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THERAPEUTIC POTENTIAL OF THREE (3) SMALL MOLECULE COMPOUNDS AND MECHANISTIC INSIGHTS INTO THE LEAD COMPOUND FOR CANCER TREATMENT

BY

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DECLARATION

The experiments in this thesis were conducted by Bernardine Tuah at the Department of Biochemistry, Cell and Molecular Biology, under the supervision of Dr Anastasia Rosebud Aikins from the Department of Biochemistry, Cell and Molecular Biology at the University of Ghana. Dr Kwabena Amofa Nketia Sarpong from the Department of Biochemistry, Cell and Molecular Biology, Prof Richard Kwamla Amewu and Dr Daniel Moscoh Ayine-Tora from the Department of Chemistry also provided guidance. All references cited in this study have been duly acknowledged.

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ABSTRACT

The need to discover novel anticancer drugs is imperative due to the high death rate associated with cancer. Conventional chemotherapy frequently lacks selectivity and induces significant adverse reactions. This study sought to identify specific small-molecule compounds that can selectively interact with cancer cells while causing minimum harm to normal cells. The cytotoxic effects of three small molecule compounds (compounds 1, 2, 3) were evaluated on different cancer cell lines. The use of computational tools, such as molecular docking and dynamics simulations, facilitated the prediction of pharmacokinetics properties and the identification of molecular targets. The effects of the most potent compound on cellular processes such as apoptosis, gene expression, cancer stemness, and migration in breast cancer cells were investigated using a combination of flow cytometry, real-time PCR, adhesion and wound healing assays. A set of 36 analogues was developed using the structure of the hit compound as a basis. Eight of these analogues were tested for their cytotoxic effects, pharmacokinetic features, and drug-likeness. Compound 2, the identified hit compound, demonstrated significant cytotoxicity against breast cancer cell lines, surpassing doxorubicin (DOX) in selectivity. The molecular targeting analysis revealed substantial interactions with cancer-related pathways, specifically EGFR, AKT1, and VEGFR2. EGFR was subsequently identified as the most likely target, with compound 2 binding to its ATP binding pocket and allosteric site. Compound 2 was shown to have the capacity to trigger apoptosis, hinder the migration of cancer cells, and modify gene expression associated with cancer metastasis, as demonstrated by functional experiments. It also modulated inflammation and immune responses differentially from DOX. Although compound 2 showed promising efficacy, its analogues did not exhibit increased activity, suggesting selectivity in its mechanism of action. This study demonstrates the therapeutic potential of Compound 2 as an anticancer agent by targeting EGFR

and possibly other pathways. The results confirm the compound's ability to regulate critical cancer-related processes and pathways, providing a basis for further development into a cancer treatment that can be used in clinical settings. The findings support further development and optimisation of Compound 2 and its analogues for further investigations.

DEDICATION

This thesis is dedicated to my children, Mborti and Nimormi Walley, my husband, Dr. Bernard Walley and my late father, Joseph Laarie. You gave me fortitude when I contemplated abandoning this endeavour.

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AKT1	AKT Serine/Threonine Kinase 1
CSC	Cancer stem cell
DOX	Doxorubicin
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
MTT	MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
P-gp	P-glycoprotein
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RoG	Radius of gyration
RT-qPCR	Quantitative reverse transcription Polymerase Chain Reaction
TNBC	Triple-negative breast cancer
VEGFR2	Vascular endothelial growth factor receptor 2
VCAM-1	Vascular cell adhesion molecule 1
MMP	Matrix Metalloproteinase
Ki67	Marker of proliferation Kiel 67
Bcl-2	B-cell lymphoma 2
ABC	ATP-binding cassette
HCC	Hepatocellular carcinoma
STAT3	Signal Transducer and Activator of Transcription 3
CD34	Cluster of Differentiation 34
CD44	Cluster of Differentiation 44
CD24	Cluster of Differentiation 24
CD133	Cluster of Differentiation 133
SOX2	SRY-box Transcription Factor 2
NANOG	Homeobox Transcription Factor NANOG
OCT4	Octamer-binding Transcription Factor 4
SALL4	Sal-like protein 4
ALDH	Aldehyde Dehydrogenase
BMI1	B lymphoma Mo-MLV insertion region 1
CXCR	C-X-C chemokine receptor
HCC	Hepatocellular Carcinoma
CADD	Computer-aided drug discovery
EM	Epithelial-mesenchymal

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Cancer is a global health concern due to the challenges such as detection, diagnosis, treatment and poor prognosis. The unregulated proliferation of malignant cells defines the disease. With millions of new instances reported each year, cancer is the world's second leading cause of illness and death. According to predictions, by the end of this decade, the number of fatalities from cancer will surpass that of cardiovascular diseases, demonstrating that cancer is a severe health concern (ReFaey et al., 2021). By 2040, the number of cancer cases globally is expected to increase by 47%, reaching a staggering 28.4 million. Several causes, such as population growth and ageing, as well as changes in cancer risk factors associated with improvements in socioeconomic situations, can be ascribed to the increase in numbers (Brenner et al., 2023). Due to changes in population distribution and the influence of globalisation and economic growth, developing nations are projected to shoulder a more significant proportion of the cancer burden than their developed counterparts (Sung et al., 2021). The varying prevalence of different cancer types across different regions reflects a complex interplay of lifestyle, environmental, and genetic factors. For example, in Ghana, breast cancer is the leading cause of cancer-related deaths, accounting for 18.4% of all cases, followed by liver and prostate cancer (Sung et al., 2021).

In Ghana, the fight against cancer involves a comprehensive strategy. The country's policy targets non-communicable diseases, including cancer, focusing on enhancing prevention and healthcare equity. The National Cancer Control Steering Committee emphasises prevention, early detection, treatment, and palliative care. Despite these efforts, one significant challenge in cancer management is the difficulty of detecting early-stage asymptomatic malignancies. Often, these

remain undetected until they have advanced, impacting the effectiveness of treatment (Serrano et al., 2019). Several factors, including limited access to screening facilities, low awareness, and inadequate screening programs, contribute to delays in cancer detection. Beyond early identification, cancer treatment encounters challenges. While chemotherapy and surgery are commonly used, a significant hurdle lies in the lack of specificity in current chemotherapy regimens. Addressing these issues is crucial for effective cancer management. Chemotherapy not only targets cancer cells but also affects healthy tissues and organs. Consequently, minimising harm to healthy cells has become a primary objective, emphasising the need for more precise and minimally invasive therapeutic approaches. While chemotherapy has long been a cornerstone of cancer treatment, ongoing research seeks to improve its effectiveness (Atlihan-Gundogdu et al., 2020).

Advancements in cancer treatment have introduced several promising therapies that offer targeted, less invasive, and more effective options for patients. Three prominent developing treatments are immunotherapy, targeted therapy, and personalised medicine. Immunotherapy, encompassing CAR T-cell therapy and checkpoint inhibitors, utilises the body's immune system to combat cancer. It has demonstrated notable efficacy in treating lung cancer and melanoma (Bicak et al., 2024; Hodi et al., 2010; June et al., 2010). Targeted therapies focus on molecules that have a role in the progression of cancer, such as HER2 in breast cancer and EGFR in lung cancer (Arteaga & Engelman, 2014; Li et al., 2022). This approach improves outcomes and has fewer side effects than other treatments. Personalised medicine customises therapies according to an individual's genetic profile, improving effectiveness and reducing adverse effects (Serrano et al., 2019). Combination therapy, where multiple drugs with distinct mechanisms of action are administered

simultaneously, also aims to enhance efficacy while reducing the risk of drug resistance (Mokhtari et al., 2017). Additionally, researchers explore synergies between new natural compounds and existing chemotherapeutic drugs to mitigate side effects associated with traditional therapies side effects (Cheon & Ko, 2022; Guo et al., 2020; Hong et al., 2019; Zare et al., 2021). These improvements are essential for enhancing the chances of survival and the overall well-being of individuals diagnosed with cancer.

Global disparities persist within and across countries and pose challenges in cancer therapy. Cancer control in developing regions faces many challenges, such as weak healthcare infrastructure, high treatment costs, and inadequate screening programs (Burstein, 2022). Specialized personnel and diagnostic tools are often scarce, delaying diagnosis and limiting treatment options (Hanna & Kangolle, 2010). Many people can't afford comprehensive care due to out-of-pocket expenses and lack of health insurance. Delayed access to advanced therapies and widespread exposure to carcinogens in unregulated environments add to the cancer burden (Omotoso et al., 2023). More robust healthcare systems, better public awareness, and reliable funding sources are necessary to improve cancer control. Addressing socio-cultural barriers, including low awareness and stigma around cancer, is essential for timely care-seeking. Empowering community leaders and influencers could shift narratives and improve early detection and care. By tackling these issues holistically and with coordinated efforts, disparities in cancer outcomes globally can be reduced, offering vulnerable populations a better chance of survival and quality of life. Survival rates for early-stage and metastatic diseases vary significantly, highlighting the need for innovative anticancer drugs that balance efficacy and minimise side effects.

The hallmarks of cancer serve as a conceptual framework for comprehending the intricate biological pathways that underlie the genesis and advancement of cancer. Two essential characteristics are the ability to avoid programmed cell death and the disruption of the normal regulation of the cell division process. Apoptosis, or programmed cell death, is an inherent biological mechanism that destroys damaged or unnecessary cells. Cancer cells frequently acquire strategies to resist apoptosis, enabling them to persist and multiply without restraint (Hanahan & Weinberg, 2011). An aberration in the cell cycle is a distinct characteristic of cancer, wherein cells circumvent regular regulatory checkpoints, resulting in unregulated cell proliferation and the development of tumours (Mir et al., 2023). Comprehending these mechanisms is crucial for developing specific treatments that can revive apoptotic pathways and control the cell division cycle to inhibit cancer progression.

Cancer metastasis, a leading cause of cancer-related deaths, involves multiple factors. Approximately 25% of all cancer cases result from abnormal cell signalling and inflammation, which play critical roles in various human cancers (Greten & Grivennikov, 2019; Korbecki et al., 2022; Majidpoor & Mortezaee, 2021). Chronic inflammation can arise from bacterial, viral, or chemical factors (Balkwill & Mantovani, 2012; Lan et al., 2021). Although the exact link between chronic inflammation and neoplastic diseases remains uncertain, the development and spread of cancer are greatly affected by the tumour's surrounding environment, specifically the presence of inflammation. Recent research highlights the significance of specific cytokines and cancer-associated stromal cells in metastasis, treatment resistance, and the spread of metastatic lesions (Jones et al., 2016; Van Der Spek et al., 2020; Zamarron & Chen, 2011). Understanding these cellular interactions enables the development of personalised medications that can disrupt the

support systems, promoting cancer spread. Granulocyte colony-stimulating factor (G-CSF) is an inflammatory cytokine that attracts neutrophils, which have immunosuppressive qualities that aid in the spread of cancer cells (Benna et al., 2020). Inflammatory mediators, such as interleukin-6 (IL-6) and prostaglandin E2 (PGE2), activate pathways essential for cancer cell growth, survival, and spread. These pathways are regulated by DNA methyltransferase 3B (DNMT3B), which emphasises the interaction between inflammation and epigenetic alterations in cancer (So et al., 2020; Wang et al., 2018). Cancer-associated fibroblasts (CAFs) and tumour-associated macrophages (TAMs) release interleukin-6 (IL-6), which triggers the signal transducer and activator of the transcription 3 (STAT3) pathway. This pathway facilitates epithelial-mesenchymal transition (EMT) and confers resistance to paclitaxel, a chemotherapeutic drug. The IL-6/JAK2/STAT3 signalling pathway illustrates the role of inflammatory signalling in developing resistance to chemotherapy (Wang et al., 2018).

Imbalances in Th1 and Th2 cells and chemokine disruptions may also contribute to cancer dissemination (Lin et al., 2020). Th1 cytokines, such as interferon-gamma (IFN-γ) and IL-2, bolster the body's immune response against tumours, but Th2 cytokines, which include IL-4, IL-5, IL-10, and IL-13, promote cancer progression and spread (Abdul-Rahman et al., 2024; Garo & Gopal, 2020). Additionally, cytokines may boost the cytotoxin sensitivity of multidrug-resistant (MDR) cancer cells, suggesting potential applications in immunotherapy (Emran et al., 2022). The transition from Th1 to Th2 dominance in advanced stages of cancer is caused by proteins produced by the tumour, such as TGF-β and IL-10. These molecules inhibit Th1 responses, making it easier for the tumour to evade the immune system (Alshaker & Matalka, 2011; Qiu et al., 2021). Chemokine-chemokine receptor interactions attract cancer cells to distant organs, driving

metastasis (Kitamura et al., 2015; Steele et al., 2016). Effective cancer treatment strategies target critical systems such as the CCL21-CCR7 chemokine ligand receptor and the CXCL12-CXCR4 axis for metastasis management (Chatterjee et al., 2014.; Lim et al., 2016; Wong & Korz, 2008).

The epithelial-to-mesenchymal transition (EMT) is crucial for metastasis (Fares et al., 2020; Liu et al., 2021; Liu et al., 2017; Peña-Romero & Orenes-Piñero, 2022; Ramamoorthi et al., 2022). During EMT, cancer cells lose cell-to-cell adhesion and gain the ability to break through the tumour environment, promoting metastasis. This process enhances invasiveness and stemness, influencing prognosis and survival outcomes (Do et al., 2020; Liu et al., 2016). Hence, research must focus on compounds that downregulate EMT regulators, modulate chemokine interactions, and address Th1/Th2 imbalances to limit metastasis. Cancer stem cells (CSCs) are a subpopulation of cancer cells with features with stem cells, such as the ability to self-renew and differentiate into several cell types within a tumour. These cells are thought to be essential in cancer initiation, progression, dissemination, and recurrence (Batlle & Clevers, 2017). CSCs have a role in therapeutic resistance by surviving therapies lethal to most tumour cells and then regenerating the tumour (Rezayatmand et al., 2022). CSCs possess distinct attributes that render them crucial in metastasis. They have heightened migratory and invasive capacities, allowing them to move to distant organs quickly (Shibue & Weinberg, 2017). In addition, CSCs are also frequently located in areas of tumours with low oxygen levels. CSCs can adjust and survive in these settings, enhancing their ability to spread to other body parts (Fares et al., 2020). Targeting CSCs has become a potential approach to prevent the spread of cancer to different parts of the body and enhance the effectiveness of treatment, thus decreasing the chances of cancer recurrence treatment (Nassar & Blanpain, 2016).

Small-molecule compounds have become crucial in drug discovery, especially in the battle against cancer. These compounds, known for their low molecular weights, can affect biological processes by targeting specific components within cells. They can block cancer-promoting pathways, trigger cell death in cancer cells, and alter the tumour environment to boost the effectiveness of other treatments (Southey & Brunavs, 2023). Many small molecules have been captured in chemical libraries and extensive collections of synthetically produced compounds. These libraries are invaluable for high-throughput screening, allowing researchers to efficiently and accurately identify potential therapeutic agents. Furthermore, medicinal chemistry and computational biology advances have enhanced the design and optimisation of small molecules and increased selectivity while reducing adverse effects (Bhullar et al., 2018). The use of small-molecule drugs in cancer therapy has recently resulted in considerable advances. Targeted medicines, such as tyrosine kinase inhibitors and proteasome inhibitors, have altered the therapy landscape for numerous cancers, bringing new hope for patients with previously untreatable illnesses (Tabana et al., 2023). As research advances, exploring small molecule compounds inside chemical libraries remains a cornerstone of novel cancer drug discovery, offering new treatments capable of effectively battling this complicated disease.

Molecular docking is a crucial tool in drug discovery for identifying small molecules that interact with cancer targets. It predicts how compounds bind to protein sites, aiding selection of leads with favourable binding, pharmacokinetics, and safety when combined with molecular dynamics (MD) simulations and ADMET data. Docking enables virtual screening for inhibitors of proteins like EGFR, common in tumour cells, helping optimise candidates quickly while reducing experimental

costs. In this study, cytotoxicity screening and molecular docking was employed to identify the most promising small-molecule compound among three candidates (compounds 1, 2 and 3) (Figure 3.1) previously reported by Sakyi et al. (2023) with known activity against *Leishmania*. Among these, compound 2—bearing a chromone-based scaffold—was identified as a potential anticancer agent based on its predicted binding affinity for EGFR. Chromones are naturally occurring flavonoid compounds with various pharmacological actions, including anti-inflammatory, antioxidant, and anticancer properties. Chromones have anticancer characteristics because they disrupt multiple cellular processes that contribute to cancer growth. They can cause apoptosis, slow cell development, and disrupt the cell cycle in cancer cells. Furthermore, chromones have been found to influence the tumour microenvironment and improve the efficacy of other therapies (Islam et al., 2023; Raj & Lee, 2020). As researchers continue to investigate chromones' many modes of action, these chemicals are promising candidates for creating innovative cancer therapeutics. Thus, this study sought to identify a promising small-molecule anticancer therapy among the three small molecule compounds found by Sakyi et al. (2023) and its possible targets, as well as investigate the impact of the treatment on important cancer characteristics.

To summarise, the continued fight against cancer necessitates both advanced research and worldwide policy initiatives to promote fair healthcare access and effective treatment. While novel therapies such as immunotherapy, targeted treatments, and personalised medicine represent tremendous advances, they are still challenging to obtain in many parts of the world. Early identification, prevention, and personalised treatment options are critical, especially as we continue to understand the hallmarks of cancer, including apoptosis resistance, cell cycle dysregulation, and the mechanisms driving metastasis. Furthermore, resolving inequities in

healthcare access, particularly in poorer countries, will be critical for lowering worldwide cancer mortality. Combining these findings with policy and preventive measures will be vital as research into cancer biology and therapeutic agents like chromone-based compounds develops. A coordinated, interdisciplinary strategy is required to tackle cancer's numerous problems, bringing hope for more effective treatments and better patient outcomes worldwide.

1.1 SPECIFIC OBJECTIVES

1.1.1 Objective 1: To identify the most potent compound among the three (3) small molecule compounds and predict its pharmacokinetics properties and molecular targets *in silico.*

a) To determine the cytotoxic effect of three (3) small molecule compounds in a panel of 4 cancer cell lines and a normal cell line.
b) To predict the pharmacokinetics properties of the hit compound using ADMETlab and SwissADME.
c) To determine the most potent compound's plausible target(s) using network pharmacology, molecular docking, and molecular dynamic simulation.

1.1.2 Objective 2: To determine the effect of the most potent compound on selected cellular and molecular processes.

a) To investigate the impact of the hit compound on apoptosis, cell cycle, cancer stemness, migration, and invasion by employing various techniques, including flow cytometry, clonogenic assays, wound healing assays, adhesion assays, and microscopy.

b) To assess the effect of the hit compounds on gene expression levels on key metastatic factors, such as EMT (*TWIST1*, *SNAI1*, and *vimentin),* migratory genes *(MMP2, MMP9, VCAM-1),* apoptotic genes *(Bcl-2, Bax, caspase 8&3)* and a proliferation marker *(Ki67)* using RT-qPCR.

c) To quantify selected chemokines and cytokines levels using the Luminex assay.

1.1.3 Objective 3: **To design analogues of the most potent compound and screen selected analogues.**

(a) To design about 30 analogues of the most potent compound.

(b) To predict their pharmacokinetic properties using SwissADME and perform docking studies against the plausible target

(c) To identify potent analogues by screening a subset of analogues in the MDA MB 468 breast cancer cell line using the MTT assay.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cancer Epidemiology

In 2022, there were projected to be 20 million new cases of cancer and 9.7 million fatalities (World Health Organization, 2024). It was estimated that 53.5 million individuals would still be living five years after being diagnosed with cancer. One in five people will, at some point in their lives, get cancer, and one in nine men and one in twelve women will die from the disease (World Health Organization, 2024). Lung cancer accounted for 2.5 million new cases, or 12.4% of all new cases, making it the most common cancer globally. Female breast cancer is second (2.3 million cases, 11.6%), followed by colon cancer (1.9 million cases, 9.6%), prostate cancer (1.5 million cases, 7.3%), and stomach cancer (970,000 cases, 4.9%) (Ferlay et al., 2024). Lung cancer is the most common cause of cancer-related mortality, accounting for an estimated 1.8 million deaths (8.7% of all cancer deaths), according to a recent study from the International Agency for Study on Cancer (World Health Organization, 2024). This is followed by colon cancer (900 000 fatalities, 9.3%), liver cancer (760 000 deaths, 7.8%), breast cancer (670 000 deaths, 6.9%), and stomach cancer (660 000 deaths, 6.8%) (Ferlay et al., 2024; Sung et al., 2021). In comparison to the projected 20 million cases in 2022, nearly 35 million additional cases of cancer are anticipated in 2050, representing a 77% increase. Lung cancer was the leading cause of cancer mortality for men, whereas breast cancer was the most common disease diagnosed and the leading cause of death for women. Globally, breast cancer is the most common cancer among women. In contrast to liver and colorectal cancers, which rank second and third in terms of cancer-related deaths, prostate and colorectal cancers are the most common cancers in men. Regarding new cases and deaths among women, colorectal and lung cancer came in second and third, respectively (Ferlay et al., 2024;

World Health Organization, 2024). The expanding number of lifestyle risk factors, such as smoking and drinking, along with an ageing population, are projected to contribute to an increase in cancer incidence (World Health Organization, 2024).

2.2 Cancer Hallmarks

Hanahan and Weinberg (2011) proposed six unique cellular phenotypic changes as the primary markers of cancer. These critical elements of neoplastic growth biological capabilities emerge at various phases during cancer formation in humans. Cancer usually occurs in 3 main steps. The first step in tumorigenesis is the initiation stage, which can induce either internal or extrinsic causes. The second step is the promotion stage, which typically involves continued cellular division and expansion of the initial cancer cells and cell survival. The final step is advancement, which leads to metastasis. These hallmarks of cancer including sustaining proliferative signalling, evading growth suppressors, resisting cell Cancer's hallmarks, such as sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing the formation of new blood vessels (angiogenesis), and activating the invasion and metastasis processes (Hanahan & Weinberg, 2011), all work together to facilitate the three stages of tumorigenesis. Since the introduction of the first six hallmarks, there has been significant progress in the conception of cancer hallmarks, resulting in the addition of two new hallmarks: reprogramming of energy metabolism and evading immune destruction. These hallmarks were added to and have since been incorporated into a list of ten hallmarks that have gained importance in recent years (Hanahan, 2022)(Figure 2.1). These cancer hallmarks are partly fuelled by genetic heterogeneity caused by genome instability and inflammation, which increases the facilitation of numerous hallmark processes. However, the distinct characteristics commonly associated with

cancer cannot encompass the complexities of cancer pathogenesis, that is, the exact molecular and cellular pathways that enable preneoplastic cells to evolve, develop, and acquire these abnormal phenotypic capabilities during tumour development and malignant progression. (Hanahan, 2022)

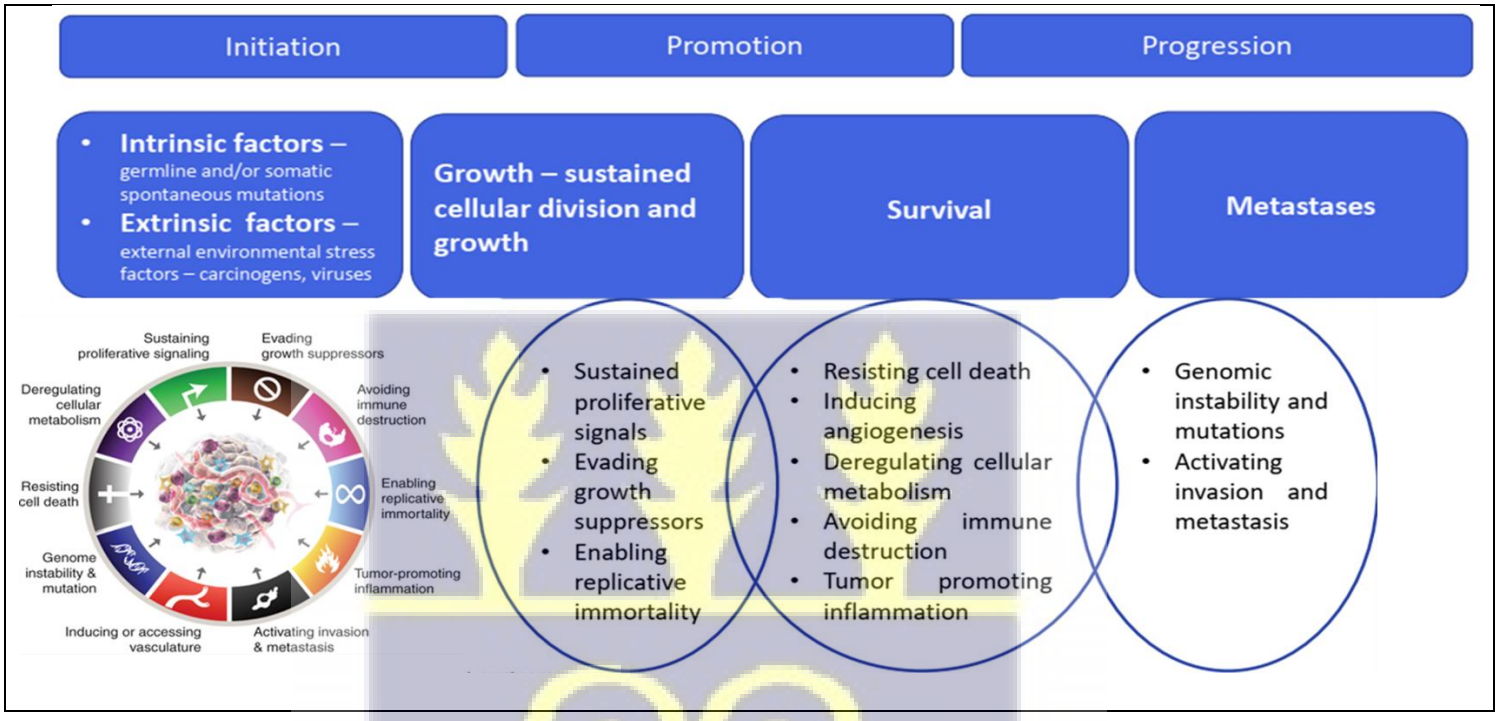


Figure 2.1: The hallmarks of cancer.
The image was adopted and modified from Hanahan, (2022)

2.3 Triple Negative Breast Cancer (TNBC) and *In Vitro* Models

TNBC is a clinically aggressive and molecularly heterogeneous subtype of breast cancer, defined by the absence of oestrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2) amplification. This lack of receptor expression renders TNBC unresponsive to endocrine or HER2-targeted therapies, resulting in limited treatment options, poor prognosis, and a high propensity for metastasis and recurrence (Conner et al., 2024; Leon-Ferre &

Goetz, 2023; Yang et al., 2022; Yin et al., 2020). To investigate the complex biology of TNBC and evaluate potential therapeutic strategies, *in vitro* models that reflect its heterogeneity are essential. Among the most widely used TNBC cell lines are MDA-MB-231 and MDA-MB-468, which represent distinct molecular and phenotypic subtypes of the disease.

MDA-MB-231 cells are classified as basal B subtype and exhibit a mesenchymal-like phenotype. These cells are highly invasive, display strong migratory capacity, and are resistant to apoptosis. They express elevated levels of mesenchymal markers such as vimentin and reduced levels of epithelial markers like E-cadherin, characteristics that are consistent with epithelial-to-mesenchymal transition (EMT). As such, MDA-MB-231 cells are frequently employed in studies investigating metastasis, drug resistance, cancer stemness, and EMT-related signalling pathways (Conner et al., 2024; Lanning et al., 2017). Their ability to form metastatic lesions *in vivo*, particularly in the lungs and brain, further underscores their utility in modelling aggressive TNBC behaviour. In contrast, MDA-MB-468 cells belong to the basal A subtype and exhibit an epithelial-like morphology. These cells are characterized by high expression of epidermal growth factor receptor (EGFR) and a heightened sensitivity to apoptosis-inducing agents. Notably, MDA-MB-468 cells are PTEN-null, resulting in constitutive activation of the PI3K/AKT signalling pathway, which is frequently dysregulated in TNBC. Due to their molecular profile, MDA-MB-468 cells are commonly used to evaluate EGFR-targeted therapies and to study apoptotic responses and cell cycle regulation (Taurin & Rosengren, 2023). Their responsiveness to EGFR inhibitors such as gefitinib and erlotinib makes them a valuable model for testing targeted therapeutic strategies.

The complementary characteristics of MDA-MB-231 and MDA-MB-468 cells provide a robust experimental platform for investigating the differential responses of TNBC subtypes to therapeutic

agents. Their combined use enables a more comprehensive understanding of TNBC biology, including mechanisms of drug resistance, EMT, cancer stemness, and signalling heterogeneity (Muthuramalingam et al., 2023). These models are instrumental in preclinical research aimed at identifying novel therapeutic targets and improving treatment outcomes for patients with TNBC.

2.4 Cell Proliferation and Proliferation Markers

According to Hanahan and Weinberg (2011), uncontrolled cell proliferation is one of the significant underlying characteristics of cancer and thus crucial to its development. Cell proliferation is a fundamental biological process involving cell growth and division. It is essential for tissue growth, development, and repair. Proliferation is tightly regulated by a series of checkpoints and signalling pathways that ensure cells divide correctly and at the right time. Dysregulation of these processes can lead to uncontrolled cell growth, a hallmark of cancer. Key regulators of cell proliferation include growth factors, cyclins, and cyclin-dependent kinases (CDKs) (Alberts et al., 2002). Growth factors bind to specific receptors on the cell surface, triggering intracellular signalling cascades that promote cell cycle progression (Yarden & Ullrich, 1988). Cyclins and CDKs form complexes that regulate the transition through different cell cycle phases, ensuring orderly cell division (Sherr, 1996).

Several molecular markers are used to assess cell proliferation in research and clinical settings. These markers are typically proteins expressed during specific phases of the cell cycle. Key proliferation markers include Ki67, proliferating cell nuclear antigen (PCNA), and minichromosome maintenance proteins (MCMs). PCNA, a protein involved in DNA replication, is another widely used marker that correlates with the S phase of the cell cycle (Miyachi et al.,

1978). MCM proteins, essential for initiating DNA replication, are also used as proliferation markers (Guzinska-Ustymowicz et al., 2009). The Ki67 protein is found in normal cells during proliferation or in the cell cycles G1 (gap 1), S (synthesis), G2 (gap 2), and M (mitosis). However, immunostaining assays show that resting differentiated cells (G0) lack Ki67, making it a reliable indicator of cell proliferation (Li et al., 2015; Sahin et al., 1994). Its expression is strongly related to cancer cell proliferation and growth. Ki67 is commonly employed as a marker for cell proliferation since it is in the nucleus and only connects to the perichromosomal layer in actively developing and dividing cells. Therefore, differences in the expression of Ki67 in proliferating malignant cells relative to healthy cells may be used as a predictor of outcomes and an early signal of therapy efficacy in cancer patients (Dowsett et al., 2007; Jones et al., 2009). Furthermore, the Ki67 labelling index has been established as an independent predictor of survival rates across all tumour stages and grades. Numerous studies have shown the predictive relevance of pKi67, indicating its potential as a reliable marker for many malignancies, including breast, large bowel, oral, and lymphoma (Dowsett et al., 2007; He et al., 2014; Jones et al., 2009; Sahin et al., 1994; Takkem et al., 2018). The results reveal that higher Ki67 expression corresponds with primary tumour size, lymphatic invasion, metastases, and tumour proliferation activity as determined by DNA flow cytometry, shorter patient survival times, and immunohistochemistry. PCNA and MCM proteins are also used to assess tumour proliferation rates and are associated with various cancers (Sahin et al., 1994; Guzinska-Ustymowicz et al., 2009). Multiple methods can be used to determine proliferative activity within tumours, including mitotic counting, flow-cytometric determination of synthesis-phase fraction, and immunohistochemistry with antibodies that react with a variety of proliferating cellular antigens (Juríková et al., 2016; Li et al., 2015). The assessment of proliferation markers can aid in selecting appropriate therapeutic strategies, such as determining

the need for aggressive treatments in patients with highly proliferative tumours (Dowsett et al., 2007).

2.5 Cell Cycle Progression and Cancer

The cell cycle is a sequential progression of cellular growth and division events. The process consists of four distinct phases: G1, S, G2, and M. During the G1 phase, cells undergo growth and prepare for DNA replication. The S phase is characterised by replicating the cell's DNA, while G2 is a phase of further development and preparation for cell division. Finally, in the M phase, the cell divides into two daughter cells through mitosis. Regulation of this process is tightly controlled by many checkpoints and proteins, including cyclins and cyclin-dependent kinases (CDKs) (Alberts et al., 2002).

Cancer is defined by the uncontrollable proliferation of cells, which frequently arises from abnormalities in the regular control of the cell cycle. Gene mutations that encode cell cycle regulators can induce disturbances, leading to a loss of cell cycle control (Hanahan & Weinberg, 2011). Crucial regulatory proteins, such as p53 and the retinoblastoma protein (Rb), are necessary to maintain the integrity of the cell cycle. Approximately 50% of human malignancies exhibit mutations in the TP53 gene, which is responsible for encoding p53. p53 is a protein that inhibits the growth of tumours by controlling the G1 checkpoint and triggering programmed cell death in response to DNA damage (Lane, 1992). Furthermore, alterations in the RB1 gene hinder the normal operation of Rb, resulting in unregulated advancement through the G1 phase (Weinberg, 1995). Cyclins and CDKs are crucial for the regulation of the cell cycle. Frequent occurrences of cyclin overexpression, specifically cyclin D1, and constant activation of CDKs are commonly

observed in many malignancies, leading to uncontrolled cell proliferation (Sherr, 1996). CDK inhibitors, such as p21 and p27, are frequently suppressed in cancer, exacerbating cell cycle disruption (Sherr & Roberts, 1999). Identifying new pharmaceuticals that specifically target cell cycle regulators has profoundly impacted the field of cancer therapeutics. Conventional chemotherapeutic medications, like paclitaxel and doxorubicin, disrupt mitosis or DNA replication in rapidly dividing cells. However, these treatments often lack specificity and can have severe side effects (Jordan & Wilson, 2004). Advancements in comprehending cell cycle control have led to the creation of targeted medicines that selectively hinder dysregulated cell cycle proteins in cancer cells, hence reducing damage to normal cells.

CDK inhibitors are a promising category of targeted pharmaceuticals. Palbociclib, ribociclib, and abemaciclib are effective in treating hormone receptor-positive breast cancer by inhibiting CDK4/6 and preventing progression from the G1 to the S phase (Knudsen & Witkiewicz, 2017). According to Finn et al. (2015), these inhibitors have improved patients' progression-free survival and are generally well-tolerated compared to traditional chemotherapy. Another potential treatment approach involves targeting the p53 pathway. Ongoing clinical trials are being conducted to examine the potential of tiny molecules like APR-246 in restoring p53 function. APR-246 could restore the function of mutant p53, leading to programmed cell death and halting the cell cycle in cancer cells (Lehmann et al., 2012). In addition, identifying MDM2 inhibitors, which hinder the breakdown of p53, has shown potential in preclinical investigations and initial-stage clinical trials (Tisato et al., 2017). In addition, ongoing research is being conducted on medications specifically targeting the mitotic checkpoint, such as inhibitors of Aurora and Polo-like kinases. These kinases are essential for the proper progression of mitosis, and suppressing their activity can lead to the

cessation and death of cancer cells (Lens et al., 2010). Alisertib, a drug that inhibits the activity of Aurora A kinase, has demonstrated its ability to suppress tumour growth in several types of cancer. This highlights the promise of using mitotic checkpoint inhibitors as a therapy for cancer (Manfredi et al., 2011).

2.6 Apoptosis

Apoptosis, or programmed cell death, is a highly selective process in multicellular organisms. Unlike necrosis (cell death due to injury), apoptosis is an orderly mechanism where the cell's contents are neatly packaged into small membrane-bound vesicles for removal by immune cells. It plays a pivotal role during normal development, eliminates potentially harmful or infected cells, and crucially maintains the delicate balance in the body. The process of apoptosis can be initiated via two main pathways: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. Understanding and harnessing the power of apoptosis is crucial in the fight against cancer.

