with BA623 (10μM) for 0, 24, 48 and 72 hour. Western blotting were performed to detect EGFR, p EGFR, ERK and AKT phosphorylation levels as described in materials and methods. EGFR, ERK and AKT phosphorylation levels were detected using phosphor specific EGFR, ERK and AKT antibodies as described in materials and methods. Cells in log-phase growth were treated with (10μM) BA623 before lysis at indicated time intervals. Tubulin was used as a loading control.

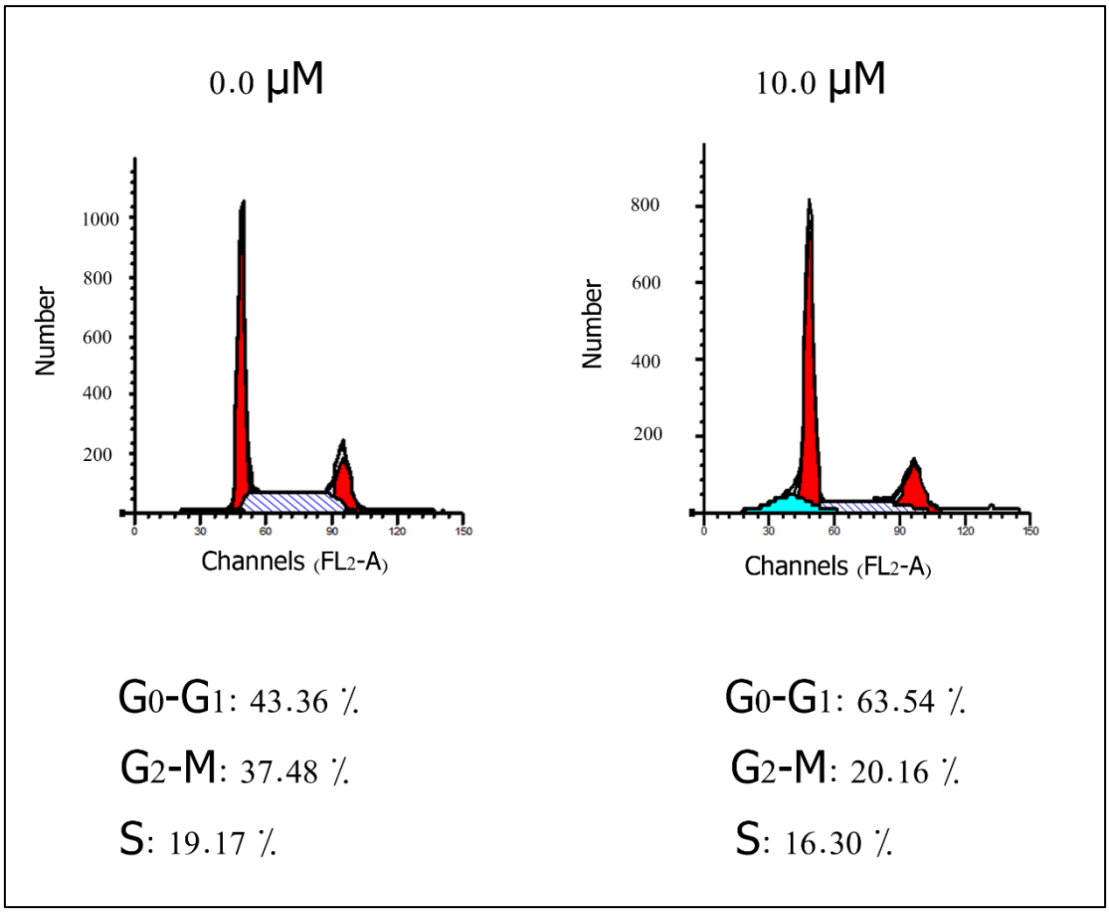

Figure (4.25): Cell cycle analysis of MDA-MB-231 cells exposed to 0.0 and 10µM of BA623. Cells were treated with vehicle (DMSO) or BA623 at the concentrations indicated and analyzed at 72 hours by flow cytometry after propidium iodide staining. The cell cycle profile was estimated by gating histograms generated with the FL2-area variable.

Chapter 5

Discussion

Chapter 5

Discussion

Cancer is a pernicious and lethal disease that records one in seven deaths globally. In spite of progression in cancer diagnosis and treatment, the number of cancer deaths is expected to elevate to reach 13.5 million deaths in 2030 (American Cancer Society, 2012). One approach to dealing with the current cancer burden has been to rationally develop targeted drugs as anti-cancer compounds. These targeted therapies are designed to target a specific features of cancer cells which make it less harmful on normal cells. Recently the strategy of targeting tyrosine kinase enzymes the core part of epidermal growth factor receptors (EGFR) is compelling. Certain cancer cells such as breast, cervical, colorectal, head and neck, gastric, lung, esophageal, prostate, bladder, renal, pancreatic, ovarian and liver cells usually express high level of epidermal growth factor receptors (EGFR) (Arteaga, 2002). The most commonly used EGFR tyrosine kinase inhibitors are Gefitinib, Erlotinib, Lapatinib, and Sorafenib (Seshacharyulu et al., 2012).

However serious side effects and multiple mechanisms of acquired resistance associated with this line of therapy necessitate the development of more efficient EGRR-TKIs as anti-tumour therapeutic drugs. This study has screened a group of pyrazolotriazolopyrimidine based (BA) compounds for selective cytotoxic activity and evaluated their inhibitory effect against EGFR in breast cancer, cervical cancer and colorectal cancer cells. Results showed that some of these compounds may be effective drugs in the treatment of breast and cervical cancer. Furthermore this study demonstrates that one of the BA-compounds namely BA623 inhibits cell survival, cell cycle progression and migration capacity of breast and cervical cancer cells. Importantly, BA compounds showed very low level of toxicity in normal cells which might indicate that these compounds are safe.

This study provides several lines of evidence that the pyrazolotriazolopyrimidine based (BA) compounds, hold a promise as novel EGFR targeted drugs to treat cervical and breast cancers and BA623 seems to be the most promising compound.

