THIAZOLIDINEDIONES PRETREATED BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AS AN ANTI-CANCER APPROACH AGAINST MCF-7 BREAST CANCER CELL LINE

LIM SHERN KWOK

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by

LIM SHERN KWOK

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LIST OF SYMBOLS

%	Percent
°C	Degree Celsius
cm^2	Square centimetre
g	Gram
kDA	Kilodalton
L	Litre
mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimetre
ng	Nanogram
nm	Nanometre
pH	Potential of hydrogen
V	Volt
x *g*	Times gravity (relative centrifugal force)
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ΔΔCt	Double delta cycle threshold
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometre

LIST OF ABBREVIATIONS

20PR	20 μM PIO + 20 μM ROSI
ACC	Acetyl-CoA carboxylase
Akt	Protein kinase B
AMPK	5’ adenosine monophosphate-activated protein kinase
APS	Ammonium persulphate
ASXL1	ASXL transcriptional regulator 1
ATCC	American Type Culture Collection
BAD	*BCL2*-associated death promoter
Bak	*BCL2* Antagonist/Killer 1
Bax	*BCL2*-associated X protein
BCA	Bicinchoninic acid
BCL2	B-cell lymphoma-2
bFGF	Basic fibroblast growth factor
BHLHB	Basic helix-loop-helix domain-containing protein B
BMP	Bone morphogenetic protein
BM-MSCs	Bone marrow-derived mesenchymal stem cells
BRCA	Breast cancer associated genes
CAP	Catabolite activator protein
CBB	Coomassie Brilliant Blue
CCL	C–C motif chemokine ligand
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CEP350	Centrosomal protein 350
CFA	Colony forming ability
CI	Combination index
cIAP-2	Cellular inhibitor of apoptosis 2
CO_2	Carbon dioxide
COPB2	Coatomer subunit beta 2
COX-2	Prostaglandin-endoperoxide synthase 2
CREB	cAMP-response element binding protein
CREBBP	CREB binding protein

CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C chemokine receptor type
ddH_2O	Deionised distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EP300	E1A-associated protein p300
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
ESC	Embryonic stem cells
Fa	Fraction affected
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
G1	Growth 1/Gap 1 phase
GA-1000	Gentamicin sulfate-amphotericin
gDNA	Genomic DNA
GLUT3	Glucose transporter 3
GLUT4	Glucose transporter type 4
GO	Gene ontology
GREM1	Gremlin-1
HBXIP	Hepatitis B X-interacting protein
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER	Human epidermal growth factor receptor

HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
IGF-1R	Insulin-like growth factor 1 receptor
IL	Interleukin
INSIG	Insulin induced gene
JAK2	Janus kinase 2
JAZF1	Juxtaposed with another zinc finger protein 1
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
LXRs	Liver X receptors
m/z	Mass to charge ratio
MALDI-TOF-TOF	Matrix Assisted Laser Desorption/Ionisation-Time of flight-Time of flight
MAPK	Mitogen-activated protein kinase
MED1	Mediator complex subunit 1
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells
MSCGM	Mesenchymal stem cells growth medium
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NCOR1	Nuclear receptor corepressor 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLK	Nemo like kinase
PANTHER	Protein Analysis Through Evolutionary Relationships
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/Streptomycin
PI3K	Phosphatidylinositol-3-kinase
PIO	Pioglitazone
PIP3	Plasma membrane intrinsic protein 3
PMF	Peptide mass fingerprinting
POU5F1	POU class 5 homeobox 1
PPAR	Peroxisome proliferator-activated receptor

PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPI	Protein-protein interaction
PPRE	Peroxisome proliferator-responsive element
PTBP1	Polypyrimidine tract-binding protein 1
PTEN	Phosphatase and tensin homolog
p-value	Probability value
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROSI	Rosiglitazone
RXR	Retinoid X receptors
SCD1	Stearoyl-CoA desaturase 1
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHH	Sonic hedgehog
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SOCE	Store-operated calcium entry
SOX2	SRY-box 2
SREBP-1c	Sterol regulatory element-binding protein 1c
STAT3	Signal transducer and activator of transcription 3
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TBE	Tris-Borate-Ethylenediamine-tetra acetic acid
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TRAIL	Tumour necrosis factor related apoptosis-inducing ligand
Tris-HCl	Trisaminomethane hydrochloride
TZDs	Thiazolidinediones
UniProtKB	Universal Protein Resources Knowledgebase
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VIM	Vimentin
WA	Withaferin A

WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis protein

LIST OF APPENDICES

PRA-RAWATAN SEL STEM MESENCHYMAL YANG BERASAL DARIPADA SUMSUM TULANG DENGAN THIAZOLIDINEDIONES SEBAGAI PENDEKATAN ANTI-KANSER TERHADAP TITISAN SEL KANSER PAYUDARA MCF-7

ABSTRAK

Kanser payudara merupakan jenis kanser yang paling lazim di kalangan wanita di seluruh dunia. Sebab kebanyakan rawatan yang sedia ada kini dikaitkan dengan kesan sampingan buruk, penemuan strategi terapeutik baharu yang melibatkan ubat yang diluluskan akan terbukti bermanfaat untuk mengatasi masalah ini. Pioglitazone (PIO) dan rosiglitazone (ROSI) ialah ubat thiazolidinediones (TZDs) yang telah diluluskan untuk rawatan diabetes mellitus jenis 2. Walaupun penggunaan PIO dan ROSI didapati boleh menyebabkan kesan sampingan buruk, terdapat kajian saintifik yang melaporkan bahawa PIO dan ROSI mempunyai kesan anti-kanser. Oleh sebab itu, kajian ini dijalankan untuk meningkatkan keberkesanan keseluruhan terapi TZDs dengan mengabungkan penggunaan PIO dengan ROSI untuk merawat sel kanser payudara MCF-7. Selain itu, terapi kombinasi PIO dan ROSI juga dikaji sebagai strategi prarawatan dengan sel stem mesechymal yang diperoleh daripada sumsum tulang (BM-MSCs) untuk merawati sel MCF-7 secara tidak langsung melalui interaksi sel-sel. Hasil kajian ini telah menunjukkan bahawa terapi kombinasi PIO dan ROSI menyebabkan perencatan kelangsungan hidup sel yang lebih tinggi dengan tahap akumulasi lipid sellular yang lebih rendah dalam sel MCF-7 pada 48 dan 96 jam kultur bersama. Tambahan pula, kelangsungan hidup BM-MSCs dengan prarawatan TZDs kekal sihat sehingga 96 jam dan tiada tanda-tanda tekanan atau kematian selular diperhatikan pada hari ke-7. BM-MSCs dengan prarawatan TZDs terutamanya 20 μM