The Bcl-2 family of proteins regulates the intrinsic pathway, including pro-apoptotic (e.g., Bax, Bak) and anti-apoptotic (e.g., Bcl-2, Bcl-xL) members. This pathway is triggered by internal signals such as DNA damage, oxidative stress, and other cellular stresses, leading to mitochondrial outer membrane permeabilisation (MOMP) and the release of cytochrome c into the cytoplasm. Cytochrome c then forms a complex with Apaf-1 and procaspase-9 to form the apoptosome, which activates caspase-9, initiating the caspase cascade that ultimately leads to cell death (Danial & Korsmeyer, 2004). The extrinsic pathway is initiated by binding extracellular death ligands (e.g., FasL, TNF-α) to their corresponding death receptors (e.g., Fas, TNFR1) on the cell surface. This

interaction leads to the formation of the death-inducing signalling complex (DISC), which recruits and activates procaspase-8. Active caspase-8 can then directly activate downstream effector caspases such as caspase-3, or it can cleave Bid, a pro-apoptotic Bcl-2 family member, to amplify the apoptotic signal via the intrinsic pathway (Ashkenazi, 2015)

The intrinsic and extrinsic pathways, two seemingly separate processes, converge at the point of caspase activation. This convergence is a crucial point, as it demonstrates the interconnectedness of these pathways and their goal: activating executioner caspases such as caspase-3, caspase-6, and caspase-7. Activating caspases, initially produced as inactive zymogens, is a critical step in apoptosis. Once activated, caspases cleave various cellular proteins, including those involved in essential cellular processes. They also dismantle the nuclear scaffold and activate DNase, an enzyme responsible for further degrading nuclear DNA. This intricate interplay of caspases contributes to the orchestrated breakdown of cellular components during apoptosis (Pfeffer & Singh, 2018; Wong, 2011; Sahoo et al., 2023). However, their activity can be inhibited by proteins from the inhibitor-of-apoptosis (IAP) family.

2.6.1 Apoptosis resistance in cancer

Cancer cells often evade apoptosis, contributing to tumour progression and treatment resistance by promoting tumour invasiveness, angiogenesis, and uncontrolled cell proliferation (Hanahan & Weinberg, 2011; Kerr et al., 1972; Pfeffer & Singh, 2018; Wong, 2011). One common strategy is for cancer cells to alter the balance between proteins that promote apoptosis (pro-apoptotic) and those that inhibit it (anti-apoptotic). Overexpression of anti-apoptotic proteins, such as Bcl-2, which prevent the initiation of the intrinsic pathway, allows cancer cells to escape cell death

signals. Its overexpression, observed in more than 60% of malignancies, presents a paradox. While elevated Bcl-2 levels shield tumour cells from intrinsic apoptotic triggers (including chemotherapy), they also fuel tumour progression and metastasis (Huang et al., 2017; Pfeffer & Singh, 2018; Perini et al., 2018).For example, Bcl-2 is frequently overexpressed in various cancers, including lymphomas and leukaemias, and is associated with resistance to chemotherapy and poor prognosis (Perini et al., 2018; Wang et al., 2017). Another mechanism by which cancer cells evade apoptosis is through the inactivation of pro-apoptotic proteins. Mutations in the TP53 gene, which encodes the tumour suppressor protein p53, are found in more than 50% of human cancers. p53 plays a critical role in inducing apoptosis in response to cellular stress and DNA damage by upregulating the expression of pro-apoptotic genes such as Bax and Puma. Loss of p53 function impairs the cell's ability to undergo apoptosis in response to genotoxic stress, contributing to cancer growth and treatment resistance (Vousden & Lane, 2007).

Caspases, critical players in apoptosis, become less effective in cancer cells. These enzymes typically initiate the breakdown of cellular components during apoptosis. Reduced caspase activity prolongs cancer cell survival. Cancer cells can upregulate inhibitors of apoptosis proteins (IAPs), which directly inhibit the activity of caspases. IAPs, such as XIAP, c-IAP1, and c-IAP2, bind to and inhibit active caspases, preventing the execution phase of apoptosis. Overexpression of IAPs is observed in various cancers and is associated with poor prognosis and resistance to apoptosis-inducing treatments (Fulda & Vucic, 2012; Liu et al., 2021). Lastly, death receptors on the cell surface trigger apoptosis when activated. Cancer cells disrupt this signalling pathway, rendering them resistant to death receptor-mediated apoptosis (Wong, 2011).

2.6.2 Therapeutic Strategies to Induce Apoptosis

Targeting the apoptotic machinery in cancer cells has emerged as a promising therapeutic strategy. One approach uses BH3 mimetics, which are small molecules that mimic the activity of pro-apoptotic BH3-only proteins. These compounds bind to and neutralise anti-apoptotic Bcl-2 family proteins, promoting the release of pro-apoptotic factors and the activation of the intrinsic pathway. Venetoclax (ABT-199) is a BH3 mimetic that specifically inhibits Bcl-2 and has shown significant efficacy in the treatment of chronic lymphocytic leukaemia (CLL) and other hematologic malignancies (Souers et al., 2013). Another strategy is activating death receptors using agonistic antibodies or recombinant ligands. For example, TRAIL (TNF-related apoptosis-inducing ligand) selectively induces apoptosis in cancer cells by binding to its receptors, DR4 and DR5. Recombinant TRAIL and agonistic antibodies targeting DR4 and DR5 are being investigated in clinical trials for their potential to induce apoptosis in cancer cells with minimal toxicity to normal tissues (Lemke et al., 2014).

Inhibition of IAPs represents another therapeutic approach. Smac mimetics are small molecules that mimic the activity of the mitochondrial protein Smac/DIABLO, which antagonises IAPs. These compounds promote apoptosis by releasing the inhibition of caspases imposed by IAPs. Several Smac mimetics are currently in clinical development and have shown promise in preclinical cancer models (Fulda, 2017). Gene therapy approaches to restore the function of tumour suppressor genes, such as TP53, are also being explored. Adenoviral vectors carrying the wild-type p53 gene have been developed and tested in clinical trials, showing some efficacy in inducing apoptosis and inhibiting tumour growth in p53-deficient cancers (Dummer et al., 2000; Osaki et al., 2000; Yen et al., 2000).

2.7 Cancer Metastasis

Cancer-related deaths are alarmingly high, primarily due to metastasis, which is the spread of cancer cells from their original site to other organs. Unfortunately, effective treatments that can halt this metastatic cascade remain scarce. With a median survival of only about a year for most cancers (Mani et al., 2024) , understanding and targeting metastasis are urgent priorities in oncology. The transformation of normal cells into malignant ones involves a multi-step process specific to each organ. Genetic and epigenetic alterations, driven by increased genomic instability, play a crucial role (Herceg & Hainaut, 2007). These changes lead to dysregulated cell cycle regulation, impaired apoptosis, and uncontrolled tissue proliferation. Moreover, cancer cells acquire the ability to migrate, invade surrounding tissues, and influence neighbouring cells and tissues. Recent data highlight the significance of specific genes and transcriptional and epigenetic regulators in cancer metastasis (Chatterjee et al., 2017; Vizoso et al., 2015; Bertucci et al., 2019; Ji et al., 2007).Targeting these regulators may hold the key to effective metastasis treatment. The stages of metastasis, according to Fontebasso & Dubinett (2015), include:

1. Escape from the Original Tumour: Invasion is the initial step, essential for propagating metastatic disease. Tumour cells interact with the stroma, either through single-cell or collective migration. The latter, although less frequent, is more effective. For metastasis to start, tumour cells must interact with stromal cells, undergo individual cell epithelial to mesenchymal transition (EMT), and undergo hybrid EMT in populations of migratory cells.
2. Intravasation: Cancer cells enter the bloodstream or lymphatic vessels, allowing them to travel to distant sites.

3. Survival Maintenance: Once in circulation, cancer cells face challenges. Some survive, while others perish due to immune responses or mechanical stress.
4. Extravasation (Seeding of a Secondary Site): Cancer cells exit the circulation and infiltrate new tissues. This step is critical for colonisation.
5. Outgrowth (Colonization): Successful colonisation leads to the establishment of secondary tumours.

Metastasis often exhibits organ selectivity. Certain tumours preferentially spread to specific organs (Chiang & Massagué, 2008). Molecular cues allow cancer cells to target specific sites for colonisation. For instance, gastrointestinal tumours readily reach the liver via the portal venous system, resulting in a high liver metastasis rate (Hess et al., 2006). Lung, breast, and melanoma tumours frequently metastasise to the brain, followed by renal-cell and colorectal carcinomas. Current prognosis and treatment decisions rely on molecular characterisation and tumour biology (Malone et al., 2020; Zhang et al., 2020). Regrettably, medications that target metastatic pathways often impose severe long-term effects on patients' quality of life. However, emerging as a beacon of hope, cancer stem cells (CSC) are promising therapeutic targets. Their pivotal role in metastasis, residual disease, and recurrence and their unique ability to self-proliferate and spread throughout the body make them a logical and compelling focus for intervention (Ghadially et al., 2022). As previously discussed, the metastatic cascade is a complex series of interdependent and sequential sub-processes. Among these, the EMT plays a significant role. It is a process that targets EMT and cancer cell migration and invasion, and it holds immense potential in our understanding of cancer metastasis.

2.8 Epithelial to Mesenchymal Transition (EMT)

EMT is a process where cell connections are disrupted in cancer cells within the primary tumour, leading to a change in cell structure from epithelial to fibroblastoid morphology(Serrano-Gomez et al., 2016). This process triggers a phenotypic change in epithelial cells, transforming them into mesenchymal cells that can migrate and invade neighbouring tissues (Chen et al., 2017; Serrano-Gomez et al., 2016; Wei et al., 2015). The EMT process is regulated by specific genes that control various cellular activities such as cell migration, breakdown of the extracellular matrix, DNA repair, and angiogenesis (Puisieux et al., 2014; Ruscetti et al., 2015). The biomarkers of EMT, including E-cadherin, α-SMA, ZEB, TWIST, vimentin, and Snail (SNAI1) (Jonckheere et al., 2022; Zeisberg & Neilson, 2009) are expressed via the TGF, SMAD, and PI3K signalling pathways (Maharati & Moghbeli, 2023). E-cadherin (CDH1) plays a critical role in inhibiting tumour transformation and proliferation by strengthening the stability of cell-cell adhesion in epithelial cells (Pećina-Šlaus, 2003). EMT is characterised by the downregulation of epithelial markers, specifically E-cadherin, and the upregulation of mesenchymal markers, including vimentin and N-cadherin. EMT transcription factors (EMT-TFs), including SNAIL, SLUG, and TWIST, are involved in suppressing the transcription of CDH1, which promotes EMT through epigenetic and post-translational mechanisms (Serrano-Gomez et al., 2016; Soini et al., 2011). During the process of EMT, cells undergo several significant changes. These include the breakdown of tight junctions, the restructuring of the cytoskeleton, and the acquisition of migratory and invasive capabilities. Epithelial cells, which typically exhibit clear apical-basolateral polarity and are attached to a basement membrane by specialised junctions, transform into mesenchymal cells that lack polarity and do not arrange into organised layers due to their focal adhesions with neighbouring cells. EMT leads to increased migratory ability, invasiveness, resistance to

programmed cell death, and enhanced production of extracellular matrix (ECM) components, highlighting the urgency and importance of understanding this process in the context of cancer metastasis.

EMT can be classified into three distinct forms. Type I EMT occurs during embryogenesis, Type II EMT is involved in wound healing, and Type III EMT is related to the advancement of cancer (Serrano-Gomez et al., 2016). Type III EMT increases the ability of cells to invade surrounding tissues and spread to distant sites, which has a detrimental effect on the prognosis and survival of individuals. Cancer cells exhibiting the EMT phenotype possess more sophisticated anti-apoptotic mechanisms and exhibit higher resistance to treatment(Williams et al., 2019; Xu et al., 2022). EMT can be triggered by several chemokines and transcription factors, such as CXCL8, CCL2, CXCL6/CXCR1/2, and CXCL1/LCN2, which activate pathways like Wnt/β-catenin, PI3K/AKT, and Src signalling (Do & Cho, 2020; Kudo-Saito et al., 2013; Liu et al., 2019; Lu et al., 2019; Sarvaiya et al., 2013). NF-κB is associated with the process of epithelial-mesenchymal transition (EMT) produced by CCL5 and CCL18 (Do & Cho, 2020). EMT enhances drug resistance by inhibiting apoptosis and prolonging the ageing process (senescence) (Xu et al., 2022).

A novel notion called partial EMT has arisen, wherein cells display a combination of epithelial and mesenchymal traits. Cancer cells can transition between two states, known as epithelial-mesenchymal plasticity. This transition allows them to move between EMT and mesenchymal-epithelial transition (MET), resulting in a "metastable" state. Hybrid epithelial-mesenchymal (EM) phenotypes display a combination of epithelial and mesenchymal characteristics. Early hybrid EM cells exhibit decreased adhesion and a rounded form, while late hybrid EM cells show more

noticeable mesenchymal markers and an elongated shape (Canciello et al., 2022; Jolly et al., 2015; Williams et al., 2019).

While all EMT biomarkers are relevant to cancer progression and metastasis, TWIST, Snail, and Vimentin are highlighted in this study because of their key roles in regulating EMT. TWIST and Snail act at the transcriptional level, while Vimentin functions structurally, making them strong indicators of mesenchymal transition and metastatic potential in cancer cells. These markers are strongly linked to breast cancer metastasis, especially in aggressive forms like triple-negative breast cancer (TNBC), which this study examined (Jacob et al., 2024; Jørgensen et al., 2020; Zhou et al., 2018).

2.8.1 TWIST

The TWIST protein family comprises TWIST1 and TWIST2, which have analogous structures and activities. TWIST1 is crucial in advancing cancer, functioning as a pivotal transcription factor for EMT. Empirical data indicates that TWIST1 can be controlled through transcriptional and post-translational mechanisms. The N-Myc and c-Myc proteins directly interact with the TWIST1 promoter, activating its transcription, specifically in neuroblastoma (Liu et al., 2021). Additional transcription factors, such as Sox12 and Sox13, can stimulate the expression of TWIST1, resulting in the occurrence of EMT and the spread of cancer cells to other areas of the body, particularly in hepatocellular carcinoma (HCC) (Feng et al., 2020). Several signalling pathways, such as STAT3, Akt/PKB, Ras, MAPK, and Wnt, influence TWIST expression and regulation (Norozi et al., 2016). TWIST functions as a downstream effector during the EMT process, playing a vital part in the molecular cascades responsible for driving EMT. In glioblastoma multiforme (GBM), there is a

significant overlap between the genes controlled by TWIST1 and those associated with cancer metastasis. This suggests a potential connection between TWIST1-mediated EMT in carcinomas and GBM (Liu et al., 2022; Mikheeva et al., 2010). In addition, TWIST can downregulate downstream genes by blocking the function of positive regulators through interactions between proteins (Serrano-Gomez et al., 2016).

One way in which TWIST causes EMT is via interacting with the histone methyltransferase (HMT) MMSET, sometimes referred to as WHSC1 or NSD2. MMSET can add two or three methyl groups to histone H3 at lysine 36 (H3K36). MMSET is highly expressed in solid tumours and is linked to a worse outcome. MMSET interacts with the TWIST promoter, leading to an increase in methylation at H3K36me2. This, in turn, activates TWIST and facilitates the evolution of prostate cancer (Ezponda et al., 2013). TWIST may induce EMT by downregulating the expression of E-cadherin (CDH1) and upregulating N-cadherin in oesophagal squamous cell carcinoma. Moreover, the prevalence of N-cadherin over CDH1 involves TWIST overexpression, and it causes upregulation of vimentin (Khales et al., 2022). TWIST expression has predictive value in colorectal, cervical, oesophageal, and lung cancers (Fattahi et al., 2021; Wang et al., 2015; Yeo et al., 2017; Zeng et al., 2015).

2.8.2 Vimentin

Vimentin, a protein belonging to the type III intermediate filament family, is predominantly found in mesenchymal cells and serves a crucial function in offering structural and functional support to cells. It functions as a mechanical transducer that connects integrins on the cell surface to cellular organelles, perhaps regulating cell migration via controlling cell adhesion (Ivaska et al., 2007). Vimentin is primarily found in the cytoplasm but can also be observed in the nucleus, which

interacts with other nuclear proteins and DNA. Recent studies have discovered many missense and frameshift mutations in vimentin linked to the advancement and spread of malignancies(Satelli & Li, 2011).

The level of vimentin expression substantially rises when cancer advances and infiltrates adjacent tissues. Overexpression of this gene is a characteristic feature of cancer metastasis, making it an essential biomarker for type-3 EMT(Mendez et al., 2010). Although the activities of vimentin are well-known, the precise mechanisms by which it contributes to various cellular processes are still being studied. Throughout the process of EMT, vimentin plays a role in controlling key transcription factors involved in inducing EMT, such as Snail, Slug, Twist, and ZEB1/2. Vimentin also influences important epigenetic modifiers throughout this process. Additionally, it has a function in suppressing cellular differentiation and augmenting the pluripotent capacity of cells, hence heightening the stemness of cancer stem cells and facilitating the spread of tumours and their resistance to treatments (Chaffer et al., 2016).

Vimentin is a downstream mediator of Snail or Slug-induced EMT, promoting cell motility. The vimentin/extracellular signal-regulated kinase (ERK) pathway promotes the attraction and addition of phosphate groups to Slug, a crucial step in the initiation of EMT (Virtakoivu et al., 2015). In addition, the connection between vimentin and Scrib, a protein essential for cell migration, safeguards vimentin from proteasomal degradation. This, in turn, maintains the levels of vimentin, enhances cell migration, and preserves cell polarity and proliferation (Pallari & Eriksson, 2006). It also facilitates the formation of new blood vessels where cancer has spread (metastatic niche) by activating calpains and signalling through NOTCH. Calpains cleave the

amino-terminal of vimentin, resulting in the production of soluble vimentin. This soluble vimentin helps move membrane type 1-matrix metalloproteinase (MT1-MMP) to the cell membrane, promoting angiogenic sprouting (Eckes et al., 2000). Initial results indicate that released vimentin may possess qualities like a ligand and interact with receptors such as NKp46 on natural killer cells. However, additional investigation is required to clarify these interactions (Kim et al., 2009).

Ultimately, vimentin plays a crucial role in maintaining the structural stability of cancer cells during their movement and facilitating their ability to invade and spread to other body parts. Although it plays a crucial role in promoting EMT and the subsequent advancement of cancer, there is still a significant need for continuous research to comprehend the intricate processes by which it operates entirely.

2.8.3 Snail family; SNAI1 and SNAI2

The Snail family of transcription factors, specifically SNAI1 (Snail) and SNAI2 (Slug) have a vital function in the process of EMT, which is critical for the spread and advancement of cancer. These factors are stimulated by many signalling pathways, including TGF-β, receptor tyrosine kinases (RTKs), Wnt, NOTCH, BMPs, and TNF (De Craene & Berx, 2013). When Snail factors are activated, they suppress the expression of CDH1, causing the breakdown of connections between cells and promoting cell movement. These effects are characteristic of EMT (Thiery et al., 2009). Snail factors have multiple functions, including their involvement in cellular differentiation and survival. They have anti-apoptotic effects and regulate the cell cycle through cyclin D/p21 pathways (Peinado et al., 2003). The intracellular distribution and abundance of Snail proteins are rigorously controlled through post-translational changes, including phosphorylation by large tumour suppressor kinase 2 (Lats2) or glycogen synthase kinase 3β (GSK3β) (Zhou et al., 2004).

Research has shown that decreased Snail expression reduces tumour growth and invasiveness in animal models of cutaneous squamous cell carcinomas. In contrast, there is a direct relationship between high levels of Snail expression and unfavourable prognosis and an increased likelihood of metastasis in patients diagnosed with ovarian, head, and neck carcinomas (Olmeda et al., 2007). Snail also impacts critical processes in embryonic development and tumour progression, including apoptosis, angiogenesis, and the production of *MMP9* (Serrano-Gomez et al., 2016). Notably, the nuclear presence of Snail is detected in a considerably lower proportion of patients with different types of tumours, such as breast cancer. For instance, the presence of nuclear Snail expression has been documented in 29% of endometrioid carcinomas and 23-38% of instances involving ovarian tumours (Blanco et al., 2002). High levels of SNAI1 expression in breast cancer are linked to tumours relapse, the spread of cancer to other parts of the body, and a reduced period without relapse, emphasising its crucial function in the advancement of cancer (Ye et al., 2015). Like ZEB1, Snail inhibits the transcription of CDH1 by attaching to E-boxes in the CDH1 promoter. It can also work together with histone methyltransferases (HMTs) and DNA methyltransferases (DNMTs) to regulate the expression of CDH1 (Batlle et al., 2000).

Snail and Slug regulate TGF-β-induced EMT in oral squamous cell cancer. According to Hirakawa et al., (2005) Snail increases the activity of *MMP2* and *MMP9* to start the EMT process. On the other hand, Slug and Snail sustain EMT over a long period by promoting the production of *MMP9*. The correlation between Snail expression and prognosis in hormone-sensitive ovarian cancer has produced inconclusive findings, highlighting the necessity for additional research (Kurrey et al., 2009). The suppression of GSK-3β increases Snail and decreases CDH1, initiating the EMT

programme (Zhou et al., 2004). In addition, SNAI1 is involved in regulating the clonogenicity, stemness, and invasiveness of prostate cancer (PCA) cells with low levels of CDH1. Increased SNAI1 expression is frequently linked to the initial development of prostate cancer and is limited to cells that can invade surrounding tissues (Ruscetti et al., 2015).

2.9 Migration and Invasion

The metastatic cascade comprises three interdependent and sequential sub-processes: invasion, intravasation, and extravasation, emphasising the crucial role of cancer cell migration and invasion in metastasis (Martin et al., 2013; Wu et al., 2021). Cancer cells' ability to invade depends on their motility, influenced by alterations in cell shape triggered by dynamic changes in actin polymerisation and cytoskeletal reorganisation. The invasive growth of tumours begins when cancer cells detach from the primary mass. This process involves a decrease in cell-cell adhesion and an increase in motility, allowing them to breach the dense structures of the surrounding stroma. This detachment is aided by reduced cell-cell adhesion, which permits the cells to produce matrix metalloproteinases that degrade the basal membrane and invade the extracellular matrix. Following the invasion of the surrounding tissue, tumour cells move through the basement membrane and extracellular matrix, entering either the lymphatic or blood vessels (intravasation), travelling through the circulatory system, and subsequently exiting into new tissues (extravasation), where they form secondary tumours (Krakhmal et al., 2015; Martin et al., 2013). Cancer cells migrate via several strategies, including individual migration in mesenchymal or amoeboid modes and collective migration as cell groups. Tumour cell migration is classified into individual and collective types based on cell junctions' morphology and genetic characteristics, the actin cytoskeleton, matrix adhesion, and protease activity. Specifically, migrating tumour cells can

exhibit mesenchymal (fibroblast-like) or amoeboid-like properties, each associated with distinct morphological traits and molecular mechanisms. Single-cell invasion, a form of invasive growth, occurs as individual tumour cells independently penetrate surrounding tissues, characterised by a breakdown of intercellular connections and dispersal as single cells. It is noted that these cells may switch between mesenchymal and amoeboid migration styles in response to environmental factors, reflecting adaptability to different molecular cues. Moreover, migrating tumour cells show a higher resistance to chemotherapy and radiotherapy compared to non-motile cells, attributed to their reduced cell division and heightened activity of anti-apoptotic genes, which shields them from treatments aimed at triggering cell death (Krakhmal et al., 2015; Martin et al., 2013; Wu et al., 2021). Tumour cell migration and invasion are essential for cancer metastasis and are regulated by enzymes and adhesion molecules. Matrix Metalloproteinase-2 (MMP2) and Matrix Metalloproteinase-9 (MMP9) are matrix metalloproteinases that degrade the basement membrane, facilitating cancer cell invasion into surrounding tissues. Vascular Cell Adhesion Molecule-1 (VCAM-1), an endothelial adhesion molecule, promotes tumour cell attachment to endothelial cells, aiding their entry into the bloodstream. Collectively, these molecules support the structural and adhesive changes required for metastatic spread (Jiang & Li, 2021; Zhang et al., 2020).

2.9.1 MMP2, MMP9 and VCAM-1

Stochastic genetic mutations, epigenetic changes within cancer cells, and a dynamic and adaptable tumour microenvironment drive the metastatic process. Distinct genes have critical roles during different phases of metastasis, which can be classified as initiation, progression, and virulence. These genes include vascular cell adhesion molecule-1 (*VCAM-1*) and matrix metalloproteinases (MMPs) such as *MMP9* and *MMP2*. Every gene group has a distinct function at various stages of

metastasis, providing distinctive benefits that impact the dissemination and target selection of cancer cells. Genes associated with metastatic progression facilitate the migration and establishment of cancer cells in remote locations. On the other hand, the genes linked to the start and severity of the disease are engaged in both the initial and final phases of the initial tumour's invasion and the development of secondary tumour sites (Chiang & Massagué, 2008).

MMPs are a group of 24 enzymes that rely on zinc for their activity. They are responsible for modifying the extracellular matrix (ECM) and are involved in various critical biological processes, including embryonic development, reproduction, angiogenesis, bone formation, wound healing, and cell movement. Nevertheless, MMPs also play a role in the development of pathological illnesses such as arthritis, inflammation, vascular problems, cancer, and metastasis (Kang & Jang, 2014; Vandooren et al., 2013). MMPs are crucial in breaking down different cell adhesion molecules and controlling connections between cells and the extracellular matrix (ECM). These proteins are crucial for the invasion of cells as they degrade various extracellular matrix proteins, allowing cancer cells to move and infiltrate neighbouring tissues. MMPs have a role in breaking down physical barriers that occur during the growth of tumours, as well as the process of cancer cells entering and exiting blood vessels and invading distant sites (Kang & Jang, 2014; Martin et al., 2013).

MMP2 and *MMP9* are often elevated in several types of human cancers and exhibit a synergistic effect in promoting cancer invasion and progression. They are involved in cancer angiogenesis by breaking down ECM components and activating angiogenic factors such as VEGF and TGF-β in various types of cancer. This includes activating latent TGF-β in a CD44-dependent manner, which

is critical for tumour growth and invasion (Quintero-Fabián et al., 2019; Sounni et al., 2011). MMP9, also known as gelatinase B or 92 kDa type IV collagenase, is primarily linked to the dissemination of cancerous cells from primary tumours to metastatic sites in several forms of human cancer. It is instrumental in modifying stromal cells, restructuring the extracellular matrix, transitioning from epithelial to mesenchymal phenotypes, cell migration, angiogenesis, and metastasis (Augoff et al., 2022; Kang & Jang, 2014). Similarly, MMP2, a 72-kDa type IV collagenase or neutrophil gelatinase, is encoded on chromosome 16 and participates in various cellular functions, including degrading type IV collagen, a crucial component of basement membranes. MMP2 is also active in menstruation, blood vessel formation, tissue repair, inflammation, and bone remodelling. It significantly impacts cancer progression, facilitating cancer cell invasion and enhancing angiogenesis (Aimest & Quigley'llll, 1995; Ali et al., 2021; Quintero-Fabián et al., 2019).

Preliminary studies indicate that the angiogenic factor IL-8 stimulates the production and function of MMP2 in melanoma cells, increasing their ability to invade surrounding tissues (Qazi et al., 2011; Quintero-Fabián et al., 2019). Experiments utilising selective inhibitors of MMP2 in mice with bladder cancer have shown a clear connection between MMP2 expression and increased support from the surrounding tissue, formation of new blood vessels, invasiveness of the tumour, and its progression. Increased levels of *MMP2* have been associated with higher expression of VEGF in gastric cancer, highlighting its crucial role in the advancement of cancer by breaking down the extracellular matrix, forming blood vessels in tumours, and facilitating metastasis (Quintero-Fabián et al., 2019). A recent study examined the impact of suppressing the *MMP2* in both normal and MCF-7 breast cancer cells exposed to radiation. This was done using MMP2

shRNA expression plasmids (pMMP-2). The findings demonstrated that pMMP-2 exerted a protective effect on human normal dermal fibroblasts (HDFs) while rendering MCF-7 cells more vulnerable to DNA damage caused by ionising radiation (Shailender et al., 2019). The contrasting reactions observed in normal cells and MCF-7 cells when the *MMP2* gene is suppressed, mainly when DNA damage occurs, could be explained by the production of reactive oxygen species. Moreover, *in vitro* investigations have indicated that combining temozolomide with MMP2 inhibitors may decrease the capacity of cancer cells to invade and survive (Gabelloni et al., 2010). The THBS2/MMP signalling pathway has been identified as a potential target for future clinical research. This is because microRNA-93-5p, involved in tumour progression, targets thrombospondin-2 (THBS2), a gene associated with the extracellular matrix, and *MMP2* and *MMP9* (Sun et al., 2019). Therefore, this pathway is relevant for studying cancer treatment.

In addition to MMPs, VCAM-1 is a crucial gene that plays a role in the invasion and migration of metastatic cells. VCAM-1, a 90-kDa glycoprotein belonging to the immunoglobulin gene family, is mainly found in active vascular endothelium (Wang et al., 2014). The primary purpose of VCAM-1 is to assist in transporting white blood cells to areas of inflamed tissue by attaching to integrin α4β1 on T lymphocytes. The cleavage of membrane-bound VCAM-1 by ADAM domain-containing enzymes, specifically ADAM17, ADAM8, and ADAM9, produces the soluble form of VCAM-1 (sVCAM-1). This soluble form is chemotactic for T cells and monocytes and stimulates angiogenesis in endothelial cells (VanHeyst et al., 2022; Wang et al., 2014; Wu, 2007). VCAM-1 has several functions in guiding the development of tumours, creating a favourable environment for metastasis, and promoting the formation of new blood vessels (Schlesinger & Bendas, 2015; Zhang et al., 2020). During migration, cancer cells form extensions of their cell membrane called

pseudopodia and invadopodia, which are rich in actin. These extensions help the cancer cells attach to the endothelium and withstand the physical stresses exerted by blood flow. VCAM-1 promotes these processes, especially in colorectal cancer cells (Zhang et al., 2020). The Oncomine database, a comprehensive microarray database for cancer research, has demonstrated notable overexpression of VCAM-1 in multiple cancer types, such as brain, breast, ovarian, renal clear cell, and oesophageal carcinomas (Rhodes et al., 2004). Notably, the presence of VCAM1 has also been linked to the reaction of tumours to chemotherapy; this highlights the potential of VCAM-1 as a molecular target for the treatment of cancer (Jacamo et al., 2014; Tas et al., 2014; VanHeyst et al., 2022). Moreover, VCAM-1 can serve as a biomarker for diseases such as clear cell renal cell carcinoma (RCC) (Wang et al., 2014). VCAM-1 exhibits a robust association with crucial genes in the epithelial-mesenchymal transition (EMT) pathway, suggesting its role in governing this biological process. Recent research indicates that VCAM-1 interacts with VLA4 on endothelial cells, enhancing the adhesion of tumour cells, facilitating their movement through blood arteries, and promoting the development of brain metastases in breast cancer. In addition, this contact generates an inflammatory environment (Zhang et al., 2020). Recent research has demonstrated that cancer-associated fibroblasts (CAFs) could secrete VCAM-1. Subsequently, this protein induces the rapid multiplication and ability to spread cancerous cells by initiating the AKT and MAPK signalling pathways, specifically within lung cancer cells (Zhou et al., 2020). Moreover, the upregulation of VCAM-1 is associated with activating many signalling pathways, including PI3K/Akt, NF-κB, and Src (Lin et al., 2015). Consequently, thoroughly assessing the significant adverse effects that may occur is crucial, including life-threatening conditions, when considering the use of chemotherapeutic agents in combination with VCAM-1 inhibitor to reduce metastasis, angiogenesis, and lymph node spread in cancer patients (Schlesinger & Bendas, 2015).

Considering these results, which emphasise the substantial potential of VCAM-1 as a focus for molecular intervention in cancer therapy, it is imperative to do additional studies on the role of VCAM-1 in cancer progression.

2.10 Cancer Stem Cells (CSC) and Stemness markers

Stemness refers to a cell's ability to continue its lineage, produce specialised cells, and maintain a balance between dormancy, growth, and renewal. This property is observed in both embryonic and adult stem cells, while CSCs exhibit similar behaviour but with a malignant nature (Pardal et al., 2003; Reya et al., 2001). Stem cells are distinguished by their stemness properties, specifically their ability to self-renew and differentiate into various lineages (Jiang et al., 2024; Liu et al., 2016). In a pathological context, the CSC hypothesis suggests that a subset of tumour cells capable of self-renewal is responsible for tumour persistence. This hypothesis explains clinical phenomena such as treatment resistance, tumour dormancy, and metastasis, defining cancer stemness as the stem-cell-like phenotype of cancer cells (Jordan & Guzman, 2004). CSCs can self-renew and generate all cell types within a tumour from a single clone, often termed tumour-initiating cells (TICs) and have the capacity to switch between stem and non-stem states (Al-Hajj et al., 2004; Clevers, 2011).

These stem-like properties enable CSCs to sustain disease progression and adapt to their environment, highlighting the crucial role of stemness in cancer cell proliferation and survival (Reya et al., 2001). Although normal stem cells and CSCs share some similarities, CSCs uniquely initiate and maintain cancer. Their persistence post-treatment is due to their stem cell characteristics, essential for cancer development and survival (Bao et al., 2006). The formation and maintenance of CSCs' stem-like properties are influenced by genetic and epigenetic factors

within cancer cells and elements from the tumour microenvironment (Mani et al., 2008). Recent studies indicate that CSC stemness is linked to their adaptability and plasticity rather than solely the EMT process (Polyak & Weinberg, 2009). CSCs exhibit cellular plasticity, enabling them to switch between epithelial and mesenchymal-like states and reverse differentiation into progenitor cells (Chaffer et al., 2013). This plasticity allows CSCs to adapt and survive therapeutic interventions and the dynamic challenges within the tumour microenvironment during progression. CSCs also display genetic mutations and epigenetic modifications that can disrupt signalling pathways involved in embryonic development (Mani et al., 2008).

CSCs are often innately resistant to various chemotherapeutic agents due to their relatively inactive and slow-dividing nature and the presence of multidrug resistance mechanisms, such as ATP-binding cassette (ABC) transporters, antiapoptotic proteins like B-cell lymphoma 2 (BCL2), and enhanced DNA repair mechanisms (Dean et al., 2005). Several dysregulated stemness pathways have been identified in cancer, contributing to CSCs' inherent resistance. For example, the transcription factor STAT3 is activated in many malignancies, playing a critical role in tumour growth and metastasis (Marotta & Polyak, 2009). Gastric CSCs with hyperactive STAT3 show increased resistance to docetaxel. CSCs also express various cell surface proteins, including CD34, CD44, CD24, and CD133, and transcriptional factors such as SOX2, NANOG, OCT4, and SALL4. Additionally, CSCs express other proteins like ALDH, BMI1, Nestin, and CXCR (Forghanifard et al., 2021; Lathia & Liu, 2017; Wei et al., 2022).

These markers, with tissue-specific expression patterns, aid in distinguishing CSCs from the rest of the cancer population. For example, CD44, CD24, and ALDH are unique to breast cancer; CD34

and CD8 to leukaemia; CD133 to colon cancer; CD44 to head and neck cancer; and CD90 to liver cancer (Al-Hajj et al., 2004). In hepatocarcinogenesis, $CD24^+$ HCC cell lines and patient-derived $CD24^+$ HCC cells show enhanced sphere formation, stemness gene expression, and tumorigenicity compared to $CD24^-$ counterparts (Lee et al., 2011). In breast cancer, the $CD24^-CD44^+$ signature is associated with a mesenchymal state with increased vimentin expression, while the ALDH+ signature indicates an epithelial state. Consequently, CD44/CD24 and ALDH1 are commonly used as CSC markers in breast cancer (Ginestier et al., 2007).

2.10.1 CD44 and Cancer Stemness

CD44 is a cell surface glycoprotein that mediates contacts between cells, promotes cell adhesion, and facilitates cell migration. It functions in multiple physiological processes, including activating lymphocytes, facilitating their movement, and guiding them to specific locations. Additionally, it is associated with a diverse array of cancer types. CD44 is present in many isoforms produced through alternative splicing, with the standard form (CD44s) and variant isoforms (CD44v) being the primary focus of cancer research (Ponta et al., 2003). CD44 is commonly linked to stem cell-like characteristics in cancer, such as increased ability to divide, resistance to programmed cell death, and the propensity to start tumour growth. CD44+ cells have been detected in breast cancer, where they display features of CSCs, including the capacity to generate mammospheres in a laboratory setting and initiate tumour growth in mice with weakened immune systems (Al-Hajj et al., 2004). These cells are furthermore linked to heightened metastatic capability and unfavourable prognosis in several types of malignancies, such as breast, colon, and pancreatic cancers (Dalerba et al., 2007).

2.10.2 CD24 and Cancer Stemness

CD24 is a small cell membrane protein connected to many cancer cells' surfaces via glycosyl-phosphatidylinositol. Initially identified as a heat-resistant antigen in mice, it distinguishes between hematopoietic and neural cells (Pirruccello & LeBien, 1986; Springer et al., 1978). The diverse glycosylation of CD24 is involved in multiple cellular processes across different cell types, while certain aspects remain incompletely understood. CD24's unique glycosylation allows it to act as a versatile ligand in various cells, including cancer cells, influencing cell adhesion, metastasis, and other physiological processes (Baumann et al., 2005; Fang et al., 2010). CD24 demonstrates elevated expression in multiple cancer types, such as ovarian, breast, prostate, bladder, renal, and non-small cell carcinomas; hence, CD24 can potentially be a significant marker for the prognosis and diagnosis of tumours (Kristiansen et al., 2003). CD24 enhances cancer cells' growth and their capacity to adhere to fibronectin, collagen, and laminin (Aigner et al., 1997). This highlights its importance as a predictive factor and a groundbreaking indicator for CSCs.