5.1 BA623 exerts potent cytotoxic effects against triple negative breast cancer cell line

Different drugs targeting EGFR are used to treat breast cancer and lapatinib is a common FDA approved therapy (Opdam et al., 2012). Several studies showed that lapatinib is effective against breast cancer cells specially those expressing HER2 with IC_{50}s range from 0.010μM to 18.6μM (**Table 5.1)** (Konecny et al., 2006; B. Liu et al., 2016). These different IC50s might be attributed to the different types and levels of receptors expression such as EGFR and HER2 in the tested cells. In comparison to this approved drug, BA623 has a similar cytotoxic level against breast cancer cell lines.

Gefitinib is one common example of EGFR targeted drugs and several studies confirmed gefitinib toxicity in different types of breast cancer cell lines (Corkery et al., 2009; Ferrer-Soler et al., 2007). For example this drug has an IC_{50} of 21μM on MCF7, 20.7μM on MDA-MB-231, 15.5μM on BT20, 8.4μM on HCC1937, 0.25μM on BT474, 0.88μM on SKBB3 (Corkery et al., 2009; Ferrer-Soler et al., 2007). In comparison to gefitinib, BA623 has a more potent cytotoxic effect on MDA-MB-231 cells with IC_{50} of 10.88μM. However BA623 has lower effect on MCF7 (IC_{50} of 44.56μM) than gefitinib.

Finally, erlotinib is another EGFR targeted drug tested in many breast cancer cells and have been shown to exhibit a wide range of toxicity with IC_{50}s range from 3.98μM on SK-BR-3 to 20μM or more on many other cell lines including MCF7 and MDA-MB-23 (Deshpande, 2016; Yamasaki et al., 2008). Once again BA623 is more effective against MDA-MB-231 cells than erlotinib but has a similar cytotoxic effect on MCF7 cells. All together BA623 has a potent cytotoxic effect against breast cancer cells similar to the approved EGFR targeted drugs.

Table (5.1): The IC50s of approved drug (B. Liu et al. 2016) **and BA623 on breast cell lines**.

Drugs	**MCF7**	**MDA-MB-231**	**Approval year**
Gefitinib	21µM	20.7µM	Approved in 2003 to treat non-small-cell lung cancer
Erlotinib	>20µM	>20µM	Approved in 2004 to treat non-small cell lung cancer
Lapatinib	7.7µM	18.6µM	Approved in 2007 to treat breast cancer
Afatinib	7.09µM	6.89µM	Approved in 2013 to treat non-small cell lung cancer
Neratinib	8.22µM	6.47µM	Approved in 2017 For HER2 breast cancer treatment
BA623	44.56µM	10.88µM	

5.2 BA623 exerts a cytotoxic effect against cervical cancer cell line

As shown in chapter 4, all BA-compounds exert different levels of toxicity on HELA cells (IC_{50} range from 28.8µM to 41µM). A among these compounds, BA623 is the most cytotoxic one (IC_{50} of 28.87µM). Erlotinib was also tested in many cervical cancer cells and has been shown to display different levels of cytotoxicity on these cells (Martinho et al., 2017). For example this drug has an IC_{50} of 2.41µM on Caski, 2.30µM of C-33A, >20µM of SiHa and >20µM on HELA cells. BA623 has a cytotoxic effect similar to erlotinib on HELA cells (Martinho et al., 2017). Lapatinib also was investigated in many cervical cancer cell lines and the results showed that lapatinib displays potent cytotoxic effects on these cell lines with 0.45µM on Caski, 1.78µM of C-33A, 2.36µM of SiHa and 2.19µM on HELA cells (Martinho et al., 2017).

In comparison to lapatinib, BA-compounds exert less toxicity on HELA cells. To our knowledge, gefitinib wasn't tested alone on cervical cancer cell lines, however it was used as a control in some studies that study novel EGFR-KI targeted compounds. Among these studies, one study was examined gefitinib on HELA cells and the IC_{50} of this drug on HELA was 20µM in the presence of EGF (Du et al., 2015).

In compression to gefitinib, BA623 displays a cytotoxic effect on HELA almost similar to gefitinib but in the absence of EGF. All together BA-compounds have a potent cytotoxic effect against cervical cancer cells almost similar to the approved EGFR targeted drugs.

5.3 BA compounds exert low cytotoxic effect against colorectal cancer cell line

Two BA compounds namely BA645 and BA623 show moderate cytotoxic effects on Caco-2 colorectal cancer cells comparing with BA642 which exert no effect on these cells. In comparison to their effects on MCF7, HELA and MDA-MB-231cells, BA compounds have less cytotoxic effects on Caco-2 cells. Although BA623 displays cytotoxic effect similar or more than gefitinib and lapatinib against breast and cervical cancer cell lines, it has a weak cytotoxic effect on Caco-2 (Giannopoulou, 2009).

5.4 BA-compounds have little cytotoxic effects on normal cells

BA-compounds have low cytotoxicity levels on normal cell lines especially BA645 compound that exerts the least cytotoxic effect with IC_{50} of (839 µM). However BA623 also has a little cytotoxicity on normal cells with IC_{50} (238.28 µM). The worst one is BA642 that has a strong cytotoxic effect on normal cells with IC_{50} (56.38 µM). All BA-compounds have lower toxicity level on normal cells than gefitinib (IC_{50} of 38.7µM) (Y. Liu et al., 2009) and Lapatinib (IC_{50} of 0.035µM) (Strecker et al., 2009).

This is very important since drugs with low IC50s on cancer cells and high IC50s on normal cells usually cause little side effects in vivo.

5.5 BA compounds reduce cancer cells migration ability

Cancer has six hallmarks which are responsible for tumour growth and metastasis (Hanahan, 2011). Invasion and metastasis is one of the principle cancer hallmarks which is considered the leading cause of death in cancer patients (Gandalovi9 et al., 2017). Due to the importance of this feature in carcinogenesis, many drugs were designed and synthesized to target and inhibit it. In this study we show that BA-compounds reduce migration ability of HELA cells significantly with 21% to 28% cells.