PIO + 20 μM ROSI (20PR) telah menurunkan keupayaan pembentukan koloni sel MCF-7 yang dikultur bersama dengan penggunaan sisipan sel dan media terkondisi. Analisis lanjut mengenai protein intraselular BM-MSCs dengan prarawatan TZDs mendedahkan bahawa vimentin memainkan peranan dalam regulasi interaksi antara BM-MSCs dan sel MCF-7. Peranan vimentin disahkan apabila fenomena bertentangan diperhatikan selepas withaferin A digunakan untuk menghalang ekspresi dan fungsi keseluruhan vimentin. Selain itu, peningkatan ekspresi vimentin ditunjukkan untuk merendahkan ekspresi FGF4 dalam BM-MSCs manakala media terkondisi daripada BM-MSCs dengan prarawatan 20PR didapati meningkatkan ekspresi PPARα dan menurunkan ekspresi PPARγ dalam sel MCF-7 yang dikultur bersama. Penyiasatan bioinformatik yang lebih mendalam mengenai mekanisme molekul yang mungkin terlibat mendedahkan bahawa vimentin mengawal ekspresi FGF4 dalam BM-MSCs dengan prarawatan 20PR melalui interaksinya dengan SOX2 dan POU5F1. Walaupun FGF4 didapati tidak berinteraksi dengan PPARα dan PPARγ secara langsung, interaksi dengan molekul lain seperti CREBBP, EP300, MED1, dan PPARGC1A mungkin berlaku melalui FGFR1. Kesimpulannya, 20PR telah ditunjukkan berkesan terhadap sel MCF-7 sama ada digunakan sebagai rawatan langsung atau secara tidak langsung sebagai bahan prarawatan untuk BM-MSCs. Oleh sebab itu, fenomena ini menunjukkan potensinya sebagai strategi rawatan untuk kanser payudara hormon positif pada masa hadapan.

THIAZOLIDINEDIONES PRETREATED BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AS AN ANTI-CANCER APPROACH AGAINST MCF-7 BREAST CANCER CELL LINE

ABSTRACT

Breast cancer is the most prevalent type of cancer among women globally. Although treatments are currently available, most of these therapies are associated with adverse effects. For that reason, new treatment strategies utilising approved drugs may prove to be beneficial. Pioglitazone (PIO) and rosiglitazone (ROSI) are thiazolidinediones (TZDs) drugs that are clinically used to treat type 2 diabetes mellitus. Although PIO and ROSI have been linked to several adverse effects, there have been reports about their anti-cancer properties. For that reason, this study was conducted to improve the overall efficacy of TZDs therapy by utilising PIO and ROSI in combination to treat MCF-7 breast cancer cells. Additionally, the study also investigated the combination therapy of PIO and ROSI as a pretreatment strategy for bone marrow-derived mesenchymal stem cells (BM-MSCs) to indirectly treat MCF-7 cells via cell-cell interaction. The results revealed that PIO and ROSI when used in combination, induced higher inhibition of cell viability with lower levels of cellular lipid accumulation in MCF-7 cells at 48 and 96 hours of co-culture. Furthermore, the viability of TZDs pretreated BM-MSCs remained uninhibited for up to 96 hours and no signs of cell stress or death was observed at Day 7. The TZDs pretreated BM-MSCs especially 20 µM PIO + 20 µM ROSI (20PR) was demonstrated to lower the colony forming ability of co-cultured MCF-7 cells using cell inserts and conditioned media. Further analysis on the intracellular protein of TZDs pretreated BM-MSCs revealed that vimentin played a role in regulating the interaction between BM-MSCs and MCF-

7 cells. This was confirmed when opposite phenomena were observed after withaferin A was used to inhibit vimentin's overall expression and function. Besides that, upregulation of vimentin was demonstrated to lower the expression of FGF4 in BM-MSCs. Subsequently, the study found that the conditioned medium from 20PR pretreated BM-MSCs upregulated PPARα and downregulated PPARγ in the co-cultured MCF-7 cells. Focusing deeper into the molecular mechanism that might be involved, bioinformatics analysis revealed that vimentin regulated the expression of FGF4 through its interaction with SOX2 and POU5F1. Although FGF4 was not found to interact with PPARα and PPARγ directly, it is possible that CREBBP, EP300, MED1, and PPARGC1A were involved via FGFR1. In conclusion, 20PR was demonstrated to be effective against MCF-7 cells when administered directly or indirectly via a pretreatment for BM-MSCs, indicating its potential as a treatment strategy for hormone positive breast cancers in the future.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cancer is a disease that arises when the cells in the body grow uncontrollably due to the uneven coordination between cell proliferation and cell death. These abnormal or damaged cells, which continuously grow and multiple, will then form non-cancerous/benign or cancerous/malignant tumours. Benign tumours are non-invasive and do not spread, while malignant tumours are able to travel and invade nearby tissues or distant places in the body, which can cause severe symptoms or even be life threatening. Globally, there has been an increase in cancer cases due to changes in lifestyle and the environment, such as smoking and drinking, higher exposure to carcinogenic compounds and radiation, stress, and physical inactivity.

It is estimated that by 2022, there will be approximately 1,918,030 cancer cases reported with 5,250 new cases diagnosed daily in America (Siegel et al., 2022). Among the numerous types of cancers, breast cancer is the foremost cause of cancer mortality in females (Noor et al., 2021). Locally in Malaysia, there is a high prevalence of breast cancer as well because it is the most detected type of cancer among women of all ethnicities. It is estimated that 1 in 19 women are at risk of being diagnosed with breast cancer (Lee et al., 2019). Currently, several options are available as treatments for breast cancer patients, which include chemotherapy, bisphosphonates, hormonal therapy, radiotherapy, and targeted therapy (Nordin et al., 2018). According to a recent study, the Malaysian public is hesitant about surgery and chemotherapy as treatment for breast cancer due to the negative side effects that are commonly associated with both therapies (Lee et al., 2019).