***2**.10.3 The Role of CD44/CD24 in Cancer Progression*

In tumour initiation and CSC inhibition, regulating CD44 and CD24 expression emerges as a promising avenue. CD44/CD24 is a commonly used CSC marker in breast cancer. Nevertheless, their manifestation is not consistently uniform, even within the same subtype of breast cancer. High CD44/CD24 ratios have also been strongly associated with cell proliferation and the development of tumours (Li et al., 2017). The $CD44^{+}/CD24^{-}$ phenotype is particularly relevant in breast cancer, where it has been shown to correlate with more aggressive disease and poorer prognosis. These cells are not only capable of driving primary tumour growth but also contribute to metastasis, the leading cause of cancer-related mortality. Studies have demonstrated that

$CD44^{+}/CD24^{-}$ cells are more resistant to chemotherapy and radiotherapy, likely due to their stem cell-like properties, including efficient DNA repair mechanisms and active drug efflux pumps (Camerlingo et al., 2014; Ponti et al., 2005; Yin & Glass, 2011). Notably, a positive correlation exists between vimentin expression and the CD44/CD24 ratio in the breast cancer stem cell population, and the experimental model has demonstrated CD44's ability to impede tumour growth and metastasis (Dehbokri et al., 2024). Additionally, targeting CD44 function has shown efficacy in reducing leukemic stem cells in acute myeloid leukaemia(Jin et al., 2006). Hence, considering CD44 as a therapeutic target for solid tumours expressing this molecule seems plausible. Antibodies and small molecules targeting CD44 have been developed, showing promise in preclinical studies by reducing tumour growth and metastasis (Jaggupilli & Elkord, 2012). Disrupting the interaction between CD44 and its hyaluronic acid ligand can inhibit tumour progression and metastasis (Toole, 2004). Furthermore, CD24, another marker, also functions as a cancer stem cell in ovarian and colorectal cancers. Although CD24 is classified as an epithelial marker, its expression varies across cancer types (Jaggupilli & Elkord, 2012). CD44 and CD24 play pivotal roles in cancer progression, influencing invasion, metastasis, and therapy resistance. Understanding their roles in cancer stemness provides valuable insights into cancer biology and highlights potential therapeutic targets. The $CD44^{+}/CD24^{-}$ phenotype is a robust marker for identifying CSCs in breast cancer and other malignancies, and targeting these markers could improve treatment outcomes by eradicating the cells responsible for tumour initiation, growth, and metastasis.

2.11 Chemokines, Cytokines and Cancer Metastasis

The cancer's inflammatory microenvironment is a complex and diverse interaction involving various cells, substances, enzymes, and signalling pathways. Chemokines and cytokines play a crucial role in the intricate process of tumour growth (Mantovani et al., 2008). Inflammatory insults trigger the activation of inflammatory responses, leading to increased production of non-specific proinflammatory cytokines such as IL-1α/β, IL-6, interferon (IFN)-α, and tumour necrosis factor (TNF)-α (Grivennikov et al., 2010). Subsequently, these cytokines play a pivotal role in triggering the production of proinflammatory chemokines, thereby setting off a cascade effect in cancer metastasis. Evidence suggests that persistent chronic inflammation is associated with an elevated rate of normal cells transforming into preneoplastic foci (Coussens & Werb, 2002).

Chemokines, a type of cytokine, are key players in cancer biology. They can attract cells and regulate their movement across tissue boundaries. They also control the placement and interactions between cells within tissues (Balkwill, 2004). They are classified into four subfamilies—CXC, CC, CX3C, and C—based on the number and position of conserved cysteine (C) residues near the N-terminus. In CC chemokines, the first two cysteines are adjacent (e.g., CCL2, CCL5); in CXC chemokines, they are separated by one amino acid (e.g., CXCL8, CXCL12). CX3C chemokines have three amino acids between the first two cysteines (e.g., CX3CL1), while C chemokines have only one conserved cysteine (e.g., XCL1). Ligands are denoted as L (e.g., CCL2: CC ligand 2), and their corresponding receptors are denoted with an R. The respective receptors (CXCR, CCR, CX3CR, and XCR) are G protein-coupled and found on immune and tumour cells. The standardized nomenclature (e.g., CXCL12 for ligand, CXCR4 for receptor) clarifies chemokine-receptor interactions in cancer processes (Wu et al., 2023).

Importantly, chemokines and cytokines guide the host's response to cancer by controlling the movement of white blood cells into the tumour microenvironment (Zlotnik et al., 2011). These proteins are present in both tumour and stromal cells. Melanoma cells, for instance, release many chemokines, such as CXCL1−3 / MGSA (Melanoma growth stimulatory activity)-α, β, and γ / GRO (Growth-regulated oncogene)-α, β, and γ, CXCL8, CCL5, and CCL2. These chemokines are crucial in promoting the proliferation and spread of melanoma cells (Dhawan & Richmond, 2002). The melanoma cells also possess chemokine receptors, including CXCR4, CXCR2, CXCR3, CCR7, and CCR10 (Weninger et al., 2003). Imbalanced chemokine signalling in the tumour microenvironment (TME) stimulates tumour growth while suppressing immune cells and promoting the buildup of immunosuppressive cells (Oppenheim & Yang, 2005). The redundancy in their chemokine receptors ensures appropriate biological responses to stress, as specific receptors can bind to multiple chemokines (Rot & Von Andrian, 2004).

2.11.1 Chemokines and Cancer

Chemokines are low molecular weight proteins (8-14 kDa) that were first discovered for their ability to guide leukocytes to sites of inflammation. Nevertheless, they are acknowledged for a wide range of functions beyond inflammation, including controlling tumour cell characteristics and activities such as forming new blood vessels, generating new nerve cells, and developing fibrous tissue (Balkwill, 2003). "Classical" chemokine receptors are G-protein-coupled transmembrane receptors (GPCRs) that stimulate cell migration by binding to chemokines. This binding activates cellular responses and frequently leads to directional migration or chemotaxis. On the other hand, "atypical" chemokine receptors (ACKRs) do not interact with G proteins, yet they nevertheless assist in cell migration (Moser et al., 2004; Nibbs & Graham, 2013).

CXCL1, a highly studied CXC chemokine, plays a crucial role in inflammatory disorders and functions as an angiogenic chemokine (Richmond, 2002). CXCL1 promotes cancer cell proliferation, migration, and metastasis. It plays a critical role in Th17 cell differentiation, reactive oxygen species (ROS) generation, and neutrophil extracellular trap (NET) creation (Acharyya et al., 2012; Wang et al., 2016; Yuan et al., 2016). During inflammatory reactions, the expression of CXCL1 is increased, which stimulates the formation of new blood vessels (angiogenesis) and attracts neutrophils as a chemotactic factor (Schraufstatter et al., 2001). The expression of CXCL1 is stimulated by carcinogens, resulting in the development of chronic inflammation and the growth of tumours (Wang et al., 2006). It is highly expressed in numerous types of malignancies and is crucial in promoting the growth and spread of cancer cells. The chemokines CXCL1 and CXCL2 promote resistance to treatment and are associated with an unfavourable prognosis. The efficiency of HER2-directed therapy is enhanced by inhibiting CXCR1 and CXCR2, which are receptors for IL8 (Singh & Lokeshwar, 2011).

CXCL2, alternatively referred to as stroma-derived factor 1 (SDF-1) and macrophage inflammatory protein 2 (MIP-2), functions as a chemical signal that attracts neutrophils (Baggiolini et al., 1997). CXCL12 is the only known molecule that binds to CXCR4 and CXCR7. It is produced by macrophages, endothelial cells, epithelial cells, and tumour cells (Sarris et al., 2012). The interaction between CXCL2 and CXCR2 on endothelial cells plays a role in controlling tumour growth and angiogenesis, as reported by Addison et al., (2000). By binding to CXCR2, it has a vital function in facilitating the movement of neutrophils from the bone marrow to areas of inflammation (Eash et al., 2010). CXCL1 and CXCL2 expression in tumours is linked to the

presence of suppressive cells and resistance to therapy (Acharyya et al., 2012). After chemotherapy, NF-kB activity in breast cancer increases the expression of CXCL1 and CXCL2. This rise attracts myeloid-derived suppressor cells (MDSCs) to the tumour microenvironment (TME), which in turn promotes the survival of cancer cells and their resistance to chemotherapy (Soria & Ben-Baruch, 2008). CXCL1/2 promotes chemoresistance in glioblastoma by attracting MDSCs and in lung cancer by attracting neutrophils that infiltrate the tumour (Hu et al., 2021; Yuan et al., 2016).

CCL2, also known as macrophage chemotactic protein-1 (MCP-1), stimulates inflammation, angiogenesis, and the proliferation of tumours (Rollins, 1997). The expression of this gene is significantly elevated in both breast tumour and stromal cells. It directly influences tumour growth by promoting the formation of new blood vessels (angiogenesis) and attracting monocytes, a kind of immune cell involved in inflammation and tissue repair. Increased levels of CCL2 might hinder the release of IFN-γ by T cells, which weakens the body's ability to fight against tumours and facilitates the growth of tumours (Salcedo et al., 2000; Steiner & Angela Murphy, 2012). CCR2, the primary receptor for CCL2, triggers the influx of monocytes and the onset of inflammation. Hepatocytes produce CCL2, which attracts myeloid suppressor cells that express CCR2 in different tumours. This process promotes tumour growth (Chun et al., 2015; Qian et al., 2011). CCL2 has been shown by Fang et al. (2015) to deleteriously impact cellular processes such as autophagy and necrosis in luminal B breast cancer cells. The interaction between CCL2 and CCR2 may play a role in developing resistance to immune checkpoint treatment (Fei et al., 2021).

The expression of CCL5 is positively associated with the advancement of tumours in various types of cancer, including breast cancer (Aldinucci & Colombatti, 2014). Tumour-derived CCL5 promotes the entry of lymphocytes into tumours, which can either enhance the immune response against the tumour or attract T cells that support tumour growth (Luboshits et al., 1999). The chemokine CCL5 selectively attracts mast cells expressing the CCR3 receptor in Hodgkin's lymphoma and monocytes expressing the CCR1 receptor in mammary tumours. Inhibiting the CCR1 receptor using an antagonist has been shown to reduce tumour growth (Aldinucci et al., 2010). The interaction between CCL5 and CCR5 triggers apoptosis in tumour-infiltrating cells (Chang et al., 2012). Gastric tumour cells acquire invasiveness by interacting with mononuclear cells, in which CCL5 plays a crucial role (Aldinucci & Casagrande, 2018).

2.11.2 Cytokines and Cancer

Granulocyte colony-stimulating factor (G-CSF) is an inflammatory protein that attracts neutrophils with immunosuppressive qualities, promoting cancer cell metastasis to distant locations (Benna et al., 2020). Interleukin-6 (IL-6) and prostaglandin E2 (PGE2), inflammatory mediators, have been linked to activating crucial pathways that promote cancer cells' growth, survival, and metastasis. The enzyme DNA methyltransferase 3B (DNMT3B) regulates these pathways (Benna et al., 2020; So et al., 2020). Cancer-associated fibroblasts (CAFs) and tumour-associated macrophages (TAMs) secrete IL-6 and trigger the activation of signal transducer and activator of transcription 3 (STAT3), which promotes EMT and resistance to the chemotherapy drug paclitaxel. This process occurs through the IL-6/JAK2/STAT3 pathway, as demonstrated by Wang et al., (2018). IL-6 and PGE2 are critical in modulating the tumour microenvironment. IL-6, released by CAFs TAMs, triggers the activation of STAT3. This activation leads to the initiation of EMT and strengthens

the resistance to chemotherapy drugs by regulating the production of genes that promote cell survival. PGE2 also stimulates pathways that promote the survival and growth of cancer cells. Both IL-6 and PGE2 demonstrate how cancers might exploit inflammatory mediators to facilitate their proliferation and metastasis (So et al., 2020; Wang et al., 2018). The IL-6/JAK2/STAT3 pathway, a significant player in EMT, increases cells' migratory and invasive capacities. EMT is also associated with acquiring stem cell-like characteristics, contributing to developing chemotherapy resistance. However, understanding the molecular mechanisms of EMT provides a promising avenue for devising approaches to combat metastasis and enhance the effectiveness of chemotherapy, offering hope for improved cancer treatments in the future.

Th1 and Th2 cytokines have a substantial impact on the tumour microenvironment. Th1 cytokines, such as IFN-γ and IL-2, enhance the body's immune response against tumours, while Th2 cytokines (IL-4, IL-5, IL-10, IL-13) are associated with the spread and invasion of tumours. In advanced cancer, there is commonly a transition from Th1 to Th2 cells, accompanied by a decline in Th1 cell-mediated immunity. This decline is linked to the advancement of cancer. The change is driven by TGF-β and IL-10 generated by the tumour, which decreases Th1 responses (Abdul-Rahman et al., 2024; Garo & Gopal, 2020). TGF-β, released by cells implicated in inflammation, exerts a substantial influence on the initiation and progression of tumours. It promotes angiogenesis, induces cellular transition towards invasiveness, aids in immune evasion by cancer cells, and inhibits apoptosis. It is prevalent in breast, prostate, and epithelial ovarian cancers, where it controls the expression of VEGF and MCP-1 to facilitate the growth of blood vessels in tumours (Ma et al., 2007; Zhang et al., 2021; Zu et al., 2012) . Monocytes secrete IL-6, which works with STAT proteins to facilitate tumour growth in various cancer types by inhibiting

apoptosis and generating reactive oxygen and nitrogen species (Hu et al., 2024; Moldogazieva et al., 2018; Qu et al., 2013; Zhang et al., 2020).

Tumour necrosis factor-alpha (TNF-α) controls signalling pathways related to inflammation, promoting epithelial-mesenchymal transition (EMT), cell proliferation, and angiogenesis (Aggarwal et al., 2006; Landskron et al., 2014). Abnormal TNF-α expressions have been detected in several forms of cancer (Cruceriu et al., 2020; Yang et al., 2011). Despite TNF-α's cancer-promoting properties, it has been employed in cancer treatments because of its capacity to reduce tumour growth (Balkwill, 2009; van Horssen et al., 2006). Interferons (IFNs), classified as categories I, II, and III, regulate inflammatory responses and influence the metabolic processes, proliferation, and programmed cell death (apoptosis) of cancer cells (Franco et al., 2017; Ivashkiv & Donlin, 2014). IFN-α, a type I IFN, exhibits potent anti-tumour properties and has been utilised in clinical interventions for cancer (Bekisz et al., 2013). Chemokines can either promote or inhibit the development of new blood vessels (angiogenesis) by affecting chemicals that encourage angiogenesis, such as VEGF and bFGF, and drawing immune cells to the tumour's surrounding environment (Keeley et al., 2011; Raman et al., 2007). The pro-inflammatory cytokines IL-2 and IL-12 have demonstrated anti-cancer capabilities, making them helpful for promoting specific anti-cancer responses (Atkins et al., 1999; Fyfe et al., 1995; Lucas et al., 2002; Rodrigo-Garzón et al., 2010).

2.12 Currently used drugs for cancer treatment

Cancer chemotherapy has been a fundamental aspect of cancer therapy for many years, including the administration of cytotoxic medications to eradicate or impede the proliferation of cancerous cells. Although chemotherapy has made considerable strides in enhancing survival rates for different types of cancer, it also poses significant obstacles, such as drug resistance, unfavourable side effects, and its impact on patient's quality of life. Chemotherapeutic medicines can be categorised extensively according to their modes of operation. The primary classifications comprise:

1. Alkylating Agents: Drugs like cyclophosphamide and cisplatin introduce alkyl groups into DNA, forming cross-links and breakage. This process hinders the replication and transcription of DNA (Chabner & Roberts, 2005; Konstantinov & Berger, 2008).
2. Antimetabolites, such as methotrexate and 5-fluorouracil, imitate the substances utilised in regular cellular metabolism, disrupting the production of DNA and RNA (Longley et al., 2003).
3. Topoisomerase inhibitors, like doxorubicin, daunorubicin, and idarubicin, hinder DNA unwinding by inhibiting topoisomerase enzymes. This action effectively stops DNA replication and transcription (Pommier, 2013).

Chemotherapy is employed as a therapeutic approach for many types of cancers, either as a sole treatment or in conjunction with other methods like surgery, radiation therapy, and immunotherapy. Cisplatin-based chemotherapy is a widely accepted and established treatment for testicular cancer, which has been shown to enhance the chances of survival significantly (Einhorn, 1981). Furthermore, the effectiveness of combination therapy, such as CHOP (cyclophosphamide,

doxorubicin, vincristine, and prednisone) has been demonstrated in treating non-Hodgkin lymphoma (Fisher et al., 1993).

Although chemotherapy has achieved notable accomplishments, its efficacy might be constrained by various circumstances, such as the emergence of drug resistance and severe adverse effects, tumour heterogeneity and CSC populations.

2.13 Therapeutic Challenges in Chemotherapy and Strategies to Overcome these Challenges

Cancer cells can develop various mechanisms to escape the cytotoxic effects of chemotherapy, such as increased drug efflux, enhanced DNA repair, and evasion of apoptosis (Holohan et al., 2013). For example, the efflux pump P-gp overexpression can reduce intracellular drug concentrations in some cancer cells like breast cancer, leading to resistance to drugs like paclitaxel (Gottesman et al., 2002). While targeting cancer cells, chemotherapy also affects rapidly dividing normal cells, leading to side effects, including myelosuppression, mucositis, alopecia, and cardiotoxicity (Nurgali et al., 2018). These side effects can significantly impact patients' quality of life and limit the dose and duration of chemotherapy that can be administered. Intra-tumoral heterogeneity poses another challenge, as different subpopulations of cancer cells within the same tumour can respond differently to chemotherapy. This heterogeneity can lead to incomplete eradication of the tumour and relapse (Dagogo-Jack & Shaw, 2018). Lastly, CSCs are often more resistant to conventional chemotherapy and can lead to tumour recurrence and metastasis (Reya et al., 2001).The persistence of CSCs, usually more resistant to traditional chemotherapy, remains a critical challenge in the quest for long-term remission. This emphasises the need for continued research and development in this area.

Several strategies have been proposed to help overcome chemotherapy challenges. These include,

1. Combination Therapy: Using multiple drugs with different mechanisms of action can help overcome resistance and improve efficacy. For example, combining chemotherapy with targeted therapies or immunotherapies has shown promise in enhancing treatment outcomes (Baudino, 2015).
2. Precision Medicine: Tailoring chemotherapy based on the genetic and molecular profiles of the tumour can improve efficacy and reduce side effects. Advances in genomic sequencing and biomarkers are enabling more personalised treatment approaches (Meyerson et al., 2010).
3. Nanotechnology: Nanoparticle-based drug delivery systems can enhance the delivery of chemotherapeutic agents to tumours, improving their therapeutic index and reducing systemic toxicity (Shi et al., 2017).
4. Targeting CSCs: Research is ongoing to develop therapies targeting CSCs, such as inhibitors of key signalling pathways involved in CSC maintenance and survival (Nassar & Blanpain, 2016).

In conclusion, cancer chemotherapy remains a vital component of cancer treatment, offering significant benefits in terms of survival and disease control. However, the challenges of drug resistance, adverse side effects, tumour heterogeneity, and the presence of CSCs highlight the need for continued research and innovation. Advances in combination therapy, precision medicine, nanotechnology, and CSC-targeted treatments hold promise for overcoming these challenges and improving the efficacy and safety of chemotherapy.

2.14 Conventional Cancer Drug Discovery Pipeline and its Challenges

The conventional cancer drug discovery pipeline is a thorough and arduous procedure encompassing the entire process, from identifying the initial target to conducting preclinical research and clinical trials and ultimately obtaining regulatory approval. Although numerous anticancer medications have succeeded, the conventional method is burdened with various difficulties, such as unreasonable expenses, lengthy durations, elevated rates of failure, and intricate biological processes.

The initial stage of the cancer drug discovery pipeline involves identifying and validating a biological target, usually a protein or gene associated with the advancement of cancer. Genomics, proteomics, and bioinformatics are utilised to identify possible targets (Wang et al., 2004). After identifying a target, high-throughput screening (HTS) is conducted to search through extensive chemical libraries to find "hits" that exhibit activity against the target (Macarron et al., 2011; Roy, 2012). The hits are further enhanced by applying medicinal chemistry techniques to produce "lead" molecules with increased potency, selectivity, and pharmacokinetic features. During the lead optimisation stage, lead compounds undergo significant chemical modifications to improve their effectiveness, decrease toxicity, and enhance their pharmacokinetic and pharmacodynamic properties. During this phase, the studies on structure-activity relationship (SAR) play a vital role. Lead compounds undergo preclinical testing involving *in vitro* assays and *in vivo* animal investigations to evaluate their safety, effectiveness, and pharmacological characteristics. Additionally, this stage includes toxicological investigations to assess possible adverse effects (Andrews & Reynolds, 2017). Promising preclinical candidates progress to clinical trials with three distinct phases. Phase I studies primarily evaluate a new drug's safety and appropriate dosage. Phase II trials are conducted to assess the effectiveness of the therapy and any potential adverse

effects. Phase III trials involve comparing the new drug to existing conventional therapies in larger groups of patients (Zanders, 2020). This process is characterised by a significant investment of time and financial resources (DiMasi et al., 2003). Following the successful completion of clinical trials, a New Drug Application (NDA) is submitted to regulatory agencies, such as the FDA, to obtain approval. This phase entails a comprehensive examination and can last multiple years (FDA, 2019).

One significant obstacle in conventional drug discovery is the substantial incidence of failure, where numerous compounds still need to pass preclinical testing or clinical trials due to ineffectiveness or harmful side effects (Arrowsmith, 2011); only a minute proportion of initial discoveries advance to be authorised pharmaceuticals. The drug research and development process is characterised by lengthy timelines and substantial costs, often spanning more than ten years and requiring billions of dollars. The requirement for thorough testing, regulatory compliance, and the high failure rate are the main factors contributing to the high costs (DiMasi et al., 2003, 2016). Cancer is a disease that exhibits a great degree of variation and complexity due to intricate biological processes. The complicated nature of cancer poses difficulties in pinpointing optimal targets and formulating medications that can efficiently combat various cancer kinds and subtypes (Dagogo-Jack & Shaw, 2018). Conventional preclinical models, such as 2D cell cultures and animal models, cannot precisely anticipate human reactions to novel medications. This disparity leads to an elevated failure rate in clinical trials (Hutchinson & Kirk, 2011). Lastly, the strict regulatory criteria for ensuring safety and effectiveness present substantial challenges, frequently resulting in delays in the approval and introduction of drugs into the market (FDA, 2019).

Given the challenges associated with the conventional drug discovery pipeline, there is an increasing need for innovative approaches to improve efficiency, reduce costs, and enhance success rates. Computer-aided drug discovery (CADD) has emerged as a powerful tool in addressing these challenges.

2.15 Computer-Aided Drug Discovery (CADD)

CADD has transformed the pharmaceutical industry using computational methods such as network pharmacology, molecular docking, molecular dynamics (MD) simulations, Quantitative structure-activity relationship (QSAR) and network analysis to simplify finding and improving new medicinal molecules. This has led to a significant decrease in the time and expense required for medication development. Essential methods in CADD include structure-based drug design (SBDD), which uses molecular docking and MD simulations to forecast how drugs would bind and their binding affinity. This approach has been successfully applied in developing HIV-1 protease inhibitors (Baassi et al., 2023; Jenwitheesuk & Samudrala, 2003; Yoosefian et al., 2022). Ligand-based drug design (LBDD) utilises established ligands to identify novel candidates through methodologies like QSAR models, making a substantial contribution to developing inhibitors/agonists for many microorganisms and diseases such as Mycobacterium tuberculosis, Staphylococcus aureus, and cancer (Adeniji & Adalumo, 2020; Mkhayar et al., 2024; Ye et al., 2023). Structure-based drug design (SBDD) involves the creation of new molecules using information about the target site. This approach has led to the discovery of novel dihydrofolate reductase inhibitors (Bohacek et al., 1996; Gehlhaar et al., 1995). Virtual screening, which includes both structure-based and ligand-based methods, has played a crucial role in the discovery of kinase inhibitors for the treatment of cancer (Shoichet et al., 2002). Significant achievements in CADD

include the creation of Imatinib for chronic myeloid leukaemia using structure-based drug design (SBDD) (Nagar et al., 2002), the enhancement of Ritonavir as an HIV protease inhibitor (Vacca et al., 1994), and the invention of Oseltamivir for influenza by simulating neuraminidase (Kim et al., 1998). However, there are difficulties in accurately forecasting the drug-likeness and ADMET features; the progress in machine learning and artificial intelligence holds the potential to improve the efficiency and predictive capability of CADD, hence speeding up the drug discovery process (Zhavoronkov et al., 2019). CADD remains highly influential in discovering new drugs, providing inventive and economically efficient methods for producing novel treatments.

CHAPTER THREE

3.0 Objective 1: Determination of the most potent compound and *in silico* prediction of pharmacokinetics properties and molecular targets.

a) To determine the cytotoxic effect of three small-molecule compounds on a panel of four cancer cell lines and a normal cell line.

b) To predict the pharmacokinetics properties of the hit compound using ADMETlab and SwissADME.

c) To determine the hit compound's plausible target(s) using network pharmacology, molecular docking, and molecular dynamic simulation.

3.1 Rationale for Objective 1

The traditional drug discovery process is linear and lengthy, presenting several challenges, such as poor pharmacokinetic properties, lack of specificity, and unexpected adverse side effects. This requires years and significant financial investment to identify lead compounds to develop commercially feasible drugs (DiMasi et al., 2003, 2016). Ongoing efforts have been made to seek efficient and timely anticancer drug development procedures that can significantly impact global health and life expectancy (Cui et al., 2020). A chemical molecule must have optimal pharmacokinetic properties to be approved as a standard drug to ensure its safe absorption, distribution, metabolism, and elimination from the body. However, many potential anticancer drugs fail in clinical trials owing to poor pharmacokinetic properties, lack of specificity, and adverse side effects (DiMasi et al., 2016). In addition, many drugs have off-target effects and interact with proteins that are not intended to cause side effects (Kim et al., 2015). This lack of specificity limits the therapeutic potential of a drug and contributes to its adverse effects(Anand et al., 2023). In response, strategies such as the "network target, multi-component", which applies cutting-edge technologies such as network pharmacology, molecular docking, and MD simulation, have been adopted approach (Lin et al., 2019; Wang et al., 2015). These technologies promise to change the drug discovery process and efficiently solve traditional barriers.

3.2 Abstract

The traditional drug discovery process for anticancer drugs is hindered by prolonged screening and off-target effects. To address these challenges, a computer-aided drug design was employed to evaluate lead compounds' pharmacological properties and molecular interactions as potential anticancer agents. This study aimed to determine the anticancer potential of three compounds and predict the likely targets of the lead compound using computer-aided drug discovery processes. Three synthetic compounds were tested for cytotoxicity against four cancer cell lines. Using the STRING database, protein-protein interactions were analysed, and functional enrichment analyses were performed using Metascape. Molecular docking, dynamic simulations, pharmacological ADME properties, and drug-likeness properties were assessed using GOLD, Amber 22, ADMETLab, and SwissADME software packages respectively. Compound 2 demonstrated significant potency among the compounds, with IC_{50} values of 9.46 µM and 18.3 µM against MDA MB 468 and MDA MB 231 cell lines, respectively. It also showed remarkable selectivity against PNT2 cells. Pathway analysis associated Compound 2 with cancer and focal adhesion pathways, identifying epidermal growth factor receptor (EGFR), AKT serine/threonine kinase 1 (AKT1), and vascular endothelial growth factor receptor 2 (VEGFR2) as potential targets. Molecular docking revealed that Compound 2 specifically binds to the ATP-binding activation site at Lys745 and the DFG motif at Asp855 of EGFR, as well as the ATP binding site of VEGFR2 at Cys919 and Phe918. Molecular dynamic simulations indicated that EGFR had the most energetically stable protein-ligand interactions, followed by VEGFR2 and AKT1. EGFR also showed the most flexible amino acid residues and strong hydrogen bond interactions. The BOILED-Egg analysis identified Compound 2 as a substrate of p-glycoprotein and a blood-brain barrier permeant with good oral

bioavailability. These results highlight the potential of Compound 2 as a targeted anticancer therapeutic, with EGFR emerging as the most promising therapeutic target.

3.3 Introduction

Cancer remains a daunting global health concern despite significant advances in medical and pharmaceutical research. In 2020, cancer, as reported by the World Health Organisation (WHO), stood as the second most prevalent cause of worldwide mortality, resulting in around 10 million deaths and accounting for about one in every six deaths (World Health Organisation, 2022). Despite advances in medical and pharmaceutical cancer research, the health crisis is still persistent. Existing treatment options for cancer include surgery, chemotherapy, radiotherapy, hormonal therapy, immunotherapy, and targeted therapies. Chemotherapy is the most widely used, alone or combined with other approaches. Despite the notable success of chemotherapy, several challenges, such as systemic toxicity, drug resistance, off-target effects, and the heterogeneity of cancer cells, are all pressing concerns (Galmarini, 2020; Maleki et al., 2023). These challenges emphasise the critical need for ongoing research to uncover safer and more effective treatment options.

Network pharmacology and MD simulations have become vital approaches to discovering new cancer drugs. These approaches help investigate the connections between biological elements to comprehensively understand diseases and identify the biological pathways, genes, and protein sets influenced by drugs (Azmi, 2012; Huang et al., 2009), as well as aid in the identification of new bioactive compounds and predict their pharmacological actions (Dai et al., 2015; Rajpoot et al., 2022; Usman et al., 2023). This method allows for more informed decisions when selecting targets, predicting mechanisms of action, and optimising drug design (Chandran et al., 2017). Success stories such as imatinib (Gleevec) and other potential kinase inhibitors development for chronic myeloid leukaemia highlight the practical applications of MD simulations (Malkhasian & Howlin, 2019; Nagar et al., 2002). MD simulations have also shed light on the potency of 16beta-hydroxy-

19s-vindolinine N-oxide and aknadicine from traditional Chinese medicine as inhibitors of BRAF for treating malignant melanoma (Tang & Chen, 2015). Potential *Leishmania donovani* Sterol Methyltransferase inhibitors have been identified using MD simulations (Sakyi et al., 2023). MD simulations have also been used to design HIV-1 protease inhibitors (Baassi et al., 2023; Jenwitheesuk & Samudrala, 2003; Yoosefian et al., 2022) and discover other potential antiviral drugs for diseases such as influenza and SARS-CoV-2 (Kawsar et al., 2022; Nichols et al., 2011; Peele et al., 2020). Furthermore, MD simulations have been employed to study the interactions of drugs with membrane proteins such as epidermal growth factor receptor (EGFR) (Cavasotto et al., 2006; Kumar et al., 2023; Prabhavathi et al., 2022), which are frequently challenging targets. Understanding the dynamics of these interactions aids the development of drugs that can successfully navigate cell membranes and reach their targets.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that plays a vital role in cell growth, proliferation, and survival; its dysregulation has been linked to a variety of cancers, including non-small cell lung cancer and squamous cell carcinoma (Cowley et al., 1986; Vealel et al., 1987). EGFR activates signalling pathways, including the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways (Bjorge et al., 1990; Gotoh et al., 1995). These pathways control essential cellular functions, and their abnormal activation contributes to cancer progression. Molecular docking, network pharmacology, and systems biology can all be used to find new compounds that target EGFR. At the same time, MD simulations allow researchers to study the dynamic interactions between drugs and EGFR at the molecular level. This allows a thorough understanding of the binding mechanisms and potential changes to improve drug efficacy. This study aimed to discover a potential anticancer compound through *in vitro* cytotoxicity screening

of three compounds that have been previously identified to have antileishmanial properties (Sakyi et al., 2023) in four human cancer cell lines. The study aimed to employ a systematic computational approach to identify the lead compound's pharmacological properties and targets. Additionally, the objective was to explore the dynamic behaviour and structural changes occurring in the potential target(s) when complexed with the lead compound.

3.4: Methods

3.4.1: Cancer Cell Lines and Cell Culture

PC3 prostate cancer, DLD-1 colorectal cancer, MDA-MB-231, and MDA-MB-468 TNBC cell lines were obtained from ATCC. The standard human prostate cell line, PNT2, was generously provided by the Department of Chemical Pathology division at the Noguchi Memorial Institute for Medical Research (NMIMR). MDA-MB-231, MDA-MB-468 and PC3 cancer cell lines were cultured in DMEM, while DLD-1 and PNT2 were cultured in RPMI 1640. All media were enriched with fetal bovine serum (10%) and penicillin-streptomycin-glutamine (1%) (Gibco-life Technologies, Carlsbad, CA, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO2.

3.4.2: Chemical Compounds

Compounds 1, 2, and 3 (Figure 3.1) were purchased from Vitas M lab, and DOX (D1515-10MG) was purchased from Sigma-Aldrich, St Louis, MO, USA).

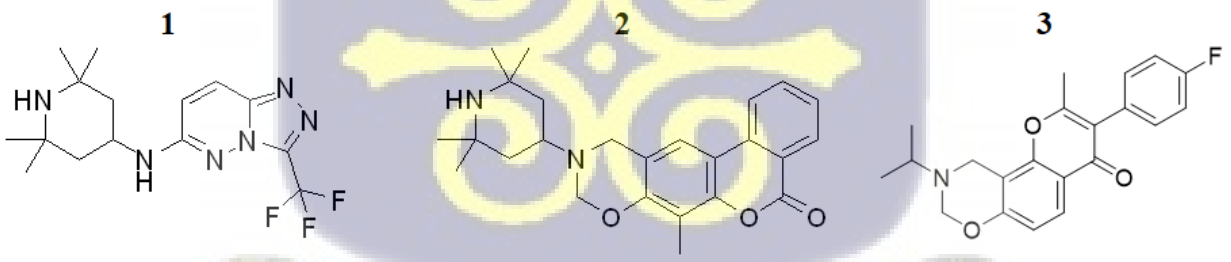


Figure 3.1: The molecular structure of the three small molecule compounds used in the anticancer screening.

3.4.3: Cytotoxicity Assay and Selectivity Index

The effects of the three compounds on cell viability were determined using MTT assay (Wang et al., 2019). Briefly, the cells were plated in 96-well plates at 1×10^4 cells/well density and incubated at 37 °C for 24 hours. The cells were treated with the compounds (0-100 µM) for 48 h. DOX (0-15 µM) was used as a positive control. In each well, 20 µL of 2.5 mg/mL (MTT) (Sigma-Aldrich, St Louis, MO, USA) was added and incubated at 37 °C for 4 hours. Subsequently, acidified isopropanol (100 µL) was added to each well and incubated at 37 °C for 30 minutes. The absorbance was measured at 570 nm using a microplate reader (Varioskan™ LUX multimode, Thermo Fisher Scientific, Carlsbad, CA, USA). The percentage of cell viability was determined based on the absorbance values, and IC_{50} values were later computed in Graphpad Prism. The selectivity index (SI) of the most potent compound was analysed following the same procedure using PNT2 normal prostate cells in a concentration range ten times more than the concentration ranges used in the four cancer cell lines (graph shown in Appendix A, figure A1). The CC_{50} of PNT2 was calculated, and the SI was computed with a good selectivity classified as SI >3 (Duarte et al., 2022). The IC_{50} values of the compounds were determined from three independent experiments, each conducted in triplicate.

$$SI = \frac{CC_{50} \text{ non-cancerous cell line}}{IC_{50} \text{ cancer cell line}}$$

3.4.4: Cancer-related genes, compound 2 target prediction and Protein-protein interactions (PPI)

Genes associated with 36 human cancers were obtained from the DisGENET (https://www.disgenet.org/, accessed on 8 February 2023) (Piñero et al., 2020) and GeneCards (https://www.genecards.org/, accessed on 8 February 2023) (Stelzer et al., 2016) databases. For the GeneCards database, a cut-off score >4 was used to obtain the most probable genes. This threshold is consistent with network pharmacology criteria, ensuring the biological reliability of the selected gene set while minimizing the inclusion of low-confidence or weakly associated genes (Muthuramalingam et al., 2023). The putative targets of compound 2 were acquired from SwissTargetPrediction (http://www.swisstargetprediction.ch/, accessed on 9 February 2023) (Daina et al., 2019) and SuperPred (https://prediction.charite.de/, accessed on 9 February 2023) (Gallo et al., 2022) webservers. The targets were converted into standardised gene names based on the UniProt database (https://www.uniprot.org/, accessed on 9 February 2023) (The UniProt Consortium, 2021), and duplicates were removed. The cancer-related genes and targets of compound 2 were intersected with a Venn diagram using the FunRich v3.1.3 software (Pathan et al., 2015) to obtain all its cancer-related targets. The cancer-related targets of compound 2 were uploaded into the STRING database (https://string-db.org/, accessed on 9 February 2023) (Jensen et al., 2009), where an interactive network was generated using "*Homo sapiens*" as the screening condition. The protein-protein interaction network was loaded into Cytoscape software v3.9.1 (Shannon et al., 2003), where the core targets of compound 2 were identified using the CytoHubba (Chin et al., 2014) and MCODE (Bader & Hogue, 2003) plugins. Specifically, the PPI network was analysed using nine algorithms in CytoHubba, and targets that ranked in the top 20 in at least five of the nine algorithms were identified. The MCODE plugin was used to group the targets into sub-clusters, and targets that belonged to the top three sub-clusters were identified. Finally, the

targets identified from the CytoHubba rankings belonging to at least one MCODE sub-cluster were selected as core targets.