Lapatinib and erlotinib anti-migration abilities were tested on C-33A and SiHa cervical cancer cell lines and results showed that they inhibit C-33A migration (around 40%) but not SiHa (Martinho et al., 2017). In compression to erlotinib and Lapatinib, BA-compounds have similar anti-migration abilities on cervical cancer cells. These findings support previous data that BA-compounds have anti-cancer effect against cervical cancer cells similar or better than some approved EGFR targeted drugs.

5.6 BA compounds activate different anti-cancer mechanisms

Anticancer agents usually inhibit cancer through specific mechanisms such as apoptosis, autophagy, inhibition of proliferation and blocking of survival and proliferation signaling pathways like ERK, AKT and PI3K pathways (Corkery et al., 2009; Dasari, 2014b; Vakifahmetoglu-Norberg, 2015). However, cancer cells normally develop mechanisms of resistance to many of these drugs and therefore more than one drug (more than one mechanism of action) is used. Importantly, BA623 induces different mechanisms to stop cancer progression, it inhibits EGFR and AKT phosphorylation which results in p53 and p21 accumulation leading cell cycle arrest.

Gefitinib was tested in many breast cancer cell lines and data showed its ability to inhibit EGFR, MAPKs, AKT and cell cycle progression in these cell lines. However, TNBC cell lines like MDA-MB-231 cells are gefitinib resistant as they show low EGFR level and gefitinib treatment showed no effect on these cells. (Corkery et al., 2009). In comparison to gefitinib, BA623 is more effective especially against the TNBC MDA-MB-231 cell line where it inhibits AKT and induces cell cycle arrest in G1 phase.

Erlotinib was also tested in many breast cancer cell lines and some of these cell lines were sensitive and the others were resistant (Yamasaki et al., 2008).This drug lead to an inhibition of EGFR, ERK1/ERK2 and AKT in sensitive cell lines. Sensitive cells for example BT-474 also exhibited G1 cell cycle arrest after treated with 10μM of erlotinib and increased number of cells in subG1, however resistant cell line like MDA-MB-231showed similar cell cycle profile before and after treatment (Yamasaki et al., 2008). Similar to BA623, erlotinib induces G1 cell cycle arrest and apoptosis in breast cancer cells but BA623 is more cytotoxic than erlotinib

MDA-MB-231which makes it superior to these approved drugs. Similar results were also observed with lapatinib in breast cancer cells where it inhibits EGFR, AKT and ERK leading to G1 cell cycle arrest and apoptosis (Konecny et al., 2006).

Taken together, BA623 similar to other approved EGFR inhibitors that induce different anticancer mechanisms including cell cycle arrest and AKT inhibition. However, BA623 is more effective than other approved EGFR inhibitors against the resistant MDA-MB-231 cells which makes it more promising compound.

Chapter 6

Conclusions and Recommendations

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6.1 Conclusions

This study describes the anti-tumour activity of BA-compounds as novel EGFR targeted agents. Moreover, the current study is the first study to provide the mechanism of action by which a pyrazolotriazolopyrimidine-based compound exerts cytotoxicity in cancer cells. Based on the data generated from the in vitro experiments of the anticancer activity of BA623, the following model is proposed **(Figure 6.1)**. BA623 initially inhibits the phosphorylation of EGFR and the activation of AKT which activates different molecular pathways including P53 and p21 leading to cell cycle arrest. This study suggests that BA623 may be an effective EGFR targeted drug in the treatment of, at the very least, breast cancer as well as cervical cancer.

6.2 Recommendations

1.We recommend to do more researches in this field.

2.We here recommend to study these compounds on other types of cancers.

