AMERICAN UNIVERSITY OF BEIRUT

CHARACTERIZATION OF THE *DE NOVO* CERAMIDE METABOLIC PATHWAY IN THE BCL-2 AND BCL-X_L-DEPENDENT RESPONSE TO TNF-α-INDUCED APOPTOSIS IN MCF-7 CELLS

by

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ABSTRACT
THE THESIS OF

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Characterization of the *de novo* Ceramide Metabolic Pathway in the Bcl-2- and Bcl-xL-Dependent Response to TNF-α-induced Apoptosis in MCF-7 cells

Several challenges remain in the treatment of breast cancer, the world's most prevalent cancer in women. One of these challenges, resistance to chemotherapeutic drugs, has been associated with the upregulation in the expression of Bcl-2 and Bcl-xL in several models. Bcl-2 and Bcl-xL, members of the Bcl-2 family, prevent apoptosis. They inhibit TNF-α-induced apoptosis in different cellular models. On the other hand, ceramide, a signaling sphingolipid that controls different cellular processes, induces apoptosis. Bcl-2 and Bcl-xL interaction with ceramide was previously studied in the TNF-α-induced response in MCF-7 breast cancer cell line where it was shown that Bcl-xL acts upstream of ceramide while Bcl-2 functions downstream of this sphingolipid. Both Bcl-2 and Bcl-xL inhibited ceramide-induced death in response to TNF-α. However, Bcl-2 did not inhibit ceramide accumulation in response to TNF-α and displayed a protective effect against ceramide-induced death. Although Bcl-xL, and to a lesser degree Bcl-2, were shown by previous literature to interfere with ceramide generation following TNF-α treatment in MCF-7 cells, disclosing regulatory mechanisms controlling this interaction remains to be essential.

In the current work, we aimed to characterize the metabolic pathways that control ceramide accumulation, particularly through *de novo* synthesis and salvage pathways, in response to TNF-α treatment in MCF-7 cells overexpressing Bcl-2 or Bcl-xL. We hypothesized that Bcl-2 and Bcl-xL regulate the *de novo* ceramide synthesis pathway and might be involved in the activation of sphingosine kinase 1 (SphK1) of the salvage pathway, thus contributing to resistance to apoptosis following TNF-α treatment.

We first validated the resistance of MCF-7 Bcl-2 and MCF-7 Bcl-xL to TNF-α-induced cytotoxicity by MTT. We then studied the transcriptional regulation of *DEGS1*, *CerS2*, *CerS4*, and *CerS6*, enzymes of the *de novo* synthesis pathway, by qRT-PCR and revealed that expression of Bcl-xL produced a downregulation in the mRNA expression of *DEGS1* and *CerS4* after 8h of TNF-α treatment whereas no change was detected with Bcl-2 expression. *SphK1* mRNA expression was also not affected in both cell lines, and this was confirmed by the inability of SK1-I to sensitize the resistant cells to TNF-α by MTT. We then aimed to interfere with the *de novo* pathway using 2 synthetic retinoids: 4-HPR and ST1926. Whereas both modulators are activators of the *de novo* synthesis pathway, 4-HPR inhibits DEGS1 enzyme leading to the accumulation of the ceramide precursor, dihydroceramide. Pre-treating the MCF-7 Vector control cells with 4-HPR partially inhibited TNF-α induced cell death. In addition,

ST1926 was able to re-sensitize MCF-7 Bcl-xL to TNF-α induced apoptotic cell death evidenced by the expression of cleaved PARP-1. MCF-7 Bcl-2, however, displayed resistance to the effect of ST1926 and nearly total inhibition of PARP-1 cleavage in response to TNF-α with or without ST1926. Further investigation of the death response induced by TNF-α with or without ST1926 revealed that ST1926 is not likely to function through p53. Interestingly, MCF-7 Vector cells displayed a significant upregulation of p53 upon TNF-α treatment at 48h suggesting the involvement of p53 in TNF-α induced apoptosis. While no change in the protein expression of p53 was detected in MCF-7 Bcl-xL at 48h, MCF-7 Bcl-2 displayed a significant increase in p53, which, in the absence of apoptosis, suggests that cells may be induced to undergo cycle arrest. Although sharing similar function, it appears that the anti-apoptotic proteins Bcl-xL and Bcl-2 function at sites that can modulate different responses to TNF-α. Understanding the exact mechanism by which these proteins resist TNF-α-induced apoptosis may help to discover new therapeutic approaches for breast cancer.

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ABBREVIATIONS

4-HPR: N-(4-hydroxyphenyl)-retinamide

AC: Adriamycin/Cyclophosphamide

AC-T: Adriamycin/Cyclophosphamide/Paclitaxel

ADH: Atypical ductal hyperplasia

ALH: Atypical lobular hyperplasia

alk-SMase: Alkaline sphingomyelinase

AP-1: Activator protein-1

APAF1: Apoptotic-activating factor 1

aSMase: Acid sphingomyelinase

ATL: Adult T-cell leukemia

ATRA: All-trans retinoic acid

BAD: Bcl-2-associated agonist of cell death

BAK: Bcl-2 agonist killer

BAX: Bcl-2-associated protein X

Bcl-2: B cell lymphoma 2

Bcl-W: B cell lymphoma W

Bcl-xL: B cell lymphoma extra large

BFL1: Bcl-2-related isolated from fetal liver 1

BH: Bcl-2 homology

BID: BH3-interacting domain of death agonist

BIK: Bcl-2-interacting killer

BIM: Bcl-2-interacting mediator of cell death

BMF: Bcl-2-modifying factor

BRCA1: Breast cancer 1

BRCA2: Breast cancer 2

BSA: Bovine serum albumin

C1P: Ceramide-1-phosphate

CAPP: Ceramide-activated protein phosphatase

CDase: Ceramidase

CDK-1: Cyclin-dependent kinase 1

cDNA: Complementary deoxyribonucleic acid

CED-9: Cell death abnormality gene 9

Cer: Ceramide

CERK: Ceramide kinase

CerS: Ceramide synthase

CERT: Ceramide transfer protein

CNL: Ceramide nanoliposome

CO_2: Carbon dioxide

c-Raf: proto-oncogene serine/threonine-protein kinase

DAG: Diacylglycerol

DCIS: Ductal carcinoma in *situ*

DD: Death domain

DEGS: Dihydroceramide desaturase

DMSO: Dimethyl sulfoxide

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

ERBB2: Human epidermal growth factor receptor 2

ER-α: Estrogen receptor alpha

FADD: Fas-associated death domain

FAS-L: Fas ligand

FBS: Fetal bovine serum

FLD: Flexible loop domain

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GBA: β-glucosidase

GCS: Glucosyl-ceramide synthase

gDNA: Genomic deoxyribonucleic acid

HAT: Histone acetyltransferase

HER2: Human epidermal growth factor receptor 2

HR+: Hormone positive

HRP: Horse Reddish Peroxidase

HTLV-1: Human T-lymphotropic virus-1

IBC: Inflammatory breast cancer

IDC: Invasive ductal carcinoma

JNK: c-Jun N-terminal kinase

KDSR: 3-ketosphinganine reductase

LASS: Longevity assurance

LCIS: Lobular carcinoma in *situ*

LC-MS: Liquid chromatography–mass spectrometry

Mcl-1: Myeloid cell leukemia 1

MOMP: Mitochondrial outer membrane permeabilization

mRNA: Messenger ribonucleic acid

mTOR: Mammalian target of rapamycin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

NF-κB: Nuclear factor kappa light chain-enhancer of activated B cell

nSMase: Neutral sphingomyelinase

OD: Optical density

OMI: serine protease

PARP-1: Poly (ADP-ribose) polymerase-1

PD1: Programmed cell death protein 1

PDL (1-2): Programmed cell death protein (1-2)

PKA: Protein kinase A

PKCα: Protein kinase C alpha

PP2A: Protein phosphatase 2A

PR: Progesterone receptor

PT: Phylloid tumor

PUMA: p53-upregulated modulator of apoptosis

PVDF: Poly vinlyne difluoride

qRT-PCR: Quantitative Real-time PCR

RA: Retinoic acid

RARE: Retinoic acid response element

RIP: Receptor-interacting protein

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RPMI: Rose Well Park Memorial Institute

RT: Reverse Transcriptase

S1P: Sphingosine-1-phosphate

S1RP (1-5): Sphingosine-1-phosphate receptor (1-5)

SAPK: Stress-activator protein kinase

SK1-I: Sphingosine kinase 1-Inhibitor

SM: Sphingomyelin

SMAC21: Second mitochondrial derived activator of caspases

SMS: Sphingomyelin synthase

SphK: Sphingosine kinase

SPL: Sphingosine-1-phosphate lyase

SPP: Sphingosine-1-phosphate phosphatase

SPT: Serine palmitoyl transferase

ST1926: (2E)-3-[3′-(1-adamantyl)-4′-hydroxy[1,1′-biphenyl]-4-yl]-2-propenoic acid

TBS: Tris-buffered saline

TBST: TBS-Tween

TGS: Tris-Glycine-SDS

TM: Transmembrane

TNBC: Triple-negative breast cancer

TNF: Tumor necrosis factor

TNFR1: Type 1 TNF receptor

TNFR2: Type 2 TNF receptor

TNF-α: Tumor necrosis factor alpha

TRADD: TNFR-associated death domain

TRAF2: TNFR-associated factor 2

TRAIL: TNF-related apoptosis-inducing ligand

XIAP: X-linked inhibition of apoptosis protein

CHAPTER I

INTRODUCTION

A. Breast cancer

1. Epidemiology and risk factors

According to the World Health Organization, breast cancer was diagnosed in 2.3 million women in 2020 with 685,000 deaths globally. It accounts for the world's most prevalent cancer in women, as 7.8 million women alive were diagnosed in the past 5 years by the end of 2020 [1]. In addition, breast cancer was shown to constitute around 20% of all cancer cases in Lebanon in an 11-year study that aimed to determine breast cancer prevalence rates from 2005 up to 2015. Lebanon was also found to have one of the highest breast cancer incidences in the world [2]. An increased risk of breast cancer in certain families is often associated with defects in several genes [3]. The best-characterized genetic mutations in breast cancer are breast cancer 1 (*BRCA1*) and breast cancer 2 (*BRCA2*), which are tumor suppressor genes responsible for maintaining DNA integrity by repairing double-stranded breaks [4]. Women with *BRCA1* mutations have a 60% risk of developing breast cancer by the age of 70 and 55% risk for *BRCA2* [5]. In addition, 80-90% lifetime risk was associated with *TP53* mutation, another tumor suppressor gene, even higher than *BRCA1/2* mutations which are the most common high penetrance germline mutations in hereditary breast cancer [6]. Collectively with other genes, such as *PTEN*, *CDH1*, and *STK11*, these known highly penetrant germline mutations are attributed to no more than 25% of cases based on previous investigations and quantitative modeling [7, 8]. Further, age, blood group, gender, smoking, obesity, ethnicity, alcohol consumption, lifestyle, and hormonal-replacement therapies are all risk factors significantly associated with the progression of breast cancer [9].

2. *Breast cancer subtypes*

Breast cancer is the abnormal growth of cells that starts in breast tissues, which are mainly composed of stromal and glandular tissues. Glandular (lobular) tissues are responsible for milk production, while the stromal tissues provide support due to their abundance in fatty and fibrous connective tissues [10] **(Figure 1)**. Before reaching an invasive state, the pre-cancerous state is associated with benign lesions confined inside the ducts or lobules referred to as atypical hyperplasia [atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH)] imposing a risk factor of developing breast cancer **(Figure 2)**. It is noteworthy to mention that women diagnosed with ADH or ALH lesions are four times more likely to advance into breast cancer [11]; however, not all ADH progress into carcinoma [12]. Two subdivisions of breast cancers are identified:

a) In situ (non-invasive) carcinoma

i. Ductal carcinoma in situ (DCIS)

DCIS differs from ADH by the abnormality of cell proliferation. DCIS develops in the milk ducts without affecting or penetrating the basement membrane, and often regarded as stage 0 breast cancer [13]. However, DCIS has a variable and non-obligate invasive potential. Furthermore, around 40% of DCIS were shown to progress to invasive ductal carcinoma (IDC) [14]. Current treatments for DCIS include surgery, followed by radiation and endocrine therapy in case it was hormone receptor positive [15].

ii. Lobular carcinoma in situ (LCIS)

LCIS develops in the milk-producing glands of the breast. Although it is considered as a risk factor for breast cancer, it is usually regarded as a benign lesion, as surgery might be recommended for some variants [16].

b) Invasive carcinoma

Invasive carcinoma can penetrate into the stroma and metastasize to the lymph nodes [17]. Invasive ductal carcinoma is the most common type of invasive carcinoma accounting for 50-75% of patients, while invasive lobular carcinoma was shown to constitute 5%-15% of patients, followed by mixed lobular/ductal mixed carcinomas and other histologies [18].

c) Rare types of breast cancer

i. Inflammatory breast cancer (IBC)

IBC is a rare type accounting for 1% to 6% of breast cancer cases with a higher metastatic potential [19, 20]. In addition, most of IBC cases arise from ductal carcinoma often accompanied with inflammatory symptoms, such as warm breast, erythema, edema, and orange peel of the breast [21].

ii. Paget's disease of the breast

It is a nipple disease that constitute around 1–4.3% of all breast cancers associated with DCIS and/or IDC in the majority of cases [22]. It is characterized by a lesion

extending between the nipple and areola. In advanced stages, it can involve the skin accompanied by ulcerations, discharge, and inflammatory symptoms [23].

iii. Phyllodes Tumors (PT) of the breast

This type of breast cancer involves the connective tissues, also known as the stroma, accounting for 0.3-0.5% of breast cancer in females [24]. Most of PTs are benign (60-75%), but there are other malignant phenotypes that can acquire metastatic potentials if untreated [24, 25].

iv. Angiosarcoma of the breast

It is a highly aggressive cancer starting from the endothelial cells that surround blood and lymphatic vessels [26]. It is extremely rare and was shown to account for around 0.04% of malignant breast cancer cases [27].