3.4.5: Functional Enrichment Analyses

The Metascape web server (https://metascape.org/gp/index.html, accessed on 13 February 2023) (Zhou et al., 2019) performed functional enrichment analyses of core compound 2 targets. The targets were annotated based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. In GO functional analyses, the functions of the targets were categorised into biological process, cellular component, and molecular function. The KEGG pathway plot was created through enrichment analysis.

3.4.6: Molecular Docking

From the KEGG pathway analysis, the significantly enriched pathways were identified. The three core proteins likely to be involved in these pathways were selected from the identified hub genes. Molecular docking was performed using the selected proteins**.** The active compound was docked to the crystal structure of EGFR (PDB: 8A27, 1.07 Å (Obst-Sander et al., 2022), AKT1 (PBD:6HHJ, 2.30 Å, (Uhlenbrock et al., 2019)), and VEGFR2 (PDB:4ASE, 1.83 Å (McTigue et al., 2012)) which were obtained from the Protein Data Bank (PDB) (Berman et al., 2003; Berman et al., 2000). The Scigress version FJ 2.6 program prepared all crystal structures for docking. This included adding hydrogen atoms and removing co-crystallized ligands. The centre of the binding pockets, *x*yz for each protein, are shown in Table 2, with a radius of 10Å. The GoldScore (GS) (G. Jones et al., 1997) and ChemScore (CS) (Eldridge et al., 1997; Verdonk et al., 2003), ChemPLP (Korb et al., 2009) and Astex statistical potential (ASP) (Mooij & Verdonk, 2005). Using the

GOLD v5.4 software suite, scoring functions were used to validate the ligands' predicted binding modes and relative energies. Initially, the co-crystallized ligand of each protein was docked, and root mean square deviation (RMSD) values for the heavy atoms were computed. The average RMSDs for each co-crystallized ligand for ASP, PLP, CS, and GS highlight the scoring functions' predictive solid capability. Detailed information on RMSD and binding scores can be found in Appendix A, Tables A1 and A2.

3.4.7: Molecular Dynamics (MD) Simulations

MD simulations were performed for the three proteins: EGFR, AKT, and VEGFR2). The MD simulation was performed using the Amber 22 software (Case et al., 2005; Salomon-Ferrer et al., 2013; Weiner & Kollman, 1981). The most feasible ligand configuration was employed in setting up the simulation. Initially, the ligand was prepared using an Antechamber to compute atomic point charges using the AM1-BCC charge model. The system was configured using the Leap program. The ligand and protein were subjected to GAFF and ff14SB force fields, respectively. The appropriate number of ions were added to each protein to neutralise receptor charges. The three-point transferable intermolecular potential (TIP3P) model with a 10 Å water solvate box was used to solvate the systems, eventually buffered to 150 mM. The non-bonded interaction cutoff value was set at 8.0 Å. Subsequently, the system was gradually heated to 300 K, with receptor atom constraints applied at 50 ps intervals. This was followed by a 50 ps equilibration period to attain density equilibrium. The system was then equilibrated under NPT conditions for 500 ps at a pressure of 1 atm and a temperature of 300 K. Finally, a 10-ns production stage was carried out. The Berendsen barostat and Langevin thermostat were used to keep pressure and temperature under control. Furthermore, with a time step of 0.002 ps, the shake algorithm restricted all

hydrogen-involved bonds. The CPPTRAJ module was used for trajectory analysis, and VMD facilitated visualisation. To compute the binding free energy of the receptor complex, both the MM-PBSA and MM-GBSA methods were employed. The RoG and maximum RoG (RoGmax), RMSD, and RMSF were analysed to assess the structural flexibility and stability. Lifetime hydrogen bond analysis was also computed.

3.4.8: Drug-likeness and ADMET studies

The pharmacokinetics, structural, and physicochemical characteristics of compound 2 were predicted using ADMETlab 2.0 (https://admetmesh.scbdd.com/, accessed on April 18, 2023)(Xiong et al., 2021) and SwissADME (http://www.swissadme.ch/, accessed on April 18, 2023) (Daina et al., 2017). To evaluate its potential for absorption in the human gastrointestinal (GI) tract and its ability to traverse the blood-brain barrier (BBB), the Egan BOILED-Egg (Brain Or IntestinaL EstimateD) permeation predictive model was utilised. Furthermore, these methodologies yielded predictions regarding the ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of compound 2.

3.4.9: Statistical and data analysis

GraphPad Prism 9.3 was used to analyse cytotoxicity data and generate all the graphs. The Kruskal-Wallis test was used for statistical analysis. To determine statistical significance, p-value < 0.05 was used. The cytotoxicity tests were carried out in triplicate with three biological replicates. Mean ± SEM was used to represent IC_{50}. The molecular dynamics data was analysed using Python 3.12.0. RoG, RoG max, RMSD, and RMSF values were shown as Mean ± SD. The MM-PBSA and MM-PBSA free energies were presented as Mean ± SEM.

3.5: Results

3.5.1: Cytotoxicity assay of compounds and selectivity index.

The impact of three small-molecule compounds on cell proliferation was assessed in various cancer cell lines and a normal cell line, with DOX as the reference anticancer drug. The goal was to determine the bioactivity of the compounds in cancer cells and evaluate the potential adverse effects of the compounds on normal cells. MDA-MB-468 and MDA-MB-231 breast cancer cell lines were susceptible to compound 2 in a dose-dependent manner ($p = 0.0011$ and $p = 0.008$, respectively) with IC_{50} of 9.46 ± 0.80 µM and 18.30 ± 1.10 µM for MDA MB 468 and MDA MB 231 respectively. Colorectal cell line DLD1 (p-value= 0.03) and prostate cell line PC3 (p-value =0.01) also showed mild susceptibility to compound 2 with IC_{50} of 40.58 ± 1.80 µM and 46.72 ± 1.64 µM respectively. Compounds 1 and 3 exhibited no significant cytotoxic effect on all the cell lines. All four cell lines had an $IC_{50} > 100$ µM except DLD-1, which had an IC_{50} of 88.4 µM (Figure 3.2). This showed that the cancer cells were resistant to the compounds 1 and 3. DOX was used as a positive control. The selectivity index (Table 3.1) demonstrates that compound 2 exerts a specific and targeted impact on the MDA-MB-468 cell line while sparing normal cells. In contrast, PC3 displays minimal selectivity when treated with compound 2. Compound 2 exhibits a favourable selectivity index compared to DOX in breast cancer cell lines.

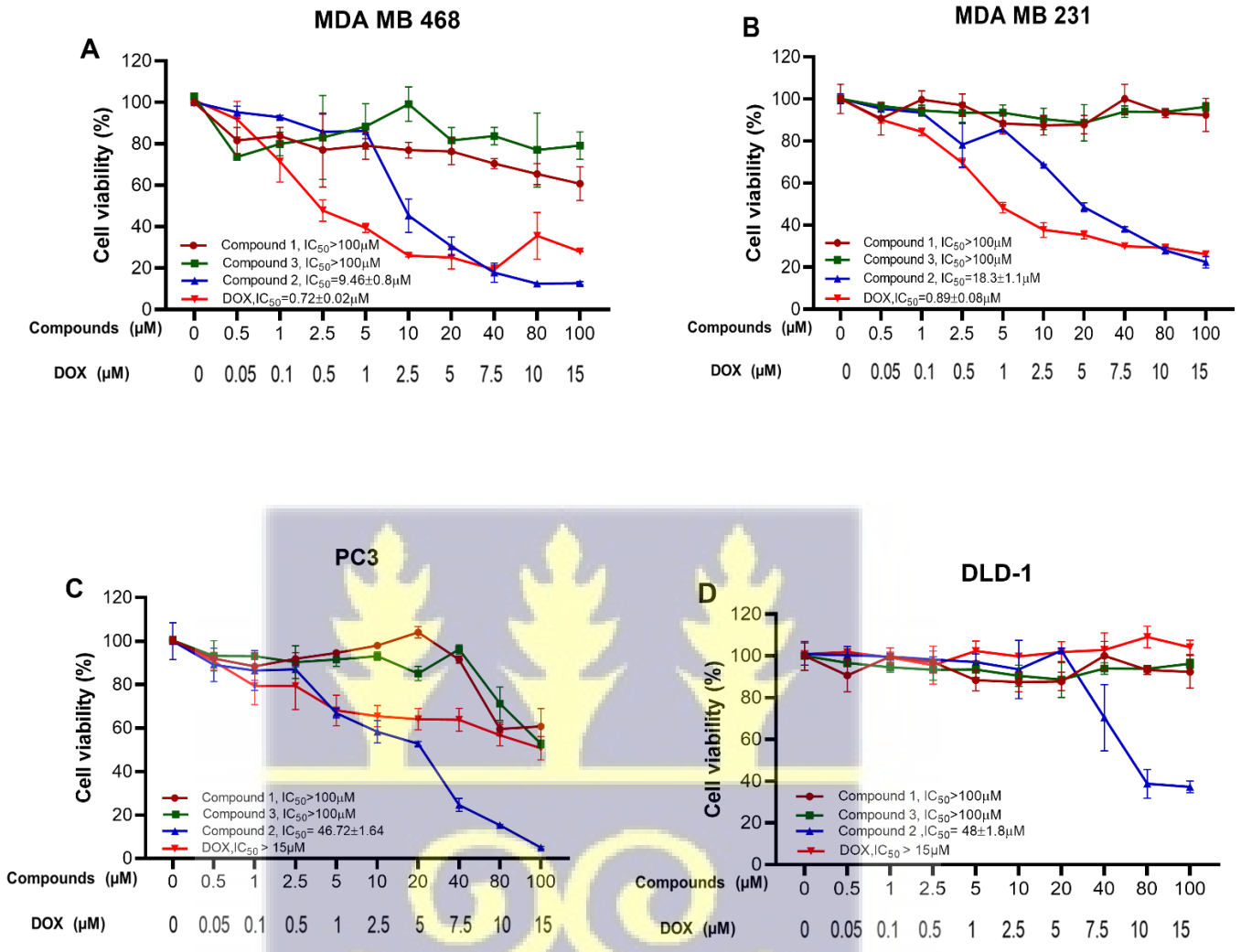


Figure 3.2: Cytotoxicity assay of the three compounds 1, 2 and 3 after 48hrs

Cytotoxicity graph of the compounds 1, 2 and 3 against four cancer cell lines. A) MDA MB 468 B) MDA MB 231 C) PC3 and D) DLD-1. The percentage cell viability was plotted against nine concentrations of each compound. MDA-MB-468 and MDA-MB-231 were treated with different concentrations; 0-100µM of compounds 1, 2, and 3 as well as 0-15µM of DOX. The negative control (untreated cells) is represented on the graph as a percentage viability against a compound concentration of zero. DOX was used as a reference drug. Data represent three independent experiments each done in triplicates.

Table 3.1: Selectivity index of compound 2 and DOX in the cancer cell line

	CC_{50} non-cancerous cell line (PNT2) (µM) ± SEM	**IC_{50} cancer cell line (µM) ± SEM**	**Selectivity index**
MDA MB 468 (compound 2)	40.61 ± 1.91	9.46 ± 0.8	4.29
MDA MB 468 (DOX)	0.88 ± 0.04	0.72 ± 0.02	1.22
MDA MB 231 (compound 2)	40.61 ± 1.91	18.30 ± 1.1	2.22
MDA MB 231 (DOX)	0.88 ± 0.04	0.89 ± 0.08	0.99
PC3 (compound 2)	40.61 ± 1.91	46.72 ± 1.64	0.87
PC3 (DOX)	0.88 ± 0.04	>15	-
DLD-1 (compound 2)	40.61 ± 1.91	40.58 ± 1.8	1.00
DLD-1 (DOX)	0.88 ± 0.04	>15	-

***S*electivity *index of compound 2 on PNT2. Data represent two independent experiments each done in triplicates*.

3.5.3: The putative targets of compound 2 and the PPI network of compound 2 cancer-related targets

From the DisGENET and GeneCards databases, 15615 cancer-related genes were identified. Using Swiss Target and SuperPred, the prediction produced 211 putative targets for compound 2. After the intersection with FunRich v3.1.3, 206 cancer-related targets for compound 2 were identified. A PPI network of the cancer-related targets of compound 2 was constructed in the STRING database. For intuitive topological analysis, 199 nodes and 1591 edges were obtained. Eighteen (18) of the 206 compound 2 cancer-related targets were selected after screening using the CytoHubba algorithm. Finally, 15 targets were selected following MCODE sub-clustering (Figure 3.3, Table 3.2).

3.5.4: Functional enrichment analysis of compound 2 and its core targets

From functional enrichment analyses, 435 biological processes, 39 cellular components, 36 molecular functions, and 122 pathways were predicted to be associated with the anticancer activity of compound 2 (Supplementary Sheet S6). The KEGG pathway analysis revealed the anticancer potential of compound 2 mainly via focal adhesion, pathways in cancer, EGFR tyrosine kinase inhibitor resistance, and ErbB signalling (Figure 3.4). From our data, regulation via focal adhesion had the highest statistical significance, with a log(q-value) of -17, a Z-score of 35, and an enrichment score of 110. Pathways in cancer, with a log(q-value) of -14 and an enrichment score of 45, were also significantly represented in the data. A high Z-score of 23 further emphasises its relevance. The ErbB signalling pathway and EGFR tyrosine kinase inhibitor resistance had high enrichment scores of 190 and 200, respectively, and Z-scores of 39 and 40, respectively, indicating their significant over-representation.

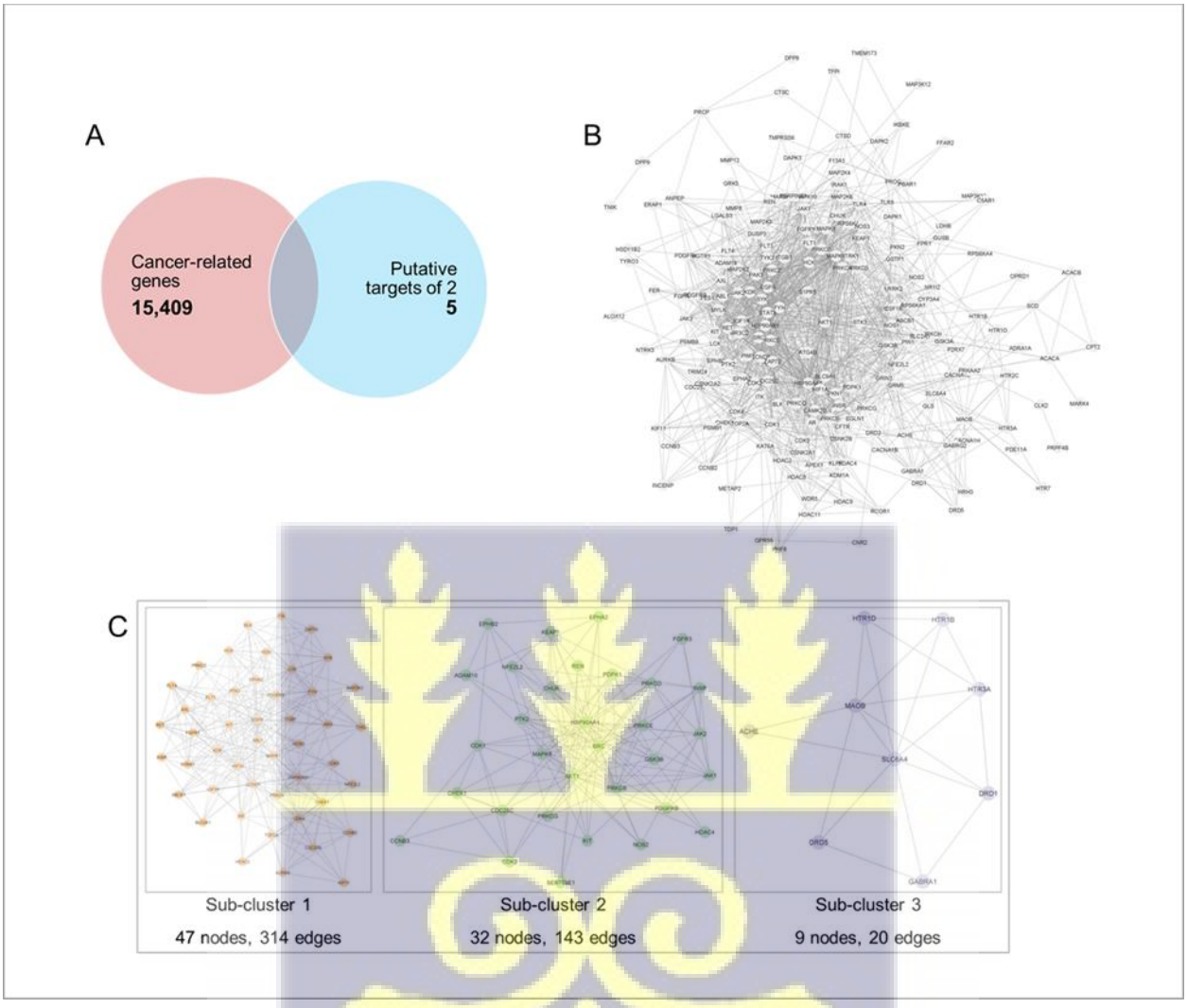


Figure 3.3: Network pharmacology between compound 2 and cancer-related targets.

(A) Venn diagram illustrating the overlap between predicted targets of compound 2 identified via SwissTargetPrediction and cancer-associated genes retrieved from GeneCards and DisGeNET.

(B) Protein–protein interaction (PPI) network of the intersecting targets, constructed using the STRING database and visualized in Cytoscape.

(C) MCODE analysis performed in Cytoscape to identify densely connected sub-clusters within the PPI network, representing potential functional modules involved in cancer-related pathways.

Table 3.2: Cancer-related targets of compound 2 and PDB sources of 3D protein structures.

Gene symbol	UniProt ID	Target name
AKT1	P31749	AKT serine/threonine kinase 1
CCND1	P24385	Cyclin D1
EGFR	P00533	Epidermal growth factor receptor
FYN	P06241	FYN proto-oncogene
GSK3B	P49841	Glycogen synthase kinase 3 beta
HIF1A	Q16665	Hypoxia-inducible factor 1 subunit alpha
HSP90AA1	P07900	Heat shock protein 90 alpha family class A member 1
HSP90AB1	P08238	Heat shock protein 90 alpha family class B member 1
JAK2	O60674	Janus kinase 2
KDR(VEGFR2)	P35968	Kinase insert domain receptor/Vascular endothelial growth factor receptor 2
MAPK1	P28482	Mitogen-activated protein kinase 1
MAPK8	P45983	Mitogen-activated protein kinase 8
PRKCB	P05771	Protein kinase C beta
PTK2	Q05397	Protein tyrosine kinase 2/ Focal adhesion kinase 1
SRC	P12931	SRC proto-oncogene

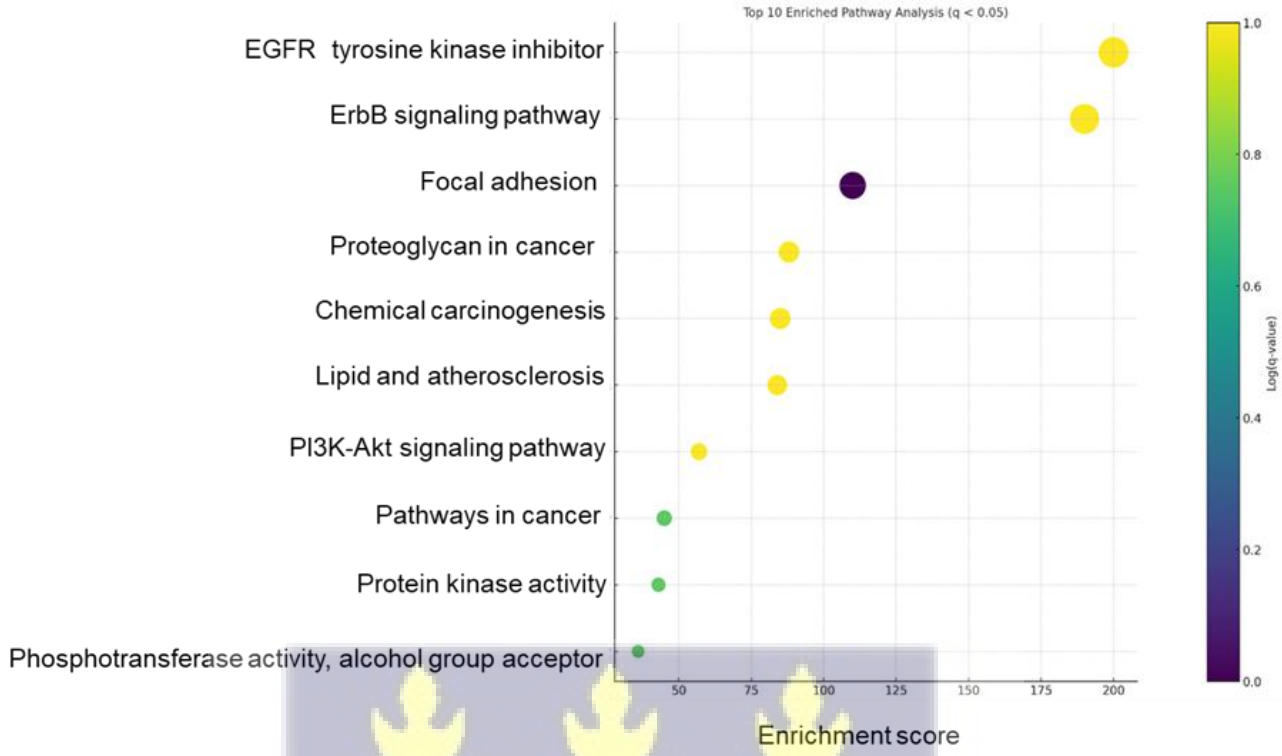


Figure 3.4: Functional enrichment analysis of compound 2 and its core target.

This is a KEGG pathway plot showing the functional enrichment analyses of core targets. The bigger bubble represents the enrichment of genes (high Z-score) in that pathway, and the colour bubble in the plot represents the statistical significance of the paths, corresponding to the colour on the log(q-value) scale bar.

3.5.5: Molecular Docking of compound 2 to the three targets shows compound 2 binds to ATP-binding and the allosteric sites of EGFR.

Molecular docking experiments revealed the binding mode of compound 2 to the three potential target proteins identified by functional enrichment analysis: AKT1, EGFR, and VEGFR2 (the three potential target proteins identified by functional enrichment analysis). With the GOLD software suite, the four scoring functions, Piecewise Linear Potential (ChemPLP), GoldScore (GS), Astex Statistical Potential (ASP), and ChemScore (CS), were used for docking studies. The binding site of AKT1 consists of a slightly hydrophilic to neutral binding pocket, whereas EGFR and VEGFR2 consist of a hydrophobic binding pocket. For AKT1, the carbonyl end of compound 2 was fitted into the binding pocket. It formed hydrophobic contacts with cysteine (Cys296) and isoleucine (Ile86). The complex can be stabilised by a catalytic water molecule that forms hydrogen bonds with the backbone carbonyl oxygen of valine (Val271) and tyrosine (Tyr272). However, AKT1 did not form a classical hydrogen bond with the ligand. For EGFR and VEGFR2, the piperidine end fitted well into the binding pocket, whereas the carbonyl portion pointed outside. For EGFR, Compound 2 formed hydrophobic contacts with leucine (leu747), isoleucine (Ile759) and leucine (Leu858) and the oxygen in the benzoxazine ring formed hydrogen bonds with side chain amine of lysine (Lys745) whereas the secondary amine in the compound forms hydrogen bond with the side chain hydroxy group and the backbone amine of aspartic acid (Asp855). Compound 2 was located at the hinge region (ATP binding site) of EGFR, specifically binding to the critical ATP-binding activation site at Lys745. Compound 2 also interacted with the DFG motif, the activation loop of EGFR at Asp855. For VEGFR2, the oxygen in the benzoxazine ring formed hydrogen bonds with the backbone amine of cysteine (Cys919), which is in the ATP binding site. Compound 2 also formed hydrophobic contacts with phenylalanine (Phe918), also in

the ATP binding site, and leucine (Leu840). The binding modes and the locations of the plausible binding pockets for compound 2 for each target are shown in Figure 3.5.

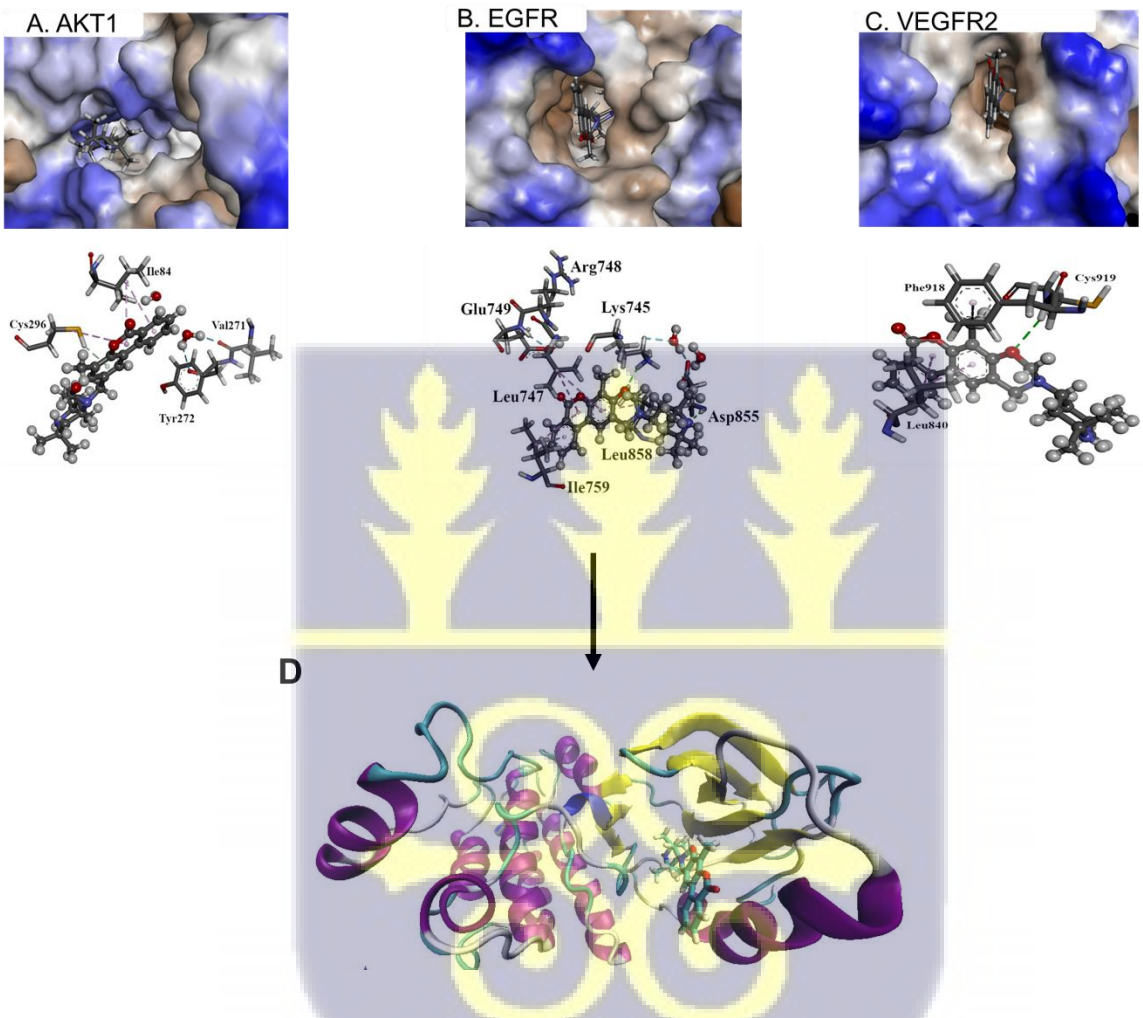


Figure 3.5: Molecular docking of compound 2 to three targets shows Compound 2 binds to the ATP-binding and the allosteric sites of EGFR

A) AKT1 (B) EGFR (C) VEGFR2. The top displays the protein surface; blue represents the hydrophilic regions, brown represents the hydrophobic regions, and grey represents the neutral areas. The bottom shows the binding interactions. The compound is in ball-and-stick format, while the amino acid residues are in stick form. Green lines represent hydrogen bonds, while purple lines indicate hydrophobic contact. (D) Image showing compound 2 in the hinge region of EGFR.

3.5.6: MD simulations of compound 2 against the three (3) protein targets show EGFR as the most plausible target

The Radius of Gyration (RoG) is an important parameter that provides information on the compactness of the protein-ligand complex. EGFR maintained a consistent and compact structure during most of the simulations, with an average RoG of 38.93± 0.03 Å and a maximum RoG of 66.84 ±0.42 Å. (Figure 3.6A & B**)**. VEGFR2 had a slightly lower average RoG of 37.09± 0.03 Å than EGFR. VEGFR2 had a RoGmax of 63.68 ± 0.42 Å and, like EGFR, displayed instances of more extended conformations during the simulation. RoG and RoGmax for AKT1 were 38.43 ± 0.02 Å and 66.07 ± 0.44 Å, respectively. AKT1 protein had a higher RoGmax value than EGFR protein. The significant difference between the average RoG and RoGmax observed for all proteins showed that each protein may have experienced moments of increased flexibility or conformational changes during the simulation. Owing to the low SD observed for all three proteins, they remained the same size and shape throughout the simulation, implying a stable conformation. The consistent compactness may indicate that compound 2 fits snugly within the binding pocket of the proteins.

The Root Mean Squared Deviation (RMSD) values for all proteins showed a similar trend: initial deviation followed by stabilisation. Throughout the simulation, the EGFR atoms deviated by about 55.11± 0.70 Å (Figure 3.6C) from their initial positions before stabilising around 1.5 Å, indicating only minor conformational changes after the initial frames. VEGFR2 atoms deviated from their starting positions by an average of 52.51± 0.52 Å and stabilised around 1.5 Å. This initial deviation is slightly less than EGFR's, implying that VEGFR2 had a somewhat more stable interaction with ligand compound 2 or had fewer conformational changes during the simulation. The RMSD of

AKT1 was 54.09 ± 0.70 Å, which is between EGFR and VEGFR2. However, AKT stabilises near 2.5 Å, indicating that it had more significant structural deviations. The findings showed that, during the simulation, all proteins initially underwent structural changes quite different from each reference structure. However, EGFR and VEGFR2 stabilised at 1.5 Å after the initial deviation, thus converging to a stimulated structure closely resembling the reference structures. Although the AKT1 structure also stabilised within a reasonable range (2.5 Å), it showed a slightly higher deviation or flexibility level than EGFR and VEGFR2. However, the low SDs observed indicate that the RMSD values fluctuated only somewhat around the averages, suggesting a relatively stable simulation.

The Root Mean Squared fluctuation (RMSF) plot revealed information about the flexibility and rigidity of different protein regions. Increased RMSF values signify increased flexibility, while decreased RMSF values indicate heightened rigidity. From the simulations (Figure 3.6D), although each protein showed unique dynamics with specific regions of flexibility, EGFR had the highest peaks compared with VEGFR2 and AKT1, indicating higher fluctuations. This showed that when EGFR is complexed with compound 2, it undergoes conformational changes. This could also mean that EGFR has certain regions that are inherently more flexible.

Hydrogen bond analysis and lifetime curve plotting were performed to further understand the strength and stability of the protein-ligand complexes, with a more extended lifetime range indicating a more stable interaction (Figure 3.6E). The hydrogen bonds in the EGFR complex had longer lifetimes, with several bonds present in more than 500 counts. The hydrogen bond between compound 2-270@O2 and the solvent had the highest lifetime with an average distance of

approximately 2.79 Å and an angle of 158.2°, existing for 1125 counts. Residues such as Asp155 and Thr154 formed hydrogen bonds with compound 2-270@N at an average distance of 2.96 Å and 2.94 Å, respectively, with corresponding average angles of 166.14° and 166.36°. For AKT1, the bond between compound 2-409@N and the solvent appeared frequently, present in over 500 counts, with an average distance of around 2.86 Å and an angle of 162.22°. Residues such as Tyr16, Arg80, and Glu242 in AKT1 were found to form hydrogen bonds with compound 2, with an average distance range of 2.83-2.91 Å and an angle range of 142.82- 159.45°. This shows that AKT1 has a more diverse set of hydrogen bonds with compound 2, which contributes to its overall hydrogen-bonding activity, and no single bond dominates the entire interaction. VEGFR2 exhibited the broadest range of lifetimes. The bond between compound 2-308@O2 and the solvent was the most prevalent, present in 1141 counts, with an average distance of approximately 2.78 Å. The residues Lys32, Cys113, and Lys62 in VEGFR2 were identified to form hydrogen bonds with compound 2-308@O2, compound 2-308@O, and compound 2-308@N, respectively, with a distance range of 2.83 - 2.91 Å and an angle range of 151.15 -159.94°. This indicated that VEGFR2 had an even more diverse set of hydrogen bonds with compound 2, and it took a more significant number of unique bonds to account for most of the hydrogen bonding activity. The interaction of each protein with its specific residues indicated that these residues are likely pivotal for the binding mode of compound 2 and may play a significant role in ligand recognition or binding. The average hydrogen distance and angles for each protein also showed that the hydrogen bonds formed between compound 2 and the proteins were quite linear and had an exceptionally short distance, indicating a robust interaction.

Finally, the free energy was calculated and quantitatively measured, and the binding affinity between compound 2 and proteins was measured. The binding of EGFR was energetically favourable, with a Molecular Mechanics–Poisson-Boltzmann Surface Area (MM-PBSA) value of -767.19 (Table 3.3), indicating a stable complex. Again, the Molecular Mechanics–Generalized-Born Surface Area (MM-GBSA) value of -807.34 revealed favourable binding, consistent with the MM-PBSA value. These energy values indicated that compound 2 strongly binds to the EGFR protein, potentially stabilising its structure. Similarly, the free energy values calculated for VEGFR2 showed that compound 2 was bound to VEGFR2, indicating a likely stable complex. Comparing AKT1 with EGFR and VEGFR2, the free energies revealed a less favourable free energy between compound 2 and the AKT complex, indicating a less stable complex.

Based on a thorough comparison of all three proteins using the five metrics examined, compound 2 appeared to have the highest affinity and most stable interaction with EGFR, closely followed by VEGFR2.

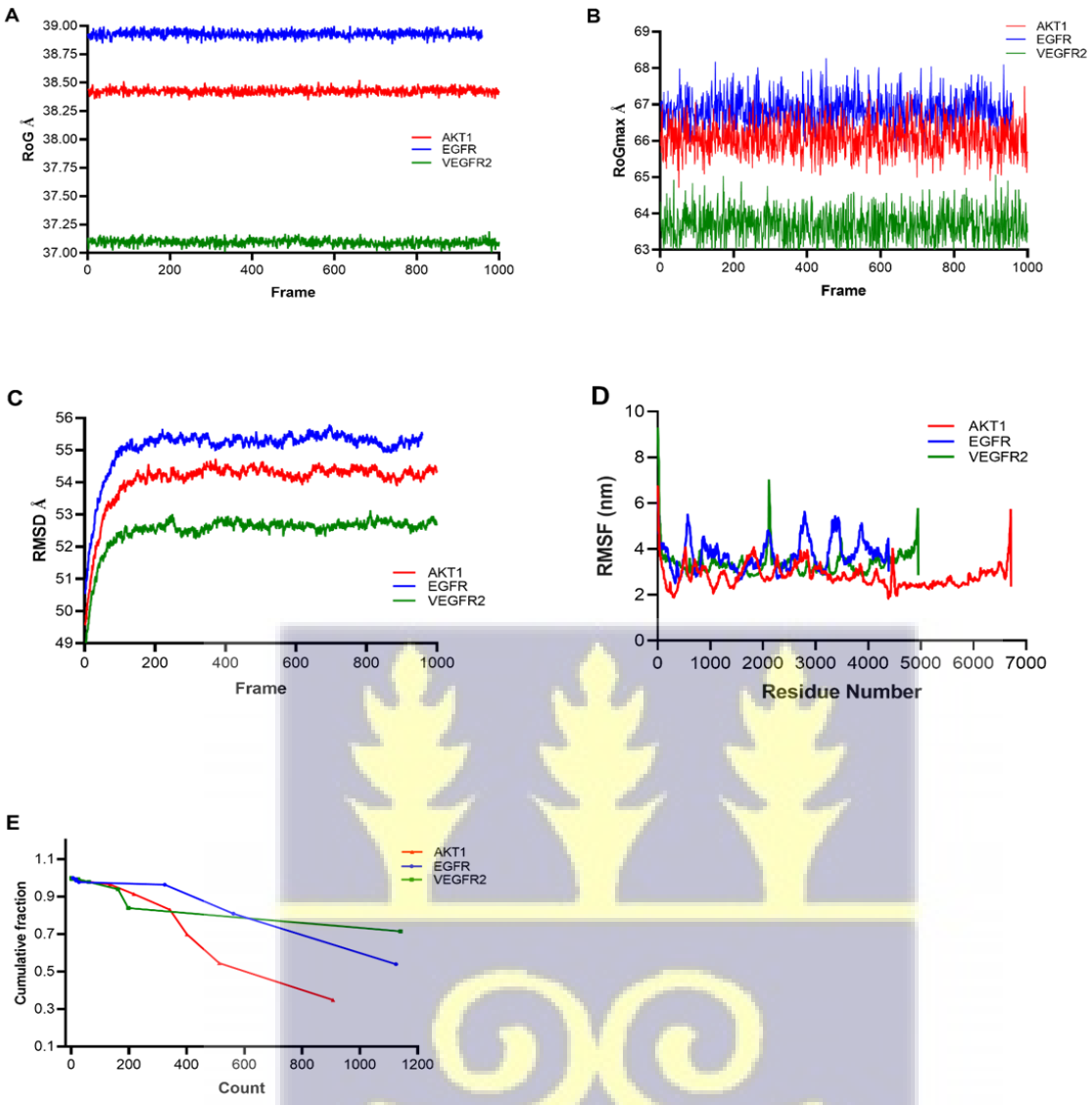


Figure 3.6: Plots showing the MD simulations of the three plausible targets

Simulations were performed for approximately 1000 frames.