Cancer therapies are usually correlated with a wide variety of side effects, including loss of appetite, vomiting, anaemia, skin irritation, pain, and fatigue. This is especially true in the case of conventional chemotherapy which non-selectively interferes with deoxyribonucleic acid (DNA) synthesis and mitosis, causing the death of rapidly dividing cancer cells and damaging healthy normal tissues. Furthermore, the high mortality rate of cancer patients is linked to the severe adverse effects caused by the chemotherapeutic medication on healthy tissues and organs (Senapati et al., 2018). For that reason, researchers have consistently tried to discover new anti-cancer compounds to be developed as an alternative that is more effective but with lesser adverse effects. However, the development of new anti-cancer therapies requires a substantial number of years with only a low success rate of 3.40% (Stewart et al., 2018; Wong et al., 2019). Because of that, it might be more beneficial to develop a new treatment strategy using approved drugs instead of researching a new compound to decrease the developmental time and increase the success rate.

Thiazolidinediones (TZDs) or glitazones are a family of drugs that are clinically prescribed to manage type 2 diabetes mellitus. TZDs are insulin sensitisers that bind to peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor, which regulates genes associated with lipid and glucose metabolism (Thangavel et al., 2017). Presently, pioglitazone (PIO) and rosiglitazone (ROSI) are both approved as monotherapy or in combination therapy with metformin or sulfonylureas by the United States Food and Drug Administration (FDA) as medications for type 2 diabetes mellitus (Eggleton & Jialal, 2021). Additionally, numerous studies have investigated the anti-cancer properties of TZDs in breast, colon, glioma, lung, and prostate cancer which revealed promising results for *in vitro* and *in vivo* models (Blanquicett et al., 2008; Fröhlich & Wahl, 2015). However, the

administration of TZDs has been associated to several adverse effects such as edema, weight gain, and osteoporosis (Singh et al., 2022). Despite that, TZDs remain as attractive targets for cancer therapy that should be studied further.

On the other hand, there have been a steady increase in the financing of global health over the past 2 decades and it is projected to continue increasing in the future (Chang et al., 2019). This is an issue that requires attention as WHO had previously identified health financing as an essential block of the healthcare system (Manyazewal, 2017). Therefore, the re-evaluation of TZDs, a cost-effective drug, as an alternative cancer therapy would help ease this financial burden. For that reason, further studies should be conducted so that TZDs can be utilised as an anti-cancer therapy in the future.

1.2 Research rationale

PIO and ROSI have been shown to possess anti-cancer effects on breast cancer cells (Zhang et al., 2008; Jiao et al., 2020) and are clinically approved by the FDA as medications for type 2 diabetes mellitus. Despite the risks associated with its usage, numerous safety data on the drugs are available, which would speed up and improve the success rate of the developmental process. Moreover, PIO and ROSI have been used in combination with other chemotherapy drugs to improve the overall efficacy of the therapeutic effect (Bunt et al., 2013; Piątkowska-Chmiel et al., 2020). Therefore, the present study aimed to study whether PIO, when combined with ROSI, will enhance the overall therapeutic effect against breast cancer cells. Additionally, the study devised an indirect method of administrating PIO and ROSI to the breast cancer cells via bone marrow-derived mesenchymal stem cells (BM-MSCs) to eliminate the

potential adverse effects of the drug interacting directly with the breast cancer cells, as shown in Figure 1.1. The idea of co-culturing TZDs pretreated BM-MSCs and breast cancer cells together was based on the "seed and soil" cancer theory which proposed that positive interactions between cancer cells (seed) and the local microenvironment (soil) leads to cancer metastasis (Liu et al., 2017). Therefore, this study aims to alter the local microenvironment via pretreated BM-MSCs to make it unfavourable for cancer cells to grow (Figure 1.2). In a nutshell, this study will provide beneficial information on the effects of PIO and ROSI when used in combination against breast cancer cells. Furthermore, the interaction between TZDs pretreated BM-MSCs and breast cancer cells will provide important data on the mechanism behind the regulation of cancer growth by stem cells. It is also believed that the current study will help to design a new strategy to treat breast cancer by utilising TZDs pretreated BM-MSCs.

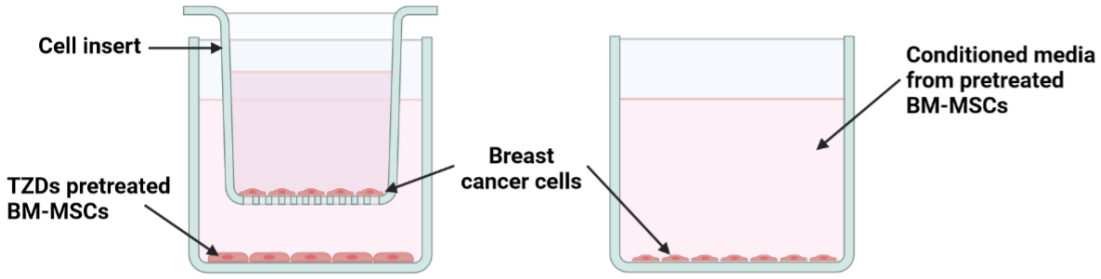


Figure 1.1: Schematic diagram depicting the indirect interactions between TZDs pretreated BM-MSCs and breast cancer cells. Indirect co-culture of breast cancer cells was accomplished with the use of cell inserts and conditioned media collected from TZDs pretreated BM-MSCs. The breast cancer cells will be "treated" with the secretome of the TZDs pretreated BM-MSCs. Figure was created with BioRender.com.

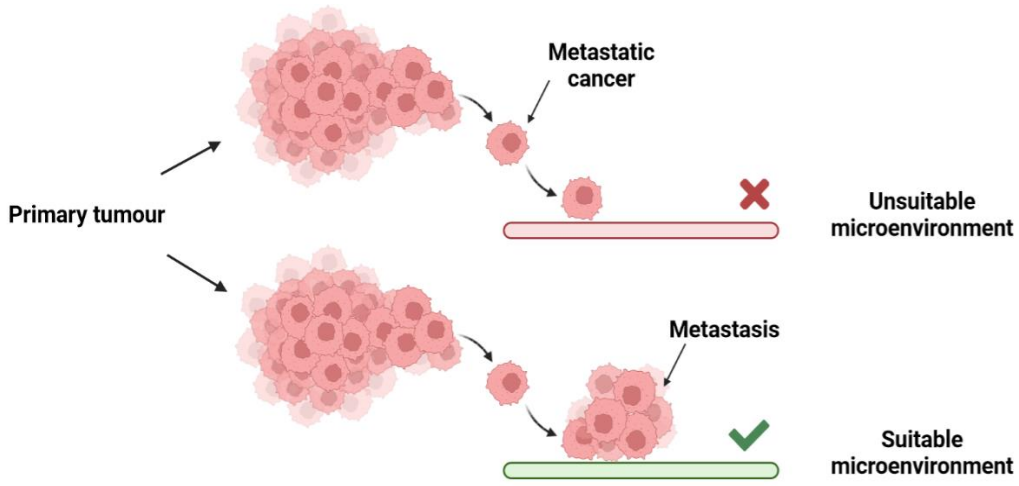


Figure 1.2: Schematic diagram representing the "seed and soil" cancer theory. The idea was to change the microenvironment via therapeutic intervention, such as 20PR pretreated BM-MSCs, making it unsuitable for the adhesion and growth of cancer cells. Figure was created with BioRender.com.