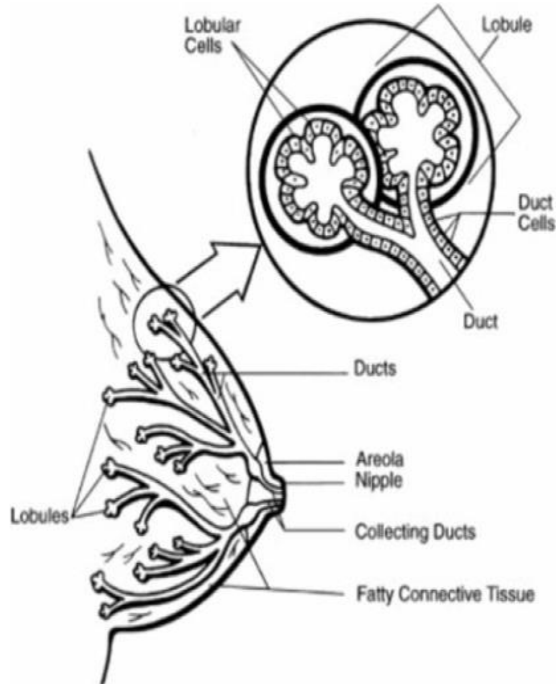

Figure 1. Breast cancer structure. Breast is composed of connective and lobular tissues. Lobular tissues are the milk producing glands. Breast cancer can arise from either the lobular tissues or their connecting ducts known as lobular and ductal carcinoma, respectively [10].

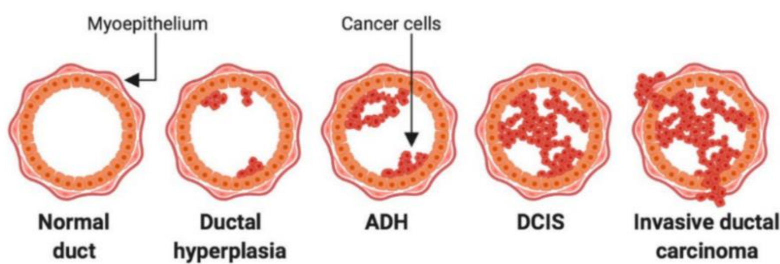

Figure 2. Progression of ductal breast carcinoma. Ductal carcinoma grows as benign lesions restricted to the ducts known as ADH. DCIS is identified once the duct is completely filled with tumor cells. This can progress into IDC where the tumor is no longer confined to the mammary duct, and has acquired metastatic potentials [28].

3. *Stages of breast cancer*

The size and type of tumor, as well as the extent to which tumor cells have invaded the breast tissues, determine the stage of breast cancer falling to into 5 different stages:

- Stage 0: the tumor is still confined to the breast tissue with a non-invasive potential. DCIS is an example of this tumor [29].
- Stage I: it is further divided into two categories: stage IA and stage IB [29].
  - Stage IA: a 2 cm tumor with negative lymph nodes.
  - Stage IB: a small group of cancer cells that have reached the lymph node. They can measure up to 0.2 mm.
- Stage II: it is further divided into two categories: stage IIA and stage IIB [29].
  - Stage IIA: axillary or sentinel lymph nodes are affected with no tumor found in the breast. The tumor can measure up to 5 cm.
  - Stage IIB: the tumor did not reach the axillary nodes. However, it might reach a size larger than 5 cm.
- Stage III: it is further divided into three categories: stage IIIA, stage IIIB, and stage IIIC [29].
  - Stage IIIA: tumor is not found in the breast and has affected 4–9 axillary or sentinel lymph nodes.
  - Stage IIIB: a tumor of any size accompanied by inflammatory markers, such as redness, breast skin ulcers and swelling. It affects up to 9 axillary or sentinel lymph nodes.
  - Stage IIIC: the tumor can be found in 10 or more axillary lymph nodes, and in the lymph nodes surrounding the clavicle.

- Stage IV: the cancer has an advanced metastatic potential. The tumor has spread to other organs of the body, such as brain, liver, lungs, bones, etc. [29].

4. *Molecular biomarkers of breast cancer*

Previously identified molecular targets in breast cancer also contribute to its sub-classification, such as estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).

a) Estrogen receptor alpha (ERα)

Estrogen receptor alpha (ERα) is a steroid hormone receptor and a transcription factor that is activated by estrogen inducing oncogenic growth. ERα signaling can also be induced via the expression of the progesterone receptor (PR) [30]. Tumors expressing either ER-α or PR are indicated as HR+ and constitute around 70% of breast cancers [31]. Current treatments for these cancers include chemotherapy and endocrine agents that work by downregulating their signaling.

b) Human epidermal growth factor receptor 2 (ERBB2)

Transmembrane receptor known as human epidermal growth factor receptor 2 (ERBB2) (formerly HER2 or HER2/neu), encoded by *ERBB2* gene, is another molecular target that promotes cancer growth, and is found positive in approximately 20% of breast cancers [32]. Chemotherapy and targeting ERBB2 using monoclonal antibodies constitute the current treatments for patients with ERBB2+ tumors [33]. Moreover, a mix of hormone and ERBB2 therapy might be prescribed for patients with HR+ and ERBB2+ tumors [34].

c) Triple-negative breast cancers (TNBCs)

Triple-negative breast cancers (TNBCs) are characterized by the downregulation or absence of ER-α, PR, and HER2; and are currently treated with chemotherapy [35]. TNBCs have a high rate of distant relapse and are considered most aggressive [36]. They constitute around 15% of breast cancer patients with relatively poor outcome [37].

5. Current therapeutic strategies

Non-metastatic breast cancers are initially treated locally by surgical resection, and removal of axillary lymph nodes to prevent metastasis. Systemic therapy consists of adjuvant and neoadjuvant types that can be pre and/or postoperative. Metastatic cancers are also treated by the same category of systemic therapies accompanied by surgery and radiation for palliation. However, metastatic breast cancer disease is currently incurable, and the main goals of the different therapeutic approaches are to prolong life span and ease symptoms [18].

a) Systemic therapies

Patients diagnosed with hormone positive (HR+) breast cancer, ie. (ER+), can benefit from endocrine therapy, in addition to chemotherapy, that target the key signaling pathways contributing to growth and carcinogenesis. For example, tamoxifen is a known selective inhibitor for ER-α receptor in the treatment of HR+ breast cancers working by competitive inhibition. Similarly, aromatase inhibitors (letrozole, anastrozole, and exemestane) are other therapeutics used in HR+ disease which act by downregulating circulating estrogen by preventing its conversion from steroids [31]. Patients with ERBB2+ tumors are also treated with chemotherapy, and targeted therapy, such as trastuzumab which is a monoclonal

antibody targeting ERBB2 receptor [38]. They may benefit from endocrine therapy in case they have HR+ disease [18]. Triple negative tumors do not express neither ER-α nor ERBB2 and are treated with conventional chemotherapy. However, disclosing their key cellular signaling pathways has identified novel therapeutic targets and future strategies [36]. Examples of chemotherapeutic regimens used in the treatment of breast cancer include Adriamycin/ cyclophosphamide (AC), Adriamycin/ cyclophosphamide/paclitaxel (AC-T), and docetaxel/cyclophosphamide [39]. Given the lack of advances in the treatment of certain breast cancers, such as the triple negative subtype, immunotherapy has emerged as a novel therapeutic strategy with promising outcomes [40]. Pembrolizumab is a monoclonal antibody and immune-checkpoint inhibitor that has recently gained FDA approval for treating early-stage triple-negative breast cancer [41]. It works by inhibiting the interaction between the programmed cell death protein 1 (PD1) of effector T-cells with its ligands (PDL1 and PDL2) expressed on cancer cells resulting in the activation of immune responses [42]. Several side effects are associated with these regimens that can range from edema, myalgias, asthenia, nausea, and neutropenia to uterine cancer, osteoporosis, febrile neutropenia, and heart failure in rare cases [18]. Although the current therapeutic strategies gained some success in the past 50 years, several challenges are still being faced in the treatment of breast cancer [43]. Furthermore, toxicities experienced in conventional therapeutic approaches, metastasis, and resistance to therapy have all encouraged the discovery of new therapeutic targets in cancer research by disclosing key novel neoplastic pathways.

B. Apoptosis resistance: a hallmark of cancer

1. Overview of apoptosis

Apoptosis, also known as programmed cell death, is a critical mechanism that aims to preserve the tissue cellular proliferation in various physiological processes including aging, embryonic development, and normal tissue homeostasis [44-46]. Deregulation of these normal processes was shown to contribute to several diseases with high morbidity and mortality worldwide, such as neurodegenerative diseases [47-49], cardiovascular diseases [50, 51], autoimmune diseases [52, 53] and infectious diseases [54]. Apoptosis evasion is one of the cancer hallmarks that is crucial for acquiring immortality through sustained proliferation and growth [55]. One of the major reasons behind drug resistance in cancer treatment is attributed to the increase in anti-apoptotic signals that play an important role in tumorigenesis and metastasis [56-59]. Thus, understanding key mechanisms promoting therapy resistance is essential for the future advancement of anti-cancer agents. Programmed cell death culminates in the degradation of cellular components following caspase activation. In contrast to necrosis which results in acute cell trauma, apoptotic mechanisms tag the cell for phagocytic clearance with minimal stress and damage to the surrounding tissue [60]. Importantly, apoptosis can be triggered by internal or external stimuli attributed to 2 distinct pathways: intrinsic and extrinsic.

a) The intrinsic pathway of apoptosis

The intrinsic apoptotic pathway is mediated through the mitochondria, and its execution is regulated by members of the Bcl-2 family, which include pro-apoptotic and anti-apoptotic proteins. In fact, cell fate decisions are determined by the balance of these 2 groups **(Figure 3)** [61]. Each member of this family is characterized by the presence of one or more BCL-2 homology (BH) domains, BH1–BH4. BH3 containing proteins, such as Bcl-2 associated agonist

of cell death (BAD), BH3-interacting mediator of cell death (BIM), BH3-interacting domain death agonist (BID), Bcl-2-interacting killer (BIK), Bcl-2-modifying factor (BMF), p53-upregulated modulator of apoptosis (PUMA), and Noxa -Latin for damage- are key regulators that trigger pro-apoptotic signals by binding and activating Bcl-2-associated X protein (BAX), and/or Bcl-2 antagonist/killer (BAK), which are apoptotic pore-forming proteins at the mitochondrial outer membrane [62]. This induces their oligomerization and macropore formation resulting in mitochondrial outer membrane permeabilization (MOMP) which leads to the release of apoptogenic factors from the intermembrane space, and subsequently caspase activation [63]. Direct activation of caspases involves cytochrome c release into the cytoplasm which in turn binds to apoptotic protease-activating factor 1 (APAF1) to form the apoptosome, while indirect activation is mediated by the release of second mitochondria-derived activator of caspases (SMAC21) and serine protease (OMI) that function to repress the activity of caspase-inhibitory proteins, such as X-linked inhibitor of apoptosis protein (XIAP) [64]. Initiator caspase 9 is activated following these events and, in turn, induces executioner caspases 3 and 7 to fragment cellular components. In fact, caspases can lunch a proteolytic cascade involving DNA fragmentation and chromatin condensation [65]. Caspase 3 and 7 also cleave poly (ADP-ribose) polymerase-1 (PARP-1) whose normal function is to repair damaged DNA [66]. PARP-1 cleavage, cell shrinkage, and nuclear condensation are all considered hallmarks of apoptosis [65, 67].