A. RoG for EGFR, VEGFR2 and AKT1 bound to the ligand, compound 2 over time.

B. RoG max for EGFR, VEGFR2 and AKT1 bound to the ligand, compound 2 over time.

C. RMSD plot showing the deviation of EGFR, VEGFR2 and AKT1 bound to compound 2 over time.

D. RMSF plot showing the residue fluctuations of EGFR, VEGFR2 and AKT1 complexed to compound 2.

E. The hydrogen bond lifetime plot of EGFR, VEGFR2 and AKT1 complexed with the ligand compound 2.

Table 3.3: Free energy calculations for EGFR, VEGFR2 and AKT1

Protein	MM-PBSA (kcal/mole) ± SD	MM-GBSA (kcal/mole) ± SD
EGFR	-767.19 ± 48.64	-807.34 ± 48.64
AKT	-229.40 ± 46.68	-177.97 ± 46.68
VEGFR2	-729.01 ± 47.75	-772.04 ± 47.73

3.5.7: Drug-likeness prediction and ADMET properties

ADMETlab 2.0 webserver's server predicted that compound 2 adhered to Lipinski's Ro5 criteria (Lipinski, 2004) with one hydrogen bond donor, five hydrogen bond acceptors, a molecular weight of 406.23 Da, and a log P value of 4.178. However, the quantitative estimate of drug-likeness (QED) score prediction for compound 2 was 0.468, which revealed that compound 2 needs to be optimised as a lead compound for drug development (Bickerton et al., 2012). The ADMET properties of compound 2 are shown in Table 3.4. The compound demonstrated excellent Caco-2 permeability (−4.944), with a low probability of poor human intestinal absorption (HIA = 0.016), indicating favourable absorption potential. Predicted probabilities for falling below 20% and 30% oral bioavailability were also low (F20% = 0.004; F30% = 0.005), suggesting good systemic exposure upon oral administration. Plasma protein binding was moderate (83.48%), and blood-brain barrier penetration was classified as moderate (0.301). Clearance was low (3.215 mL/min/kg), and the predicted half-life was short (0.078 h). Toxicity predictions flagged potential concerns, including hERG inhibition (0.839), hepatotoxicity (0.942), and skin sensitization (0.817), though the compound was non-mutagenic (Ames = 0.115).

Furthermore, the SwissADME BOILED-Egg model indicated how closely a compound aligns with the ideal conditions for optimal absorption. Compound 2 was predicted to have the ability to be absorbed from the GI tract and traverse the BBB, as illustrated in Figure 3.7A. In Figure 3.7B, the drug-likeness radar for compound 2 presented details of its compatibility with six physicochemical characteristics. Notably, the physicochemical characteristics of compound 2 fell within the pink area, as shown in Figure 3.7B. A bioavailability score of 0.55 was predicted for compound 2, indicating that approximately 55% of the compound would be absorbed into the bloodstream after oral administration. This pinpoints the compound's potential for oral administration. In this model, compound 2 was shown to be a p-glycoprotein (P-gp) substrate, implying that it could be transported back into the GI lumen after absorption.

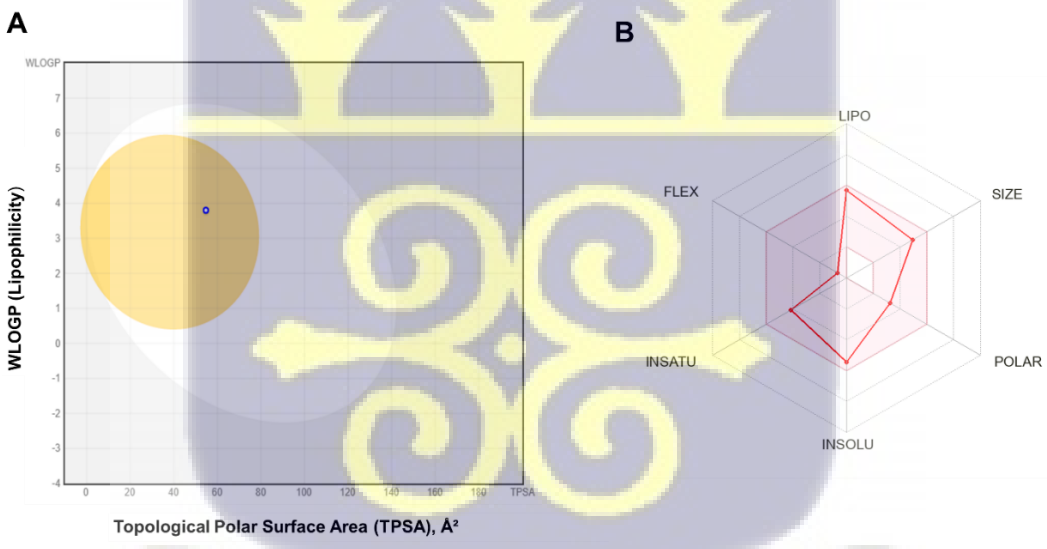


Figure 3.7: SwissADME and Druglikeness Analysis

(A) Egan BOILED-Egg model of compound 2. The white region predicts gastrointestinal absorption, and yellow region predicts blood-brain barrier penetration. Red dots indicate non-P-gp substrates; blue dots indicate P-gp substrates. (B) Drug-likess radar of compound 2. The plot includes six parameters: lipophilicity (LIPO), size (SIZE), polarity (POLAR), solubility (INSOLU), flexibility (FLEX), and saturation (INSATU). The pink area represents the optimal range for drug-likeness.

Table 3.4: ADMET properties of compound 2

Property	Value	Reference	Interpretation
Absorption			
Caco-2 permeability	-4.944	> −5.15 = high; −5.15 to −6.0 = moderate < −6.0 = low	Excellent
Human intestinal absorption	p=0.016	HIA+ = poor absorption, p>0.3. (HIA- = high absorption, p<0.3	Excellent
20% bioavailability	p=0.004	$F_{20\%}$+ =poor bioavailability <20%, p>0.05 $F_{20\%}$- = good bioavailability ≥ 20%), p<0.05	Excellent
30% bioavailability	p=0.005	$F_{30\%}$+ =poor bioavailability <30%, p>0.05 $F_{30\%}$- = good bioavailability ≥ 30%), p<0.05	Excellent
Distribution			
Plasma protein binding	83.48%	Optimal: < 90%	Excellent
Blood-brain barrier penetration	0.301	> 0.30= high; −1.0 to 0.30= moderate < −1.0= low/negligible	Moderate
Metabolism			
CYP 2C19/2C9/2D6/3A4 inhibitor	-	-	Inhibitor
Excretion			
Clearance	3.215	High: >15 mL/min/kg; moderate: 5-15 mL/min/kg; low: <5 mL/min/kg	Low clearance
Half-life	p=0.078	p> 0.5=long (desirable); p< 0.5=short	Excellent
Toxicity			
hERG blocker	p=0.839	> 0.7= hERG blocker (cardiotoxic risk) < 0.7= non-blocker	hERG blocker
Human hepatotoxicity	p=0.942	> 0.7= likely toxic; < 0.7= non-toxic	Toxic
AMES toxicity	0.115	> 0.5= likely Mutagen < 0.5= negative (Non-mutagenic)	Ames negative
Rat oral acute toxicity (LD50)	0.698	> 0.7= high; 0.3–0.7= moderate; < 0.3=low	Moderate toxicity
Skin sensitisation	p=0.817	> 0.7= sensitizer; < 0.7= non-sensitizer	Sensitizer

3.6: Discussion

The study was conducted to assess the *in vitro* anticancer potential of three synthetic c.1ompounds that had previously been identified as potentially antileishmanial using molecular modelling and *in vitro* assay approach (Sakyi et al., 2023).In addition, in-silico approaches were employed to predict the potential anticancer targets of the most potent compound. Compound 2 was more potent against breast cancer cell lines using a cytotoxicity assay. The IC_{50} values found for both breast cancer cell lines were lower than values obtained for antileishmanial activity (21.9 µM). This suggests that compound 2 may be a potential anticancer candidate.

In the network pharmacology analysis of compound 2, fifteen (15) hub genes were identified, primarily associated with two fundamental pathways: the focal adhesion pathway and 'pathways in cancer'. Focal adhesions, crucial for cell migration and survival, may be influenced by compound 2, impacting cancer cell invasion and metastasis. The 'Pathways in Cancer' encompass cell cycle regulation, apoptosis, and survival mechanisms. EGFR, a member of the ErbB family, is frequently overexpressed or mutated in several cancers, including lung, breast, colorectal, and head and neck cancers. Aberrant activation of the ErbB pathway contributes to uncontrolled cell growth, evasion of apoptosis, angiogenesis, and metastasis (Lindsey & Langhans, 2015). Targeting this pathway has become a significant therapeutic strategy. EGFR inhibitors such as gefitinib, erlotinib, and cetuximab have shown potential in treating certain cancers, such as non-small cell lung cancer(Yang et al., 2017). Tumours also often overexpress VEGF, promoting angiogenesis and ensuring an adequate blood supply for tumour growth(Niu & Chen, 2010). VEGRF2 signalling is involved in focal adhesions, emphasising its role in endothelial cell behaviour and angiogenesis. Inhibiting VEGFR2 has been a successful strategy to impede angiogenesis and limit tumour

progression. Anti-angiogenic drugs, such as bevacizumab, target VEGFR and are used in treating various cancer types, including colorectal, breast, and renal cancers (Hsu & Wakelee, 2009). From our studies, Compound 2 can potentially target both EGFR and VEGFR2 for more specific and effective treatments for patients with different types of cancer. The PI3K/AKT pathway was also highlighted as a potential target for compound 2. The PI3K/AKT pathway is frequently dysregulated in cancer and drives uncontrolled cell growth and survival. Targeting PI3K, AKT, or mTOR shows promise in cancer therapy (Glaviano et al., 2023). The PI3K/AKT pathway is also involved in chemoresistance, emphasising its importance for research and therapeutic development (Kaboli et al., 2021; Liu et al., 2020). From these studies, Compound 2 demonstrated broad, multi-targeted effects on cancer cells, impacting diverse biological processes.

Molecular docking and dynamics simulations focused on AKT1, EGFR, and VEGFR2 to elucidate their interactions with compound 2, providing additional insights. From the molecular docking, the interacting residues of AKT1 with compound 2 were not in the ATP-binding site. For VEGFR2, compound 2 was found to occupy the ATP binding region and form a crucial hydrogen bond with the backbone NH of the Cys919 residue at the hinge region. This interaction was also seen in some approved VEGFR2 inhibitors, such as sorafenib and some previously identified novel VEGFR2 inhibitors (Aziz et al., 2016; Bauer et al., 2008; Liu et al., 2022). Compound 2 also interacted with Phe918 in the ATP binding site, as seen in some approved VEGFR2 inhibitors (Liu et al., 2022). Likewise, compound 2 also interacted with Lys745, a critical ATP-binding activation site of EGFR (Honegger et al., 1987). Gefitinib and Erlotinib, potent EGFR inhibitors, have also been shown to interact with EGFR at the same site. This reveals compound 2 as likely an ATP-competitive inhibitor, directly competing with ATP and inhibiting the phosphorylation activity

necessary for VEGFR2 and EGFR signalling. While ATP-competitive inhibitors are effective, the risk of drug resistance exists due to mutations occurring at the same ATP binding site. Additionally, this type of inhibition may impact other kinases with similar ATP-binding pockets. Notably, the interaction of compound 2 with Asp855, which is part of the DFG motif, the activation loop of EGFR could block the activation of EGFR's kinase domain, preventing the transmission of growth signals within the cell. Ultimately, this inhibition strategy of compound 2 binding to both the ATP site and the DFG motif presents a robust inhibition of EGFR and holds promise for preventing cell proliferation and survival in cancers where EGFR dysregulation occurs.

From the MD simulation, AKT1 displayed diverse hydrogen bonds with compound 2, suggesting varied interactions contributing to its overall hydrogen bonding activity. VEGFR2 showed a balance of specific and solvent-mediated bonds, implying a more flexible binding mode, potentially making it more tolerant to ligand mutations (Sarabipour et al., 2016). Compound 2 may be more involved in water-mediated interactions in the AKT1 and VEGFR2 complexes, implying that the binding pockets are more solvent-exposed or that water molecules play an essential role in stabilising the ligand within the pocket (Schiebel et al., 2018). The observed conformational change or flexibility for EGFR could be necessary to accommodate the ligand, compound 2, or to help the protein function. This conformational change in EGFR upon binding has been attributed to the extracellular domains of EGFR family members dimerising to activate the protein (Ferguson et al., 2003). The high stability of interactions between compound 2 and EGFR, characterised by strong hydrogen bonds, indicates a specific binding pocket. Compound 2 formed an energetically favourable bond and strong and geometrically favourable hydrogen bonds with EGFR, suggesting a robust binding mechanism. This has implications for drug development because compound 2

may be a better inhibitor or modulator of EGFR and possibly VEGFR2 than AKT. This finding from the MD simulation analysis is consistent with our KEGG pathway enrichment profile.

EGFR inhibitors represent a promising strategy to combat EGFR-driven malignancies. These inhibitors bind to the EGFR protein, blocking its activation and downstream signalling. Consequently, they impede the uncontrolled growth and division of cancer cells, ultimately leading to cell death. However, existing EGFR inhibitors have limitations, including efficacy challenges and the emergence of resistance. While first-generation inhibitors like erlotinib and gefitinib show promise in treating specific EGFR-mutant cancers, resistance often develops over time (Pao et al., 2005; Shi et al., 2022). Osimertinib, a third-generation irreversible EGFR-Tyrosine kinase Inhibitor (TKI), has effectively treated non-small cell lung cancer (NSCLC) in patients with EGFR T790M mutations. Unfortunately, even osimertinib faces resistance, limiting its long-term effectiveness (Du et al., 2021; Li et al., 2023). Blueprint's lead EGFR inhibitor, BLU-945, has shown safety in phase I trials, and fourth-generation inhibitors hold the potential for overcoming resistance mechanisms (Mullard, 2022; Shum, 2022). However, continuing research and developing improved EGFR inhibitors is essential to enhance therapeutic outcomes. Our study highlights compound 2 as a promising targeted therapeutic option for anticancer treatment. The dual binding mechanism of compound 2 may enhance specificity, minimising off-target effects often associated with broader ATP-competitive inhibitors (Wittlinger et al., 2024). Furthermore, compound 2 could prove valuable in treating cancers resistant to other EGFR inhibitors that solely target the ATP binding site. We may overcome or delay resistance by targeting multiple sites, as cancer cells would need to accumulate mutations at various locations to evade inhibition.

The compound shows favourable oral pharmacokinetics, with strong Caco-2 permeability and low risk of poor absorption or bioavailability. Its low HIA (0.016) and bioavailability thresholds suggest efficient intestinal uptake. The log P value of 4.138 indicates that the compound is highly lipophilic, meaning it preferentially dissolves in non-polar solvents rather than in water. This value supports membrane permeability but may reduce solubility and raise concerns about off-target effects, and metabolic instability, poor formulation behaviour. While its polar surface area is suitable, predicted P-gp substrate status, hERG inhibition, and hepatotoxicity present safety challenges. To address these, strategies such as structural modification, nanoparticle formulation, or prodrug design could improve safety and bioavailability while maintaining positive pharmacokinetic properties.

3.7: Conclusion

In conclusion, the cytotoxicity assay helped spotlight the therapeutic promise of compound 2, as it provided a direct measure of its potential antitumor activity. The network pharmacology, molecular docking, MD simulations, and the BOILED-Egg analysis offered a comprehensive picture of plausible targets of compound 2, pharmacokinetics properties, and its interactions with EGFR, AKT1, and VEGFR2. These findings pave the way for future research to validate and expand on the simulation data. They also clarify compound 2's binding specificity, affinity, and availability, which are crucial for drug development and optimisation. The favourable binding of possibly drug development.

CHAPTER FOUR

4.0 Objective 2: To determine the effect of the hit compound on selected cellular and molecular processes.

a. To determine the effect of the hit compound on gene expression levels on key metastatic factors, such as EMT (*TWIST1*, *SNAI1*, and *vimentin),* invasion and metastasis genes *(MMP2, MMP9, VCAM-1),* apoptotic genes *(Bcl-2, Bax, caspase 8&3)* and a proliferation marker *(Ki67)* using RT-qPCR.

b. To investigate the impact of the hit compound on apoptosis, cell cycle, cancer stemness, migration, and invasion.

c. To quantify levels of selected chemokines and cytokines using the Luminex assay

4.1 Rationale for Objective 2

Due to the lack of biomarkers for targeted therapies and an aggressive, often invasive, disease progression, there are few FDA-approved drug treatments for TNBC. TNBC is extremely difficult to treat due to the absence of ERs, PRs, and HER2; therefore, it does not respond to drugs targeting these receptors. However, the biological and pathological characteristics of TNBC provide insight into several potential molecular targets for current and future therapeutics. Hence, targeted therapies, such as those targeting DNA repair pathways, androgen receptor signalling pathways, and kinases, represent promising treatment options against TNBC. In addition, immunotherapy has also been demonstrated to improve overall survival and response in TNBC. Collaboration among researchers, pharmaceutical companies, and healthcare providers is pivotal in accelerating the discovery and advancement of novel treatments for TNBC. These collaborative efforts aim to enhance survival rates and enhance the quality of life for patients facing this aggressive form of breast cancer.

4.2 Abstract

TNBC is difficult to treat due to its aggressive nature and lack of hormone receptors, making conventional therapies ineffective. This study evaluated the efficacy of a novel compound, compound 2, in modulating TNBC cell behaviours through a series of in vitro assays on MDA-MB-468 and MDA-MB-231 TNBC cell lines. This study used various research methods to explore how Compound 2 affects breast cancer. Our investigation involved analysing apoptosis using detection kits, studying cell cycle dynamics with flow cytometry, assessing clonogenic potential, profiling gene expression via qRT-PCR, examining wound healing, and characterising cancer immunophenotypes using flow cytometry. Additionally, cytokine and chemokine levels in MDA-MB-468 cells were measured using Luminex assay. Compound 2 demonstrated a dual impact on cancer cells, particularly in MDA-MB-468 cells. Firstly, it significantly increased both early and late apoptotic phases, indicating its pro-apoptotic activity, particularly in MDA-MB-468. Gene expression analyses further validated this effect, revealing the downregulation of anti-apoptotic genes and the upregulation of pro-apoptotic genes. Second, compound 2 induced G1-phase arrest in MDA-MB-468. Compound 2 also reduced cancer stem cell populations and inhibited clonogenic growth. Compound 2 demonstrated an inhibitory effect on cell migration, especially at IC_{50} concentration. Furthermore, significant changes in gene expression profiles are observed in genes related to EMT. Lastly, the Luminex assay revealed that compound 2 significantly decreased the expression of IL4 and IL8 while increasing the expression of CCL2 and CXCL1. However, it had no significant effect on IL6, IL10, CXCL2, CCL5, TNF-α, IFN-γ and IL-1β, IL2. Compound 2 demonstrates a multifaceted anticancer profile, indicating its potential to prevent cancer relapse and limit cell proliferation. As a result, it emerges as a promising candidate for targeted therapy in

TNBC. This study provides a basis for further *in vivo* studies and potential clinical applications to explore the full therapeutic potential of Compound 2 in aggressive breast cancer types.

4.3 Introduction

Breast cancer (BC) is a prevalent malignancy affecting young women worldwide. In 2022, reported BC cases reached 2.3 million, resulting in 670,000 deaths (Ferlay et al., 2024). Like any other disease, these figures represent only reported cases, and the actual number of individuals affected by breast cancer may be higher, as some cases may go undiagnosed or unreported. However, these figures underestimate the impact, as some cases remain undiagnosed or unreported. Breast cancer affects women post-puberty across all countries, emphasising its pervasive nature (World Health Organization, 2024). The disease encompasses various subtypes with distinct molecular characteristics, prognosis, and treatment responses (He et al., 2019; Yersal & Barutca, 2014),

TNBC stands out as a highly aggressive and heterogeneous subtype of breast cancer. It is characterised by the absence of ER, progesterone receptor PR, and HER2 expression. TNBC accounts for approximately 10-15% of all breast cancer cases globally and presents significant challenges due to its aggressive behaviour, high relapse rates, limited targets, and limited treatment options (Leon-Ferre & Goetz, 2023; Yang et al., 2022; Yin et al., 2020). While chemotherapy remains the primary approach for TNBC, it faces obstacles such as drug resistance and side effects affecting both healthy and cancerous cells (Anand et al., 2023; Emran et al., 2022). Notably, TNBC's molecular landscape contributes to varying responses to chemotherapy(van der Noord et al., 2022). Targeted therapies aimed at specific pathways may enhance sensitivity to chemotherapy but can also suppress the immune system, posing additional complexities in finding effective TNBC treatments (Li et al., 2022; van der Noord et al., 2022). Hormone therapies like tamoxifen and targeted treatments such as trastuzumab have proven non-responsive in this subtype (Yao et al., 2017).

TNBC disproportionately affects younger women, particularly those of African descent (Carey et al., 2006; Hercules et al., 2022). Unfortunately, our understanding of TNBC in these age groups remains limited. We urgently need to identify novel and effective therapeutic strategies for managing TNBC. Current research focuses on discovering new drugs and repurposing existing ones for TNBC treatment. Targeted therapies that block specific molecules and pathways associated with TNBC are gaining prominence. These include inhibitors targeting PI3K/AKT/mTOR, epidermal growth factor receptor, Notch, PARP, and antibody-drug conjugates (Leon-Ferre & Goetz, 2023; Zhu et al., 2023).

Standard chemotherapy for treating TNBC typically includes anthracyclines, alkylating agents, taxanes, and fluorouracil. This regimen is administered as a neoadjuvant, followed by surgery for early-stage cases. However, in cases of relapsed or refractory TNBC, there is no established standard treatment. Responses to existing options are often short-lived, and metastases remain common. Available therapies for advanced TNBC include capecitabine, gemcitabine, eribulin, and platinum-based agents (Won & Spruck, 2020).

Findings from the previous objective showed that a small molecule compound, compound 2, exhibited cytotoxicity against TNBC cell lines, particularly MDA MB 468 cells. Additionally, *in silico* analysis pointed to EGFR as a potential target. To validate its anticancer potential, further *in vitro* experiments were conducted. In this study, the impact of compound 2 on key cancer hallmarks, including apoptosis, cell cycle regulation, migration, and metastasis, was explored. Compound 2 caused a G1-cell arrest in MDA MB 468 cells and triggered significant late apoptosis

in most cell populations. Furthermore, it showed variably effect on EMT markers, inhibited cell migration and cancer stemness, all of which have implications for cancer metastasis. These findings emphasise the potential of compound 2 in positively affecting significant cancer hallmark markers.

4.4 Methods

4.4.1 Apoptosis

Following the manufacturer's protocol, the apoptosis assessment was conducted using the annexin V-FITC apoptosis detection kit (Sigma, APOAF-20TST). Briefly, MDA-MB-468 and MDA-MB-231 cells (1 x 10^6) were seeded in 100 mm tissue cultured (TC)-treated culture dishes overnight and treated for 48 hours with the respective IC_{50} values of compound 2. The 48-hour incubation period was chosen in accordance with established protocols, which indicate that this timeframe is adequate for the manifestation of drug-induced apoptotic signaling, especially in epithelial cancer cell lines. This time frame captures both early and late apoptotic events while minimizing confounding effects from secondary necrosis or non-specific cytotoxicity (Lakshmanan & Batra, 2013). After trypsinisation and centrifugation, 1 x 10^6 cells were resuspended in 1 mL of 1X binding buffer. Subsequently, 500 µL of each cell suspension was pipetted into labelled FACS tubes. Cells were stained with 5 µL of Annexin V and 10 µL of propidium iodide (PI), followed by incubation in the dark for 10 minutes. Apoptosis was quantified using the BD FACS LSR FoetessaTM X-20 flow cytometer (BD Biosciences), and results were analysed in Flowjo v10.9. Cells positive for Annexin V were only considered early apoptotic, while those positive for both Annexin V and PI were considered late apoptotic. PI staining alone indicated mechanically damaged cells. Duplicate-independent experiments were performed for each cell line, with DOX as a positive control in all assays.

4.4.2 Gene expression analysis

4.4.2.1 RNA extraction and reverse transcription real-time PCR

MDA-MB-231 and MDA-MB-468 cells (1 x 10^6) were seeded in a 100 mm TC culture dish and treated with the IC_{50} concentrations of either compound 2 or DOX for 48 hours. RNA extraction followed the Qiagen RNeasy Mini kit protocol. Cells were lysed, and the lysate was mixed with 70% ethanol. After centrifugation, buffer RW1 and buffer RPE were added successively. The spin column was then dried, and RNA was eluted using RNase-free water. RNA yield was quantified using a nanodrop. Approximately 10 ng of RNA was used for RT-qPCR experiments with the Luna Universal One-Step RT-qPCR kit (New England Biolabs). The mRNA expression of proliferation marker (*Ki67*), apoptotic genes (*Bcl-2*, *Bax*, *caspases 3* and *8*), EMT genes (*SNAI1*, *TWIST1* and *Vimentin*), and invasion and metastasis genes (*MMP2*, *MMP9*, *VCAM1*) was assessed in triplicates. *GADPH* served as the housekeeping gene. The primer sequences of each gene are shown in Table 4.1

4.4.3 Clonogenic assay

A colony formation assay following instructions by Fu et al., (2019) was conducted to interrogate the impact of compound 2 on breast cancer cell stemness. In a 6-well plate, MDA-MB-468 cells were seeded at a density of 1 × 10^3 cells per well and incubated at 37°C for 24 hours. Subsequently, the cells were treated with compound 2 or DOX at ¼ IC_{50}, ½ IC_{50}, IC_{50}, and 2xIC_{50} concentrations for 48 hours. The multiple drug concentrations were used to evaluate dose-dependent effects on long-term cell survival and to identify the minimum concentration required to inhibit clonogenic growth. This approach enables accurate assessment of drug potency and sensitivity (Franken et al., 2006). After two days, the medium was removed, and the fresh medium was replaced every three

days for 14 days. The cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet dye. Images were captured using an inverted light microscope (OPTIKA® Ponteranica, Italy). The expansion of clonogenic cells was quantified using the ImageJ plugin ColonyArea, and a graph of colony area percentage versus drug concentration was generated.

4.4.4 Cancer stemness immunophenotyping

MDA-MB-468 cells (1 x 10^6) were seeded in 100 mm TC-treated culture dishes overnight. They were treated for 48 hours with the respective IC_{50} values of compound 2 and DOX. Detection of the cell surface markers $CD44^+/CD24^+$ was performed following protocol by Gupta et al., 2011. The results were subsequently analysed using Flowjo v10.9. A duplicate independent experiment was conducted.

Table 4.1: Sequences and annealing temperature of primers used for gene expression analysis

Gene	Primer Sequences		Annealing temperature (°C)
	Forward (5′-3′)	**Reverse (3′-5′)**	
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	56
Caspase 3	GCTGCCTGTAACTTGAGAGTAG	GTATGGAGAAATGGGCTGTAGG	60
Caspase 8	AGAAGAGGGTCATCCTGGGAGA	TCAGGACTTCCTTCAAGGCTGC	60
Bcl-2	TGCACCTGACGCCCTTCAC	AGACAGCCAGGAGAAATCAAACAG	58
Bax	TGACATGTTTTCTGACGGCA	CACCCTGGTCTTGGATCCA	60
Vimentin	TCTCTGAGGCTGCCAACCG	CGAAGGTGACGAGCCATTTCC	60
TWIST1	AGGCATCACTATGGACTTTCTC	GGCCAGTTTGATCCCAGTAT	58
SNAI1	CCACGAGGTGTGACTAACTATG	ACCAAACAGGAGGCTGAAATA	56
MMP2	CTCATCGCAGATGCCTGGAA	TTCAGGTAATAGGCACCCTTGAAGA	60
MMP9	ACGCACGACGTCTTCCAGTA	CCACCTGGTTCAACTCACTCC	60
VCAM-1	GATTGGTGACTCCGTCTCATT	CCTTCCCATTCAGTGGACTATC	57
Ki67	AATTCAGACTCCATGTGCCTGAG	CTTGACACACACATTGTCCTCAGC	60

4.4.5 Cell cycle analysis

MDA-MB-468 cells (1 x 10^6) were seeded in 100 mm TC-treated dishes overnight. Subsequently, the cells were treated with the respective IC_{50} concentrations of compound 2 and DOX for 24 hours. A 24-hour incubation allows cells to complete at least one full cycle, making it sufficient to detect drug-induced changes in cell cycle distribution such as G1 arrest or S-phase delay. This time frame is widely used in protocols involving flow cytometry or live-cell imaging (Brown et al., 2010). After treatment, the cells were trypsinised and collected as a suspension. Following centrifugation, the cells were fixed using 70% ice-cold ethanol and added dropwise while gently vortexing. The fixed cells were then incubated at -20°C overnight. The cells were washed twice with cold phosphate-buffered saline (PBS) to remove residual ethanol. Next, the cells were treated with 50 µL of 1 mg/mL RNase A. Subsequently, PI staining was performed, and the cells were incubated for at least 4 hours at 4°C. Flow cytometry analysis was conducted, and the results were analysed using Flowjo v10.9. Additionally, a duplicate independent experiment was carried out.

4.4.6 Adhesion assay

MDA MB 468 cells were seeded in a 12-well plate and incubated for 6 hours. Subsequently, the cells were treated with varying compound 2 and DOX concentrations and incubated for 24 hours. This incubation time ensures stable attachment and spreading. This duration allows for integrin engagement, cytoskeletal reorganization, and downstream signalling relevant to adhesion strength and cell morphology (Pijuan et al., 2019). After treatment, the cells were collected, and 1000 cells were reseeded in fresh media onto a gelatin-coated 96-well plate. This reseeding step allowed the cells to adhere over 6 hours. Following adhesion, the plate was washed to remove any non-adherent cells. The adherent cells were then fixed using 5% paraformaldehyde and stained with crystal

violet. Three random images of adherent cells were captured for each treatment from the 96-well plate. Finally, crystal violet was solubilized using DMSO, and absorbance was measured at 570 nm.

4.4.7 Wound healing (scratch) assay

A wound healing assay was conducted to assess the impact of the compounds on cell migration (Wang et al., 2019). MDA-MB-468 cells were utilised for this study. Specifically, 70 µL of a cell suspension containing 2×10^4 cells were seeded into a 35mm µ-dish chamber equipped with a culture insert (Ibidi GmbH, Munich, Germany) and placed in a 6-well plate. The cells were incubated at 37°C for 24 hours. Subsequently, the cells were treated with the IC_{50} and IC_{25} concentrations of either compound 2 or DOX. Using these concentrations helps ensure that observed reductions in cell migration are not simply due to cell death but rather reflect true anti-migratory effects. Cell migration was monitored using an inverted light microscope (OPTIKA® Ponteranica, Italy) at 0, 12-, 24-, 36-, and 48-hours post-treatment. The acquired images were analysed using the Wound Healing Size Tool plugin in ImageJ to determine the percentage of wound closure. The experiment was performed in triplicates.

4.4.8 Cytokines concentration determination

MDA MB 468 cells (1×10^6) were seeded in 100 mm TC-treated dishes overnight. Subsequently, the cells were treated with the respective IC_{50} concentrations of compound 2 and DOX for 48 hours. After treatment, the cells were trypsinised and collected as a suspension. Following centrifugation, the supernatant was harvested for cytokine concentration determination using a 12-plex Luminex Discovery Assay, Human Premixed Multi-Analyte Kit (R&D System), following

the manufacturer`s protocol. Briefly, all reagents and samples were maintained at room temperature before use, as per the protocol's requirements, cytokine standards (TNF-α, IL-6, IL-8, IL-1β, IFN-δ, IL-2, CCL2, CCL5, CXCL1, CXCL2 were reconstituted, and serial dilutions were prepared. Microparticle and biotin-antibody cocktails were diluted. To each well, 50uL of compound 2 or DOX treated cells supernatant, the untreated cells supernatant, and standards were allocated to wells, followed by incubation. After washing steps, streptavidin-PE was added. Finally, fluorescence data acquisition allowed the determination of cytokine concentrations. The concentration of each cytokine was determined by comparing the sample fluorescence against the generated standard curve. The concentration of each cytokine was determined by comparing the sample fluorescence against the generated standard curve. This assay was done in duplicate.

4.4.9 Data analysis

The data analysis used GraphPad Prism 9.1.2 (GraphPad Software, San Diego, USA). A one-way ANOVA was conducted, followed by comparisons among multiple groups. The results were presented as the mean ± standard error of the mean (SEM), derived from at least three or two independent experiments. Statistically significant group differences were defined as $P<0.05$.

4.4.10 Ethical clearance

This study involved exclusively *in vitro* experiments using established human cancer and normal cell lines obtained from accredited cell repositories. No human participants, identifiable personal data, or patient-derived primary tissues were used during the research. Similarly, no animal models were employed. As such, in accordance with the ethical guidelines of the University of Ghana and international research standards, ethical clearance was not required for this study.

4.5 Results

4.5.1 Compound 2 induced apoptosis in breast cancer cell lines

Flow cytometry and gene expression analysis of selected genes were used to determine the effect of compound 2 on apoptosis in MDA-MB-468 and MDA-MB-231 cell lines. Flow cytometry results revealed that compound 2 significantly induced early and late apoptosis in MDA-MB-468 (82.4%) and MDA-MB-231 (55.1%) cells compared to untreated cells. Compound 2 exhibited a more pronounced apoptotic effect, with 40.5% of MDA-MB-468 cells in the late apoptosis phase, compared to 11.5% in MDA-MB-231 cells (Figure 4.1, Q2). Gene expression analysis further demonstrated that compound 2 downregulated the expression of *Bcl-2* in both breast cancer cell lines. Additionally, compound 2 led to an upregulation of *Bax* expression in MDA-MB-231 cells, marginally higher than in MDA-MB-468 cells and untreated cells (Figure 4.2). The *Bcl-2*/*Bax* ratios for compound 2-treated cells were 0.30 in MDA MB 468 cells and 0.79 in MDA-MB-231 cells (Table 4.2). Interestingly, a similar trend was observed for DOX, although the *Bcl-2*/*Bax* ratios were higher in DOX-treated cells compared to compound 2-treated cells. Compound 2 also induced an upregulation of Caspase 3 in MDA-MB-468 treated cells. These findings indicate that compound 2-treated cells may exhibit reduced cellular resistance to apoptosis stimuli.