1.3 Research objectives

The general objective of this study was to evaluate the drug-drug interaction between PIO and ROSI on breast cancer cells and to utilise it indirectly as a treatment strategy on breast cancer cells with the help of BM-MSCs. The specific objectives of this study were:

1. To investigate the *in vitro* effects and drug synergism of TZDs on MDA-MB-231 and MCF-7 cells.
2. To study the *in vitro* effects of TZDs on BM-MSCs and the interaction of TZDs pretreated BM-MSCs with MCF-7 cells.
3. To identify intracellular proteins isolated from TZDs pretreated BM-MSCs and the effects of the protein on the interaction between TZDs pretreated BM-MSCs and MCF-7 cells.
4. To investigate the role of the identified protein in changing the interaction between TZDs pretreated BM-MSCs and MCF-7 cells and the potential molecular mechanism involved.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a common term that covers a substantial group of diseases that can originate from and affect any part of the human body. Despite the varied types of diseases it covers, a distinguishing characteristic of cancer is the rapid growth of abnormal or damaged cells that spread and invade other parts of the body, a process referred to as metastasis. In recent years, the incidence of cancers has been on the rise, which hugely impacted the different aspects of human life, making cancer a major health and economic burden globally. Annually, millions of people are diagnosed with cancer, making it one of the most prominent causes of mortality worldwide. According to the World Health Organization (WHO), there were approximately 10 million deaths in 2020, and nearly 1 in 6 deaths was attributed to cancer (Ferlay et al., 2021).

Compared to normal healthy cells, cancer cells can grow despite the absence of growth signals or the presence of signals that stops cell growth or cause apoptosis (programmed cell death). Additionally, cancer cells are capable of immunomodulation (regulates the body's immune system), promoting angiogenesis (formation of new blood vessels), and utilise different kinds of nutrients, allowing the cancer cells to grow more rapidly (National Cancer Institute, 2021). Fundamentally, cancer is a type of genetic disease since it occurs primarily due to the changes in genes that regulate basic cell functions. There are 3 main categories of genes that contribute to cancer growth, which are tumour suppressor genes (Joyce et al., 2022), proto-onco-genes (Amaya Arbeláez et al., 2021), and DNA repair genes (Li et al., 2021). These genes work

together in balance to ensure normal cell growth and survival, whereby mutations may lead to the development of cancerous cells.

Since cancer is a type of genetic disease, it can be inherited but cancer-causing genetic changes may arise as well through accumulated DNA replication errors that occur during cell division (National Cancer Institute, 2021). Besides that, DNA in the cells may also be damaged due to exposure to physical (such as ultraviolet and ionising radiation), chemical (such as asbestos, tobacco smoke, and alcohol), and biological carcinogens (such as viral, bacterial, and parasitic infections) (World Health Organization, 2022). These damaged cells are normally removed by the body, but the effectiveness of the cellular repair mechanism is reduced as a person ages. For that reason, adoption of a healthier lifestyle, minimising exposure to carcinogens, and regular screening have been given more attention to minimise the incidence of cancer (Lewandowska et al., 2021). However, the discovery and development of new anti-cancer compounds and therapy should not be overlooked since there will always be a demand for a more effective treatment.

There are numerous types of cancers, usually named for the organ or tissue of origin. Broadly speaking, cancers can be categorised into 4 major types based on the specific type of cell, which includes carcinoma (epithelial cells), sarcoma (supportive and connective tissues), leukemia (blood cells), and lymphoma (lymphocytes) (American Society of Clinical Oncology, 2019). These classifications are important as a correct diagnosis is required for appropriate and effective treatment since each cancer type requires a specific course of therapy. Furthermore, the condition of the patient is taken into consideration as well when determining the appropriate treatment regimen.

2.2 Breast cancer

2.2.1 General introduction

Breast cancer is a type of tissue cancer that originates from the epithelium of the ducts or lobules in the glandular tissue of the breast (World Health Organization, 2021). It is the most prevalent and frequently diagnosed malignancy among women where 2.26 million new cases and 685,000 deaths were reported globally in 2020 (Ferlay et al., 2021). Breast cancer was also the primary cause of death among women aged 45 – 84 years in the United States (Centers for Disease Control and Prevention, 2021). In developing nations especially at South-Eastern Asia, the mortality rate is higher than the global average despite having a lower occurrence rate due to the large variations in socioeconomic disparities (Francies et al., 2020). Therefore, the development of cheaper and more effective treatments would improve the overall outlook of this global issue.

Breast cancer is a complex and multifactorial disease that is attributed to genetic and environmental factors. The pathogenesis of the disease usually begins from hyperproliferation of the ductal or lobular cells which progresses into benign tumours or metastatic carcinomas after stimulation from carcinogenic factors (Sun et al., 2017). Currently, the hierarchical model and stochastic model are the 2 hypothetical models that are used to explain breast cancer tumorigenesis and progression. The first model suggests that all breast tumour subtypes originate from a single mutated progenitor cell (stem cell). On the other hand, the second model postulates that breast cancer originates from the sufficient accumulation of random mutations by any breast epithelial cells (Sgroi, 2010; Wang et al., 2014).

2.2.2 Risk factors of breast cancer

There are numerous factors that increases the chance of a women developing breast cancer such as family history, age, reproductive factors, and lifestyle choices. A recent study in the UK found that women had an average of 1.75-fold higher risk of being diagnosed with breast cancer if they had a single first-degree relative with history of breast cancer. Furthermore, the risk increased to 2.5-fold higher if the women had 2 or more relatives with history of breast cancer (Brewer et al., 2017). Besides family history, age is one of the most important risk factors because the incidence of breast cancer was found to be highly correlated with increasing age (Siegel et al., 2022).