In contrast to pro-apoptotic elements, pro-survival proteins contain all BH domains, and these include B cell lymphoma 2 (Bcl-2), B cell lymphoma extra-large (Bcl-xL), B cell lymphoma W (Bcl-W), Bcl-2-related isolated from fetal liver 1 (BFL1), and Myeloid cell leukemia 1 (Mcl-1) [62]. These proteins function by sequestering monomeric activated BAK or

BAX, preventing the release of pro-apoptotic factors from the mitochondria allowing sustained growth and proliferation. The intrinsic apoptotic pathway can be initiated following exposure to diverse stimuli, including mitochondrial damage [68], cellular organelle damage [69], genotoxic stress (ionizing radiation) [70], chemotherapy [71], and hypoxia [72]. Depriving cells of growth factors [73] and nutrients [74] can also trigger programmed cell death. However, the mechanisms of the induction may differ depending on the type of stimulus. Furthermore, p53 was shown to induce the transcription of BAX [75], PUMA [76], Noxa [77], and repress Bcl-2 in response to genotoxic stress (chemotherapeutics and ionizing radiation) [78]. It was also found to act on the Bcl-2 family post-translationally through forming inhibitory complexes with Bcl-2 and Bcl-xL at the mitochondria further promoting its destabilization [79]. In contrast, growth factors deprivation or kinase inhibition trigger apoptosis through the upregulation of BIM [80], and dephosphorylation of BAD [81], respectively. Thus, cell fate decisions are modulated by the specificity of the responses where each can be triggered by a different stimulus.

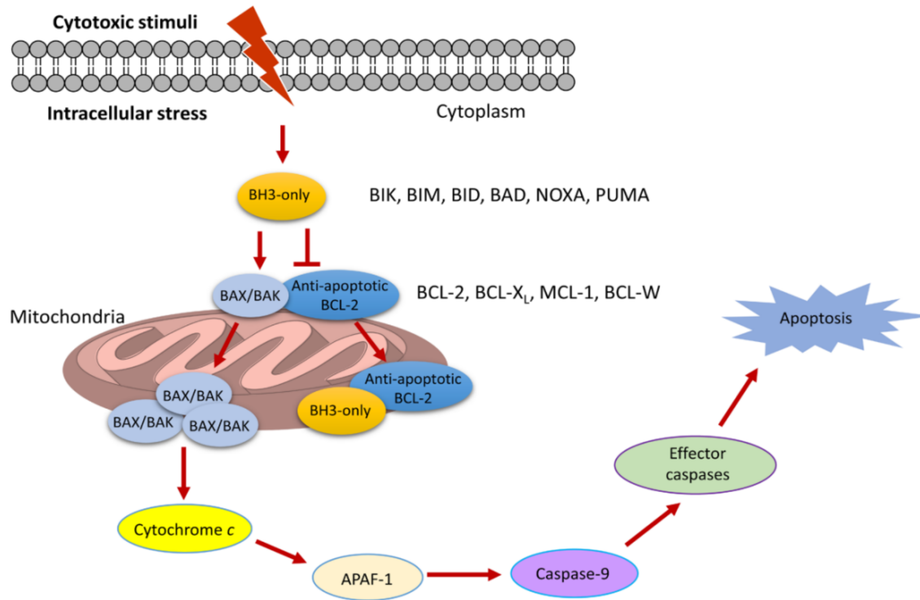

Figure 3. The intrinsic apoptotic pathway mediated by Bcl-2 family through the mitochondria. Cell fate decision is regulated by members of the Bcl-2 family which is divided into two groups: pro-apoptotic and anti-apoptotic. Intracellular stresses induce the activation of pro-apoptotic elements, such as BIK, BIM, BID, BAD, NOXA, and PUMA which are BH3-containing only proteins. These members will trigger BAX/BAK oligomerization resulting in MOMP, and the release of cytochrome c which will bind to APAF-1 forming the apoptosome. This will in turn activate initiator caspase 9, and subsequently effector caspases inducing apoptosis. In contrast, anti-apoptotic members, such as Bcl-2, Bcl-xL, Mcl-1, and Bcl-W function to sequester the activity of pro-apoptotic members BAX and BAK preventing MOMP formation [82].

b) <u>The extrinsic pathway of apoptosis</u>

The extrinsic pathway is initiated by the binding of external death ligands, such as Fas ligand (Fas-L), TNF-related apoptosis-inducing ligand (TRAIL), and tumor necrosis factor α (TNF-α), to the death domain of their corresponding transmembrane receptors which belong to the TNF

receptor family. They are characterized by the presence of cytoplasmic death domain (DD) consisting of 80 amino acid sequence essential for apoptosis, and these include Fas/CD95/Apo-1, TRAIL receptors (DR4 and DR5), and the type 1 TNF receptor (TNFR1) [83].

i. TNF-α signaling pathway

The signaling pathway involved in extrinsic apoptosis is best characterized in the TNF-α/TNFR1 **(Figure 4)**. TNF-α is a cytokine produced by immune cells to regulate a variety of physiological processes, such as apoptosis, inflammation, and immunity [84]. Upon binding to its receptor TNFR1, it will induce a conformational change exposing the DD domain to TRADD (TNFR-associated death domain). This will recruit FADD (Fas-associated death domain), which is an adaptor protein essential to initiate cellular death signals. FADD will then bind to pro-caspase 8 inducing its activation. Caspase 8 will, in turn, induce a protease cascade activation involving executioner caspases 3 and 7. It will also activate the mitochondrial pathway through BID cleavage leading to the release of apoptogenic factors from the mitochondria [85].

Another pathway involving TNFR1 signaling is apoptosis suppression through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein- 1 (AP-1) by c-Jun N-terminal kinases (JNK) signaling. This anti-apoptotic effect is mediated by the recruitment of other adaptor molecules, such as TNFR-associated factor 2 (TRAF2), and receptor-interacting protein (RIP) [86]. It is also noteworthy to mention that the overexpression of some proteins, including Bcl-2 and Bcl-xL inhibit TNF-α induced apoptosis [87, 88].

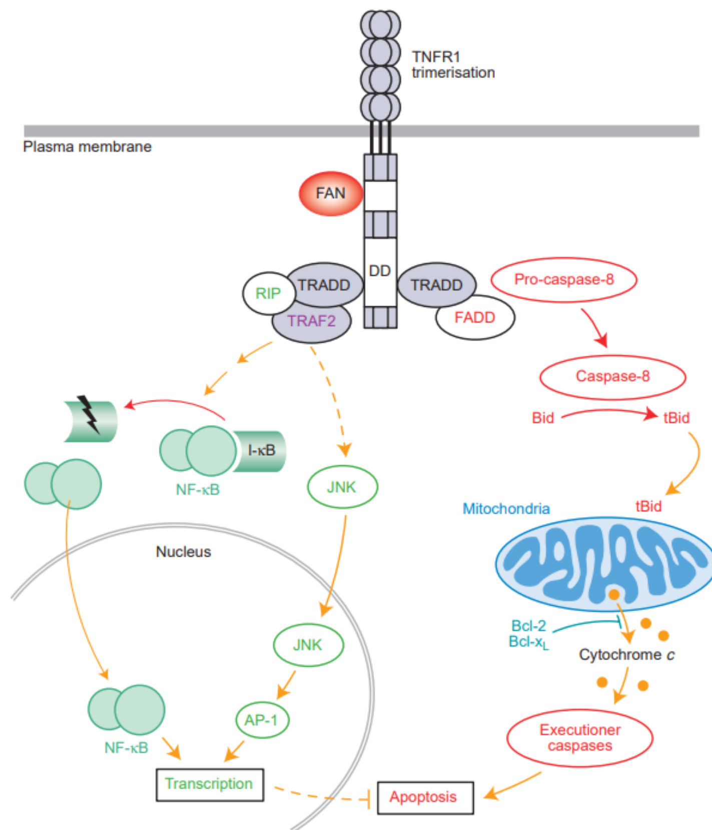

Figure 4. Schematic representation of TNFR1 signaling [89]. Binding of TNF-α to its receptor TNFR1 will recruit adaptor proteins, such as TRADD and FADD which will in turn promote the cleavage of procaspase 8. Caspase 8 will launch a series of caspase activation events involving executioner caspases 3 and 7, thereby activating the cell death cascade. It will also cleave the pro-apoptotic Bcl-2 family member BID to t-BID further promoting the apoptotic event through the mitochondria [85]. In contrast, the overexpression of anti-apoptotic Bcl-2 members, such as Bcl-2 and Bcl-xL will hinder TNF-α induced apoptosis by inhibiting the release of apoptogenic factors from the mitochondria (cytochrome c) and caspase activation [87, 88]. The recruitment of other adaptor molecules, such as TRAF2 and RIP will promote anti-apoptotic signals through the activation of AP-1 by JNK signaling, and the activation NF-κB [86].

2. Bcl-2 family and apoptosis resistance in cancer

To ensure sustained growth and proliferation, one of the mechanisms acquired by cancer is apoptosis evasion through altering the expression of the anti-apoptotic and/or pro-apoptotic Bcl-2 family. Gene mutations affecting the regulation of the mitochondrial pathway are exceptionally common in carcinogenesis [90]. As previously stated, cell fate decision is mediated through an equilibrium established by members of the Bcl-2 family which can be anti- or pro-apoptotic. For cancer to successfully evade apoptosis, it is no surprise that it will increase anti-apoptotic molecules and/or downregulate the pro-apoptotic members which will prevent the release of cytochrome c from the mitochondria.

a) Bcl-2 and Bcl-xL overexpression in cancer

The Bcl-2 family members are master regulators of drug-induced apoptosis in the treatment of cancers due to the upregulation of the anti-apoptotic proteins and/or the downregulation of pro-apoptotic ones [91, 92]. Early observation of Bcl-2 overexpression in cancer was significantly associated with the pathogenesis of human follicular lymphoma as a result of Bcl-2 oncogene chromosomal translocation into the immunoglobulin heavy chain gene locus [93, 94]. In addition, cells overexpressing Bcl-2 were shown to confer apoptosis resistance to a wide variety of stimuli, such as TNF-α [87], chemotherapy [95], and γ-irradiation [96]. Beside evading apoptosis, Bcl-2 overexpression was found to promote metastatic potential in breast cancer [97], glioma [98], colorectal cancer [99], melanoma [100], neuroblastoma [101], and lung cancer [102] cell lines. Analysis of patient samples exhibiting an upregulation of Bcl-2 revealed a role in invasion and metastasis in several

cancers (lung cancer [103], laryngeal squamous cell carcinoma [104], and colorectal cancer [105]).

Similar to Bcl-2, Bcl-xL overexpressing cells also restricted apoptosis following the treatment with several stimuli, including γ-irradiation [106], TNF-α [88], and chemotherapy [107]. Upregulation of Bcl-xL was also shown to be significantly associated with increased invasiveness in various cellular models, such as lung cancer [108], glioma [109], and colorectal cancer [99]. Breast cancer overexpressing Bcl-xL cellular model implanted into mice fat pad enhanced tissue invasiveness and distant metastasis to various organs, such as liver, brain, bones, lymph node, lungs, and kidneys [110]. In addition, increased Bcl-xL levels were significantly associated with tumor progression and poor prognosis in colorectal cancer as well as in hepatocellular carcinoma patients [111, 112]. Breast cancer nodal metastasis, therapeutic resistance [113, 114], and invasive potential of tongue cancer [115] were found to be closely associated with upregulated Bcl-xL expression. More importantly, Bcl-xL was reported to be more potent in preventing apoptosis than Bcl-2 in response to doxorubicin and taxol [116, 117].

i. Bcl-2 and Bcl-xL in breast cancer

In metastatic human breast cancer cell lines, overexpression of Bcl-2 renders the cells less susceptible to taxane [118]. Additionally, estrogen promotes Adriamycin resistance in breast cancer cells by a mechanism that involve Bcl-2 [119]. In fact, estrogen was found to upregulate Bcl-2 expression by inducing its transcription in MCF-7 cell lines [120]. Around 75% of primary breast tumors express significant levels of Bcl-2 with the majority in ER+ (~85%) followed by HER2+ (50%) and TNBCs (41%) tumors [121-123]. Long term resistance to tamoxifen has been associated with the upregulation

in the expression of Bcl-2 and Bcl-xL [124]. Despite having similar function, Bcl-xL has been identified as a more potent inhibitor to doxorubicin in MCF-7 cell line [116], and was associated with poor outcome as compared with Bcl-2 [113, 125-128].