3. Chemical modifications are needed to improve the activity of BA-compounds.

4. In vivo studies are also recommended.

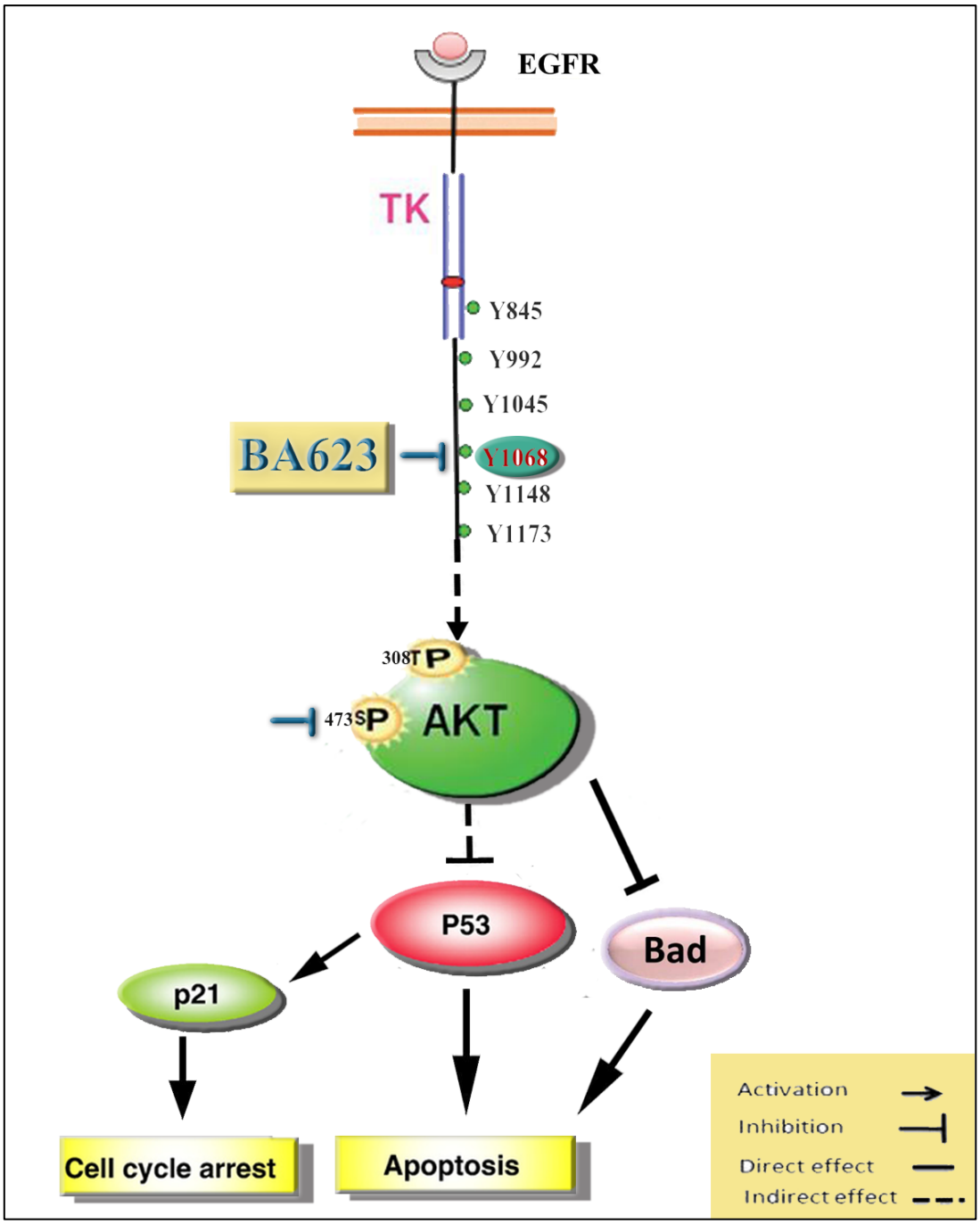

Figure (6.1): A proposed model for the mechanism by which BA623 exerts its anti-cancer activity.

References

References

Abraham, A. G., & O'Neill, E. (2014). PI3K/Akt-mediated regulation of p53 in cancer. *Biochemical Society Transactions*, *42*(4), 798–803.

Abraham, J., & Staffurth, J. (2016). Hormonal therapy for cancer. *Medicine*, (2015), 1–4.

Adams, J., & Kauffman, M. (2004). Development of the Proteasome Inhibitor Velcade TM (Bortezomib), *22*(2), 304–311.

Aliwaini, S., Peres, J., Kröger, W. L., Blanckenberg, A., de la Mare, J., Edkins, A. L., … Prince, S. (2015). The palladacycle, AJ-5, exhibits anti-tumour and anti-cancer stem cell activity in breast cancer cells. *Cancer Letters*, *357*(1), 206–218.

Aliwaini, S., Swarts, A. J., Blanckenberg, A., Mapolie, S., & Prince, S. (2013). A novel binuclear palladacycle complex inhibits melanoma growth in vitro and in vivo through apoptosis and autophagy. *Biochemical Pharmacology*, *86*(12),

Allred, D. C., Mohsin, S. K., & Fuqua, S. a. (2001). Histological and biological evolution of human premalignant breast disease. *Endocrine-Related Cancer*, *8*(1), 47–61.

American Cancer Society. (2012). Estimated Number of New Cancer Cases by World Area.

American Cancer Society. (2013a). Targeted Therapy. *American Cancer Society*, 27.

American Cancer Society. (2013b). Targeted Therapy What is targeted therapy ? *American Cancer Society*, 27.

American Cancer Society. (2014). The Science Behind Radiation Therapy.

American Cancer Society. (2015). Cancer Immunotherapy.

American Cancer Society. (2015). Chemotherapy Drugs: How They Work, 17.

American Cancer Society. (2016a). *Cancer Treatment & Survivorship Facts & Figures 2016-2017*.

American Cancer Society. (2016b). *Chemotherapy | American Cancer Society*.

American Cancer Society. (2016c). Chemotherapy Side Effects.

American Cancer Society. (2016d). Risks of Cancer Surgery.

American Cancer Society. (2017). *Understanding Your Pathology Report: Colon Polyps (Sessile or Traditional Serrated Adenomas)*.

Arora, a, & Scholar, E. M. (2005). Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther*, *315*(3), 971–979.

Arteaga, C. L. (2002). Epidermal growth factor receptor dependence in human tumours: more than just expression? *The Oncologist*, *7 Suppl 4*(Supplement 4), 31–9.

Arvelo, F., Sojo, F., & Cotte, C. (2015). Biology of colorectal cancer. *Ecancermedicalscience*, *9*, 1–20.

Baker, L., Quinlan, P. R., Patten, N., Ashfield, a, Birse-Stewart-Bell, L.-J., McCowan, C., … Thompson, a M. (2010). P53 Mutation, Deprivation and Poor Prognosis in Primary Breast Cancer. *British Journal of Cancer*, *102*(4), 719–26.

Baskar, R., Lee, K. A., Yeo, R., & Yeoh, K.-W. (2012). Cancer and Radiation Therapy: Current Advances and Future Directions Rajamanickam. *International Journal of Medical Sciences*.

Beck, J. T., Ismail, A., & Tolomeo, C. (2014). Targeting the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway: An emerging treatment strategy for squamous cell lung carcinoma. *Cancer Treatment Reviews*, *40*(8), 980–989.

Behray, M., Webster, C. A., Pereira, S., Krishnamurthy, S., Al-jamal, W. T., Chao, Y., … Wafa, T. (2016). Synthesis of novel diagnostic silicon nanoparticles for targeted delivery of thiourea to EGFR-expressing cancer cells Synthesis of novel diagnostic silicon nanoparticles for targeted de- livery of thiourea to EGFR-expressing cancer cells. *American Chemical Society*.

Bosco, E. E., & Knudsen, E. S. (2007). RB in breast cancer: at the crossroads of tumourigenesis and treatment. *Cell Cycle (Georgetown, Tex.)*, *6*(6), 667–71.

Brosh, R., & Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. *Nature Reviews. Cancer*, *9*(10), 701–13.