Reproductive factors, including early menstruation (menarche), late menopause, late age of first pregnancy, and low pregnancy rates (parity) may increase the risk of developing breast cancer as well. Studies have found that each delay of 1 year in menarche decreases the developing breast cancer by 5% while each additional parity lowers the risk by 10% (Dall & Britt, 2017; Horn & Vatten, 2017). Such reproductive factors are likely associated with the level of sex hormones such as estrogen. Both endogenous (Endogenous Hormones and Breast Cancer Collaborative Group et al., 2013) and exogenous estrogens such as hormone replacement therapy (Liu et al., 2016) have been reported to increase the risk of breast cancer. However, a recent review article postulate that the duration of estrogen exposure is not the cause of increased breast cancer risk, rather it is the timing of the exposure (Dall & Britt, 2017).

Modern lifestyle choices have also been shown to increase the incidence of breast cancer. As an example, excessive alcohol intake and smoking was found to

increase the risk of contralateral breast cancer by 1.5 – 1.62-fold (Knight et al., 2017). A separate study reported that alcohol consumption of ≥ 30 g/day increased the risk of estrogen receptor positive breast cancer by 1.35-fold and the 1.28-fold for estrogen receptor negative breast cancer (Jung et al., 2016). Physical inactivity and high consumption of dietary fat may increase the risk of breast cancer as well. A study associated higher consumption of industrial and ruminant trans fatty acid with elevated breast cancer risk by 1.14-fold and 1.09-fold, respectively (Matta et al., 2021). Furthermore, numerous studies have strongly associated obesity with higher incidence of breast cancer, especially in postmenopausal women (Engin, 2017; Picon-Ruiz et al., 2017).

2.2.3 Current breast cancer prevention therapy

The most accessible method for breast cancer prevention is adoption of a healthier lifestyle. Risk of breast cancer can be reduced through dietary modification and exercise. A meta-analysis of 174 studies found that individuals that exercise and had total physical activity level of approximately 13 times higher than the minimum recommended amount had risk reduction of 14% for breast cancer (Kyu et al., 2016). A separate meta-analysis on 68,416 breast cancer cases, significantly associated physical activities with a decrease of breast cancer risk by 3% and 2% for every 10 metabolic equivalent of energy hours per week increment for recreational activity and physical activity, respectively (Chen et al., 2019b). Besides that, reduced consumption of alcohol and tobacco would also lower the risk of breast cancer incident due to their carcinogenic properties.

Regular screening is another successful strategy in combating breast cancer-related mortality since tumour metastasis is linked to approximately 90% of cancer

deaths. This is because breast tumour can be removed by surgery if it was detected as a primary tumour or at an early stage of metastasis and chemotherapy would work more effectively (Sun et al., 2017). In a randomised controlled trial on 53,883 women aged 39 – 41 years, it was reported that annual screening for breast cancer resulted in a relative reduction in mortality (Duffy et al., 2020). Screening for genetic markers with deleterious mutation such as breast cancer associated genes, *BRCA1* and *BRCA2*, is an acceptable strategy as well. According to the American Society of Breast Surgeons, bilateral mastectomy for healthy women with deleterious mutation in the *BRCA1* and *BRCA2* genes is a reasonable approach to reduce the risk of developing breast tumour (Sauter, 2018). However, studies have shown that mastectomy has a negative impact on the quality of life and body image of women (Türk & Yılmaz, 2018) and breast-conserving surgery was preferrable as it leads to better outcome in body image with less systemic side effects (Ng et al., 2019).

Besides that, the use of pharmacological or natural agents to inhibit the growth and development of breast cancer are utilised as well. For example, FDA approved selective estrogen receptor modulator medications such as tamoxifen and raloxifene have been used to treat breast cancer in high-risk women (Sun et al., 2017; Sauter, 2018). However, similar to other cancer drugs, administration of tamoxifen is also associated with several side effects such as uterine malignancies, hypertension, pulmonary embolism, and stroke (Farrar & Jacobs, 2022). Aromatase inhibitors are also used to treat postmenopausal breast cancer patients. It functions by inhibiting aromatase that catalyses the conversion of estrogen from androgen, thus lowering the levels of circulating estrogen in the plasma (Sun et al., 2017). A meta-analysis of the randomised trials regarding aromatase inhibitors and tamoxifen found that aromatase inhibitors reduce the mortality rate by approximately 15% and the recurrence rate by

about 30% compared to tamoxifen. It was also reported that aromatase inhibitors reduced the mortality rate by about 40% when compared to patients that did not receive endocrine treatment (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2015). Usage of aromatase inhibitors are commonly associated with bone loss due to estrogen deficiency, arthritis, ulcers and blisters, sexual dysfunction, and abnormal liver function due to the inflammation of the liver (Peters & Tadi, 2022).

Targeted therapy or immunotherapy utilising monoclonal antibodies to target human epidermal growth factor receptor (HER) 2 is also an effective approach for breast cancer treatment since approximately 15 – 20% of breast cancer overexpress the *HER2* gene (Elizalde et al., 2016). Trastuzumab (Herceptin®) is an FDA approved recombinant humanised monoclonal antibody that downregulates the expression of *HER2*, inhibits its signalling pathway, and trigger immune-mediated responses against HER2-overexpressing cells (Gajria & Chandarlapaty, 2011). A meta-analysis on 7 randomised controlled trials involving 1,497 patients, reported that administration of trastuzumab increased the survival rate of HER2-positive breast cancer patients (Balduzzi et al., 2014). The final analysis of the HERceptin Adjuvant trial also demonstrated that HER2-positive early breast cancer patients had significantly improved long-term disease-free survival when treated with 1 year of adjuvant trastuzumab (Cameron et al., 2017). Side effects of trastuzumab therapy are often mild and manageable but it may also cause congestive heart failure (Van den Nest et al., 2019). Despite all the advancements in the field, the occurrence of drug resistance and severe side effects has hindered the effectiveness of these treatments. Furthermore, the cost of the available treatment makes it a huge financial burden for the middle- and lower-income populace. Therefore, new alternatives that are more effective and accessible are required to combat this health issue.