3. *Bcl-2 and Bcl-xL: structure and function*

Bcl-2 and Bcl-xL are two proteins playing important roles in many physiologic and developmental processes [129]. Their 3D structure is composed of 8 α-helices with a hydrophobic groove on the surface [130, 131]. Further, BH regions, including BH1, BH2, and BH3 domains define the top of this groove which was illustrated to be responsible for the interaction with pro-apoptotic members of the Bcl-2 family, such as BAD and BAK to inhibit apoptosis. Bcl-2 and Bcl-xL also possess carboxy-terminal hydrophobic region that give them the ability to localize to the cytoplasmic side of the mitochondrial membrane affecting its integrity [132]. This region, also referred to as the hydrophobic transmembrane anchoring (TM), allows them to integrate in other intracellular membranes, such as the endoplasmic reticulum where they play an important role in regulating calcium homeostasis and signaling [133]. Although sharing similar homology, some structural differences could explain distinct interaction specificities with each of the BH3 family members. For example, Bcl-xL displayed a greater binding affinity towards BAK and BAD proteins compared to Bcl-2 [130, 134, 135]. In addition, Bcl-xL was found to interfere with apoptosis signaling by specifically binding and interacting with cytochrome c [136].

a) <u>Post-translational modifications of Bcl-2 and Bcl-xL</u>

Bcl-2 possesses a flexible loop domain (FLD) which is a major site for its regulation by phosphorylation **(Figure 5)** [137]. Several kinases contribute to the phosphorylation of Bcl-2 at distinct amino acids of the FLD region (T56, T69, S70, T74, and S87) [138-141], and these include, mitogen-activated protein kinase (MAPK) p38 [142], mammalian target of rapamycin (mTOR) [143], proto-oncogene serine/threonine-protein kinase (c-Raf) [144], cyclin-dependent kinase 1 (CDK1) [145, 146], protein kinase A (PKA) [147], Jun N-terminal kinase (JNK) [148, 149], and protein kinase Cα (PKCα) [150]. The phosphorylation level of these residues has been implicated in the regulation of cell cycle and mitotic division. For instance, Bcl-2 was shown to be normally phosphorylated during mitosis and to become hyperphosphorylated upon the treatment with microtubule-damaging agents that induce cell cycle arrest and apoptosis [147, 151]. Phosphorylation of Bcl-2 triggered the dissociation of the Bcl-2/BAX, and subsequent cell death upon paclitaxel treatment [152]. Similarly, Bcl-xL has an analogue of the FLD region whose amino acids are also subjected to phosphorylation [153, 154]. Its phosphorylation at S62 by JNK enhances apoptosis accompanied by a reduced BAX binding [155]. Additionally, in response to ionizing radiation, the phosphorylation of Bcl-xL at T47 and T115 by stress activated

protein kinase (SAPK/JNK) was found to trigger apoptotic response while the mutant Bcl-xL (A47, A115) conferred resistance to DNA damage [156].

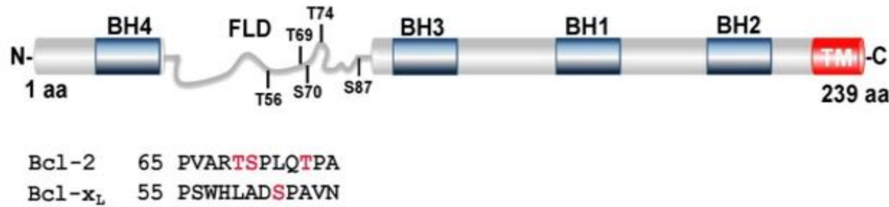

Figure 5. Schematic representation of the Bcl-2 protein structure and its phosphorylation sites. Bcl-2 or Bcl-xL structure is composed of four BH domains (blue), a transmembrane domain (TM) (red), and a flexible loop region (FLD) (gray). T56, T69, S70, T74 and S87 of Bcl-2 and S62 of Bcl-xL are residues within the FLD region targeted for phosphorylation [137].

C. Ceramide sphingolipid

1. General overview on sphingolipids

Sphingolipids are a group of lipids discovered by J. L.W. Thudichum in 1876 in brain extracts and were considered merely as structural molecules. The backbone of sphingolipids consists of sphingosine, and was named after the Greek mythical creature, the Sphinx due to their mysterious functions [157]. Its chemical structure was disclosed in the 20th century followed by identifying the function of other constituents of the plasma membrane (sphingomyelins, glycosphingolipids, and gangliosides) in cell recognition and cell-cell interaction. Further, sphingolipid metabolism and signaling have been widely appreciated in cellular signaling, and have been implicated in wide variety of human diseases [158].

Sphingolipids are important components of the plasma membrane contributing to its fluidity and barrier function. In fact, the plasma membrane is a dynamic physical structure that participates in many physiological processes [159]. Ceramide, the building block of sphingolipids, is a neutral lipid composed of a fatty acid chain attached to a sphingosine base (18C long-chain amino alcohol) via an amide bond **(Figure 6)**. The fatty acid chain can be characterized by its length (16C – 24C) as well as its saturation level, forming several ceramide species that contribute to different biological functions [160, 161]. Sphingolipids are considered amphipathic molecules due to their structure containing hydrophobic and hydrophilic residues. Sphingolipids hydrophobic region consists of a sphingosine base attached to a fatty acid while the hydrophilic portion can include phosphate, phosphorylcholine, and sugar residues forming more complex sphingolipids, such as [sphingosine-1-phosphate (S1P) or ceramide-1-phosphate (C1P)], sphingomyelin, and glycosphingolipids, respectively [162] **(Figure 7)**.

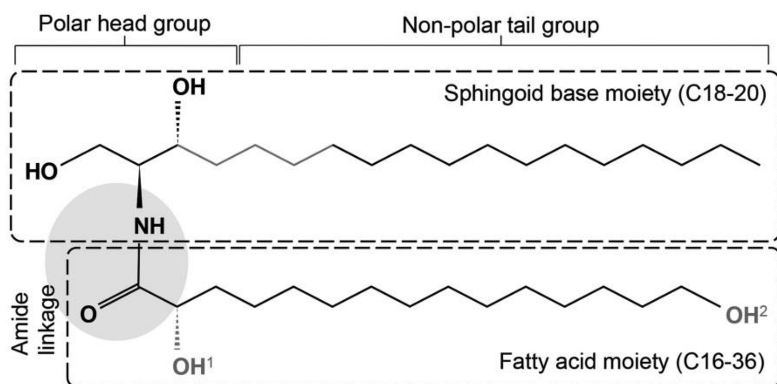

Figure 6. Ceramide structure. Ceramide is composed of sphingosine (2-amino-4-trans-octadecene-1,3-diol), an unsaturated 18 -hydrocarbons long-chain amino alcohol, joined to a fatty acyl chain moiety by amide linkage [163].

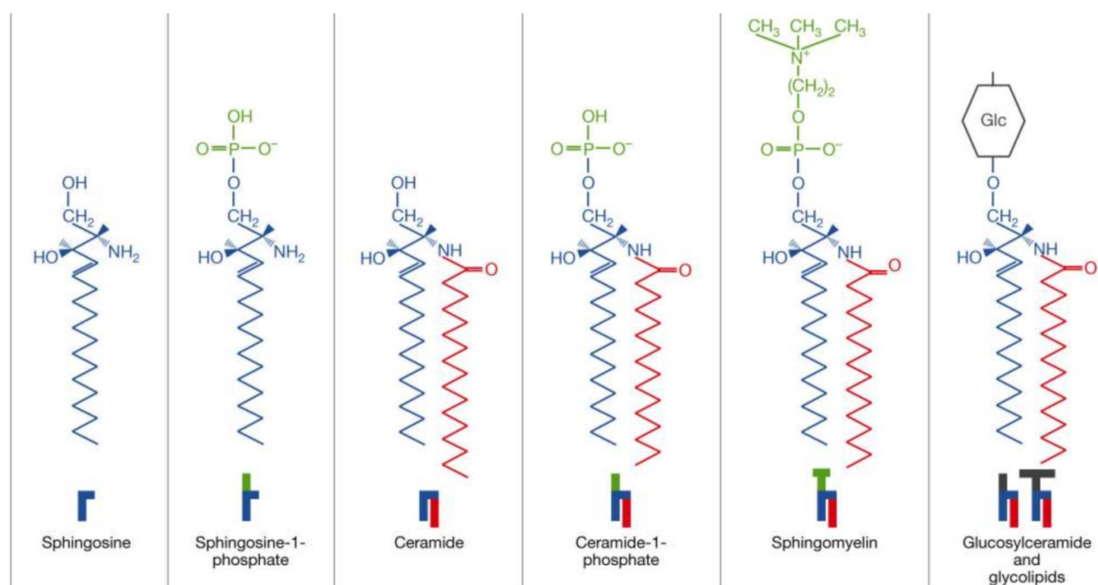

Figure 7. Sphingolipids structures. Ceramide is a central sphingolipid composed of a sphingosine base (in blue) attached to a fatty acid (in red) and is considered the building block of more complex ones (C1P, sphingomyelin, glycolipids, and glucosylceramide). Phosphate group (in green) represents the hydrophilic group of S1P and C1P, while phosphocholine (in green) represents the head group for sphingomyelin. Glucosylceramide and glycolipids have sugar residues as head groups [162].

2. *Ceramide: a bioactive lipid*

Ceramide is not only a structural molecule, but also a bioactive lipid that participates in signal transduction regulating various cellular function, such as cell migration [164, 165], cell differentiation [166], apoptosis [160], and cell cycle arrest [167]. Furthermore, ceramide has played an important aspect in disclosing signaling pathways regulating cancer biology [160], neurological disorders [168], host-pathogen interaction [169], cardiovascular diseases, and diabetes [161]. Endogenous ceramide accumulation was observed following the treatment with several stress agents, such as TNF- α [170-174], FAS [175-177], chemotherapeutic agents [178-

183], growth factors deprivation [167], irradiation [184, 185], hypoxia [186, 187], and heat shock [188].

a) Ceramide and apoptosis

A significant body of evidence highlights the activation of ceramide metabolic pathways as a downstream effector of programmed cell death.

i. Ceramide and the extrinsic pathway of apoptosis

As previously mentioned, the extrinsic pathway is mediated by the activation of TNFR family (TNFR1, FAS/CD95, and TRAIL) through ligand binding. Decreased ceramide accumulation was observed in CD95 [189], TRAIL [190, 191], and TNF-α [192, 193] -resistant cancer cells that can be reversed by the addition of an exogenous ceramide source which confirms the function of ceramide as a downstream effector of the extrinsic pathway. In response to TNF-α, ceramide can directly bind to cathepsin-D (the endo-lysosomal aspartic protease CTSD) to induce its proteolytic activation. Cathepsin-D can in turn cleave and activate the pro-apoptotic Bcl-2 family member Bid [194-196].

ii. Ceramide and the intrinsic pathway of apoptosis

Ceramide was also shown to interact with the mitochondrial pathway in apoptosis. In fact, ceramide induces MOMP, and ceramide channel formation at the mitochondrial membrane resulting in the release of apoptogenic factors, such as cytochrome c [197-199]. BAX insertion and oligomerization required ceramide rich domains at the mitochondrial outer membrane [200]. Furthermore, BAX and ceramide synergistically

acted on disrupting the mitochondrial outer membrane in other studies leading to a more efficient apoptosis and MOMP formation [201, 202]. Another role of ceramide in apoptosis signal transduction is the activation of ceramide-activated protein phosphatase (CAPP), a protein phosphatase 2A (PP2A), that can function to dephosphorylate anti-apoptotic and pro-apoptotic Bcl-2 family members resulting in their deactivation and activation, respectively to induce apoptosis [203, 204].

iii. Ceramide and p53

p53, also identified as guardian of the genome, is a tumor suppressor and a transcription factor that regulates a wide variety of cellular process, such as DNA repair, cell cycle arrest, and apoptosis in response to stress [205]. Given the role of ceramide in stress response mechanisms, such as senescence, cell cycle arrest, and death, its connection with p53 has been regularly studied [206-208]. Following genotoxic stress, Dbaibo *et al.* have shown ceramide accumulation to be induced in p53 dependent and independent stress responses [209]. Moreover, multiple reports have revealed that ceramide induction can be downstream, upstream or even independent of p53 in several cellular models under different types of stimuli [210]. In addition, ceramide metabolic enzymes were found to be transcriptionally regulated by p53. For example, CerS6 [211] and nSMase [212] were identified as direct transcriptional targets of p53.

3. Synthesis pathways of ceramide

Ceramide synthesis is highly compartmentalized, and its continuous flux can be achieved through 3 major metabolic pathways that depend on cell type, and nature of the stimulus: The *de novo* pathway, the salvage pathway, and the sphingomyelin hydrolysis pathway [213].

a) The *de novo* synthesis pathway

The *de novo* pathway synthesizes endogenous ceramide at the level of endoplasmic reticulum (ER) starting with the condensation of serine and palmitoyl-CoA in a reaction catalyzed by serine palmitoyl transferase (SPT) to form 3-ketosphinganine which will be reduced to sphinganine by 3-ketosphinganine reductase (KDSR) [214, 215]. This is followed by its acylation to form dihydroceramide by ceramide synthases (CerS1-6) [216]. A desaturation is then inserted between C4 and C5 in a reaction catalyzed by dihydroceramide desaturase (DEGS 1-2) [217] (**Figure 8**). The generated ceramide can then be transported to the Golgi by the non-vesicular transporter Ceramide Transporter (CERT) or vesicular trafficking to act as a precursor for sphingomyelin and other complex glycosphingolipids by sphingomyelin synthase (SMS) and glucosylceramide synthase (GCS), respectively. Vesicular flow will later shuttle these molecules to the plasma membrane [218]. The *de novo* pathway was found to regulate apoptotic processes in multiple cellular models following the treatment with a variety of stimuli, such as TNF-α, hypoxia, and chemotherapeutic agents [172, 219-221].

i. Dihydroceramide desaturase (DEGS)

DEGS is the enzyme catalyzing the conversion of dihydroceramide into ceramide, the last step of the *de novo* synthesis pathway of ceramide, by introducing a 4,5 trans double bond to the sphinganine base [217].