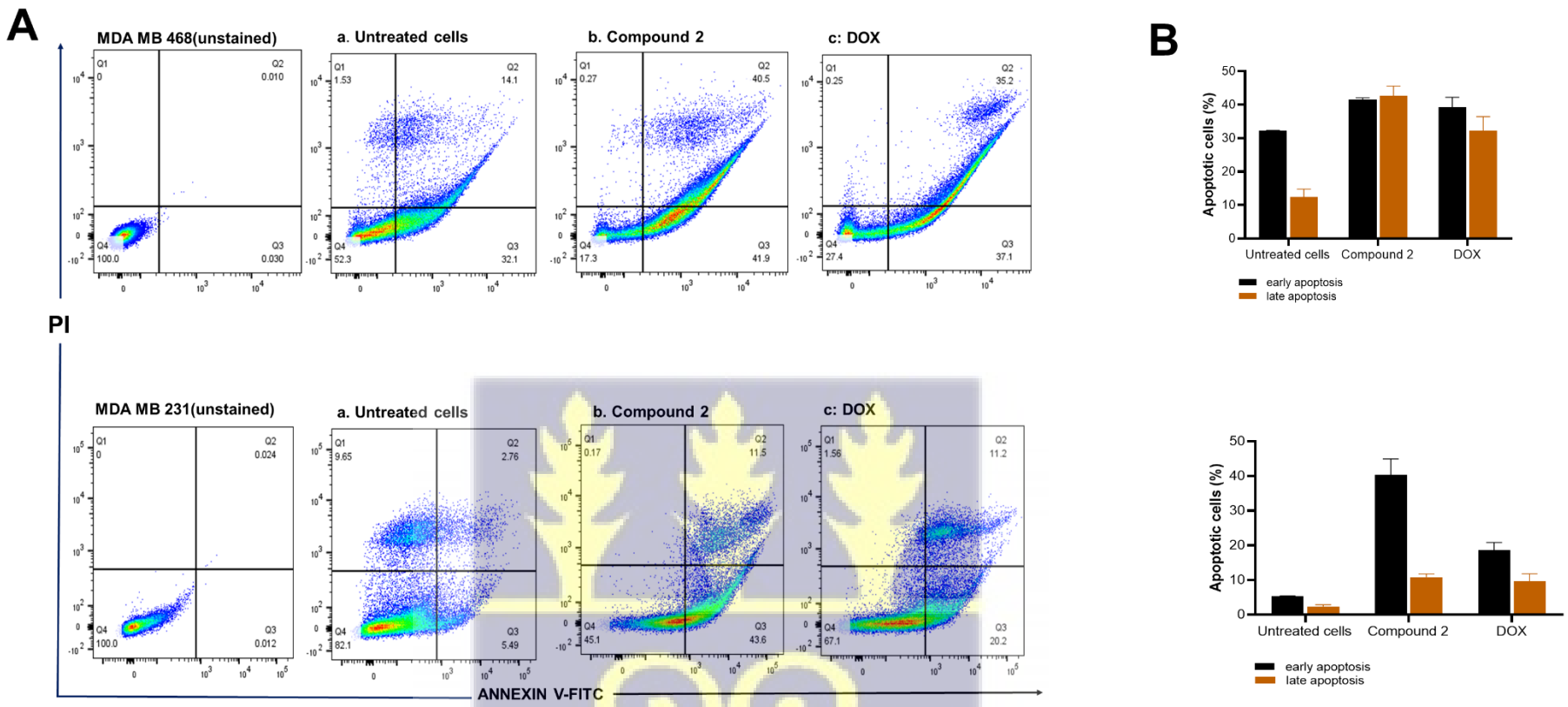


Figure 4. 1: Compound 2 induced apoptosis in breast cancer cell lines

A. Flow cytometric analysis of MDA-MB-468 and MDA-MB-231 cells treated with IC_{50} concentrations of either compound 2 or DOX. Based on the staining study, four groups were identified: necrotic/mechanically damaged cells (Q1, annexin V^-PI^+), late apoptotic cells (Q2, annexin V^+ PI^+), early apoptotic cells (Q3, annexin V^+PI^-), viable cells (Q4, annexin V^- PI^-).

B. Bar chart representing the percentage of early and late apoptotic cells in MDA-MB-468 and MDA-MB-231 cells following 48-hour treatment with IC_{50} concentrations of either compound 2 or DOX. Data are expressed as mean ± SEM from at least two independent experiments, each conducted in triplicates.

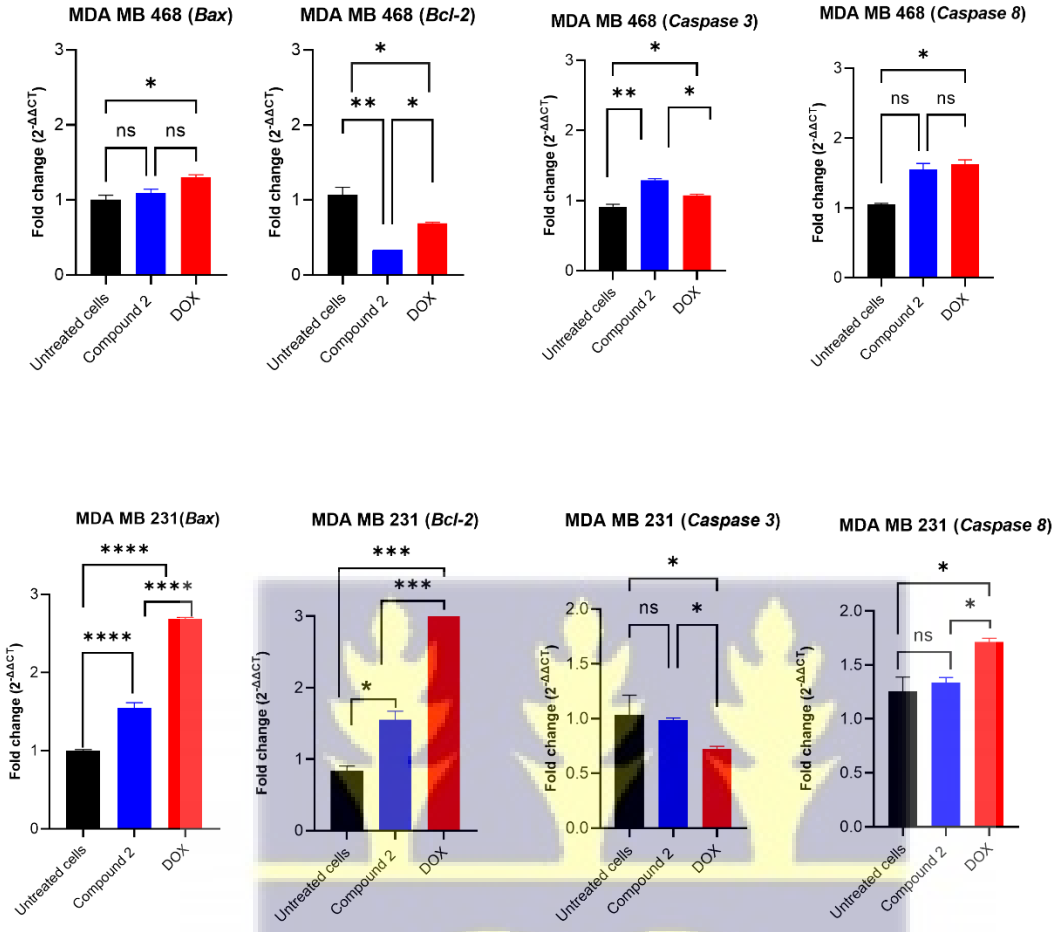


Figure 4.2: Gene expression analysis of apoptotic genes; *Bcl-2, Bax, Caspase 3 and Caspase 8*

MDA-MB-468 and MDA-MB-231 were treated with IC_{50} concentrations of compound 2 and DOX independently for 48 h. Total RNA was extracted and quantified prior to RT-qPCR analysis. GADPH was used as a housekeeping gene. The alterations in gene expression within each cell line are quantified as fold changes. Each data point represent mean ± SEM of two independent experiments, each conducted in triplicates. Asterisks (*) denoted statistically significant differences, ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$ by turkey multiple comparison test.

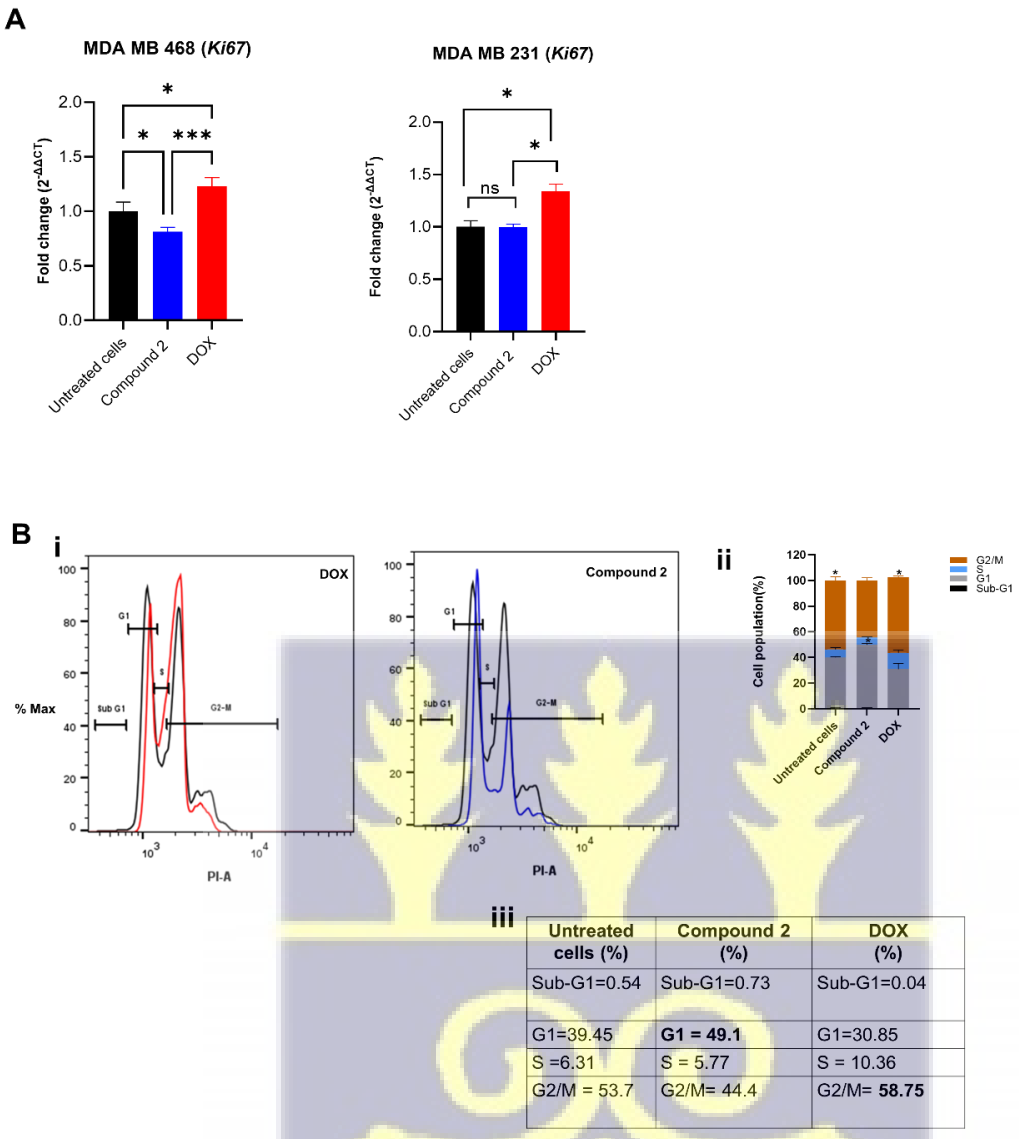
Table 4.2: *Bcl-2/Bax* ratio of the breast cancer cells

	MDA MB 468 *Bcl-2/Bax* ratio	**MDA MB 231 *Bcl-2/Bax* ratio**
Untreated cells (control)	1.0	1.04
Compound 2- treated cells	0.30	0.79
DOX-treated cells	0.53	1.20

Bcl-2/Bax <1 means the cells are susceptible to apoptosis.

4.5.2 Compound 2 induced a downregulation of *Ki67* and G1-phase cell arrest in MDA-MB -468

In the MDA MB 468 cells, treatment with compound 2 resulted in downregulation of *Ki67* expression compared to untreated cells (p-value = 0.04) (as shown in Figure 4.3A). When cells were treated with compound 2 and DOX at their IC_{50} concentration for 24 hours, a typical DNA pattern representing sub-G1, G1, S, and G2/M phases of the cell cycle was observed. Specifically, the compound 2-treated cells exhibited a significantly higher G1 population (49.1%) than the untreated control cells (39.45%). Additionally, there was a decrease in the proportion of cells in the G2/M phase from 53.7% in untreated cells to 44.4% in compound 2-treated cells (as shown in Figure 4.3B). Interestingly, DOX-treated cells had a higher proportion of cells in the G2/M phase of the cell cycle. Furthermore, the compound 2-treated cells showed a higher proportion of cells in the sub-G1 phase (indicating apoptotic cells) at 0.73%. These findings suggest that compound 2 induces G1-phase cell cycle arrest. However, in MDA MB 231 cells, there was no significant difference (p-value = 0.9) in *Ki67* expression between untreated cells and those treated with compound 2 (as shown in Figure 4.3A). Due to this, cells were not subjected to cell cycle analysis.



Untreated cells (%)	Compound 2 (%)	DOX (%)
Sub-G1=0.54	Sub-G1=0.73	Sub-G1=0.04
G1=39.45	**G1 = 49.1**	G1=30.85
S =6.31	S = 5.77	S = 10.36
G2/M = 53.7	G2/M= 44.4	G2/M= **58.75**

Figure 4.3 Compound 2 induced a downregulation of Ki67 and G1-phase cell arrest in MDA MB 468

A. Gene expression analysis of Ki67 in MDA-MB-468 and MDA-MB-231 cell lines. Cells were treated with IC_{50} concentrations of compound 2 and DOX independently for 48 h. Total RNA was extracted and quantified prior to RT-qPCR analysis. GADPH was used as a housekeeping gene. Gene expression within each cell line are quantified as fold changes. **B.(i)** Cell cycle analysis of MDA MB 468 cell line. Cells were treated with IC_{50} concentrations of compound 2 and DOX independently for 24 h. **(ii)** Analysis of cell population number % of each cell cycle phase relative to total cells. **(iii)** Distribution of cell population number % of each cell cycle phase. Data point represent mean ± SEM of two independent experiments, each conducted in triplicates. ****p < .0001; ***p < .001; **p < .01; *p < .05

4.5.3 Compound 2 suppresses stem-like properties in MDA MB 468 cells

In the immunophenotyping study, compound 2 demonstrated a significant reduction ($p=0.0012$) in the number of CSCs within the MDA MB 468 cell line compared to untreated cancer cells. In compound 2-treated cells, the CSCs expressed the $CD44^{+}/CD24^{+}$ markers at a percentage of 43.3, whereas the untreated group had a percentage of 54.2. Notably, DOX cells induced a more pronounced reduction in the CSC population than compound 2 (Figure 4.4A). A clonogenic assay was employed to assess the cells' potential for re-proliferation after exposure to different concentrations of compound 2. The results revealed that compound 2 exhibited a dose-dependent inhibition of cell re-proliferation ($p<0.0001$) (as shown in Figure 4.4B). However, there was no significant difference ($p>0.05$) in re-proliferation between the IC_{50}-treated group and the 2x IC_{50}-treated cells. This indicates that compound 2 may have the potential to prevent cancer relapse.

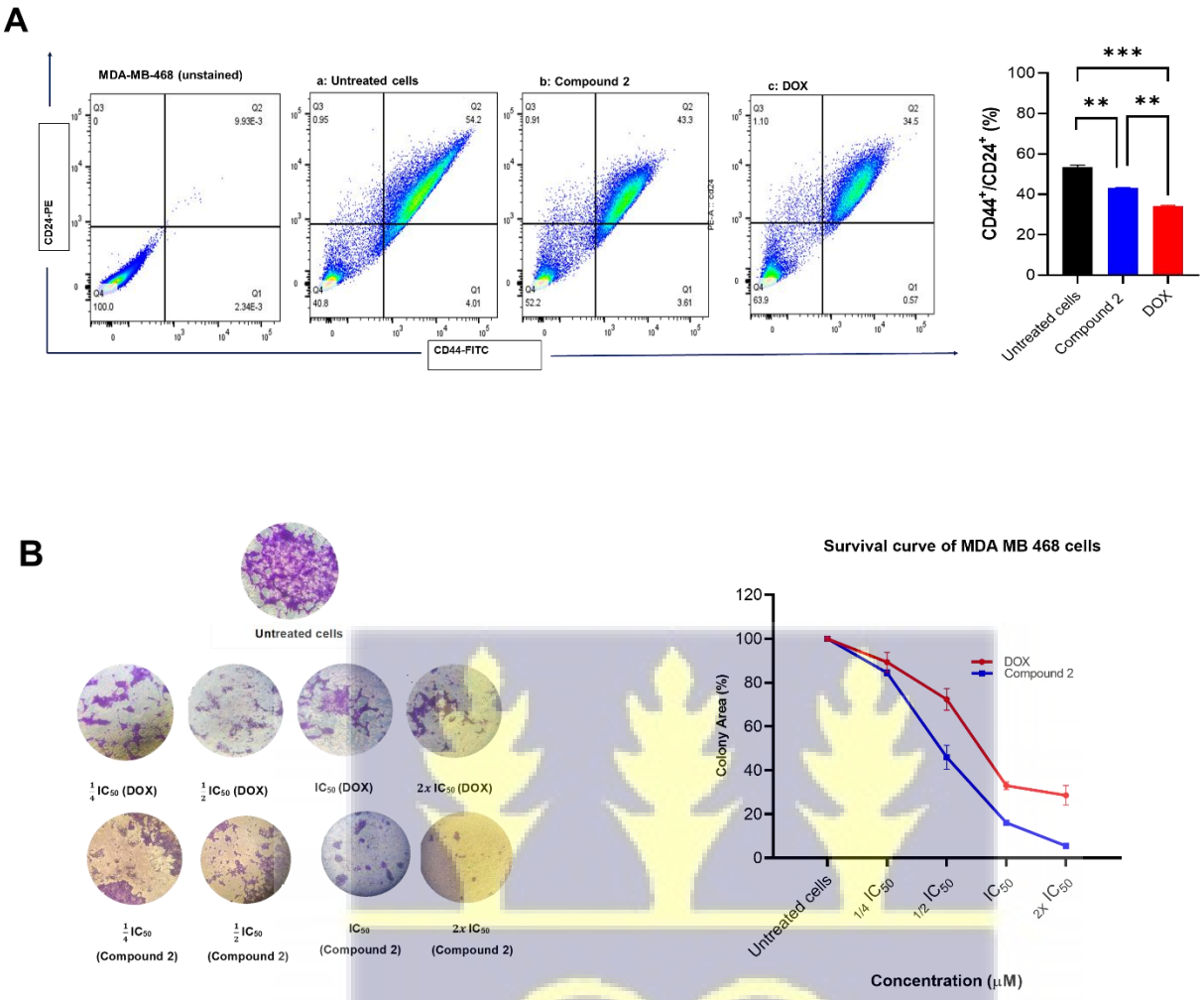


Figure 4.4: Compound 2 suppresses stem-like properties in MDA MB 468 cells

A. Flow cytometric analysis revealed that compound 2 decreased the $CD44^+/CD24^+$ expression in MDA MB 468 cells compared to untreated cells. The staining study allowed the identification of four distinct groups: Q1, $CD44^+/CD24^-$ cells, Q2, $CD44^+/CD24^+$ cells, Q3, $CD44^-/CD24^+$ cells, and Q4, $CD44^-/ CD24^-$ cells.

B. The number of MDA-MB 468 colonies formed after treatment with 0 (untreated), ¼ IC_{50}, ½ IC_{50}, IC_{50}, and 2xIC_{50} concentrations of compound 2 or DOX for 48 h. Cells were incubated for 10–14 days post-treatment to allow colony formation, followed by fixation and staining with 0.5% crystal violet. Colonies were counted and a survival curve of colony area against concentration was plotted.

4.5.4 Compound 2 exhibited diverse effects on the expression of three genes associated with EMT

Metastasis involves sequential steps, including critical events such as EMT, cell migration, and invasion. The expression of EMT-related genes exhibited variable effects between the two breast cancer cell lines studied (Figure 4.5). In the MDA MB 468 compound 2-treated group, *TWIST1* was upregulated compared to the untreated group. However, this difference was not statistically significant (p-value=0.07). No significant difference in *TWIST1* expression was observed between MDA MB 231 compound 2-treated cells and the untreated group. In MDA MB 468 compound 2-treated cells, *SNAI1* showed significant downregulation (p-value=0.035) compared to untreated cells. However, no significant difference (p-value=0.68) in *SNAI1* expression was observed in MDA MB 231 compound 2-treated cells compared to untreated cells. *Vimentin* was significantly upregulated (p-value=0.03) in MDA MB 468 compound 2-treated cells. Conversely, compared to the untreated group, vimentin was downregulated considerably in MDA MB 231 compound 2-treated cells (p-value=0.03). These findings imply that compound 2 may impact EMT-related processes differently in distinct breast cancer cell lines.

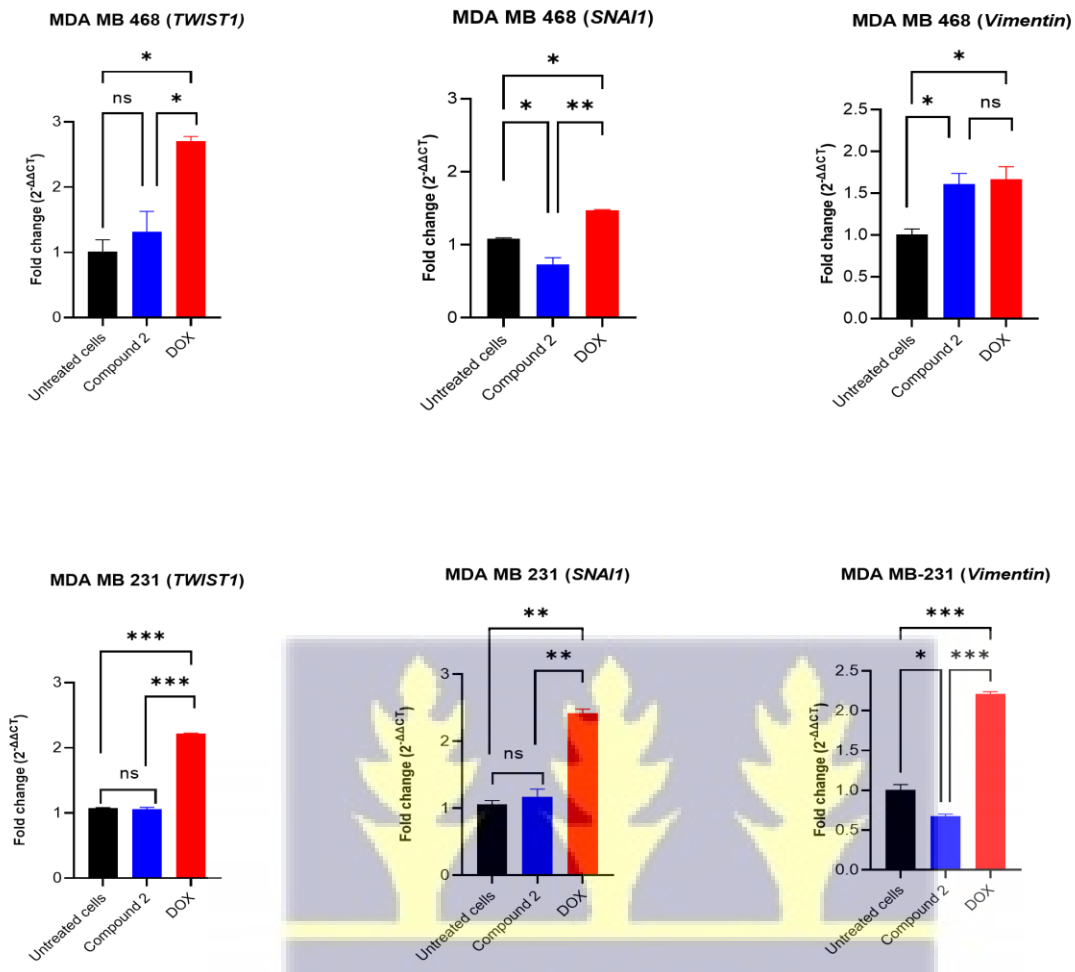


Figure 4.5: Compound 2 exhibited diverse effects on the expression of three genes associated with EMT

MDA-MB-468 and MDA-MB-231 were treated with IC_{50} concentrations of compound 2 and DOX independently for 48 h. Total RNA was extracted and quantified prior to RT-qPCR analysis. GADPH was used as a housekeeping gene. The alterations in gene expression within each cell line are quantified as fold changes. Each data point represent mean ± SEM of two independent experiments, each conducted in triplicates. Asterisks (*) denoted statistically significant differences, ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$ by turkey multiple comparison test.

4.5.5: Compound 2 inhibited cellular adhesion of MDA MB 468 cells.

Cells were cultured with two doses (IC_{50} and IC_{25}) of compound 2 and DOX for 24 hours, then reseeding the pretreated cells for an additional 3 hours. Both compound 2 and DOX demonstrated significant dose-dependent inhibition of cellular adhesion in MDA MB 468 cells compared to the untreated control (Figure 4.6A&B). Remarkably, cells treated with the IC_{50} concentration of compound 2 exhibited the most pronounced inhibition, with only approximately 29.77% of adhered cells remaining post-treatment (Figure 4.6A). While this difference was not statistically significant (p-value=0.93) compared to the percentage of adhered cells (31.5%) in the IC_{50} DOX-treated group, a statistically significant difference (p-value < 0.001) was evident when comparing compound 2-treated cells to the untreated cells. This observation aligns with the image of cells treated with the IC_{50} of compound 2 (Figure 4.6B), which depicts fewer attached cells compared to the untreated cells. These data indicate that compound 2 may help prevent cancer growth and spread.

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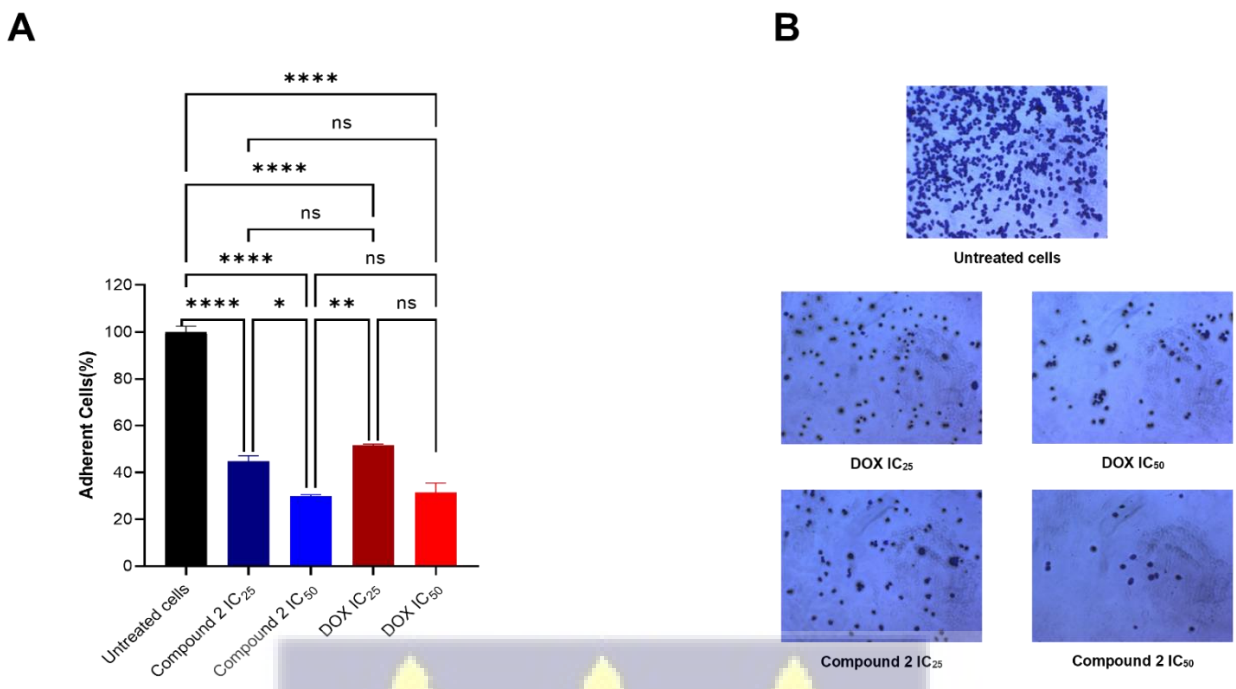


Figure 4.6: Compound 2 suppressed cellular adhesion of MDA MB 468 cells

A. Quantification of adherent MDA-MB-468 cells following 24-hour treatment with the IC_{50} concentration of compound 2 or doxorubicin (DOX). After treatment, 1,000 cells were reseeded, fixed with 5% paraformaldehyde, stained with crystal violet, and absorbance was measured at 570 nm to assess cell attachment. The experiment was performed three times independently, and the data shown are the average of all three replicates. Asterisks (*) denoted statistically significant differences, ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$ by turkey multiple comparison test.

B. Representative images depict cell adherence following reseeding after IC_{50} treatment for 24 h with compound 2 or DOX. Reduced attachment may reflect treatment-induced impairment adhesion capacity. Representative images from the three independent experiments. Scale bar: 200 µm.

4.5.6 Inhibitory effects of compound 2 on cell migration

Compound 2 had varying effects on gene expression levels in the two breast cancer cell lines. In MDA MB 468 cells, *MMP2*, *MMP9*, and *VCAM-1* expression were downregulated compared to untreated cells. However, MDA MB 231 cells led to the downregulation of *MMP2* and the upregulation of *MMP9*. The expression of *VCAM-1* remained unchanged between untreated cells and MDA MB 231 cells. Interestingly, all the genes were upregulated in DOX-treated cells, with significant overexpression of *VCAM-1* (as shown in Figure 4.7A). Additionally, the wound healing assay was employed to study cell migration. This experiment treated MDA MB 468 cells with either the IC_{50} or IC_{25} concentration of compound 2 and DOX. Initially, the IC_{50} dose of compound 2 inhibited migration at 12 and 24 hours, followed by cell death observed at 36-48 hours. Consequently, the wound area increased. In contrast, untreated cells exhibited closure of the wound in a time-dependent manner. Notably, cells treated with the IC_{25} dose of compound 2 consistently demonstrated inhibition of wound closure (as depicted in Figure 4.7B). Specifically, at 48 hours, the wound area was 50.54% ± 1.31% for compound 2-treated cells, compared to 26.76% ± 0.9% for untreated cells. These findings suggest that compound 2 can inhibit cell migration and wound closure.

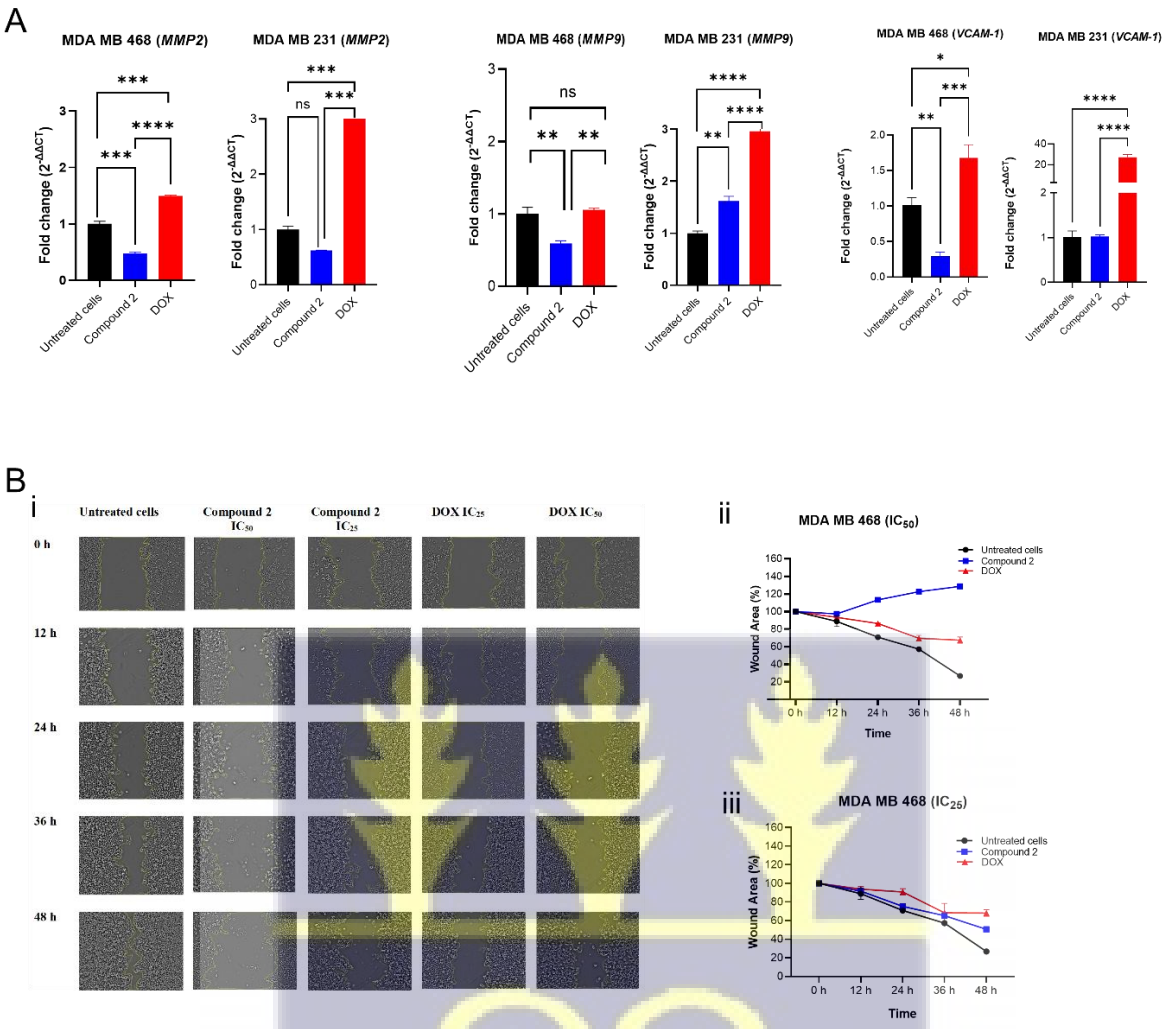


Figure 4.7: Inhibitory effects of compound 2 on cell migration

A. Gene expression analysis of invasion and metastasis genes; *MMP2*, *MMP9*, *VCAM-1*. MDA-MB-468 and MDA-MB-231 were treated with IC_{50} concentrations of compound 2 and DOX independently for 48 h. Total RNA was extracted and quantified prior to RT-qPCR analysis. GADPH was used as a housekeeping gene. The alterations in gene expression within each cell line are quantified as fold changes. Each data point represent mean ± SEM of two independent experiments, each conducted in triplicates. Asterisks (*) denoted statistically significant differences, ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$ by turkey multiple comparison test.
B.(i) Wound healing assay to study cell migration of MDA-MB-468 BC cell line treated IC_{25} and IC_{50} concentration of Compound 2 and DOX independently. **(ii)** & **(iii)** Plot of wound area over time for IC_{50}-treated cells and IC_{25} treated cells respectively. The wound area at different time points is a percentage relative to the initial measurement at 0 hours. Two independent experiments were done. The original magnification used was ×100.

4.5.7 Effect of compound 2 and DOX on expression of cytokines

From this study, Compound 2 increased the expression of TNF and IL-1B; however, these expressions were not significant when compared to the control. DOX significantly upregulated the expression of IL-6, IL-8, IL-4, and IL-2. Though DOX increased the expression of IL-10 and IFN, the expression was insignificant compared to the untreated cells. Compound 2 only significantly affected the expression of IL-8. DOX induced higher cytokine expression compared to Compound 2. Compound 2 was found to upregulate CCL2 and CXCL1 expression; however, there was no significant difference between CXCL2 and CCL5 expression for all treatments in the breast cancer cell line. DOX significantly increased the CCL2 and CXCL1 expression in the breast cancer cells compared to the untreated cells and Compound 2 (Figure 4.8).