2.2.4 Predictive markers related to breast cancer

2.2.4(a) Hormone receptors

The expression of hormone receptors (estrogen and progesterone receptor) has clinical and biological significance since it can be used as a prognostic marker and a factor for treatment determination (Chan et al., 2015). Globally, approximately 70% of all breast tumour population are hormone receptor positive, making it the most common subtype (Waks & Winer, 2019). The estrogen and progesterone receptors are protein surface markers found on mammalian cells that are stimulated by sex hormone, estrogen, and progesterone (Mohanty et al., 2020). In breast cancer cells, stimulation of the receptors by the hormones upregulates cyclin G1 which promotes cell proliferation (Tian et al., 2018). The recommended treatment for this subtype of breast cancer is usually endocrine therapy but resistance development is unavoidable in advanced stages (Turner et al., 2017), thus driving the need to develop alternative therapies. Compared to single hormone receptor positive and hormone receptors negative subtypes, double hormone receptors positive breast cancer patients generally experience lower risks of mortality (Bae et al., 2015; Li et al., 2020).

2.2.4(b) Genetic markers

There have been several genes that were identified to be involved in the initiation and development of breast cancer. *BRCA1* and *BRCA2* are anti-oncogenes that are closely associated with the development of breast tumour. *BRCA1* and *BRCA2* encodes for protein that repairs DNA damage (Mehrgou & Akouchekian, 2016). Specifically, *BRCA1* activates DNA damaged-induced cell cycle checkpoints (Wu et al., 2010) while *BRCA2* regulates the repair for stalled replication forks (Fradet-Turcotte et al., 2016). Studies have found that inheritance of deleterious mutations in either *BRCA1* or *BRCA2* significantly increased the incidence of breast cancer (Paluch-

Shimon et al., 2016; Kuchenbaecker et al., 2017) and the patients had poorer prognosis as well (Liu et al., 2021).

HER1, or also known as ERBB1, is a cell surface glycoprotein that is involved with several signalling pathways, including phosphatidylinositol-3-kinase (PI3K), c-Jun N-terminal kinases (JNK), and Ras-Raf-mitogen-activated protein kinase (MAPK). The activation of *HER1* has been found to protect cells against apoptosis and promote cell proliferation, invasion, and angiogenesis (Appert-Collin et al., 2015; Ali & Wendt, 2017). In a clinical setting, overexpression of *HER1* in breast tumours has been linked to poorer prognosis (Alanazi & Khan, 2016).

Concurrently, *HER2*, or also known as ERBB2, is an oncogene that promotes the growth and metastasis of breast cancer cells (Gutierrez & Schiff, 2011). Historically, overexpression of *HER2* or HER2-positive breast cancer patients represents approximately 15% – 20% of all breast cancer cases (Furrer et al., 2018). Studies have also reported that overexpression of *HER2* increases the population of cancer stem cells through the phosphatase and tensin homolog (PTEN)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) complex 1 signalling pathway and results in clinically aggressive subtypes with poor prognosis (Sun et al., 2017; Martínez-Sáez & Prat, 2021).

2.2.5 Breast cancer subtypes

Breast cancer are commonly categorised into 6 main molecular subgroup which includes luminal A, luminal B, triple negative, claudin-low, HER2 type, and normal-like (Mehrgou & Akouchekian, 2016). Luminal A breast cancer tend to be estrogen and progesterone receptor-positive, but HER-2 negative. This subtype tends

to have low recurrence rate with the best prognosis. MCF-7, T47D, and SUM185 cells lines are example of this subtype (Smith et al., 2017). On the other hand, luminal B cells are estrogen receptor and HER2 positive, and/or progesterone receptor positive. Luminal B patients are often younger with poorer prognosis compared to luminal A patients. Examples of cell lines include BT474 and ZR-75 (Dai et al., 2017).

Triple negative breast cancer cells are negative for estrogen and progesterone receptor and HER2. This subtype of tumour is often aggressive with poorer prognosis than the other subtypes. MDA-MB-468 and SUM190 are examples of cell lines with these properties (Smith et al., 2017). Similarly, claudin-low breast tumour are triple negatives as well but with lower expression of Ki67 and cell-cell adhesion proteins such as E-cadherin. Examples of cell lines with these features include MDA-MB-231, BT549, Hs578T and SUM1315 (Holliday & Speirs, 2011; Dai et al., 2017). Next, HER2 type breast cancer cells are estrogen and progesterone receptor negative but HER2 positive. Patients with this tumour subtype tend to have poorer clinical outcome and are prone for recurrence. MDA-MB-453 and SKBR3 are cell line examples of this subtype (Holliday & Speirs, 2011). Finally, normal-like breast tumours are normally small in size with good prognosis (Mehrgou & Akouchekian, 2016).

2.2.6 Breast cancer cell lines

2.2.6(a) MDA-MB-231

MDA-MB-231 cells are epithelial cells isolated from a 51-year-old White female patient with invasive ductal carcinoma. This cell line has a claudin-low subtype phenotype that expresses mutated p53 and is commonly used to model late-stage breast cancer (Welsh, 2013). It is the second most popular breast cancer cell line and the most studied triple negative cell line in metastatic breast cancer research (Liu et al., 2019).

MDA-MB-231 is an aggressive and invasive cell line due to the functional Rac3/extracellular signal-regulated kinases (ERK) 2/ nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling pathway in the cells (Gest et al., 2013).

2.2.6(b) MCF-7 cells

MCF-7 cells are epithelial cells derived from a 69-year-old White woman with metastatic adenocarcinoma. This cell line has a luminal A subtype phenotype (estrogen and progesterone receptor positive but HER2 negative) and is commonly used to represent a model of early-stage breast cancer (Welsh, 2013). It is the most popular breast cancer cell line in metastatic breast cancer research (Liu et al., 2019) as its subtype accounts for 70% of the breast cancer population (Waks & Winer, 2019). MCF-7 is a poorly aggressive and non-invasive cell line (Gest et al., 2013) that expresses high levels of estrogen receptor alpha (ER-α) and low levels of estrogen receptor beta (ER-β) (Comşa et al., 2015).

2.2.6(c) ZR-75-30

ZR-75-30 cells are epithelial cells isolated from malignant ascites fluid from a 47-year-old premenopausal Black woman with infiltrating ductal carcinoma. This cell line has a luminal B subtype phenotype (estrogen, progesterone, and HER2 receptor positive) and is highly aggressive (Zhang et al., 2019). ZR-75-30 cells have been studied as a model of estrogen receptor positive breast cancer that is insensitive to tamoxifen and possesses fusion genes with high-copy-number of coamplified parts of chromosomes, 8 and 17 (Schulte et al., 2012).