DEGS exists in 2 isoforms: DEGS1 and DEGS2. They differ in tissue distribution, and function whereby DEGS1 is ubiquitously expressed and accounts for ceramide generation in most tissues. In addition to the desaturase function, DEGS2 also has a c-4 hydroxylase activity synthesizing phytoceramides that are confined to kidney, skin, and intestine [222]. Mouse heart and cultured cells subjected to hypoxia displayed a downregulation of DEGS enzymes accompanied by the accumulation of dihydroceramide, and adaptation to the hypoxic condition [223]. Similarly, the knockdown of DEGS1-2 under normal oxygen condition reproduced the inhibitory activity of the hypoxic environment on cellular proliferation, while their overexpression displayed a protective effect in hypoxia which highlights their function as oxygen sensors [224].

ii. Ceramide synthases (CerSs)

Ceramide synthases are encoded by a family of six genes referred to as longevity assurance homologues *(LASS)* [225]. Multiple forms of CerSs have been identified (CerS1-6). The function of these enzymes is to generate ceramide in the *de novo* and the salvage pathways via the acylation of sphingoid bases with fatty acyl-CoA. Despite sharing similar enzymatic mechanisms, intracellular localization, and structural features, they differ in their tissue distribution, and in their preference

towards different fatty acyl-CoA chain length **(Table 1)** [216]. CerSs have been widely studied, and were regarded as important regulators of the programmed cell death implicated in several diseases, such as cancer [226]. CerS6 was shown to be an important mediator of TNF-α induced apoptosis in MCF-7 cells [227]. In addition, ER+ tumors were reported to upregulate CerS4 and CerS6 [228].

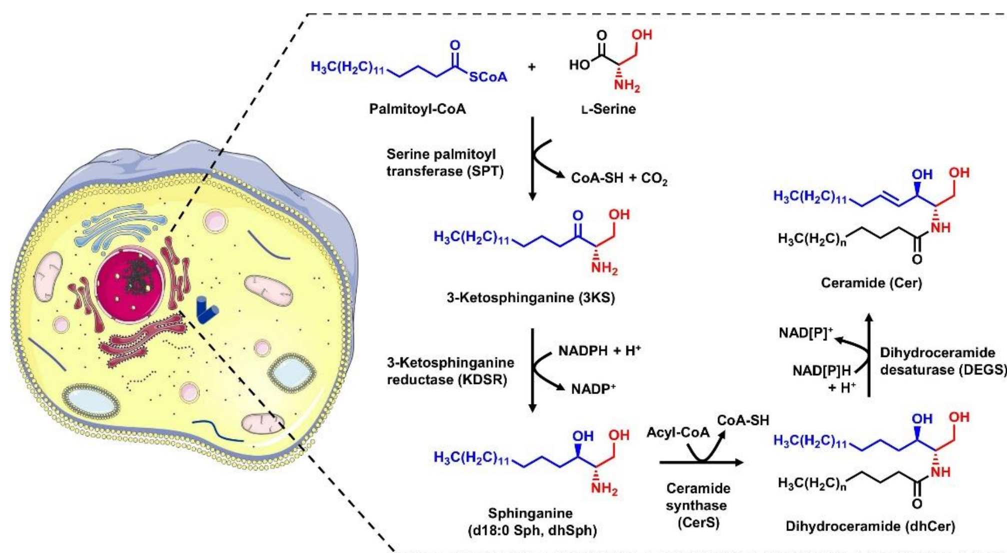

Figure 8. Schematic representation of the *de novo* synthesis pathway of ceramide. It begins with a condensation reaction of serine and palmitoyl-CoA to form 3-ketosphinganine by serine palmitoyl transferase (SPT), an enzyme which localizes to the endoplasmic reticulum. This is followed by 3-ketosphinganine reduction by 3-ketosphinganine reductase (KDSR) to form the sphinganine base. Ceramide synthases (CerS1-6) will catalyze the formation of dihydroceramide by attaching a variable length fatty acid to the sphinganine molecule via amide linkage. Ceramide is finally synthesized by the insertion of a 4,5 trans double bond to dihydroceramide in a reaction catalyzed by dihydroceramide desaturase (DEGS) [229].

Ceramide synthase	Tissue expression	Acyl-chain lengh
CerS1	Brain, skeletal muscle, testis	C18
CerS2	Kidney, liver	C20-C26
CerS3	Testis, skin	C22-C26
CerS4	Low level of expression in various tissues	C18-C20
CerS5	Low level of expression in various tissues	C16
CerS6	Low level of expression in various tissues	C14 and C16

Table 1. Ceramide Synthases isoforms. CerSs differ by their tissue distribution and specificity towards different acyl-chain length [230].

b) <u>The salvage pathway</u>

The salvage pathway accounts for around 50-90% of the sphingolipid biosynthesis suggesting an essential role for sphingolipid turnover/synthesis, and signal transduction [231, 232]. Ceramide can be generated through the degradation of complex sphingolipids, such as sphingomyelin, and glucosylceramide by acid sphingomyelinase (aSMase) and acid β-glucosidase, respectively. The salvage pathway takes place at the level of lysosomes and/or late endosomes that provide an acidic pH contributing to a constitutive degradation of sphingomyelin [233]. First, aSMase will catalyze the hydrolysis of sphingomyelin to ceramide. In order, to be translocated outside the lysosome, acid ceramidase will further hydrolyze ceramide to sphingosine and its corresponding fatty acyl chain. These can exit the lysosome to be rejoined by the action of CerSs at the level of ER to recycle ceramide that can re-enter in the synthesis of complex sphingolipids, such as sphingomyelin formation in a reaction catalyzed by sphingomyelin synthase (SMS), and glycosphingolipids by glucosylceramide synthase (GCS)

[234]. In contrast, sphingosine can also be phosphorylated by sphingosine kinases (SphK) to form sphingosine-1-phosphate (S1P) that can be either dephosphorylated by sphingosine-1-phosphate phosphatase (SPP) or degraded by S1P lyase (SPL), the only irreversible reaction in sphingolipid metabolism, forming hexadecenal and phosphoethanolamine to exit the sphingolipid metabolism pathway **(Figure 9)** [218].

c) Sphingomyelin hydrolysis pathway

Sphingolipids are important components of the plasma membrane contributing to its fluidity and barrier function. Sphingomyelin (SM) is one of the major functional constituents of the plasma membrane that can serve as a pool of sphingolipids, and its metabolism has been shown to play essential roles in homeostasis and cellular signaling [235, 236].

The formation of ceramide from sphingomyelin is accompanied by the release of diacylglycerol (DAG) in reactions catalyzed by sphingomyelinases (SMases). SMases exist in 3 isoforms that can differ in subcellular localization, organ distribution, and optimum pH: neutral (nSMase), acid (aSMase), and alkaline SMase (alk-SMase). Most tissues ubiquitously express nSMase and aSMase; however, alk-SMase is limited to the intestine and liver where it contributes to the digestion of dietary sphingomyelin. aSMase takes part of the salvage pathway and catabolizes sphingomyelin inside the lysosomes/endosomes; whereas nSMase hydrolyzes the plasma membrane sphingomyelin **(Figure 9)** [218]. Further, a wide variety of stimuli appeared to activate SMases including UV-A/ionizing radiation [184, 237] and serum withdrawal [167]. In addition, nSMase1 is considered as a housekeeping enzyme with no particular involvement in signaling, while nSMase2 was reported to mediate IL-1β and TNF cytokine responses [238-241]. MCF-7 cells treated with TNF-α were shown to

accumulate 75% of ceramide through *de novo* pathway, however, the activation of nSMase remained crucial for the achievement of a full apoptotic program [242].

4. Anti-apoptotic role of the sphingolipid metabolites S1P and C1P

In contrast to sphingosine and ceramide, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P) were found to oppose apoptotic signals and promote tumor progression, inflammation, metastasis, and angiogenesis [243].

S1P can be generated by the phosphorylation of sphingosine by sphingosine kinases (SphKs), which exist in 2 isoforms: cytosolic sphingosine kinase 1 (SphK1), and sphingosine kinase 2 (SphK2) mainly localized to the nucleus [244]. SphK1 is anti-apoptotic while SphK2 appears to have growth inhibitory and pro-apoptotic effects, but can display neoplastic properties in some cancers [245]. S1P can be intra or extracellularly secreted by acting in an autocrine and paracrine manner through 5 G-protein coupled receptors (S1PR1-5). More importantly, therapeutic resistance was shown to be associated with upregulated SphK/S1P signaling in several cancers [244].

Once generated in the ER, ceramide can be transported to the Golgi via ceramide transport protein (CERT) to generate C1P by ceramide kinase (CERK). C1P will later be transferred by C1P transfer protein (CPTP) to the plasma membrane to mediate its intra or extracellular signaling though G proteins receptors [246]. CERK/C1P signaling was shown to be upregulated in breast cancer with poor prognostic value and treatment resistance [247, 248].

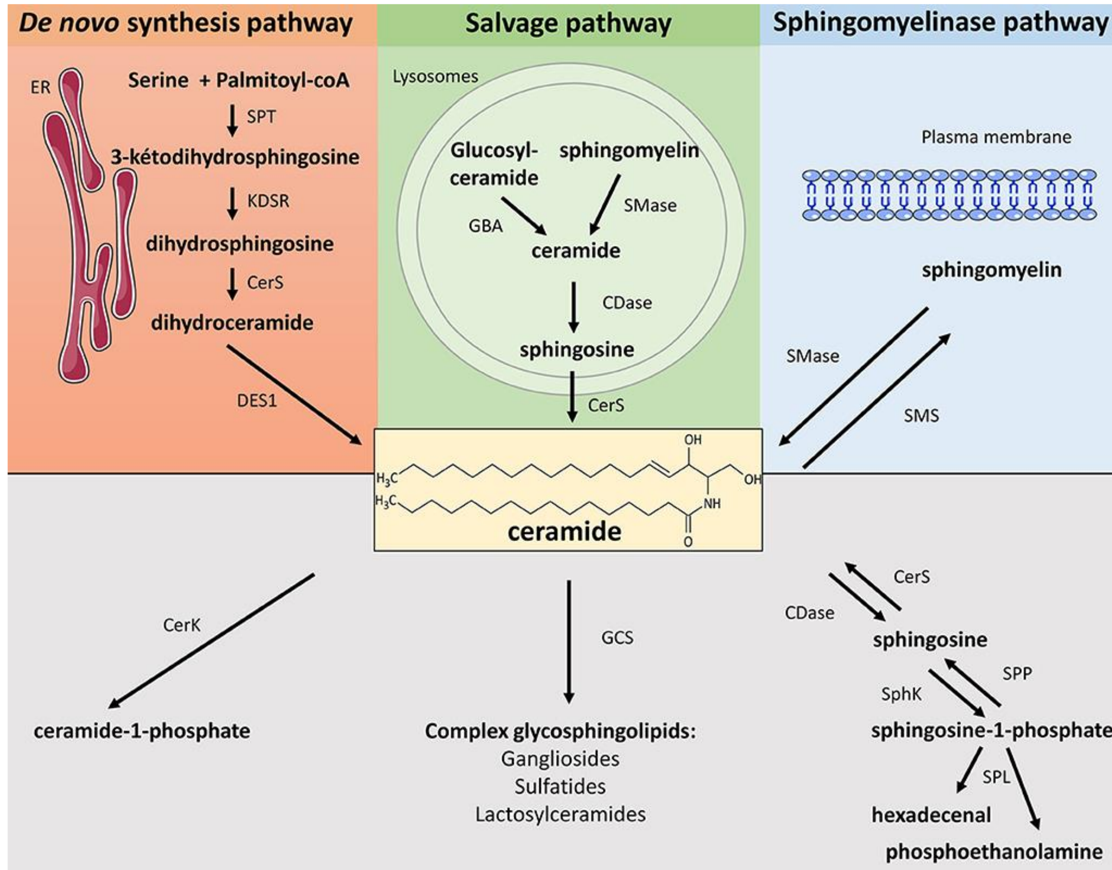

Figure 9. Schematic representation of the pathways involved in ceramide metabolism. Ceramide can be synthesized from 3 major pathways: the *de novo* pathway at the level of ER, sphingomyelin hydrolysis at the level of the plasma membrane or in the lysosomes, and the salvage pathway in the lysosomes. Ceramide can be used for the production of complex sphingolipids, such as sphingomyelin, ceramide-1-phosphate, and glycosphingolipids (gangliosides, sulfatides, and lactosylceramides) in reactions catalyzed by sphingomyelin synthase (SMS), ceramide kinase (CerK), and glucosylceramide synthase (GCS), respectively. In the salvage pathway, ceramide can be produced from the hydrolysis of glucosylceramide and sphingomyelin by β-glucosidase (GBA), and acid-sphingomyelinase (aSMase). Ceramide is then cleaved by ceramidase (CDase), and shuttled outside the lysosome to the ER as sphingosine with a free fatty-acyl chain to be rejoined by the activity of ceramide synthase (CerS) that regenerates

ceramide. Sphingosine can also be subjected to phosphorylation by sphingosine kinase (SphK) to produce sphingosine-1-phosphate (S1P). S1P can be either recycled back to sphingosine by sphingosine-1-phosphate phosphatase (SPP), or irreversibly degraded by sphingosine-1-phosphate lyase (SPL) to hexadecenal and phosphoethanolamine exiting the pathway. Adapted from [218].