Bumrungthai, S., Munjal, K., Nandekar, S., Cooper, K., Ekalaksananan, T., Pientong, C., & Evans, M. F. (2015). Epidermal growth factor receptor pathway mutation and expression profiles in cervical squamous cell carcinoma: therapeutic implications. *Journal of Translational Medicine*, *13*, 244.

Burd, E. M. (2003). Human Papillomavirus and Cervical Cancer. *CLINICAL MICROBIOLOGY REVIEWS*, *16*(1), 1–17.

Chakraborty, S., Li, L., Puliyappadamba, V. T., Guo, G., Hatanpaa, K. J., Mickey, B., … Habib, A. A. (2014). downstream signalling networks, (May).

Changavi, A. A., Shashikala, A., & Ramji, A. S. (2015). Epidermal Growth Factor Receptor Expression in Triple Negative and Nontriple Negative Breast Carcinomas. *Journal of Laboratory Physicians*, *7*(2), 79–83.

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., & Liu, L. F. (1984). Nonintercalative antitumour drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *The Journal of Biological Chemistry*, *259*(21), 13560–6.

Cheung-Ong, K., Giaever, G., & Nislow, C. (2013). DNA-Damaging Agents in Cancer Chemotherapy: Serendipity and Chemical Biology. *Chemistry & Biology*, *20*(5), 648–659.

Cohen, M. H., Johnson, J. R., Chen, Y.-F., Sridhara, R., & Pazdur, R. (2005). FDA drug approval summary: erlotinib (Tarceva) tablets. *The Oncologist*, *10*(7), 461–6.

Cohen, M. H., Williams, G. A., Sridhara, R., Chen, G., & Pazdur, R. (2003). FDA drug approval summary: gefitinib (ZD1839) (Iressa) tablets. *The Oncologist*, *8*(4), 303–6.

Colagiuri, B., Dhillon, H., Butow, P. N., Jansen, J., Cox, K., & Jacquet, J. (2013). Does Assessing Patients' Expectancies About Chemotherapy Side Effects Influence Their Occurrence? *Journal of Pain and Symptom Management*, *46*(2), 275–281.

Corkery, B., Crown, J., Clynes, M., & O'Donovan, N. (2009). Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. *Annals of Oncology*, *20*(5), 862–867.

Crown, J., O'Shaughnessy, J., & Gullo, G. (2012). Emerging targeted therapies in triple-negative breast cancer. *Annals of Oncology*, *23*(suppl 6), vi56-vi65.

Dai, C. -l., Tiwari, A. K., Wu, C.-P., Su, X. -d., Wang, S.-R., Liu, D. -g., … Fu, L. -w. (2008). Lapatinib (Tykerb, GW572016) Reverses Multidrug Resistance in Cancer Cells by Inhibiting the Activity of ATP-Binding Cassette Subfamily B Member 1 and G Member 2. *Cancer Research*, *68*(19), 7905–7914.

Dasari, S., & Tchounwou, P. B. (2014a). Cisplatin in cancer therapy: molecular mechanisms of action. *European Journal of Pharmacology*, *740*, 364–78.

Dasari, S., & Tchounwou, P. B. (2014b). Cisplatin in cancer therapy: molecular mechanisms of action. *European Journal of Pharmacology*, *740*, 364–78.

Davies, C., Godwin, J., Gray, R., Clarke, M., Cutter, D., Darby, S., … Peto, R. (2011). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet*, *378*(9793), 771–84.

Delaney, G., Jacob, S., Featherstone, C., & Barton, M. (2005). The role of radiotherapy in cancer treatment. *Cancer*, *104*(6), 1129–1137. http://doi.org/10.1002/cncr.21324

Deshpande, G. (2016). Comparative Effect of Tyrosine Kinase Inhibitors in Human Cancer Cell Lines. *Journal of Pharmacology & Clinical Research*, *1*(2).

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene*, *26*(22), 3279–3290.

Dow, L. W., Sinkule, J. A., Look, A. T., Horvath, A., & Evans, W. E. (1983). Comparative cytotoxic and cytokinetic effects of the epipodophyllotoxins 4'-demethylepipodophyllotoxin-9-(4,6-O-2-ethylidene-beta-D-glucopyranoside) and 4'-demethylepipodophyllotoxin-9-(4,6-O-2-thenylidene-beta-D-glucopyranoside) and their metabolites on . *Cancer Research*, *43*(12 Pt 1), 5699–706.

Du, J., Zhang, E., Zhao, Y., Zheng, W., Zhang, Y., Lin, Y., … Wang, F. (2015). Discovery of a dual-targeting organometallic ruthenium complex with high activity inducing early stage apoptosis of cancer cells. *Metallomics*, *7*(12), 1573–1583.

Enock, A., & Ndefo, U. (2011). Sipuleucel-T (Provenge) Injection The First Immunotherapy Agent (Vaccine) For Hormone-Refractory Prostate Cance. *Drug Forecast*, *36*(4), 197–202.

Erickson, K. F., Chertow, G. M., & Goldhaber-Fiebert, J. D. (2013). Cost-Effectiveness of Tolvaptan in Autosomal Dominant Polycystic Kidney Disease. *Annals of Internal Medicine*, *159*(6), 382.

Ferrer-Soler, L., Vazquez-Martin, A., Brunet, J., Menendez, J. A., De Llorens, R., & Colomer, R. (2007). An update of the mechanisms of resistance to EGFR-tyrosine kinase inhibitors in breast cancer: Gefitinib (Iressa^{TM})-induced changes in the expression and nucleo-cytoplasmic trafficking of HER-ligands (Review). *International Journal of Molecular Medicine*, *20*(1), 3–10.

Field-Smith, A., Morgan, G. J., & Davies, F. E. (2006). Bortezomib (Velcadetrade mark) in the Treatment of Multiple Myeloma. *Therapeutics and Clinical Risk Management*, *2*(3), 271–9.