2.2.6(d) MDA-MB-468

MDA-MB-468 is a cell line with epithelial morphology isolated from a pleural effusion of a 51-year-old Black female patient with metastatic adenocarcinoma. This cell line has a triple-negative phenotype with a high expression of Ki67 (Holliday & Speirs, 2011). MDA-MB-468 cells are highly aggressive and invasive with a mutated p53 gene (Rasti & Azimi, 2015) and are widely studied as the model of triple negative breast cancer (Wojtowicz et al., 2020).

2.2.6(e) MDA-MB-453

MDA-MB-453 cells are epithelial cells derived from a 48-year-old female patient with metastatic carcinoma of the breast. This cell line has a HER2 and androgen receptor positive phenotype that acquires mesenchymal features when treated with dihydrotestosterone (Ahram et al., 2021). MDA-MB-453 cells are commonly used as a model for the molecular apocrine breast cancer subtype (Vranic et al., 2011).

2.2.6(f) Selected breast cancer cell line

Among the 5 breast cancer cells that were discussed, as summarized in Table 2.3, MDA-MB-231 and MCF-7 cells were selected for the current study. This is because both cell lines are commonly used for *in vitro* studies with different mutation profiles that covers a wide range of breast cancer phenotypes. For this study, MDA-MB-231 cells were used to represent hormone receptor negative, late-stage breast cancers, while MCF-7 cells were used to represent hormone receptor positive, early-stage breast cancers.

Table 2.1: Summary of MDA-MB-231, MCF-7, ZR-75-30, MDA-MB-468, and MDA-MB-453 cell lines.

Cell line	Source	Characteristics	Use in research
MDA-MB-231	51-year-old White female patient with invasive ductal carcinoma	Claudin-low subtype phenotype that expresses mutated p53	Model for late-stage cancer
MCF-7	69-year-old White woman with metastatic adenocarcinoma	Luminal A subtype phenotype with high levels of ER-α and low levels of ER-β	Model for early-stage cancer
ZR-75-30	Malignant ascites fluid from a 47-year-old premenopausal Black woman with infiltrating ductal carcinoma.	Luminal B subtype phenotype with progesterone receptor positive and possesses fusion genes with high-copy-number of coamplified parts of chromosomes, 8 and 17	Model for estrogen receptor positive breast cancer that is insensitive to tamoxifen
MDA-MB-468	pleural effusion of a 51-year-old Black female patient with metastatic adenocarcinoma.	Triple negative phenotype with a high expression of Ki67 and mutated p53	Model for triple negative breast cancer
MDA-MB-453	48-year-old female patient with metastatic carcinoma of the breast	HER2 and androgen receptor positive phenotype	Model for molecular apocrine breast cancer subtype

2.3 TZDs

2.3.1 General introduction

TZDs or chemically known as 1,3-thiazolidine-2,4-diones with a molecular formula of $C_3H_3NO_2S$ are a class of heterocyclic compounds which consists of a 5-membered C_3NS ring (Figure 2.1). At room temperature, TZDs exists as a crystalline white solid that is only sparingly soluble in common organic solvents such as water and dimethyl sulfoxide (DMSO) (Long et al., 2021). TZDs were initially developed by Takeda Pharmaceuticals and introduced as an insulin-sensitising drug for treating type 2 diabetes mellitus and obesity in the early-1980s (Fujita et al., 1983). The exact mechanism of TZDs remained unknown until it was discovered to be a potent and selective activator for PPARγ in the mid-1990s (Lehmann et al., 1995). After its introduction in the United States, the adoption of TZDs to treat type 2 diabetes mellitus became prevalent due to the growing recognition of the role of insulin resistance in the progression of the disease (Arnold et al., 2019). However, the use of TZDs became less frequent after a meta-analysis found that ROSI significantly increased the risk of heart attack and mortality due to cardiovascular events by 1.43-fold and 1.64-fold, respectively (Nissen & Wolski, 2007).

TZDs became even less popular when studies found other safety issues regarding the drug especially its association with heart failure (Jiang et al., 2018; Yang et al., 2019; Yen et al., 2021). As a result of this, the FDA issued new restrictions to the use of ROSI whereby only patients that were successfully treated previously and patients that cannot be treated with other medication are allowed prescription (Center for Drug Evaluation and Research, 2021). Historically, FDA have previously approved 3 TZDs for the treatment of type 2 diabetes mellitus, including troglitazone, PIO, and

ROSI (Soccio et al., 2014). However, troglitazone was withdrawn from the market by the FDA in 2000, 3 years after its initial approval in 1997 due to its frequent association with liver injury which included acute liver failure (Yang et al., 2014; Mazerbourg et al., 2016). Although the precise mechanism is inadequately understood, the hepatotoxicity was not correlated to the activation of PPARγ, rather it is an idiosyncratic effect of the drug itself. For that reason, PIO and ROSI was approved by the FDA later in 1999 (Soccio et al., 2014).

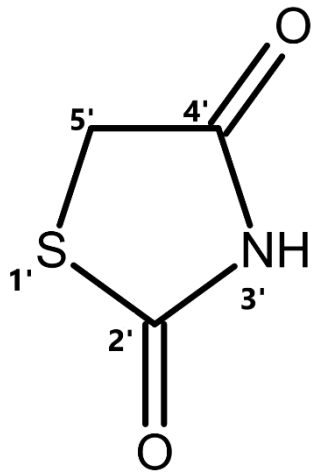


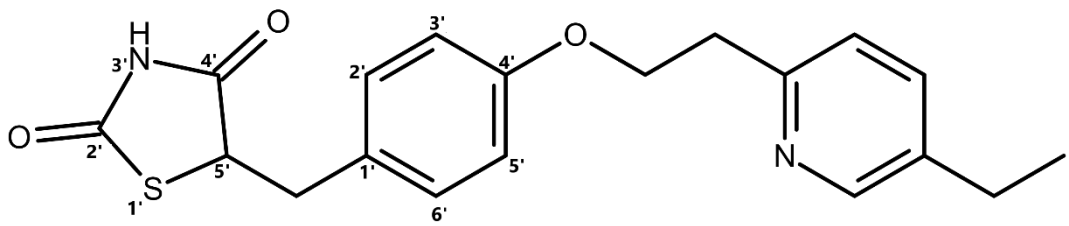
Figure 2.1: Chemical structure of TZDs. The structure is made up of thiazolidine, a non-aromatic analogue of a 5-membered ring heterocycle (thiazole) with 2 carbonyl groups as position 2 and 4. The overall ring structure is termed 1,3-thiazolidine-2,4-diones (TZDs) (Long et al., 2021).