5. *Sphingolipid rheostat*

Cellular fate is dictated by ceramide/S1P ratio, which is described as one of the sphingolipid rheostats. Four enzymes regulate the balance between survival and apoptosis in sphingolipid metabolism: ceramide synthase, ceramidase, sphingosine kinase (SphK), and sphingosine-1-phosphate phosphatase (S1PP) **(Figure 10).** This is not, however, the only balance, a balance with the production of C1P is also crucial due its emerging role in promoting oncogenic pathways [249]. As previously mentioned, a shift towards the production of S1P or C1P promotes cell survival while the increase in ceramide activates apoptotic signals.

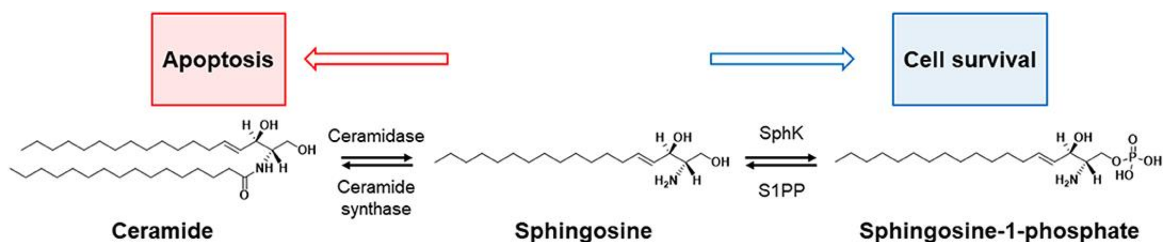

Figure 10. Sphingolipid rheostat theory. It corresponds to a balance that exists between ceramide and sphingosine-1-phosphate (S1P) lipids which dictates the cell fate. Favoring the production of ceramide by ceramide synthase (CerS) mediates a pro-apoptotic response while the production of sphingosine from ceramide by ceramidase (CDase), and its subsequent phosphorylation by

sphingosine kinase (SphK) to generate S1P promote cell survival. Sphingosine-1-phosphate phosphatase (S1PP) can reform sphingosine which can be recycled back to ceramide [249].

6. Ceramide species: structure and function

Six different isoforms of ceramide synthases (CerSs) synthesize ceramides of specific chain length. In fact, ceramides biological function is dictated by their molecular species, and cellular context [250]. In human head and neck squamous carcinoma, C18 ceramide displayed a pro-apoptotic activity [251] while C16 ceramide was reported to have an anti-apoptotic one [252], and pro-apoptotic in other cell lines [253, 254]. Similarly, very-long-chain ceramides (C20 and C20:1) synthesized by CerS2 promoted mitochondrial dysfunction and apoptosis in cardiomyocytes while long chain ceramide (C16) produced by CerS5 could not [255]. More importantly, ceramide ratio of C16/C18 and C24/C18 was higher in malignant breast cancer tissues compared to normal tissues with C16 elevation being associated with increased metastatic potential [256].

a) Dihydroceramide

As previously mentioned, the last step of the *de novo* synthesis pathway of ceramide involves the insertion of a trans double bond between C4 and C5 of dihydroceramide by DEGS enzyme to generate ceramide. In fact, this trans unsaturation has been shown to modify ceramide packaging behavior, biophysical, and elastic properties [222]. Early studies have regarded dihydroceramide as an ineffective or biologically inert molecule due to its inability to induce cell death compared to ceramide [170, 257, 258]. However, this dogma was mistaken especially because dihydroceramide has been recently implicated in several physiological processes, such as

hypoxia [223, 224, 259], cell proliferation [260, 261], and autophagy [261-263]. Also, short and long chain dihydroceramides have been shown to inhibit ceramide channel formation in mitochondria to induce apoptosis [264].

7. Ceramide in cancer treatment

Over many years, molecular research advancement has disclosed key biological mechanisms associated with cancer growth and progression. These discoveries have encouraged the development of novel treatment approaches targeting factors involving DNA repair [265], cell cycle [266], and immunity [267]. Sphingolipid metabolism is one of the emerging areas of research that has been attracting cancer scientists given its role in controlling the cell fate through the accumulation of bioactive lipids such as ceramide inside the cell. Regulation of ceramide generation and its mechanism of action have gained significant attention in cancer research due to its critical role as effector intracellular molecule in programed cell death, cell cycle arrest, and autophagy [160]. Further, ceramide generation can be inhibited by the upregulation of sphingomyelin synthase (SMS) and/or glucosyl-ceramide synthase (GCS), or its clearance by ceramidase (CDase), all of which were described in several cancers decreasing its pro-apoptotic signaling [160]. In fact, ceramide has been termed "tumor suppressor lipid" due to its ability to promote cell death following the exposure to conventional anti-cancer treatments such as radiotherapy [268] and chemotherapeutic agents (*daunorubicin*, *etoposide, hesperidin, fludarabine, taxol, and gemcitabine)* [178-183]. Cancer therapy has also regarded ceramide as a significant biomarker. For example, an enhanced tumor response was associated with total plasma ceramide accumulation in cancer patients treated with a combination of radiation therapy and the chemotherapeutic agent irinotecan [269]. Similarly, CerS6 was identified as prognostic

biomarker among 19 genes predicting the survival of colorectal cancer patients [270]. However, cancer resistance to therapy has been attributed to the overexpression of some ceramide metabolic enzymes promoting growth [244, 271]. For example, SphK1 overexpression was reported to promote cetuximab resistance in human colorectal cancer models, and their sensitivity to the drug was restored in resistant cells after *SphK1* knockdown by siRNA or its inhibition by N,N-dimethyl-sphingosine [272].

Therefore, targeting ceramide metabolizing-enzymes emerged as a novel therapeutic approach in the treatment of cancer [249]. For instance, targeting the ceramide-S1P pathway by sphingosine kinase inhibitors displayed anti-neoplastic effects against different types of cancers, such as glioblastoma [273], leukemia [274], and hepatocellular carcinoma [275]. In addition, the administration of ceramide nanoliposome (CNL) *in vivo* and in cellular models was reported to intensify the effect of chemotherapeutic agents [276-278], and to inhibit tumor growth by inducing apoptosis [279-281]. However, these therapeutic approaches are still facing many challenges in their delivery and bioavailability inside tumors due the hydrophobic and hemolytic nature of ceramide [282]. Thus, understanding the catabolism and synthesis of ceramide may help identify novel therapeutic targets for the treatment of cancer.

a) Ceramide and breast cancer

One of the ways by which cancer cells resist apoptosis is by increasing the expression of ceramide metabolic enzymes resulting in the downregulation of ceramide [283, 284]. However, ceramide metabolism in breast cancer has been debatable. In an attempt to study the differential expression profiles of sphingolipids in breast tumors and normal tissues, Schiffmann et al. showed a significant elevation of endogenous ceramide inside malignant tissues correlating it

with the disease status [256]. Further, a high expression of ceramide synthases (CerS2/4/6), associated with increased levels of C16:0-Cer, C24:1-Cer and C24:0-Cer, was reported in malignant tissues compared to normal ones [256]. More importantly, ER+ tumors were linked with increased levels of C18:0-Cer and C20:0-Cer, while C16-Cer elevation was associated with metastatic potentials [256, 285]. In fact, ER+ tumors displayed an upregulation of CerS4 and CerS6 [228]. In addition, the downregulation of SphK1 and SphK2 promoted the sensitivity of MCF-7 cells to doxorubicin [286, 287]. Similarly, high SphK1 expression was associated with poor prognosis in breast tumors [228]. CERK expression was reported to confer chemoresistance, migration, and growth of cells in TNBC [248].

TNF-α was shown to mediate apoptotic processes through the accumulation of ceramide that can involve the *de novo* synthesis and the sphingomyelin hydrolysis pathways in MCF-7 cells [172].

8. *Bcl-2, Bcl-xL, and ceramide*

Bcl-2 and Bcl-xL interaction with ceramide was previously studied in TNF-α-induced response in MCF-7 breast cancer cell line where it was demonstrated that Bcl-xL acts upstream of ceramide while Bcl-2 functions downstream of this sphingolipid [288]. Moreover, both Bcl-2 and Bcl-xL inhibited cell death in response to TNF-α. However, Bcl-2 did not significantly inhibit endogenous ceramide accumulation in response to TNF-α compared to Bcl-xL, but inhibited ceramide-induced death [288, 289]. In addition, caspase-8 was cleaved in MCF-7 Vector and MCF-7 Bcl-2 cells (overexpressing Bcl-2), but cleavage was completely inhibited in MCF-7 Bcl-xL cells (overexpressing Bcl-xL) accompanied by a potent inhibition of ceramide accumulation [288]. Interestingly, Bcl-2 overexpression was found to inhibit ceramide-induced

apoptosis without interfering with ceramide generation which further demonstrates that Bcl-2 acts downstream [290-292]. Conflicting studies, however, have stated that Bcl-2 prevents both ceramide accumulation and ceramide-induced cell death [187, 293, 294]. Targeting bacterial sphingomyelinase to the mitochondria increased ceramide accumulation and apoptosis in MCF-7 cells, while this was resisted by the overexpression of Bcl-2 [292]. Several studies have reported the function of Bcl-2 and Bcl-xL in inhibiting ceramide channel formation and cytochrome c release [295-297]. While the majority of published research emphasizes the involvement of Bcl-xL, however, a study on Bcl-2 and cell death abnormality gene 9 (CED-9) (a Bcl-2 homolog) also supports this claim [297]. More importantly, Bcl-xL displayed a protective effect by directly interacting with ceramide preventing channel formation [295]. In addition, S1P and C1P were found to induce the expression of Bcl-xL or Bcl-2 modulating anti-apoptotic effects [298-300].

D. Modulators of ceramide metabolism

1. Synthetic retinoids

Retinoids are a class of signaling molecules belonging to the vitamin A family. Dietary-derived all-trans retinoic acid (ATRA), the main bioactive retinoid in the human body, mediates its signaling though through RAR–RXR heterodimers [301]. In the nucleus, retinoic acid (RA) mediates its effects through RAR-RXR heterodimers that are bound by DNA in regions known as retinoic acid response elements (RAREs). This will subsequently recruit coactivators, such as HAT (histone acetyltransferase) to induce the transcription of target genes [302-304]. Given their roles in regulating cell cycle and apoptotic processes, retinoids have gained importance in the treatment of cancer. RA was able to induce apoptosis in several cancers, including human

medulloblastoma [305] and metastatic melanoma cells [306]. In addition, the overexpression of RARα and RARγ sensitizes neoplastic epidermal keratinocytes to growth suppression and apoptosis following RA treatment [307]. Retinoids combined with trastuzumab or tamoxifen displayed an enhanced anti-tumor effect in breast cancer [308]. The usage of natural retinoids, however, is limited in the treatment of most solid tumors in the long-term due to the vitamin-A associated cytoxicity, and the development of retinoid resistance. Thus, much effort has been invested in the development of selective retinoid derivatives to improve cancer therapy and reduce their associated side effects [309, 310].

a) Fenretinide (4-HPR)

Fenretinide [N-(4-hydroxyphenyl) retinamide; 4-HPR] **(Figure 11)**, is a synthetic retinoid derived from ATRA that has been defined as an effective anti-cancer agent, especially due to its pro-apoptotic activity in a wide variety of cancers, such as ovarian carcinoma, breast carcinoma, prostate adenocarcinoma, lymphomas, leukemias, and others [311]. Furthermore, its enhanced efficacy and low toxicity have been associated with the modification of the carboxyl end of all-trans RA with an N-4-hydroxyphenyl [312, 313]. Interestingly, 4-HPR mediates its effect through both receptor-dependent and -independent pathways [311]. Another cytotoxic mechanism induced by 4-HPR involves the generation of reactive oxygen species (ROS) [313]. Further, this 4-HPR-induced ROS is inhibited following the treatment with anti-oxidants [314-316], Bcl-2 overexpression [317], and BAX/BAK knockout [317]. Also, fenretinide was shown to activate caspase-8 independently of Fas ligand, TNF-α, or TRAIL activation in ovarian carcinoma cell line [318]. ATRA-resistant T lymphoma cells and adult T-cell leukemia (ATL) displayed anti-proliferative and pro-apoptotic effects upon treatment with 4-HPR [319, 320].