Fumagalli, D., Andre, F., Piccart-Gebhart, M. J., Sotiriou, C., & Desmedt, C. (2012). Molecular biology in breast cancer: should molecular classifiers be assessed by conventional tools or by gene expression arrays? *Critical Reviews in Oncology/hematology*, *84 Suppl 1*, e58-69.

Gandalovi9, A., Rosel, D., Fernandes, M., Veselý, P., Heneberg, P., Cermák, V., … Brábek, J. (2017). Migrastatics— Anti-metastatic and Anti-invasion Drugs:

Promises and Challenges.

García-Claver, A., Lorente, M., Mur, P., Campos-Martín, Y., Mollejo, M., Velasco, G., & Meléndez, B. (2013). Gene expression changes associated with erlotinib response in glioma cell lines. *European Journal of Cancer*, *49*(7), 1641–1653.

Giannopoulou, E., Antonacopoulou, A., Floratou, K., Papavassiliou, A. G., & Kalofonos, H. P. (2009). Dual targeting of EGFR and HER-2 in colon cancer cell lines. *Cancer Chemotherapy and Pharmacology*, *63*(6), 973–981.

Giglia, M. D., & Chu, D. I. (2016). Familial Colorectal Cancer: Understanding the Alphabet Soup. *Clinics in Colon and Rectal Surgery*, *29*(3), 185–195.

González Martín, A. (2007). Molecular biology of cervical cancer. *Clinical and Translational Oncology*, *9*(6), 347–354.

Gottesman, M. M. (2002). MECHANISMS OF CANCER DRUG RESISTANCE. *Annual Review of Medicine*, *53*, 615–27.

Grady, W. (2007). *Colorectal Cancer Evidence-Based Chemotherapy Strategies*. (L. B. SALTZ, Ed.).

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, *144*(5), 646–674.

Hima, B. A. (2011). Breast Cancer – Therapies – Challenges Ahead. *Cancer Science & Therapy*, *S2*, 1–5.

Holstege, H., Joosse, S. A., van Oostrom, C. T. M., Nederlof, P. M., de Vries, A., & Jonkers, J. (2009). High incidence of protein-truncating TP53 mutations in BRCA1-related breast cancer. *Cancer Research*, *69*(8), 3625–33.

Ibeanu, O. A. (2017). Molecular pathogenesis of cervical cancer, *4047*.

Ivanovich, J. L., Read, T. E., Ciske, D. J., Kodner, I. J., & Whelan, A. J. (1999). A practical approach to familial and hereditary colorectal cancer. *The American Journal of Medicine*, *107*(1), 68–77.

Jeter, J. M. W. K. S. B. G. (2006). Genetics of Colorectal Cancer.

Kaufman, B., Trudeau, M., Awada, A., Blackwell, K., Bachelot, T., Salazar, V., … Westlund, R. (2009). Lapatinib monotherapy in patients with HER2-overexpressing relapsed or refractory infl ammatory breast cancer : fi nal results and survival of the expanded HER2 + cohort in EGF103009 , a phase II study. *Lancet Oncology*, *10*(6), 581–588.

Kheirelseid, E. A. H., Miller, N., & Kerin, M. J. (2013). Molecular biology of colorectal cancer: Review of the literature. *American Journal of Molecular Biology*, *3*, 72–80.

Khokhar, A. (2012). Breast cancer in India: where do we stand and where do we go? *Asian Pacific Journal of Cancer Prevention : APJCP*, *13*(10), 4861–6.

Klein, C. A. (2009). Parallel progression of primary tumours and metastases. *Nature Reviews. Cancer*, *9*(4), 302–12.

Klein, C. a, & Stoecklein, N. H. (2009). Lessons from an aggressive cancer: evolutionary dynamics in esophageal carcinoma. *Cancer Research*, *69*(13), 5285–8.

Klein, G. (1998). Foulds' dangerous idea revisited: the multistep development of tumours 40 years later. *Advances in Cancer Research*, *72*, 1–23.

Konecny, G. E., Pegram, M. D., Venkatesan, N., Finn, R., Yang, G., Rahmeh, M., … Slamon, D. J. (2006). Activity of the Dual Kinase Inhibitor Lapatinib (GW572016) against HER-2-Overexpressing and Trastuzumab-Treated Breast Cancer Cells. *Cancer Res*, *66*(3), 1630–9.

Krasinskas, A. M., & M., A. (2011). EGFR Signaling in Colorectal Carcinoma. *Pathology Research International*, *2011*, 932932.

Le Tourneau, C., Raymond, E., & Faivre, S. (2007). Sunitinib: a novel tyrosine kinase inhibitor. A brief review of its therapeutic potential in the treatment of renal carcinoma and gastrointestinal stromal tumours (GIST). *Therapeutics and Clinical Risk Management*, *3*(2), 341–8.

Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr,

Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of Clinical Investigation*, *121*(7), 2750–67.

Leu, W.-J., Swain, S. P., Chan, S.-H., Hsu, J.-L., Liu, S.-P., Chan, M.-L., … Guh, J.-H. (2016). Non-immunosuppressive triazole-based small molecule induces anticancer activity against human hormone-refractory prostate cancers: the role in inhibition of PI3K/AKT/mTOR and c-Myc signaling pathways. *Oncotarget*, *7*(47), 76995–77009.

Liu, B., Huang, X., Hu, Y., Chen, T., Peng, B., Gao, N., … Jin, G. (2016). Ethacrynic acid improves the antitumour effects of irreversible epidermal growth factor receptor tyrosine kinase inhibitors in breast cancer. *Oncotarget*, *7*(36), 58038–58050.

Liu, Y., Sakagami, H., Amano, O., Kikuchi, H., Nakamura, Y., Ishihara, M., … Yu, G. (2009). Tumour-specific cytotoxicity and type of cell death induced by peplomycin in oral squamous cell carcinoma cell lines. *Anticancer Research*, *28*, 2197–2204.