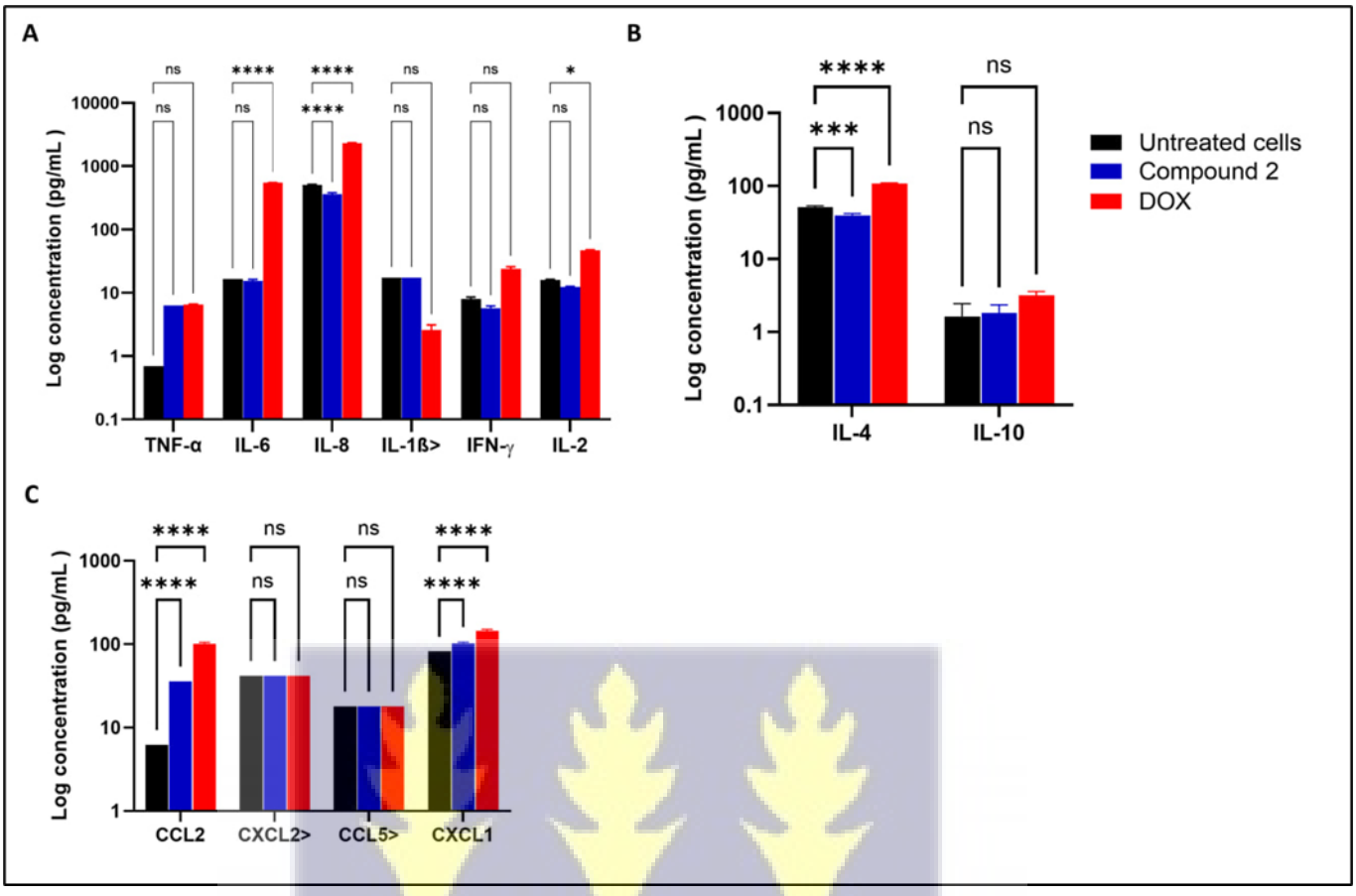


Figure 4.8: Effect of compound 2 and DOX on cytokines concentration in MDA-MB-468 cells

A. Plot showing the effect of Compound 2 and DOX on selected pro-inflammatory cytokines levels.

B. Plot showing the effect of Compound 2 and DOX on selected anti-inflammatory cytokines levels.

C. Plot showing the effect of Compound 2 and DOX on selected chemokines levels.

MDA-MB-468 were treated with IC_{50} concentrations of compound 2 and DOX independently for 48 h. Luminex determined the protein levels of all the cytokines. Data are presented as mean ± SD of three independent experiments performed in duplicate. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ vs. untreated. ns: not significant.

4.6 Discussion

This study investigated the impact of Compound 2 on the expression of key markers associated with breast cancer. The objective was to discover a new cancer drug candidate by studying compound 2`s effect on cell behaviour, gene expression, and cancer stem cells.

In the cell cycle analysis, the results obtained for DOX align with previous research findings (Ling et al., 1996; Nicoletto & Ofner, 2022; Shin et al., 2015)**.** During the G1 phase of the cell cycle, cells encountering arrest face two possible outcomes: either they undergo DNA repair facilitated by the p53 gatekeeper, or they proceed toward apoptosis if DNA repair is unfeasible (Pellegata et al., 1996). Notably, the cells treated with compound 2 arrested a significant population in the G1 phase, which may contribute to the induction of apoptosis in MDA-MB-468 cells. G1 arrest can prevent cells from entering the S phase, causing an inhibition of cell proliferation (Bertoli et al., 2013). The flow cytometry apoptotic analysis revealed that most compound 2-treated cells were in the late apoptotic phase (Figure 4.1A). Additionally, the expression levels of Ki67, a proliferation marker, were downregulated in the MD- MB-468 cells. Ki67 is a tumour biomarker in breast cancer diagnosis, staging, and monitoring (Davey et al., 2021; Jones et al., 2009). It also aids in predicting chemotherapy response by estimating cancer growth and spread. The observed downregulation of *Ki67* aligns positively with the cell cycle and apoptosis analysis findings, indicating that MDA-MB-468 cells were more susceptible to compound 2. Furthermore, the G1-phase cell cycle arrest induced by compound 2 hints at its potential interference with cell cycle progression, potentially inhibiting cell proliferation in cancer cells. The *Bcl-2/Bax* ratio is pivotal in regulating apoptosis, a genetically controlled cell death process. Bcl-2 inhibits cell death, while BAX promotes it (Cory & Adams, 2002). The balance between cell survival and cell death hinges

on the ratio of Bcl-2 to BAX. A higher *Bcl-2/Bax* ratio favours cell survival by inhibiting apoptosis, whereas a lower ratio shifts the balance toward apoptosis and cell death. Compound 2, with its lower *Bcl-2/Bax* ratio, may promote apoptosis. This effect could be advantageous in cancer treatment because it targets cancer cells potentially limiting tumour growth and enhancing therapeutic efficacy. By reducing anti-apoptotic *Bcl-2* levels relative to pro-apoptotic *Bax*, compound 2 may enhance cancer cell death. In contrast, DOX likely has a distinct impact on this ratio. A lower *Bcl-2/Bax* ratio in cancer cells indicates a potential therapeutic benefit. It implies that compound 2 might selectively induce apoptosis in cancer cells while sparing normal cells. However, further investigation is necessary to validate this finding and explore the precise underlying mechanisms.

Compound 2, like the standard chemotherapeutic agent DOX, suppressed cancer cell adhesion to the extracellular matrix (ECM) component known as gelatin. The significance of cancer cells adhering to the ECM extends to critical aspects of cancer progression, invasion, and metastasis. Cancer cells rely on anchorage-dependent growth, which involves adhering to a surface (often the ECM) for division and survival. This process plays a pivotal role in forming and maintaining solid tumours ("Cell Adhesion and the Extracellular Matrix," 2021; Farahani et al., 2014). Cancer cells must first degrade the ECM and move through it to become invasive and metastatic. However, this degradative process requires initial attachment to the ECM (Kai et al., 2019). Adhesion to the ECM provides a platform for cancer cells to secrete enzymes that break down ECM components, facilitating their invasion into surrounding and distant tissues. Additionally, cancer cells can interact with the ECM to stimulate angiogenesis by releasing pro-angiogenic factors and adhering to endothelial cells lining blood vessels (Neve et al., 2014; Paolillo & Schinelli, 2019). Compound

2 can influence cell survival by modulating interactions between cancer cells and the ECM. The observed effects on cell migration and adhesion indicate that Compound 2 might inhibit cancer progression and metastasis. Additionally, it appears that Compound 2 interferes with focal adhesions, which were identified as plausible targets through network pharmacology in Chapter 3. Focal adhesions play a critical role in cell attachment and migration. By targeting adhesion molecules, such as integrins and cadherins, Compound 2 could disrupt these adhesive structures, resulting in reduced cell attachment and altered signalling pathways (Yayan et al., 2024).

The varying effects of Compound 2 on gene expression levels in the two breast cancer cell lines (MDA-MB-468 and MDA-MB-231) suggest that it may have distinct mechanisms of action depending on the cellular context. The downregulation of *MMP2*, *MMP9*, and *VCAM-1* in MDA-MB-468 cells may indicate a potential role in suppressing cancer cell invasion and metastasis. However, the upregulation of *MMP9* in MDA-MB -231 cells warrants further investigation. For the wound healing assay, treatment with the IC_{50} dose of compound 2 initially inhibited cell migration, followed by cell death. This suggests that compound 2 affects both migration and viability. Notably, the IC_{25} dose of compound 2 consistently inhibited wound closure, reinforcing its potential as an anti-migratory agent. EMT is a process where epithelial cells acquire mesenchymal properties, promoting cell migration and invasion. Compound 2 might modulate EMT-related pathways, affecting cancer cell behaviour. EMT is closely linked to metabolic changes in cancer cells. *TWIST1* and *SNAI1* are key transcription factors involved in EMT regulation. Compound 2 may directly or indirectly affect the expression of these factors. The observed downregulation of *SNAI1* in MDA-MB-468 cells suggests that compound 2 could inhibit EMT by suppressing *SNAI1*, which promotes mesenchymal characteristics (Huang et al., 2022).

Vimentin, an intermediate filament protein, is associated with mesenchymal properties. Upregulation of *Vimentin* in MDA-MB-468 cells upon compound 2 treatment indicates a potential shift toward a mesenchymal state. Conversely, the downregulation of *Vimentin* in MDA-MB-231 cells suggests an opposing effect on EMT. Compound 2 differently regulates *Vimentin* in MDA-MB-468 and MDA-MB-231 TNBC cells, reflecting their distinct EMT statuses. MDA-MB-231 cells are inherently mesenchymal-like with high vimentin expression, so compound 2 may induce a mesenchymal-to-epithelial transition (MET), leading to vimentin downregulation; in contrast, MDA-MB-468 cells are more epithelial-like and may undergo partial EMT upon treatment, resulting in vimentin upregulation. These opposing responses may also stem from differences in pathway activation (e.g., PI3K/AKT, MAPK), epigenetic regulation, or post-transcriptional control mechanisms such as miRNA activity. These results highlight that intrinsic subtype differences impact EMT dynamics and influence the therapeutic potential of compound 2, stressing the importance of tailored approaches for TNBC treatment (Dongre & Weinberg, 2019). EMT is closely connected with metabolic alterations in cancer cells. It is plausible that compound 2 may modify metabolic pathways (such as glycolysis and oxidative phosphorylation) that impact EMT progression (Georgakopoulos-Soares et al., 2020). EMT involves changes in cell adhesion molecules and cytoskeletal components. Compound 2 could impact these structures, affecting cell motility and invasiveness. Compound 2 modulates EMT differently in distinct breast cancer cell lines. Its differential impact on different breast cancer cell lines highlights the complexity of EMT regulation and the need for further investigation (Cho et al., 2019; Huang et al., 2022). Understanding these differential effects can guide personalised treatment strategies. Compound 2 could be combined with existing chemotherapeutic agents or targeted therapies.

Reducing CSCs is a promising discovery, as they play crucial roles in tumour initiation, progression, and therapy resistance. Targeting CSCs holds the potential for inhibiting cancer growth and minimising recurrence risk. Typically, the $CD44^{+}/CD24^{-/low}$ expression pattern is associated with cancer stem cell characteristics in breast cancer and correlates with poor clinical outcomes (Camerlingo et al., 2014; Ponti et al., 2005). However, MDA-MB-468 cells have shown a different profile in some studies, predominantly featuring $CD44^{+}/CD24^{+}$ cells and a low population of $CD44^{+}/CD24^{-}$ cells (Jaggupilli & Elkord, 2012; Li et al., 2017; Ricardo et al., 2011; Sheridan et al., 2006). This observation aligns with the CSC flow cytometric analysis of the MDA-MB-468 cells used in our study, which also exhibited a predominance of $CD44^{+}/CD24^{+}$ and low $CD44^{+}/CD24^{-}$ cells. The noteworthy feature is that epithelial cell lines such as MDA-MB- 468 exhibit high levels of CD24 while inducing tumours. The mechanism behind this phenomenon remains uncertain. Although MDA-MB-468 is abundant in non-invasive $CD44^{+}/CD24^{+}$ cells, it is suspected that a subset of $CD44^{+}/CD24^{-}$ cells within this cell line undergoes an interconversion to the $CD44^{+}/CD24^{+}$ phenotype following metastasis (Sheridan et al., 2006). Study by Croker et al., (2009) supported this concept, demonstrating migration, colony formation, and invasion by $CD44^{+}$ cells in MDA-MB-468. A recent study by Fernando et al., (2024) also supports the concept of interconversion between $CD44^{+}/CD24^{-}$ and $CD44^{+}/CD24^{+}$ phenotypes in MDA-MB-468 cells. Additionally, Meyer et al., (2009) proposed the possibility of interconversion between these phenotypes, suggesting that epithelial-like $CD44^{+}/CD24^{+}$ cells can readily give rise to $CD44^{+}/CD24^{-}$ cells during tumour initiation. Our findings show that DOX significantly decreased the CSC population more than compound 2. This suggests that DOX might be particularly effective in targeting CSCs, possibly through distinct mechanisms. Further investigation is needed to understand better the differential impact of compound 2 and DOX on CSCs. Additionally, the

clonogenic assay results indicate compound 2 effectively suppresses cancer cell re-growth, especially at concentrations near its IC_{50} value. Collectively, these data suggest that compound 2 may have a role in preventing cancer relapse by targeting CSCs and inhibiting cell re-proliferation. However, additional studies are necessary to validate these observations and explore the precise molecular pathways involved. Furthermore, considering its impact on EMT and CSCs, compound 2 may enhance improve the efficacy of traditional cancer treatment (Sharifi-Rad et al., 2023).

Cytokines are critical mediators of the immune response and play a pivotal role in cancer progression by influencing tumour growth, metastasis, and the tumour microenvironment. Our findings provide significant insights into how compound 2 and DOX treatments modulate the inflammatory responses, which can, in turn, impact breast cancer progression. From this study, IL-6 levels remained similar between untreated cells and Compound 2-treated cells but showed a substantial increase with DOX treatment. The increase in IL-6 expression upon treatment with DOX is consistent with findings from other studies (Elsea et al., 2008; Srdic-Rajic et al., 2018). This upregulation is believed to be mediated through the activation of the NF-κB signalling pathway, a crucial regulator of inflammatory responses and cancer cell survival (Esparza-López et al., 2013). Furthermore, IL-6 has been shown to promote EMT (Wang et al., 2018). In addition to its direct effects on cancer cells, DOX-induced IL-6 secretion also impacts the tumour microenvironment and elevated IL-6 levels are linked with the development of chemoresistance, further complicating the therapeutic management of breast cancer (Bent et al., 2021; Srdic-Rajic et al., 2018). The significant increase in IL-6 levels with DOX treatment indicates a robust inflammatory reaction and potential activation of pathways that can lead to more aggressive cancer behaviour. Compound 2 did not significantly elevate IL-6 levels from this study, suggesting a

potentially less aggressive inflammatory response. Compound 2 may selectively target pathways or molecules that do not directly interact with IL-6 signalling. The absence of an effect on IL-6 levels suggests that Compound 2 could be a candidate for combination therapy. Drugs that specifically reduce IL-6 or inhibit its signalling pathways could be used alongside Compound 2 to achieve a more comprehensive anti-tumour effect. DOX treatment resulted in elevated levels of IL-8, a cytokine associated with angiogenesis and EMT, potentially promoting cancer aggressiveness (Long et al., 2016; Zhao et al., 2017). In contrast, Compound 2 exhibited lower IL-8 levels, suggesting a less aggressive inflammatory profile. While DOX reduced IL-1β levels contrary to some studies, indicating complex inflammatory responses (Rébé & Ghiringhelli, 2020; Sauter et al., 2011), Compound 2 showed no significant change in IL-1β levels, implying different inflammatory modulation. Both DOX and Compound 2 increased TNF-α, a cytokine involved in inflammation and tumour progression, with a milder effect observed than controls. DOX increased IL-2 levels compared to compound 2, suggesting activation of anti-tumour immune responses. IL-10 levels were detectable but not significant for either treatment, indicating minimal anti-inflammatory response. DOX increased IL-4, which may be associated with tumour growth promotion, whereas Compound 2 did not significantly elevate IL-4 levels, suggesting a less tumour-promoting immune profile. IFN-γ level was decreased in compound 2 treated cells even though this was not statistically significant, DOX showed decreased IFN-γ levels, which was also not significant compared to the untreated cells. Both treatments, however, significantly elevated CCL2. This might hinder the release of IFN-γ by T cells, which weakens the body's ability to fight against tumours and facilitates the growth of tumours (Salcedo et al., 2000; Steiner & Angela Murphy, 2012). CXCL1 levels were highest with DOX, promoting angiogenesis and tumour growth (Schraufstatter et al., 2001; Wang et al., 2006), while Compound 2 resulted in a moderate

increase in CXCL1 levels. These findings highlight distinct inflammatory and immune responses to Compound 2, suggesting potential as an alternative or complementary therapy in breast cancer treatment strategies. Its ability to modulate inflammation and immune responses differently from DOX suggests it may offer therapeutic benefits with potentially reduced side effects related to inflammation and immune suppression.

In summary, compound 2 shows promise as a targeted cancer therapy by inhibiting proliferation, migration, and EMT-related gene expression, while promoting apoptosis through a reduced Bcl-2/Bax ratio. It significantly lowers cancer stemness, suggesting a role in preventing relapse. Unlike DOX, compound 2 induces a milder inflammatory response with lower levels of tumour-promoting cytokines, indicating fewer side effects. Its immune modulation profile supports its potential as an alternative or complementary therapy for breast cancer. This study corroborates in *silico* findings in chapter 3 identifying EGFR as a key target of compound 2. EGFR is frequently overexpressed in TNBC, especially in basal-like subtypes such as MDA-MB-468. Compound 2 likely impairs EGFR kinase activity by blocking ATP binding. This inhibition may disrupt downstream signalling pathways, including PI3K/AKT and MAPK/ERK. These pathways are critical for promoting cell proliferation, survival, and migration in TNBC. Further studies are needed to explore the molecular mechanisms and clinical potential of compound 2 in breast cancer treatment as well as direct biochemical validation of EGFR inhibition by compound 2. This study's limitation is relying on mRNA data without validating protein level changes. Future research should confirm these results using methods like Western blotting, focusing on apoptosis markers and EMT-related proteins.

4.7 Conclusion

The observed effects of compound 2 on cancer cells highlight its potential as a therapeutic agent. Specifically, it influences cancer cell behaviour regarding proliferation, adhesion, migration, and gene expression related to EMT. The inhibition observed with compound 2 in cancer cells may be attributed to several mechanisms. The lower *Bcl-2*/*Bax* ratio observed for compound 2 -treated cells suggests it may promote apoptosis. Reducing anti-apoptotic *Bcl-2* levels relative to pro-apoptotic *Bax* enhances cancer cell death. Compound 2 significantly reduces CSCs within the MDA MB 468 cell line. Targeting CSCs can potentially hinder tumour growth and reduce the likelihood of recurrence. Compound 2's effects on CSCs and inhibition of cell re-propagation suggest it may prevent cancer relapse. Compound 2 may alter metabolic pathways, suppress programmed cell death, and affect cancer stem cell self-renewal. Compound 2 also shows promise as a therapeutic agent with a unique inflammatory and immune modulation profile compared to DOX. While these mechanisms are promising, additional research is essential to validate compound 2's efficacy and explore the precise molecular pathways involved. In summary, compound 2's diverse actions make it a potential targeted cancer therapy. Further research will clarify its therapeutic value and clinical use.

CHAPTER FIVE

5.0 Objective 3: To design analogues of the most potent compound and screen selected analogues.

1. To design about 36 analogues of the potent compound, compound 2,
2. To predict their pharmacokinetics properties using SwissADME and performing docking studies against the plausible target, EGFR.
3. To identify potent analogues by screening a selected subset of analogues in the MDA MB 468 breast cancer cell line using the MTT assay

5.1 Rationale for Objective 3

The rationale behind designing lead compound analogues lies in the potential benefits of improving EGFR inhibitor efficacy and safety. In the quest for more effective cancer treatments, scientists meticulously modify the chemical structures of lead compounds to enhance crucial drug-like properties, including solubility, stability, and bioavailability (Fischer & Ganellin, 2010a, 2010b). These modifications pave the way for targeted therapies that hold promise in the fight against cancer. Researchers can selectively create analogues based on structural insights into EGFR-inhibitor interactions, explicitly targeting specific EGFR variants and overcoming resistance mechanisms.

5.2 Abstract

EGFR is an essential druggable target in most cancers. Despite the presence of several generations of EGFR inhibitors, the development of resistance to these inhibitors is still a challenge. Compound 2 was found to be a potential inhibitor of EGFR through MD simulation. In this study, we aimed to generate analogues of Compound 2 as potential inhibitors of EGFR with improved therapeutic efficacy based on its IC_{50} value. Thirty-six (36) analogues were generated via the scaffold-hopping analogue approach. Substructure and Tanimoto similarity search methods were employed to identify the structurally related compounds of compound 2, and plausible analogues were determined via molecular docking. Pharmacokinetic analysis of all analogues was done using Swiss ADME. Eight selected analogues were then screened against MDA-MB 468 cells. Among the 36 analogues generated, 20 exhibited similarities to existing compounds, while the remaining 16 showed no similarity to known compounds. These 16 compounds require further investigation to validate their potential novelty. Among the analogues, compound E and compound B13 were not substrates of P-glycoprotein (P-gp), suggesting potential resistance-invading properties. Most analogues adhered to Lipinski's Ro5, and all had an excellent bioavailability score of 0.55. The eight analogues screened against MDA MB 468 showed no anticancer effect. The data suggest that the screened analogues do not have any potent anticancer activity.

5.3 Introduction

The epidermal growth factor receptor (EGFR) is a type of receptor tyrosine kinase crucial for cell growth, proliferation, and survival. Dysregulation of EGFR has been associated with several cancers, including non-small cell lung cancer, breast cancer and squamous cell carcinoma (Cowley et al., 1986; Vealel et al., 1987). EGFR activates vital signalling pathways, such as Ras/Raf/MEK/ERK and PI3K/AKT/mTOR, which govern essential cellular functions (Bjorge et al., 1990; Gotoh et al., 1995). Abnormal activation and dysregulation of EGFR signalling contribute to the development and progression of various cancer types, making it an attractive target for therapeutic interventions.

EGFR inhibitors represent a promising strategy to combat EGFR-driven malignancies. These inhibitors bind to the EGFR receptor, blocking its activation and downstream signalling. Consequently, they impede the uncontrolled growth and division of cancer cells, ultimately leading to cell death. However, existing EGFR inhibitors have limitations, including efficacy challenges and the emergence of resistance. While first-generation inhibitors like erlotinib and gefitinib show promise in treating specific EGFR-mutant cancers, resistance often develops over time (Shi et al., 2022). A typical resistance mechanism is the acquisition of secondary mutations in the EGFR gene, such as the T790M mutation in exon 20, which alters the binding affinity of TKIs, rendering them ineffective (Kobayashi et al., 2005). Third-generation irreversible EGFR-Tyrosine kinase Inhibitors (TKIs), such as osimertinib, have successfully treated non-small cell lung cancer (NSCLC) with EGFR T790M mutations. Unfortunately, even osimertinib, a commonly used drug, eventually faces resistance, which limits its long-term effectiveness (Du et al., 2021; Li et al., 2023). Some cancers, particularly NSCLC, can undergo histological transformation to a more

aggressive phenotype, such as small cell lung cancer (SCLC), which is less responsive to EGFR TKIs (Sequist et al., 2011). Overexpression of drug efflux transporters, such as ABCB1 (P-glycoprotein), and changes in drug metabolism can reduce the intracellular concentration of TKIs, diminishing their efficacy (Ambudkar et al., 2003). Lastly, aside from developing resistance of cancer cells to TKI, cancer cells can also activate compensatory signalling pathways to bypass EGFR inhibition. For example, amplification of the MET gene or activation of the PI3K/Akt and MAPK pathways can sustain cell survival and proliferation despite EGFR blockade(Engelman et al., 2007).

Blueprint's lead EGFR inhibitor, BLU-945, has shown safety in early clinical trials (Shum et al., 2022). Fourth-generation inhibitors are being explored to overcome resistance mechanisms (Mullard, 2022). Also, combining EGFR TKIs with other targeted therapies, such as MET inhibitors or PI3K/Akt pathway inhibitors, may block multiple survival pathways and prevent compensatory mechanisms (Terp et al., 2021; Wang et al., 2019). Additionally, combining EGFR TKIs with immune checkpoint inhibitors, such as anti-PD-1/PD-L1 antibodies, is being explored to enhance antitumor immune responses and overcome resistance (Rizvi et al., 2015). However, continuing research and developing improved EGFR inhibitors is essential to enhance therapeutic outcomes. In summary, EGFR plays a pivotal role in cancer biology, and the quest for novel agents targeting this receptor holds immense potential for treating EGFR-driven malignancies. Researchers can propel next-generation medications by screening for innovative EGFR inhibitors and employing rational analogue design strategies to enhance drug efficacy and specificity (Mavromouaboyashitakos et al., 2011). This approach represents a substantial leap toward more effective and personalised cancer therapies.

Previously, compound 2 was identified as a promising EGFR inhibitor based on MD simulations. Significant cytotoxicity against the MDA MB 468 breast cancer cell line was demonstrated by compound 2 at an IC_{50} of 9.46µM. However, compared to the reference anticancer drug DOX, a higher IC_{50} concentration was observed for compound 2, indicating that DOX is more cytotoxic against MDA MB 468 cells. Furthermore, SwissADME analysis revealed that compound 2 is a substrate for P-gp, an active efflux pump associated with drug resistance. Monitoring the potential development of resistance to compound 2 over time is crucial. Hence, this study aims to identify analogues of compound 2 that could potentially exhibit improved or comparable cytotoxic activity compared to conventional anticancer drugs like DOX. Additionally, analogues with favourable pharmacokinetic properties are sought to minimise the risk of drug resistance. The analogue-based drug discovery approach was employed to achieve this, a powerful approach in medicinal chemistry where researchers modify existing drug molecules (known as leads) to create new compounds with improved therapeutic properties. Scientists aim to enhance drug efficacy, safety, and specificity by making subtle changes to the chemical structure (Fischer & Ganellin, 2010a, 2010b). This strategy builds upon the success of known drugs while exploring novel variations. In this approach, the scaffold hopping analogue design approach was employed to create 36 analogues of compound 2. Subsequently, pharmacokinetic analysis using Swiss ADME was conducted, and molecular docking to EGFR was performed for all analogues. Finally, eight analogues were purchased and screened for their anticancer activity. This research holds great promise in advancing the field of breast cancer treatment, particularly for TNBC, where targeted therapies are urgently needed. The contributions made may lead to breakthroughs in breast cancer therapy.

5.4 Materials and Methods

5.4.1 Designs of Analogues

Specifically, substructure and Tanimoto similarity search methods (Leach & Gillet, 2007) were employed to identify the structurally related compounds of compound 2 using the Emolecules web-based compound collection with a similarity threshold of 0.8.

5.4.2 Molecular Docking

Molecular docking was done using GOLD for all analogues of the EGFR proteins, as previously described in Chapter 3, section 3.4.6.

5.4.3 Drug-likeness and ADMET studies

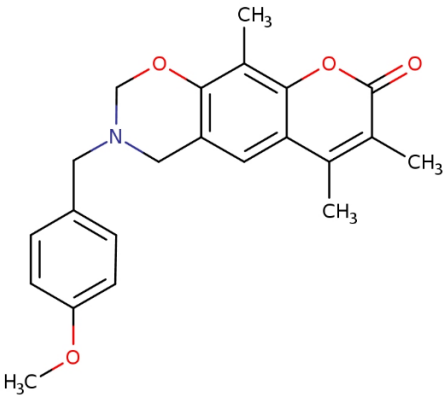
This was done in SwissADME as described in Chapter 3, section 3.4.

5.4.4 Cancer cell lines and cell culture

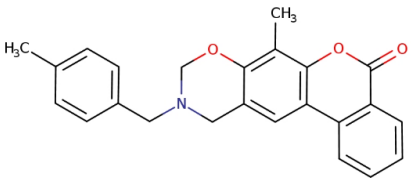
The cancer cell, MDA-MB-468, was obtained and cultured in DMEM as described in Chapter 3, under section 3.4.1.

5.4.5 Test compounds

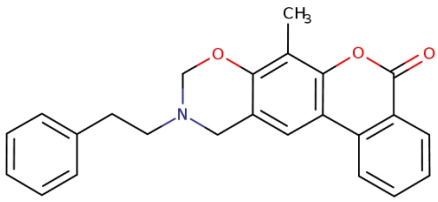
Eight commercially available analogues (as shown in Figure 5.1) were purchased from Vitas M lab.



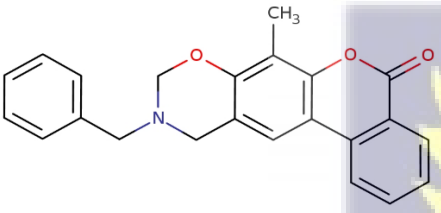
Analogue A
M.W = 365.429g/mol



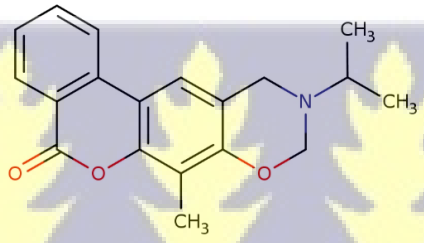
Analogue B
M. W = 349.240g/mol



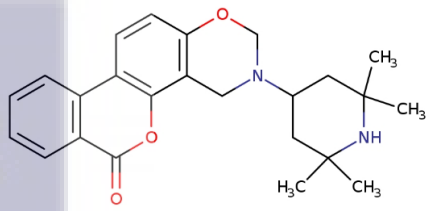
Analogue C
M. W = 371.430g/mol



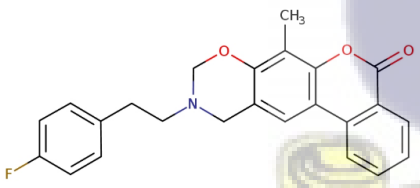
Analogue D
M. W = 357.409g/mol



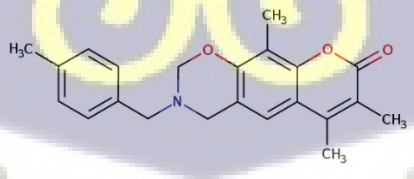
Analogue E
M. W = 309.365g/mol



Analogue F
M. W = 392.499g/mol



Analogue G
M. W = 389.426g/mol



Analogue H
M. W =349.430g/mol

Figure 5.1: Molecular structure and molecular weight of the eight (8) analogues

5.4.6 Cytotoxicity screening

MDA-MB-468 cells were treated to varying analogue concentrations (0 to 100 µM) for 48 hours. The MTT assay was performed using the protocol outlined in Chapter 3, section 3.4.3. Two-independent assays were conducted.

5.5 Results

5.5.1 Thirty-six (36) analogues of compound 2 were designed

A total of thirty-six (36) compounds were designed. Twenty (20) designed compounds successfully passed a similarity check with existing small molecule compounds in a chemical library (as detailed in Appendix B). The remaining sixteen analogues (Appendix B) exhibited no similarity to known compounds, indicating their potential novelty.

5.5.2 Molecular Docking revealed 12 analogues with high scores and consensus docking poses

Following the docking protocol, the potential inhibitors based on high scores and consensus docking poses among the four top-scoring compounds. Twelve (12) commercially available analogues (A-H, J, K, O, and S) were selected as potential inhibitors. Due to financial constraints, the top eight out of the twelve analogues with high docking score and docking poses most similar to compound 2 against EGFR were purchased for subsequent cytotoxicity screening. Additionally, among the remaining sixteen analogues, B5, B6, B7, B8, B9, and B13 demonstrated potential inhibitory activity.

5.5.3 Drug-likeness and ADMET studies of analogues

The pharmacokinetic properties and drug-likeness of all the analogues using SwissADME. Notably, all analogues were substrates of P-glycoprotein (P-gp), except for analogues E and B13. The bioavailability score for all analogues was 0.55, indicating high human intestinal absorption and blood-brain permeability. Additionally, most of the analogues adhered to Lipinski's Rule of Five except analogues B3-B8, G, P, Q (MLOGP>4.15) and J (MR>130). The log P values ranged from 2.36 to 4.46 for the analogues. Among the eight analogues selected for cytotoxicity screening, only analogues B, E, and F fell within the drug-like region on the bioavailability radar (Figure

5.2B). Analogues D, A, C, D, G, and H extended beyond the drug-like boundary (as shown in Appendix C).

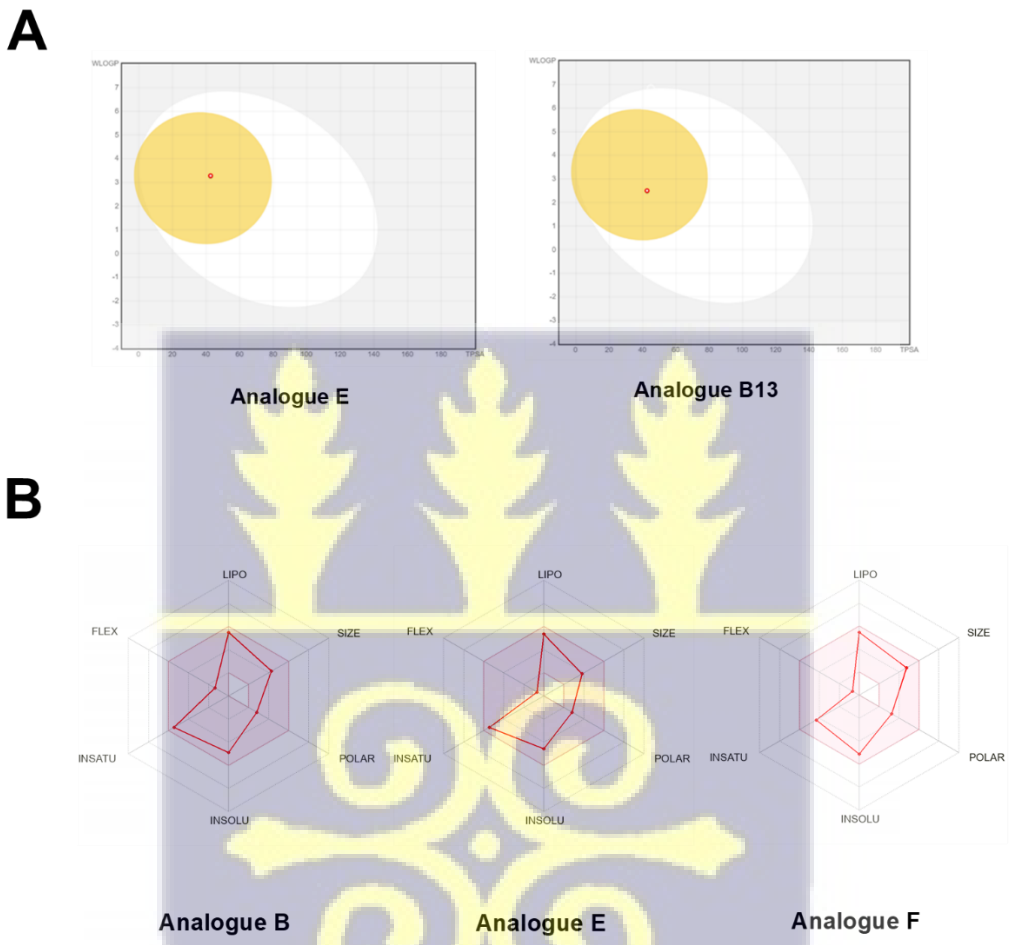


Figure 5.2: Drug-likeness and ADMET studies of analogues

A. The boiled egg analysis of analogues which were not substrate of P-gp . This was done using the SwissADME database.

B. Bioavailability radar of the eight (8) analogues which adhered to the six physiochemical properties of drug-likeness

5.5.3 MDA MB 468 cells were resistant to all the screened analogues

Considering the increased susceptibility of MDA-MB-468 cells to compound 2 (as demonstrated in Chapter 3), the selected analogues were screened against MDA-MB-468 cells. Our primary objective was identifying an analogue with a significantly lower IC_{50} value than compound 2. However, the results revealed that MDA-MB-468 cells were resistant to all the analogues. The IC_{50} values for these analogues ranged from 44.38 µM (Analog G) to 106.2 µM (Analog E). These values are higher than the IC_{50} value of 9.46 µM observed for compound 2 against MDA-MB-468. In other words, none of the screened analogues demonstrated therapeutic efficacy superior to compound 2 against MDA MB 468 cells. These findings emphasise the need for further exploration and optimisation to develop more effective treatments for TNBC.