Furthermore, 4-HPR was able to induce cell death through ceramide accumulation, altering mitochondrial membrane potential, and caspase activation in the human T-lymphotropic virus type 1 (HTLV-1) negative T cells with no effect on normal T lymphocytes [320]. In addition, ROS generation upon 4-HPR treatment mediated cell cycle arrest, ceramide accumulation, cytochrome c release, and apoptosis [321]. DEGS1 enzyme is another identified molecular target for 4-HPR [322]. However, it is considered as an enhancer of the *de novo* pathway of ceramide synthesis due to its ability to activate SPT and/or CerSs with simultaneous inhibition of DEGS1 resulting in the accumulation of dihydroceramides [323].

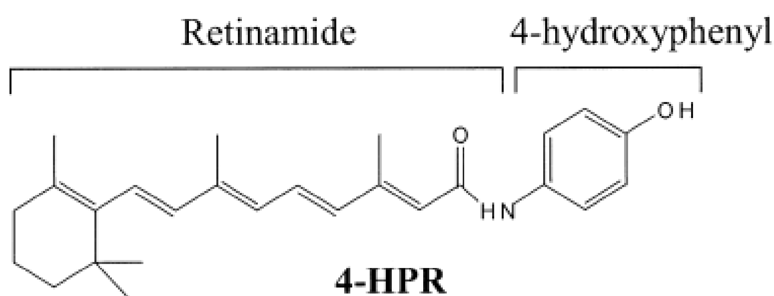

Figure 11. Chemical structure of fenretinide (4-HPR) [324].

b) Adarotrene (ST1926) synthetic retinoid

(2E)-3-[3′-(1-adamantyl)-4′-hydroxy[1,1′-biphenyl]-4-yl]-2-propenoic acid known as ST1926 **(Figure 12)**, is another synthetic retinoid that gained significant attention due to its anti-tumor activity studied *in vitro* and *in vivo* models [325-329]. Its mechanisms of action include early DNA double stranded breaks (6h), G1/S phase cell cycle arrest, and apoptosis [329]. In an attempt to study its DNA damaging effect, Fratelli *et al*. have identified H2A.Z histone variant, which is an active player in maintaining the genome integrity [330, 331], as a direct nuclear

target of ST1926, among others [332]. DNA polymerase-α was also suggested as a molecular target of ST1926, as cells with *POLA1* mutations displayed a protective effect against ST1926-induced DNA damage response [328]. In addition, ST1926 was reported to restore the *de novo* synthesis pathway of ceramide in ATL cells, and to induce the expression of a distinct set of CerSs without inhibiting DEGS1, resulting in ceramide accumulation rather than dihydroceramide followed by cell death [325].

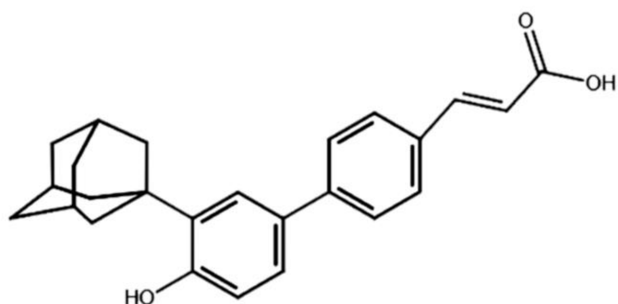

Figure 12. Chemical structure of the synthetic retinoid ST1926 [333].

2. *Sphingosine kinase 1-Inhibitor (SK1-I)*

SphK1 is an important enzyme in sphingolipid metabolism that functions to phosphorylate sphingosine to generate S1P. In contrast to ceramide, S1P has a proinflammatory and anti-apoptotic functions that are associated with tumor therapeutic resistance, angiogensis, and metastasis [244]. Thus, targeting SphK1 gained significant attention in cancer research with the aim to interfere with its neoplastic properties. SK1-I [(BML258) or 1 (2R, 3S, 4E)-N-methyl-5-(4-pentylphenyl)-2-aminopent-4-ene-1, 3-diol] **(Figure 13)** is a SphK1 selective inhibitor, and a sphingosine analogue functioning by competitive inhibition [334]. In fact, its anti-neoplastic effect was reported in several tumors, such as glioblastoma, breast, colon, and lung cancer [335].

In an *in vivo* model of breast cancer, SK1-I administration decreased S1P levels in serum, and this was associated with reduced metastasis, angiogensis, and an enhanced apoptotic response [336]. Similarly, SK1-I was shown to render breast and prostate cancer cells susceptible to the chemotherapeutic agent docetaxel [337].

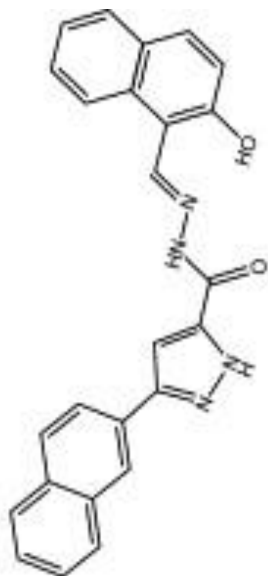

Figure 13. Chemical structure of sphingosine kinase 1-Inhibitor (SK1-I) [338].

CHAPTER II

AIMS OF THIS STUDY

Bcl-2 and Bcl-xL, anti-apoptotic members of the Bcl-2 family, prevent apoptosis by sequestering pro-apoptotic members of the Bcl-2 family or blocking the mitochondrial release of apoptogenic factors. They inhibit TNF-α-induced apoptosis in different cellular models [87, 88, 288, 339-341]. On the other hand, ceramide, a signaling sphingolipid that controls different cellular processes, induces apoptosis. A previous work done by our lab group investigated ceramide interaction with Bcl-2/Bcl-xL in the TNF-α-induced response in MCF-7 cells and demonstrated that Bcl-xL acts upstream of ceramide and Bcl-2 functions downstream of this sphingolipid. While Bcl-2 inhibited ceramide-induced death in response to TNF-α but not ceramide generation, Bcl-xL prevented ceramide accumulation but did not protect from ceramide-induced apoptosis [288]. Furthermore, one of the mechanisms mediating TNF-α induced apoptosis in MCF-7 cells is the *de novo* synthesis pathway of ceramide [172]. In fact, this pathway was found to mediate apoptotic processes in multiple cellular models following the treatment with a variety of stimuli [172, 219-221].

Thus, in this study we aim to:

Characterize the interaction of Bcl-2 and Bcl-xL with the de novo synthesis pathway of ceramide and with the ceramide catabolism pathway upon exposure of MCF-7 human breast cancer cells to TNF-α.

1. We first assessed the viability of MCF-7 cells overexpressing Bcl-2 and Bcl-xL upon treatment with TNF-α using MTT.

2. We then studied the effect of Bcl-xL and Bcl-2 overexpression in MCF-7 cells on the transcription of key enzymes involved in ceramide metabolism [*DEGS1*, *SphK1* and *CerS(2, 4,* and *6)]* in response to TNF-α by quantitative Real-time PCR (qRT-PCR).
3. We next assessed the viability of MCF-7 cells overexpressing Bcl-2 and Bcl-xL upon treatment with TNF-α in combination with modulators of ceramide metabolic enzymes (ST1926, 4-HPR, and SK1-I) using MTT.
4. We finally studied the apoptotic response of these cells through western blot analysis of apoptotic proteins expression (cleaved PARP-1) after treatment with TNF-α alone or in combination with ST1926, the enhancer of the *de novo* synthesis pathway of ceramide. We also investigated the protein expression of p53 in these cells.

CHAPTER III

MATERIALS AND METHODS

A. Cell culture

MCF-7 Vector, MCF-7 overexpressing Bcl-2 or Bcl-xL (MCF-7 Bcl-2 or MCF-7 Bcl-xL) cells were derived from TNFα-sensitive MCF-7 parental breast cancer cell lines, and were a kind gift from VM Dixit laboratory [339]. MCF-7 cells are ER+ and naturally express p53. MCF-7 Vector, MCF-7 Bcl-2, and MCF-7 Bcl-xL cells were cultured in Rose Well Park Memorial Institute (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS), and 1% Penicillin/Streptomycin; and were incubated at 37 °C, and 5% CO_2. Cell passaging was done twice a week and Hygromycin (150 µg/mL, Toku-E) was added to the culture medium for selection. Experiments were then performed without Hygromycin.

For cell treatments, 4-HPR (10^4 µM, Sigma) and ST1926 (10^{-2} M, Sigma) were kindly provided by the laboratory of Dr. Nadine Darwiche (American University of Beirut) and SK1-I (50 mM) was purchased from Enzo Life Sciences. The three drugs were dissolved with dimethyl sulfoxide [(DMSO), Sigma]. All of the drugs, including human recombinant TNF-α (14367 nM, Sino Biological) were diluted with RPMI medium supplemented with 10% FBS according to the desired concentrations.

B. MTT: cell viability assay

MCF-7 Vector, MCF-7 Bcl-2, and MCF-7 Bcl-xL cells were seeded in 96 well plates at a density of 8000 cells/well in 100 µL of FBS-supplemented medium and pretreated with 90 µL of

ST1926 (125 nM) or of 4-HPR (11.1 μM) or of SK1-I (5.5 μM) for 3 hours and then treated with 10 μL of TNF-α (12 nM) or 10 μL of media. After the addition of TNF-α, we would obtain a final concentration of 112.5 nM for ST1926, 10 μM for 4-HPR, 5 μM for SK1-I, and 1.2 nM for TNF-α. (Media + DMSO) solution in the presence or absence of TNF-α was used as a control. For model validation, cells were cultured in media in absence of TNF-α (control) or treated with 1.2 nM of TNF-α. At the required time point, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (stock solution: 5mg/mL powder dissolved in distilled water, Sigma) was added to 100 μL of culture medium/well. The mixture was then incubated for 3 hours at 37 °C in a humidified atmosphere containing 5% CO_2. MTT assay determines cell viability based on the mitochondrial activity of viable cells which converts the yellow tetrazolium salt (MTT) into a purple formazan dye. Formazan crystals were then dissolved by 100 μL of DMSO, and the amount of reduced MTT was measured at 595 nm using mini enzyme-linked immunosorbent assay (ELISA) plate reader.

C. Protein extraction and western blot

MCF-7 Vector, MCF-7 Bcl-2, and MCF-7 Bcl-xL cells were seeded in 6 well plates at a density of 300,000 cells/well, rested overnight, and then pre-treated with 1.8 mL of ST1926 (125 nM) for 3 hours. 200 μL of TNF-α (12 nM) was then added and incubated for 24h and 48h. After the addition of TNF-α, we would obtain a final concentration of 112.5 nM for ST1926, and 1.2 nM for TNF-α. (Media + DMSO) solution in the presence or absence of TNF-α was used as a control. Cells were scraped and pelleted along with their supernatant at the indicated time points. Proteins were extracted using Laemmli buffer and were quantified according to the company's instructions by Bio-Rad dye-binding assay with bovine serum albumin (BSA) used as standard.

30µg of total cell lysate was resolved on polyacrylamide gel with Prism Ultra Protein Ladder as standard for molecular weight. Migration was performed for 90 minutes at 100 mV in 1X *TGS* (Tris-Glycine-SDS) migration buffer. Proteins were then electrically transferred in Bio-Rad trans-blot cell into poly vinlyne diluoride (PVDF) membrane (Bio-Rad), and then blocked overnight with 5% fat-free milk prepared in (1X TBST). The latter was prepared as follows: Tween (1 ml) (0.1%) + 100 ml 1X TBS [prepared from of 10X TBS: 12 g Tris (hydroxymethyl)-aminomethane and 87.8 g NaCl, pH.8) dissolved in 1 L distilled water] + 900 ml distilled water. This was followed by immunoblotting with the relevant primary antibody **(Table 2)** overnight at 4°C. The next day, membranes were subjected to 3 serial washes by 1X TBST, and then incubated with the appropriate Horse Reddish Peroxidase (HRP) conjugated secondary antibody for 1 hour at room temperature. Serial washes were performed again using 1X TBST. Protein visualization was conducted on Bio-Rad ChemiDocMP imaging system by applying Clarity^{TM} ECL Western Blotting substrate (Bio-Rad). ImageJ software was used to quantify the protein bands with β-tubulin or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalization.

Antibody	Supplier	Batch number	Origin
PARP-1 (F-2)	Santa Cruz	Sc-8007	Mouse
p53 (Do-1)	Santa Cruz	Sc-126	Mouse
Bcl-x $_{S/L}$ (S-18)	Santa Cruz	Sc-634	Rabbit
Bcl-2 (C-2)	Santa Cruz	Sc-7382	Mouse
GAPDH (0411)	Santa Cruz	Sc-47724	Mouse
β-tubulin	Sigma-Aldrich	16-230	Mouse

Table 2. List of primary antibodies.