Lockhart, A. C., Cropp, G. F., Berlin, J. D., Donnelly, E., Schumaker, R. D., Schaaf, L. J., … Rothenberg, M. L. (2006). Phase I/pilot study of SU5416 (semaxinib) in combination with irinotecan/bolus 5-FU/LV (IFL) in patients with metastatic colorectal cancer. *American Journal of Clinical Oncology*, *29*(2), 109–15.

Maemondo, M., Inoue, A., Kobayashi, K., Sugawara, S., Oizumi, S., Isobe, H., … Nukiwa, T. (2010). Gefitinib or Chemotherapy for Non–Small- Cell Lung Cancer with Mutated EGFR, *25*(24).

Manuscript, A., & Syndromes, G. P. (2013). NIH Public Access, *48*(Suppl 2), 1–28.

Martinho, O., Silva-Oliveira, R., Cury, F. P., Martins Barbosa, A., Granja, S., Feijó Evangelista, A., … Manuel Reis, R. (2017). HER Family Receptors are Important Theranostic Biomarkers for Cervical Cancer: Blocking Glucose Metabolism Enhances the Therapeutic Effect of HER Inhibitors. *Theranostics*, *7*(73).

Massarweh, S., & Schiff, R. (2006). Resistance to endocrine therapy in breast cancer: exploiting estrogen receptor/growth factor signaling crosstalk. In *Endocrine-related cancer* (Vol. 13 Suppl 1, pp. S15-24).

McDermott, J., & Jimeno, A. (2015). Pembrolizumab: PD-1 inhibition as a therapeutic strategy in cancer. *Drugs of Today*, *51*(1), 7.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., & Gianni, L. (2004). Anthracyclines : Molecular Advances and Pharmacologic Developments in Antitumour Activity and Cardiotoxicity. *PHARMACOLOGICAL REVIEWS*, *56*(2), 185–229.

Molina, M. A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J., & Baselga, J. (2001). Trastuzumab (Herceptin), a Humanized Anti-HER2 Receptor Monoclonal Antibody, Inhibits Basal and Activated HER2 Ectodomain Cleavage in Breast Cancer Cells. *Cancer Research*, *61*(12).

Morgillo, F., Della Corte, C. M., Fasano, M., & Ciardiello, F. (2016). Mechanisms of resistance to EGFR-targeted drugs: lung cancer. *ESMO Open*, *1*(3), e000060.

National Cancer Institute. (2015). Types of treatment-surgery.

Nyati, M. K., Morgan, M. A., Feng, F. Y., & Lawrence, T. S. (2006). Integration of EGFR inhibitors with radiochemotherapy. *Nature Reviews Cancer*, *6*(11), 876–885.

Ono, M., & Kuwano, M. (2006). Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. *Clinical Cancer Research*, *12*(24), 7242–7251.

Opdam, F. L., Guchelaar, H. J., Beijnen, J. H., Schellens, J. H., F.L., O., H.-J., G., … J.H.M., S. (2012). Lapatinib for advanced or metastatic breast cancer. *The Oncologist*, *17*(4), 536–542.

Padma, V. V. (2015). An overview of targeted cancer therapy. *BioMedicine*, *5*(4), 19.

Paul, M. K., & Mukhopadhyay, A. K. (2004). Tyrosine kinase - Role and significance in Cancer. *International Journal of Medical Sciences*, *1*(2), 101–

115.

Polyak, K. (2008). Is breast tumour progression really linear? *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *14*(2), 339–41.

Priyadarshini, K., & Keerthi Aparajitha, U. (2012). Medicinal chemistry Paclitaxel Against Cancer: A Short Review. *Medicinal Chemistry*, *2*(7), 139–141.

Reis-Filho, J. S., & Tutt, A. N. J. (2008). Triple negative tumours: a critical review. *Histopathology*, *52*(1), 108–18.

Ross, J. S., & Fletcher, J. A. (1998). The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. *Stem Cells (Dayton, Ohio)*, *16*(6), 413–28.

Sawicka, M., Kalinowska, M., Skierski, J., & Lewandowski, W. (2004). A review of selected anti-tumour therapeutic agents and reasons for multidrug resistance occurrence. *The Journal of Pharmacy and Pharmacology*, *56*(9), 1067–81.

Segovia-Mendoza, M., González-González, M. E., Barrera, D., Díaz, L., & García-Becerra, R. (2015). Efficacy and mechanism of action of the tyrosine kinase inhibitors gefitinib, lapatinib and neratinib in the treatment of her2-positive breast cancer: Preclinical and clinical evidence. *American Journal of Cancer Research*, *5*(9), 2531–2561.

Selivanova, G., & Wiman, K. G. (2007). Reactivation of mutant p53: molecular mechanisms and therapeutic potential. *Oncogene*, *26*(15), 2243–54.

Seshacharyulu, P., Ponnusamy, M., Haridas, D., Jain, M., Ganti, A., & Batra, S. (2012). Targeting the EGFR signaling pathway in cancer therapy. *Expert Opinion on Therapeutic Targets*, *16*(1), 15–31.

Sette, G., Salvati, V., Mottolese, M., Visca, P., Gallo, E., Fecchi, K., … Eramo, A. (2015). Tyr1068-phosphorylated epidermal growth factor receptor (EGFR) predicts cancer stem cell targeting by erlotinib in preclinical models of wild-type EGFR lung cancer. *Cell Death & Disease*, *6*(8), e1850.

Simon, N., Antignani, A., Sarnovsky, R., Hewitt, S. M., & FitzGerald, D. (2016). Targeting a Cancer-Specific Epitope of the Epidermal Growth Factor Receptor in Triple-Negative Breast Cancer. *J. Natl. Cancer Inst.*, *108*(8), djw028.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., & McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science (New York, N.Y.)*, *235*(4785), 177–82.

Soonthornthum, T., Arias-Pulido, H., Joste, N., Lomo, L., Muller, C., Rutledge, T., & Verschraegen, C. (2011). Epidermal growth factor receptor as a biomarker for cervical cancer. *Annals of Oncology*, *22*(10), 2166–2178.