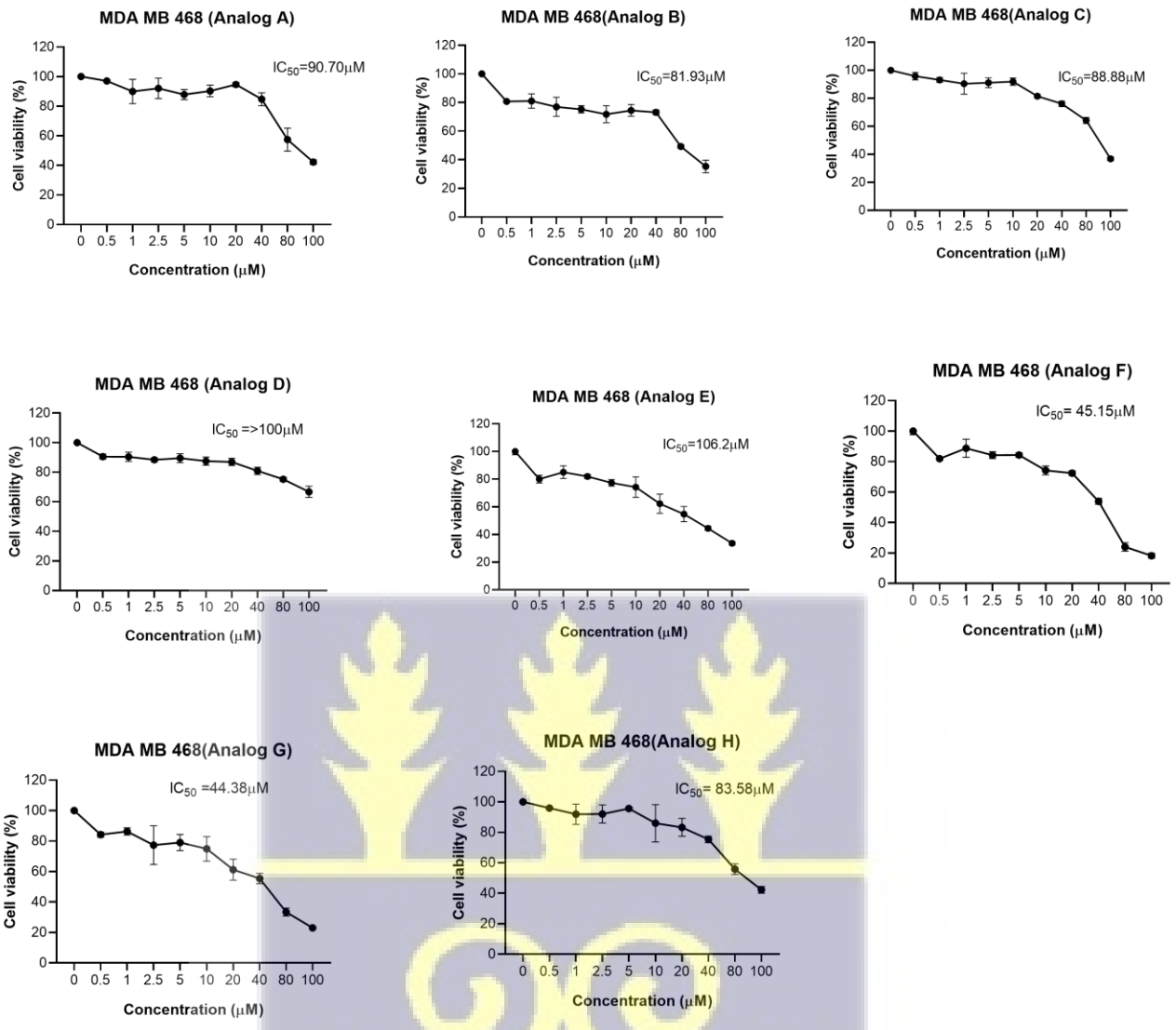


Figure 5.3: MDA MB 468 cell were resistant to all the screened analogues

The percentage cell viability was plotted against nine concentrations of each compound. MDA-MB-468 and MDA-MB-231 were treated with different concentrations (0-100µM) of eight analogues as well as 0-15µM of DOX. The negative control (untreated cells) is represented on the graph as a percentage viability against a compound concentration of zero. DOX was used as a reference drug. Data represent two independent experiments each done in triplicates.

5.6 Discussion

This study aimed to identify analogues for compound 2 with improved therapeutic efficacy in terms of IC_{50}. Thirty-six analogues were generated, of which 20 exhibited similarities to existing compounds in chemical libraries, while the remaining 16 showed no similarity with known compounds.

Given that these 16 compounds stand out due to their dissimilarity to other compounds, it is imperative to conduct further investigations to validate their potential novelty. This validation hinges on their unique chemical structure and factors such as biological activity, safety, and patentability. A comprehensive approach that combines computational predictions and experimental validation is necessary to achieve this. Strategies for assessing the potential novelty of these 16 compounds include detailed structural analysis, comparison of core structures, functional groups, and substituents with known compounds in databases, and an extensive search in scientific literature and patents. Additionally, evaluating the pharmacological properties of each analogue is crucial, as novel analogues may exhibit distinct pharmacokinetic profiles. Target prediction using computational methods can provide information on likely molecular targets (Li et al., 2020; Shimizu et al., 2023). Conducting *in vitro* assays to assess and validate biological activity is crucial in this research. Comparing the effects of analogues on cellular processes (e.g., apoptosis, cell cycle, migration) with known compounds will reveal their uniqueness and potential therapeutic efficacy. This practical validation is a significant step towards the potential clinical application of these analogues (Hoeger et al., 2014).

Among the analogues, E and B13 were not substrates of P-gp, showing their potential for invading resistance. However, analogue E did not exhibit any anticancer activity. Most analogues adhered

to Lipinski's Rule of Five, which predicts drug-likeness based on molecular properties. Although analogues B3-B8, G, P, Q, and J disobeyed the Lipinski rule, they could still be potential compounds with good therapeutic activity. Analogues D, A, C, D, G, and H extend beyond this boundary, indicating potential challenges. Analogues falling within the drug-like region have better chances of success in clinical trials. Those extending beyond the boundary may require further optimisation or careful evaluation (Lipinski, 2004; Lipinski et al., 2001).

Although MDA MB 468 cells serve as a valuable model system for understanding breast cancer biology and advancing targeted therapies (Holliday & Speirs, 2011; Hugo et al., 2017), screening the eight analogues did not reveal any anticancer activity for the compound.

5.7 Conclusion

In summary, while the novel analogues hold promise, further investigations, optimisation, and validation are essential to identify potential drug candidates.

CHAPTER SIX

6.0 General Discussion, Conclusions, Limitations and Recommendations

6.1 General Discussion

The primary objective of this study was to identify a potential anticancer compound, predict its potential targets and pharmacokinetic properties, investigate its impact on crucial cancer hallmarks, and explore optimisation strategies through an analogue-based drug discovery approach. Three small molecules previously identified as possible hits for inhibiting *Leishmania donovani* sterol methyltransferase using computational analysis and later screened on *Leishmania* (Sakyi et al., 2023). These compounds were later screened in four cancer cell lines and a normal cell line, PNT2, to determine their anticancer potential and toxicity on normal cells. Compound 2 was identified as the most potent compound in objective 1 (Chapter 3) and used for further analysis.

The molecular structure of compound 2 is represented by the SMILES string "O=C(OC1=C2C=C3C(OCN(C4CC(C)(C)NC(C)(C)C4)C3)=C1C)C5=C2C=CC=C5" and shows potential for targeted drug discovery, particularly against EGFR. The structure of compound 2 had a fused ring system, which, according to molecular docking and MD simulation results, aligned well with the binding pockets of both EGFR and likely VEGFR2. Compound 2 interacted with key residues within EGFR, including Lys745 in the ATP binding pocket and Asp855 in the activation loop (DFG motif). This dual interaction strategy may overcome resistance observed with most known EGFR inhibitors. SwissADME analysis revealed favourable pharmacokinetic properties for compound 2, except for its classification as a substrate of P-gp. P-gp, an active efflux pump, could limit compound accumulation in cells, potentially impacting therapeutic efficacy

(Callaghan et al., 2014; Ling, 1992). Compound was predicted to affect focal adhesion and pathways in cancer, and the KEGG pathway analysis further indicated enrichment of the ErbB Signalling Pathway and EGFR Tyrosine Kinase Inhibitor Resistance. Remarkably, this aligns with the results from MD simulations, which identified EGFR as the optimal target for Compound 2 based on free energy binding and hydrogen bond analysis. These findings highlight EGFR's significance in the context of Compound 2, a potential therapeutic agent which has demonstrated significant efficacy in treating breast cancer. *In vitro* screening has shown robust activity against breast cancer cells (MDA-MB-468 and MDA-MB-231), particularly on MDA-MB-468 cells. Notably, compound 2 demonstrated greater selectivity in vitro compared to DOX, a conventional chemotherapeutic agent. This suggests a potential advantage in minimizing off-target toxicity, although further in vivo validation is required to confirm this benefit.

Building on the initial findings of this study, compound 2 was further screened against critical hallmarks of cancer, such as apoptosis, cell cycle, migration, and metastasis in objective 2 (Chapter 4). The results were significant. Compound 2 affected the proliferation of MDA MB 468 by exhibiting several promising features: G1-phase cell cycle arrest, induction of apoptosis, downregulation of the proliferation marker Ki67, and a lower *Bcl-2*/*Bax* ratio. These findings suggest that Compound 2 selectively targets cancer cells, potentially enhancing cancer cell death. It may play a role in suppressing cancer cell adhesion to the ECM, a critical factor in cancer progression, invasion, and metastasis. Compound 2 disrupts adhesive structures and alters signalling pathways by interfering with focal adhesions, creating new avenues for cancer research (Yayan et al., 2024). Its differential effects on gene expression levels in different breast cancer cell lines highlight its complexity. Notably, Compound 2 modulates EMT, impacting cancer cell

behaviour. The observed downregulation of *SNAI1* and upregulation of *Vimentin* suggest distinct mechanisms of action, highlighting the complexity of EMT regulation. Further investigation is needed to validate these findings and explore the precise mechanisms. Compound 2 effectively suppressed cancer cell re-growth, especially near its IC_{50} value and significantly decreased the CSC population compared to the untreated cells. This implies that compound 2 could prevent cancer relapse by targeting CSCs and inhibiting cell re-proliferation. Generally, compound 2 exhibited inflammation and immune responses differently from DOX. Compound 2 showed a significantly lower increase in IL-6 levels compared to DOX. IL-6 is known to promote cancer aggressiveness, EMT, and chemoresistance, indicating that Compound 2 may induce a less aggressive inflammatory response (Srdic-Rajic et al., 2017; Esparza-López et al., 2013; Bent et al., 2021).

While compound 2 exhibited potent suppression of critical hallmarks of cancer, its cytotoxic effect was less than that of DOX, as seen in its IC_{50} value. This led to the aim of improving the biological activity of compound 2 through the analogues-based drug discovery approach. Among the 36 analogues generated, 20 exhibited similarities to existing compounds, while the remaining 16 showed no similarity to known compounds. These 16 compounds require further investigation to validate their potential novelty, considering factors such as chemical structure, biological activity, safety, and patentability. Significantly, out of the analogues, E and B13 did not function as substrates of P-gp, indicating their potential ability to overcome resistance. While analogue E did not demonstrate any anticancer properties, most analogues conformed to Lipinski's Rule of Five, which suggests drug-like properties. Analogues beyond this boundary may encounter limitations(Lipinski et al., 2001); however, further exploration and validation of these findings

remains a priority for future research. Although the analogues did not demonstrate improved activity compared to the original compound, this evaluation phase provided important information about the relationship between the compound's structure and activity. The fact that the analogues did not show increased activity suggests that the original compound has a specific interaction with its targets.

6.2 General Conclusions

This study highlights the significant therapeutic potential of small molecule compounds, particularly compound 2, in targeting cancer cells while minimising harm to normal cells. By employing a comprehensive approach that integrated computational tools, molecular docking, and dynamics simulations, the research identified EGFR as the primary target of compound 2. This compound exhibited notable cytotoxicity in vitro and showed improved selectivity compared to conventional agents like DOX, suggesting potential therapeutic advantages. Functional experiments revealed that compound 2 might play a role in inducing apoptosis, inhibiting cell migration, and modulating gene expression linked to cancer progression, particularly in breast cancer cells. Compound 2`s *in silico* ADMET analysis showed strong Caco-2 permeability (−4.944), indicating good intestinal absorption. However, predicted human intestinal absorption (HIA = 0.016) and low bioavailability at 20% (0.004) and 30% (0.005) suggest limited systemic exposure, which could be improved with better formulation. Its log P of 4.178 points to moderate-to-high lipophilicity, aiding membrane permeability but raising concerns about solubility and tissue accumulation. Interestingly, structural analogues of compound 2 did not demonstrate enhanced activity, implying that the parent compound may have a unique pharmacophore responsible for its selective mechanism of action. These findings offer a preliminary basis for the continued exploration and potential optimisation of compound 2 as a therapeutic candidate. This

study highlights the importance of targeted molecular therapies in improving cancer treatment efficacy and reducing adverse effects. Future research should focus on the detailed mechanistic pathways of compound 2, its long-term effectiveness, and safety in clinical settings, as well as the exploration of synergistic effects with other anticancer agents. In conclusion, this research contributes valuable insights into developing novel, selective anticancer therapies and reinforces the promise of targeted molecular interventions in the fight against cancer

6.3 Limitation of Work

The study yielded promising results and conducted thorough analyses, but it has several limitations. Most tests were performed in a controlled laboratory setting, which may not accurately replicate the complex environment of a living organism. The research focused on a limited set of cancer cell lines, which do not capture cancer heterogeneity. This study relied heavily on *in silico* methods for predicting pharmacokinetic parameters, which require experimental validation through thorough ADME studies. However, the study needed more *in vivo* investigations to understand pharmacokinetics, biodistribution, metabolism, and toxicity in a whole organism. Additionally, an enzymatic or binding assay was not performed to validate computational simulation results, and investigating potential off-target effects was not comprehensive. Despite designing and testing analogues of the hit compound, the study examined a limited number of potential analogues. Lastly, the molecular mechanisms by which compound 2 induces cell death, inhibits migration and alters gene expression have not been fully elucidated.

6.4 Recommendations for Future Work

An enzyme or binding assay is recommended to confirm that compound 2 explicitly targets EGFR. Following this, compound 2 should be evaluated for its pharmacodynamics, pharmacokinetics, and toxicological profile using animal models in an *in vivo* experiment. This involves assessing the drug's effectiveness in decreasing tumour growth, its presence in various tissues, its breakdown and elimination from the body, and any possible harmful effects. A more extensive molecular profiling technique should be employed to conduct an off-target study of compound 2 to discover all possible binding targets. This will aid in understanding any unexpected interactions that may lead to side effects. Various methodologies can investigate off-target interactions, including affinity chromatography combined with mass spectrometry, phage display, cross-linking mass spectrometry, protein microarrays, and cell-based assays such as RNA interference and CRISPR-Cas9 screens. Long-term stability tests on compound 2 are necessary to assess its chemical and physical stability. These studies should investigate its performance in different solvents, temperatures, and pH levels. Potential formulations could be created, such as encapsulating compound 2 in liposomes or nanoparticles that improve its stability, solubility, and delivery. Additional investigations employing transcriptomics, proteomics, and metabolomics are recommended to help clarify the exact molecular processes by which compound 2 triggers apoptosis, hinders migration, and modifies gene expression. This can help identify the precise binding sites and signalling routes modulated by compound 2. Furthermore, it is necessary to examine the potential of compound 2 in combination with current chemotherapy, targeted therapy, or immunotherapy. Combinations may enhance therapeutic efficacy, overcome resistance mechanisms, and reduce the required doses of conventional agents. Given the selective cytotoxicity observed *in vitro*, exploring synergistic interactions could provide a more comprehensive strategy for treating aggressive subtypes like TNBC. Also, expanding the analogue

library could lead to molecules with improved activity and pharmacokinetic properties. Finally, it is crucial to collaborate with interdisciplinary research teams, such as molecular biologists, pharmacologists, and bioinformaticians, to carry out thorough research on the signalling pathways impacted by compound 2.

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APPENDIX A

Table A1: Mean RMSD values for heavy atoms between the co-crystallized ligand and the docked molecule.

PDB	ASP	PLP	CS	GS
6HHJ	0.8371	0.6432	1.0030	1.0900
4ASE	0.6773	0.3783	0.3914	0.6043
8A27	14.2807	13.9609	13.8974	1.5400

Table A2: Results of the scoring function for the ligands.

PDB	ASP	PLP	CS	GS
6HHJ	38.26	65.31	29.15	53.81
4ASE	27.59	52.3	31.25	48.08
8A27	26.83	76.42	38.77	69.96

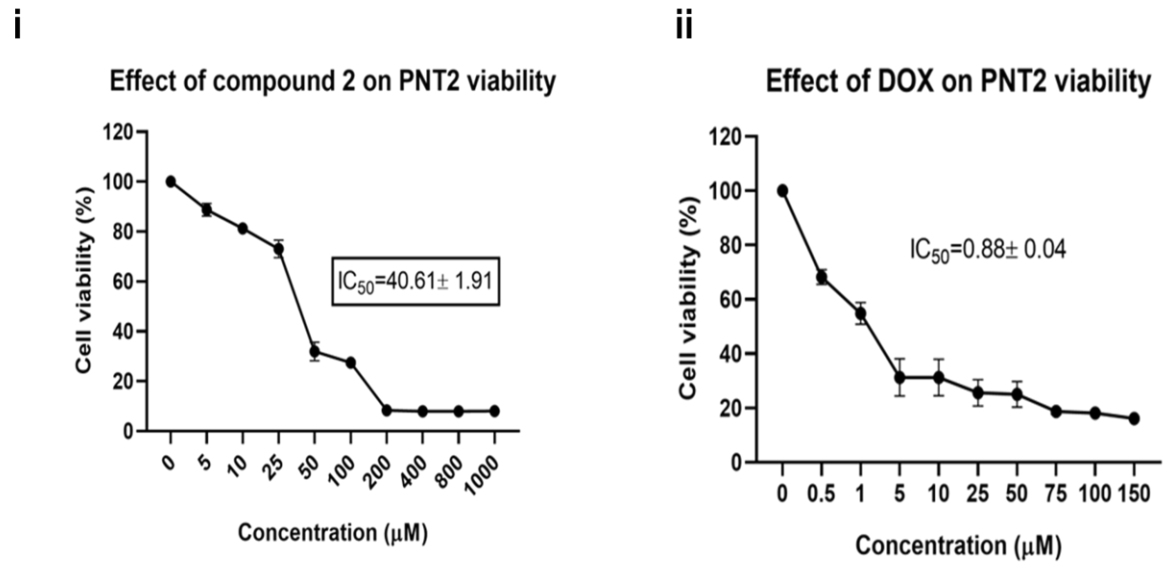
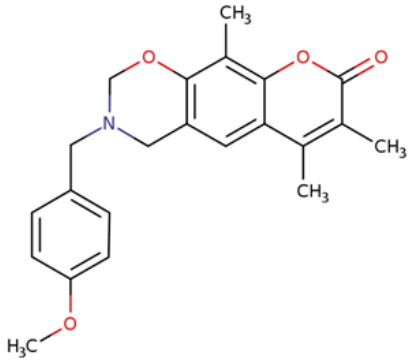


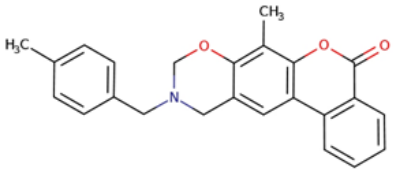
Figure A1: Cytotoxic effect of compound 2 on normal cell line, PNT2

(i) Graph showing effect of compound 2 on PNT2. (ii) graph showing effect of compound 2 on DOX. The percentage cell viability was plotted against nine concentrations of each compound. PNT2 cells were treated with different concentrations (0-1000µM) of eight analogues as well as 0-150µM of DOX. The negative control (untreated cells) is represented on the graph as a percentage viability against a compound concentration of zero. DOX was used as a reference drug. Data represent two independent experiments each done in triplicates

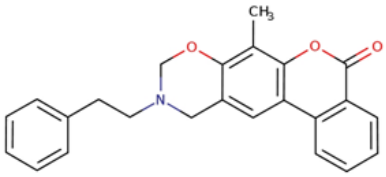
APPENDIX B



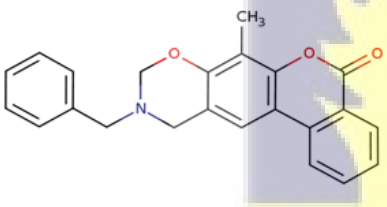
Analogue A
M.W = 365.429g/mol



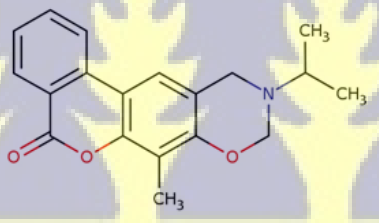
Analogue B
M. W = 371.436g/mol



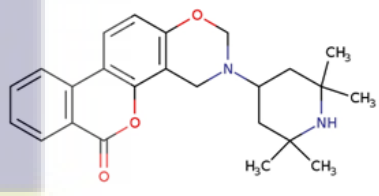
Analogue C
M. W = 371.436g/mol



Analogue D
M. W = 357.409g/mol



Analogue E
M. W = 309.365g/mol



Analogue F
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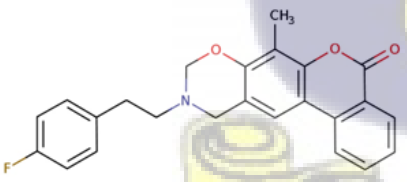
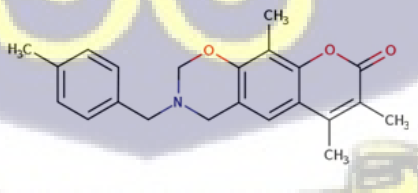
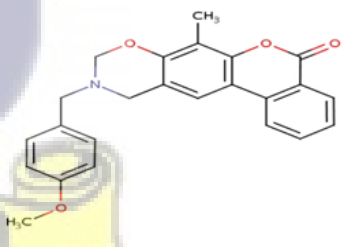


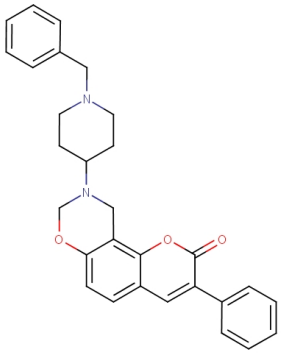
Analogue G
M. W = 389.426g/mol



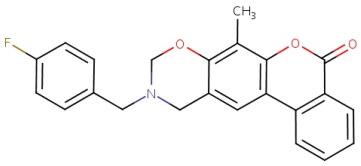
Analogue H
M. W =349.43g/mol



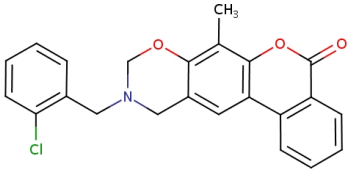
Analogue I
M. W = 387.43 g/mol



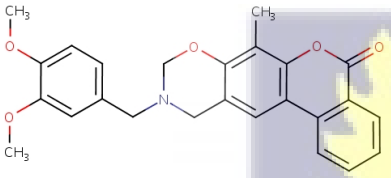
Analogue J
M.W = 452.54g/mol



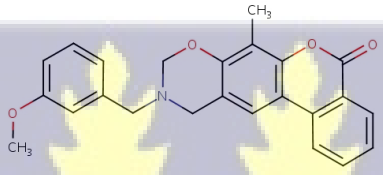
Analogue K
M. W = 375.39g/mol



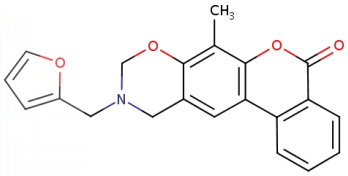
Analogue L
M. W = 391.85g/mol



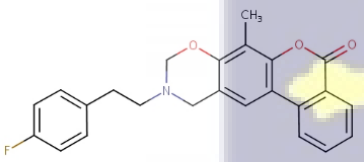
Analogue M
M. W = 417.45g/mol



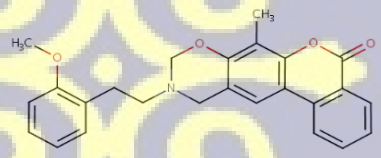
Analogue N
M. W = 387.43g/mol



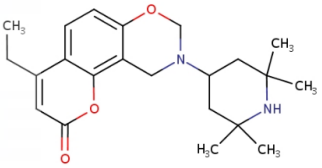
Analogue O
M. W = 347.37g/mol



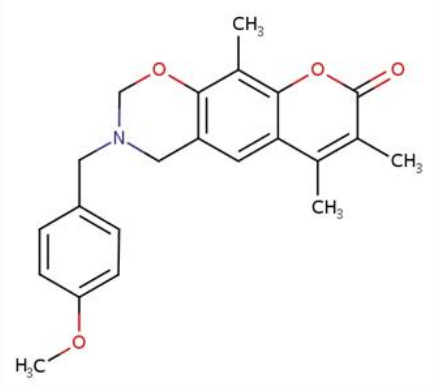
Analogue P
M. W = 389.42g/mol



Analogue Q
M. W = 401.45 g/mol

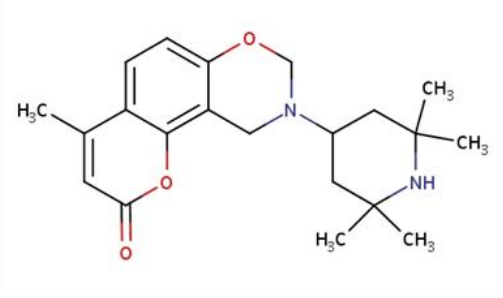


Analogue R
M. W = 370.493 g/mol



Analogue S

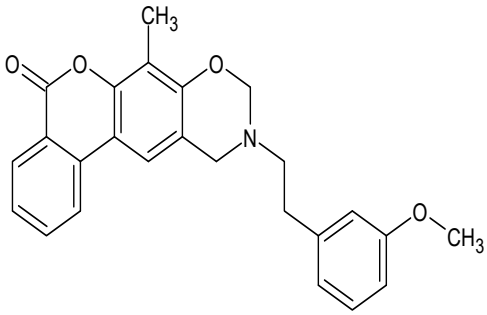
M. W = 365.429 g/mol



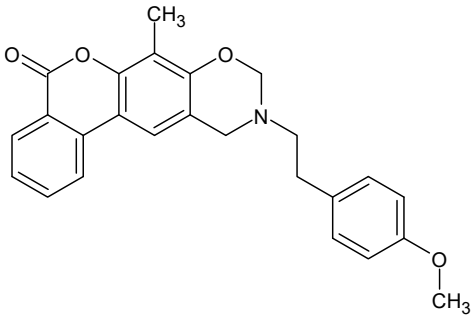
Analogue T

M. W = 356.466g/mol

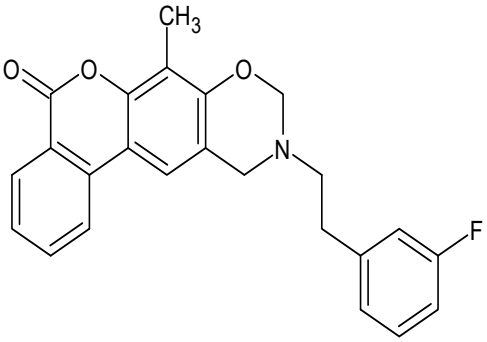
Figure B1: Molecular structure of the twenty (20) analogues with similarities to preexisting compounds in the chemical library



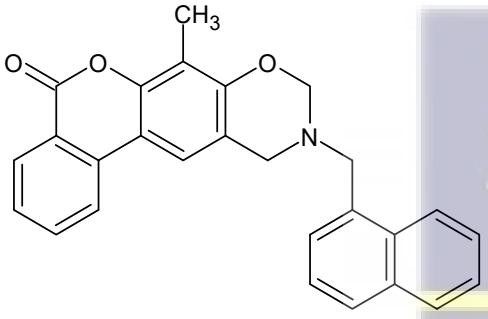
Analogue B1
M.W = 401.45g/mol



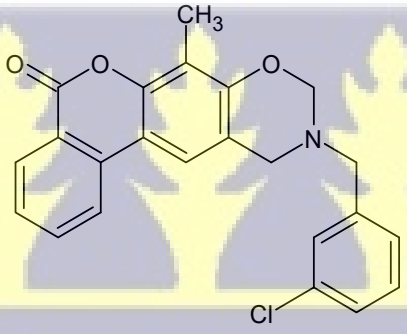
Analogue B2
M. W = 401.45g/mol



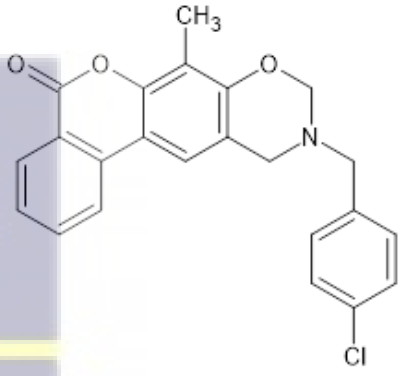
Analogue B3
M. W = 389.42g/mol



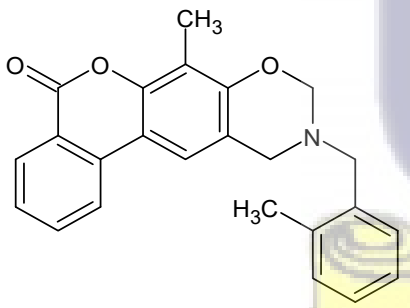
Analogue B4
M. W 407.46g/mol



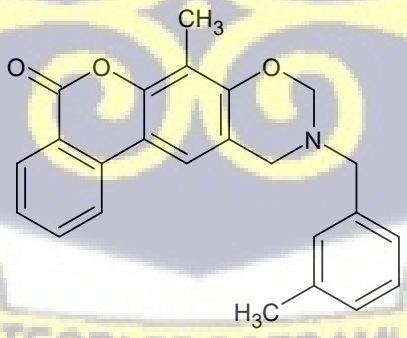
Analogue B5
M. W = 391.85g/mol



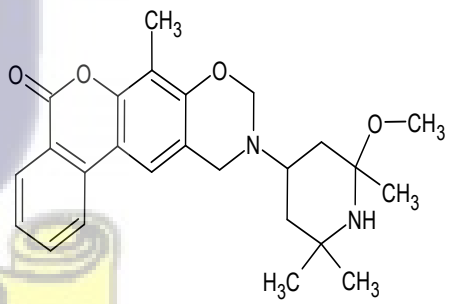
Analogue B6
M. W = 391.85g/mol



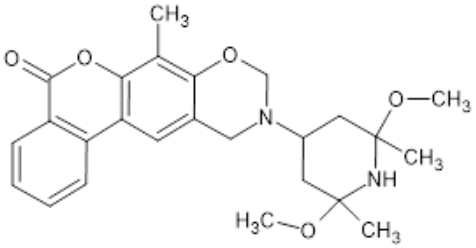
Analogue B7
M. W = 389.42g/mol



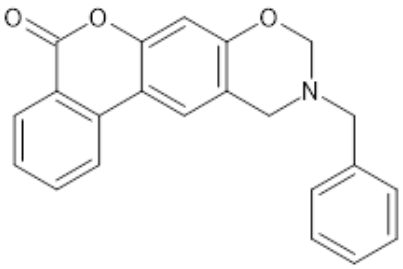
Analogue B8
M. W = 371.43g/mol



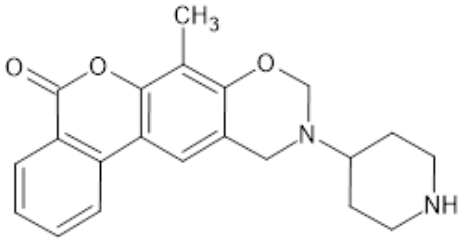
Analogue B9
M. W = 422.52g/mol



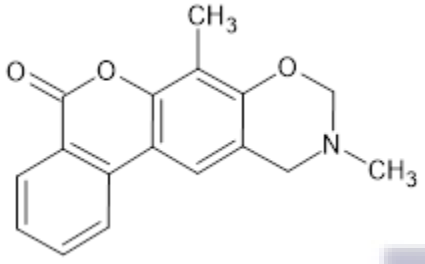
Analogue B10
M.W = 438.52g/mol



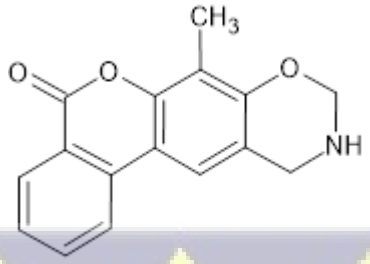
Analogue B11
M. W = 343.38 g/mol



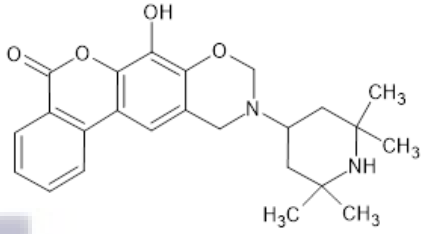
Analogue B12
M. W = 350.41g/mol



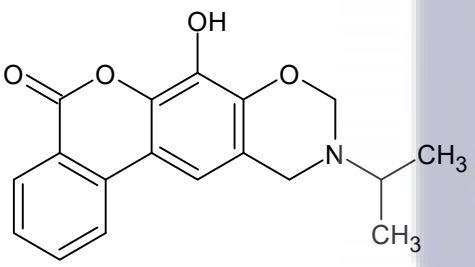
Analogue B13
M. W = 281.31g/mol



Analogue B14
M. W = 267.28g/mol



Analogue B15
M. W = 408.49g/mol



Analogue B16
M. W = 311.33g/mol

Figure B2: Molecular structure of the 16 analogues, which had no similarity with any compound in chemical libraries

APPENDIX C

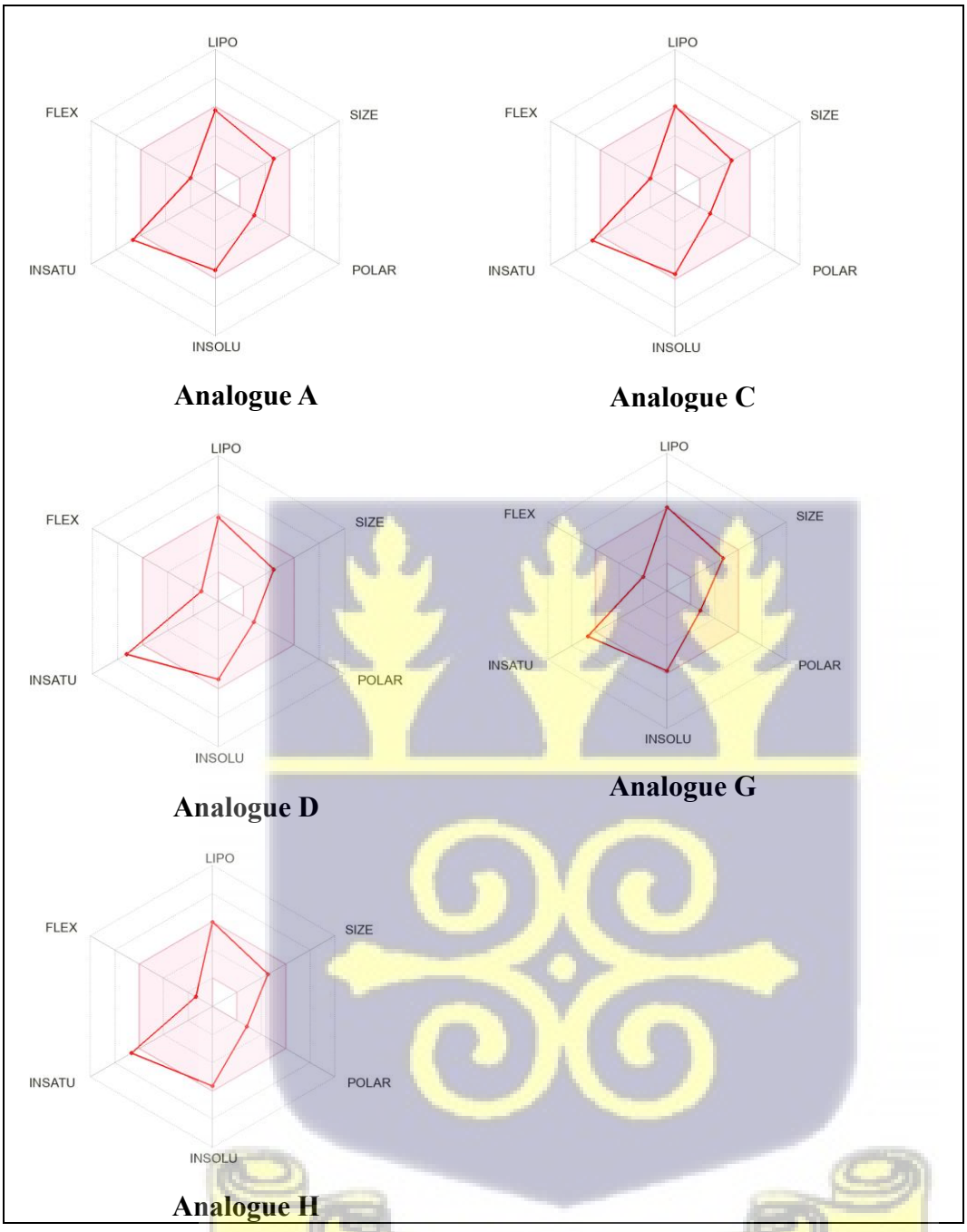


Figure C1: Bioavailability radar for five (5) analogues. Analogues A, C, D, G and H have their saturation property deviating from the pink area.