2.3.2 Chemistry of PIO and ROSI

PIO is an oral anti-hyperglycemic medication (Actos) that is chemically known as 5-[[4-[2-(5-ethylpyridin-2-yl)ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione, with a molecular formula of $C_{19}H_{20}N_{2O}O_3S$ (Figure 2.2A). PIO is an agonist for both PPARα and PPARγ (Sakamoto et al., 2000; Orasanu et al., 2008; Nicholls & Uno, 2012) which are mainly expressed in different parts of the body, thus having different results on lipid levels and cardiovascular outcome when compared to ROSI which is

primarily a PPARγ agonist (Singh et al., 2022). On the other hand, ROSI is an oral anti-diabetic medication (Avandia) that is chemically known as 5-[[4-[2-[methyl(pyridin-2-yl)amino]ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione, with a chemical formula of $C_{18}H_{19}N_3O_3S$ (Figure 2.2B). ROSI is a very potent TZDs with a strong binding affinity for PPARγ that is 30-fold higher than PIO (Della-Morte et al., 2014).

A



B

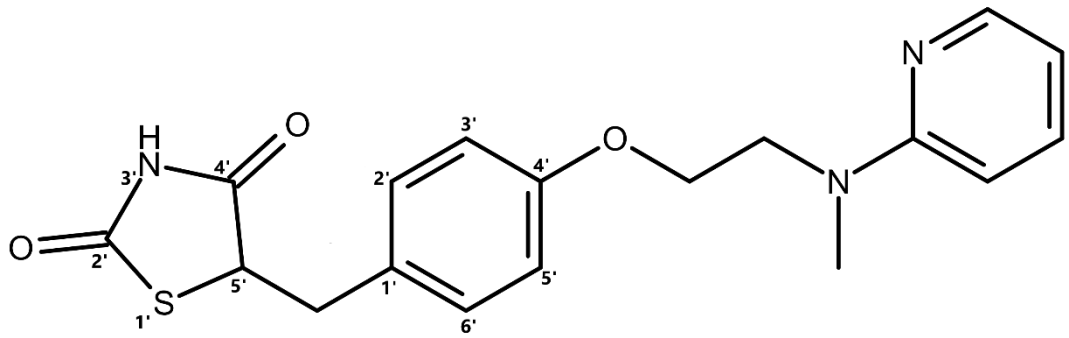


Figure 2.2: Chemical structure of (A) PIO and (B) ROSI. The structure of PIO is made up of TZDs with the addition of a benzyl group at position 5 which in turn is substituted by a 2-(5-ethylpyridin-2-yl)ethoxy group at position 4 of the phenyl ring. The structure of ROSI is made up of TZDs with the addition of a benzyl group at position 5 which in turn is substituted by a 2-[methyl(pyridin-2-yl)amino]ethoxy group at position 4 of the phenyl ring (Long et al., 2021).

2.3.3 Mechanism of action

TZDs function as an insulin sensitiser that influence the intracellular metabolic pathways by increasing insulin sensitivity of critical tissues and enhancing insulin action (Eggleton & Jialal, 2021). The TZDs-mediated insulin sensitisation increases adiponectin levels, insulin-dependent uptake of glucose in fat and muscle tissue, and fatty acid oxidation while decreasing hepatic gluconeogenesis (Yau et al., 2013; Li et al., 2018; Vieira et al., 2019). On a molecular level, TZDs bind to PPARγ to trigger the downstream signalling that regulates numerous pathways involved in metabolism regulation (Choi et al., 2014). The activation of PPARγ then upregulates the expression of genes involved in the insulin signalling cascade such as glucose transporter type 4 (*GLUT4*) and catabolite activator protein (*CAP*), stimulating the production of more insulin sensitive adipocytes, and activation of the PI3K, plasma membrane intrinsic protein 3 (PIP3), and Akt signalling pathways which improves insulin sensitivity at the cellular level (Della-Morte et al., 2014).

2.3.4 Side effects of TZDs

TZDs therapy is commonly associated with several adverse effects especially when used for a long-term. Edema or fluid-retention is one of the most common side effects is associated with TZDs. Analysis of several studies and data from large databases have found that monotherapy of PIO or ROSI was linked to 3% – 5% incidence of peripheral edema and the risk increased to approximately 8% and approximately 16% when administered in conjunction with sulfonylureas and insulin, respectively (Lebovitz, 2019). In fact, up to almost 20% of patients have been found to experience dose-related fluid retention due to the PPARγ mediated stimulation of sodium reabsorption (Eggleton & Jialal, 2021). Besides that, weight gain is another side effect that is commonly linked to TZDs. Although fluid retention is considered to

be responsible (Singh et al., 2022), it is probable that use of TZDs increased the mass of adipose tissue as well. Activation of PPARγ would increase the maturation rate of preadipocytes into mature adipocytes and increase fat storage in the subcutaneous tissues (Ma et al., 2018).

Administration of TZDs have also been correlated with higher risk of heart failure. However, PIO and ROSI have been reported to have differential effects on cardiovascular diseases. The study found that PIO had beneficial effect on cardiovascular diseases while ROSI increased the risk of cardiovascular incidence (Juurlink et al., 2009; Simo et al., 2010). A recent review paper concluded that PIO slowed down the atherosclerosis process and lowered the incidence of cardiovascular event (DeFronzo et al., 2019). However, a separate review found that TZDs usage by patients with history of cardiovascular disease had an increased risk of edema and heart failure. The author also noted that patients treated with ROSI had an increase in mortality rate while patients treated with PIO did not despite the increased risk of heart failure (Lebovitz, 2019). Patients with recent history of ischemic stroke or transient ischemic stroke was also found have lower risk of stroke and myocardial infarction when treated with PIO (Kernan et al., 2016).

Another major complication that is associated with TZDs therapy is the increased risk of bone fractures and decrease in bone density especially in women. It is proposed that PPARγ activation diverted the differentiation of osteoblasts into adipocytes which lead to bone loss (Eggleton & Jialal, 2021). In a clinical trial involving non-diabetic patients with insulin resistance, PIO was found to increase the risk of bone fracture requiring surgery or hospitalisation especially men (Kernan et al., 2016). In a separate long-term pragmatic trial, PIO was not observed to increase the