Soonthornthum, T., Joste, N., Lomo, L., Muller, C., Rutledge, T., & Verschraegen, C. (2011). Epidermal growth factor receptor as a biomarker for cervical cancer, (February), 2166–2178.

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., … Børresen-Dale, A.-L. (2001). Gene expression patterns of breast carcinomas distinguish tumour subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(19), 10869–74.

Soulieres, D., Senzer, N. N., Vokes, E. E., Hidalgo, M., Agarwala, S. S., & Siu, L. L. (2004). Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *22*(1), 77–85.

Spangle, J. M., Munger, K., Munger, K., Hubbert, N., & Haas, J. (2013). The HPV16 E6 Oncoprotein Causes Prolonged Receptor Protein Tyrosine Kinase Signaling and Enhances Internalization of Phosphorylated Receptor Species. *PLoS Pathogens*, *9*(3), e1003237.

Spano, J. P., Fagard, R., Soria, J.-C., Rixe, O., Khayat, D., & Milano, G. (2005). Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives. *Annals of Oncology*, *16*(2), 189–194.

Stoecklein, N. H., & Klein, C. A. (2010). Genetic disparity between primary tumours, disseminated tumour cells, and manifest metastasis. *International Journal of Cancer. Journal International Du Cancer*, *126*(3), 589–98.

Strecker, T. E., Shen, Q., Zhang, Y., Hill, J. L., Li, Y., Wang, C., … Brown, P. H. (2009). Effect of lapatinib on the development of estrogen receptor-negative mammary tumours in mice. *Journal of the National Cancer Institute*, *101*(2), 107–13.

Takagi, S., Hayashi, a, & Ohta, Y. (2009). HER2 and HER3 cooperatively regulate tumour cell growth and determine sensitivity to the HER kinase inhibitor TAK-285. *Cancer Research*, *69*(2 Supplement), 3155–0.

The Palestinian Ministry of Health. (2015). *Cancer in Gaza during the years 2009-2014*.

Tourneau, C. Le. (2007). Sunitinib : a novel tyrosine kinase inhibitor . A brief review of its therapeutic potential in the treatment of renal carcinoma and gastrointestinal stromal tumours (GIST), *3*(2), 341–348.

Vakifahmetoglu-Norberg, H., Xia, H., & Yuan, J. (2015). Pharmacologic agents targeting autophagy. *The Journal of Clinical Investigation*, *125*(1), 5–13.

Vijaya, V. (2015). Review article An overview of targeted cancer therapy, *5*(4), 1–6.

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature Medicine*, *10*(8), 789–99.

Wang, D., & Lippard, S. J. (2005). Cellular processing of platinum anticancer drugs. *Nature Reviews. Drug Discovery*, *4*(4), 307–20.

Wang, H. C., Yan, X. Q., Yan, T. L., Li, H. X., Wang, Z. C., & Zhu, H. L. (2016). Design, synthesis and biological evaluation of benzohydrazide derivatives containing dihydropyrazoles as potential EGFR kinase inhibitors. *Molecules*, *21*(8).

Ware, K. E., Hinz, T. K., Kleczko, E., Singleton, K. R., Marek, L. A., Helfrich, B. A., … Heasley, L. E. (2013). A mechanism of resistance to gefitinib mediated

by cellular reprogramming and the acquisition of an FGF2-FGFR1 autocrine growth loop. *Oncogenesis*, *2*(3), e39.

Weigelt, B., Geyer, F. C., & Reis-Filho, J. S. (2010). Histological types of breast cancer: how special are they? *Molecular Oncology*, *4*(3), 192–208.

West, H. (Jack). (2015). Immune Checkpoint Inhibitors. *JAMA Oncology*, *1*(1), 115.

Widakowich, C., de Castro, G., de Azambuja, E., Dinh, P., & Awada, A. (2007). Review: side effects of approved molecular targeted therapies in solid cancers. *The Oncologist*, *12*(12), 1443–55.

Wilhelmsen, K., Litjens, S. H. M., & Sonnenberg, A. (2006). Multiple Functions of the Integrin α6β4 in Epidermal Homeostasis and Tumourigenesis. *MOLECULAR AND CELLULAR BIOLOGY*, *26*(8), 2877–2886.

World Health organization. (2006). *Cervical cancer - WHO | Regional Office for Africa*.

World Health organization. (2014). WHO | Cancer. *word cancer report*.

World Health Organization. Fact sheet, 297. (2011). *Cancer fact sheet*.

Yamasaki, F., Zhang, D., Bartholomeusz, C., Hortobagyi, G. N., Kurisu, K., & Ueno, N. T. (2008). Sensitivity of breast cancer cells to erlotinib depends on cyclin-dependent kinase 2 activity. *Molecular Cancer Therapeutics*, *6*(8), 2168–2177.

Yang, J. J., Zhou, Q., Yan, H. H., Zhang, X. C., Chen, H. J., Tu, H. Y., … Wu, Y. L. (2017). A phase III randomised controlled trial of erlotinib vs gefitinib in advanced non-small cell lung cancer with EGFR mutations. *British Journal of Cancer*, *116*(5), 568–574.

Yewale, C., Baradia, D., Vhora, I., Patil, S., & Misra, A. (2013). Epidermal growth factor receptor targeting in cancer: A review of trends and strategies. *Biomaterials*, *34*(34), 8690–8707.

Yi, Y. W., Wang, A., Seong, Y., & Bae, I. (2013). Inhibition of the PI3K / AKT pathway potentiates cytotoxicity of EGFR kinase inhibitors in triple-negative

breast cancer cells, *17*(5), 648–656.

Zhou, C., Wu, Y.-L., Chen, G., Feng, J., Liu, X.-Q., Wang, C., … You, C. (2011). Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *The Lancet Oncology*, *12*(8), 735–742.

Zilfou, J. T., & Lowe, S. W. (2009). Tumour suppressive functions of p53. *Cold Spring Harbor Perspectives in Biology*, *1*(5), a001883.