D. RNA extraction, reverse transcription and quantitative Real-Time PCR

1. RNA extraction

Cells were seeded in 6 well plates at a density of 300,000 cells/ well. On the next day, cells were treated with TNF-α (1.2 nM) for 8 hours. Cells were collected with a cell scraper and 1 ml of TRIzol (Sigma) was added to each pellet and then incubated for 5 minutes at room temperature. This was followed by the addition of 200 µl of chloroform and incubation for 3 minutes at room temperature. The obtained mixture was centrifuged at 15,000xg for 15 minutes at 4°C. The resulting aqueous phase was subsequently collected and transferred to a fresh tube. Isopropanol was added to the samples in a 1:1 ratio, and then incubated for 1 hour at -20°C. The samples were centrifuged at 15,000xg for 15 minutes at 4°C to allow RNA precipitation. The supernatant was removed, and the RNA pellet was washed with 70% ethanol to get rid of any

remaining isopropanol. After centrifugation at 12,000xg for 5 minutes at 4°C, the RNA pellet was left to air-dry and then was resuspended in nuclease-free water. Quantification of RNA was performed on Nanodrop (DeNovix tc-11).

2. Reverse transcription of RNA to cDNA

QuantiTect Reverse Transcription Kit (QIAGEN) was used to reverse transcribe 500 ng of RNA to cDNA according to the manufacturer's instructions. Briefly, gDNA Wipeout Buffer was used to remove any possible genomic DNA (gDNA) from the RNA sample. A master mix containing [Quantiscript reverse transcriptase (RT), Quantiscript RT buffer, and RT primer mix] was used to conduct the reverse transcription reaction at 42 °C for 30 minutes on PCR T100 thermal cycler (Bio-Rad). Temperature was automatically adjusted to 95°C for 3 minutes in order to deactivate the reverse transcriptase enzyme.

3. Quantitative Real-Time PCR (qRT-PCR)

A master mix containing 1 µL of each forward and reverse primer (10 uM), 5 µL of Bio-iTaq Rad's Universal SYBR Green Supermix, and 2 µL of nuclease-free was prepared for each cDNA sample. In each well of the 96 well plates (Bio-Rad), 9 µL of the master mix were dispensed followed by 1 µL of cDNA with concentration of 10 ng/µL. Each sample was loaded in duplicates. The PCR plate was sealed with an adhesive sealer and was centrifuged for 1 minute. Quantitative real-time PCR was performed in Bio-Rad CFX 96 thermocycler according to the relevant heat cycle protocols. The obtained data were analyzed using the $2^{-\Delta Ct}$ method and were normalized to the expression of *β-actin* gene, which was used as an internal reference control. The mRNA expression levels of *DEGS1*, *CerS2*, *CerS4*, *CerS6*,

and *SphK1*, encoding for five key enzymes involved in ceramide metabolism, were evaluated upon treatment of MCF-7 Vector, MCF-7 Bcl-2, and MCF-7 Bcl-xL cells with 1.2 nM of TNF-α. To this end the following human primers **(Table 3)** were ordered from Sigma.

Gene	**Sequence**
β-actin	FP: CTGGCACCACACETTCTA RP: AGCACAGCCTGGATAGCAAC
DEGS1	FP: CCAACATTCCTGGAAAAGTCTTC RP: GCCTCTTCATTCTTGAGTAGGGA
CerS2	FP: CCGATTACCTGCTGGAGTCAG RP: GGCGAAGACGATGAAGATGTTG
CerS4	FP: CTTCGTGGCGGTCATCCTG RP: TGTAACAGCAGCACCAGAGAG
CerS6	FP: ACATTCCTTCAGCCTCCTGGAGTT RP: GCTCCCTGGTTTCCAGGCCAC
SphK1	FP: CTGGCAGCTTCCTTGAACCAT RP: TGTGCAGAGACAGCAGGTTCA

Table 3. Primer sequences of *β-actin*, *DEGS1*, *CerS2*, *CerS4*, *CerS6*, and *SphK1*.

E. Statistical analysis

Independent experiments were performed for MTT, qRT-PCR and western blot twice or thrice. Data represent the mean of independent experiments ± S.D. Statistical analysis was

conducted using Excel software where the unpaired Student's t-test was used to calculate p-values. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

CHAPTER IV

RESULTS

A. Validation of MCF-7 cellular models: Overexpression of Bcl-2 and Bcl-xL

Western blot analysis was performed on each of the MCF-7 cell lines (MCF-7 Bcl-2 and MCF-7 Bcl-xL) to confirm the overexpression of the proteins Bcl-2 and Bcl-xL, respectively. Bcl-xL and Bcl-2 were upregulated with difference changes 5.33 and 12.90 in MCF-7 Bcl-xL and MCF-7 Bcl-2, respectively compared to MCF-7 Vector; whereas very low levels of Bcl-xL and Bcl-2 were detected in MCF-7 Vector, and hence the overexpression model was validated (**Figure 14**).

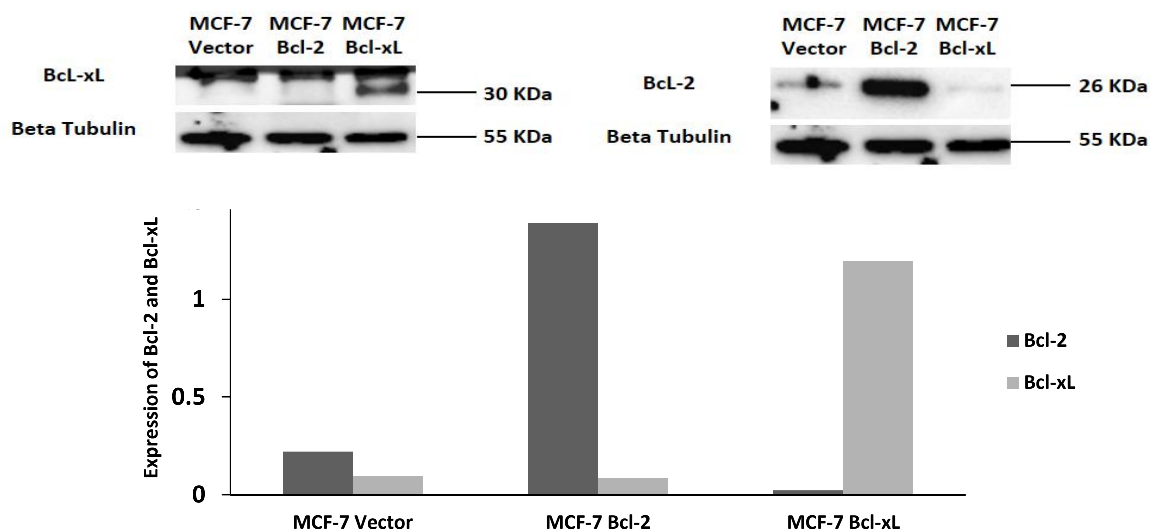

Figure 14. Overexpression of Bcl-xL and Bcl-2 in MCF-7 cells. Western blot analysis was performed in order to confirm the overexpression of Bcl-xL and Bcl-2 in MCF-7 Bcl-xL and MCF-7 Bcl-2 cells respectively. Beta-tubulin was used as internal reference control.

B. Validation of the resistance of MCF-7 Bcl-xL and MCF-7 Bcl-2 cells to TNF-α-induced cytotoxicity

TNF-α was able to induce cell death by mechanisms that are typical for apoptotic processes in MCF-7 Vector cells while MCF-7 Bcl-xL and MCF-7 Bcl-2 cells were resistant to it [288]. Here, MTT assay was performed after treating MCF-7 Vector, MCF-7 Bcl-xL and MCF-7 Bcl-2 cells with 1.2 nM of TNF-α for 24 and 48 hours. At 24h, the viability of MCF-7 Vector cells was reduced very significantly to reach 82% compared to untreated control while MCF-7 Bcl-xL and MCF-7 Bcl-2 cells displayed 92% and 90% of viability, respectively. At 48h, the effect of TNF-α on MCF-7 Vector cell viability was more prominent where it was reduced to around 40% with extreme significance while MCF-7 Bcl-xL and MCF-7 Bcl-2 were still 86% and 76% viable, respectively, with a minimal significant decrease in both cell lines (**Figure 15**).

These results confirm that MCF-7 Bcl-xL and MCF-7 Bcl-2 are resistant to the cytotoxicity induced by TNF-α. Furthermore, a slight selectivity to TNF-α on the viability is observed in MCF-7 Bcl-2 compared to MCF-7 Bcl-xL.

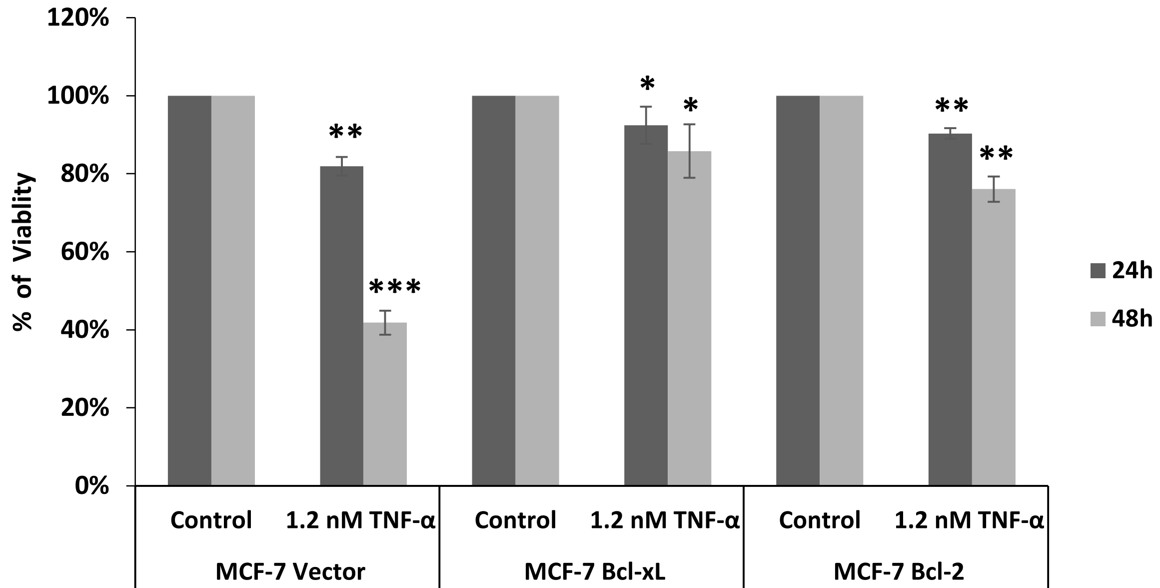

Figure 15. Evaluation of TNF-α induced cytotoxicity in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2. Figure representing the percentage of the cell viability in TNF-α-treated cells and untreated controls. Cell viability was analyzed by MTT and optical density (OD) was measured at 595 nm. Values are represented by the ratio of OD of treated cells over untreated cells. Each column represents the mean ± S.D. of three independent experiments done in triplicates. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

C. Effect of Bcl-xL and Bcl-2 overexpression in MCF-7 cells on the transcription of *de novo* and salvage ceramide metabolic enzymes in response to TNF-α

Ceramide accumulation in response to TNF-α was previously studied in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 and showed that Bcl-xL acts upstream while Bcl-2 acts downstream of ceramide [288]. In this section, we investigated the interference of Bcl-xL and Bcl-2 with the *de novo* pathway of ceramide synthesis in response to TNF-α. MCF-7 Vector,

MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells were treated with 1.2 nM of TNF-α for 8 hours followed by analyzing the mRNA expression of *DEGS1*, the enzyme catalyzing the last step of the *de novo* pathway, and of ceramide synthases including *CerS2*, *CerS4*, and *CerS6* by qRT-PCR. This time point was previously shown to precede ceramide accumulation in these cell lines [288]. The mRNA expression of *DEGS1* in MCF-7 Bcl-xL was significantly downregulated by 60% upon TNF-α treatment compared to the untreated control **(Figure 16)**. In contrast, MCF-7 Bcl-2 did not show a significant change in *DEGS1* mRNA expression in response to TNF-α compared to the untreated control. While *CerS2* and *CerS6* mRNA did not show a significant change in either of the cell lines, *CerS4* mRNA expression was significantly reduced in MCF-7 Bcl-xL by 16% and not in MCF-7 Bcl-2 **(Figure 16)**. These results suggest that the overexpression of Bcl-xL in MCF-7 cells reduces the mRNA expression of *DEGS1* and *CerS4* that play a role in *de novo* ceramide synthesis pathway which might be contributing for their resistance to TNF-α while the overexpression of Bcl-2 displayed TNF-α resistance independently of any transcriptional regulation of these enzymes at 8h.

We also investigated the involvement of the salvage pathway in promoting resistance of Bcl-2 or Bcl-xL to TNF-α in MCF-7 cells. *SphK1* mRNA expression was studied following TNF-α treatment for 8h but did not show any significant change in all three cell lines **(Figure 17)**. This result indicates that Bcl-2 and Bcl-xL do not interfere with the transcriptional regulation of *SphK1*, enzyme of the salvage pathway, upon exposure to TNF-α.

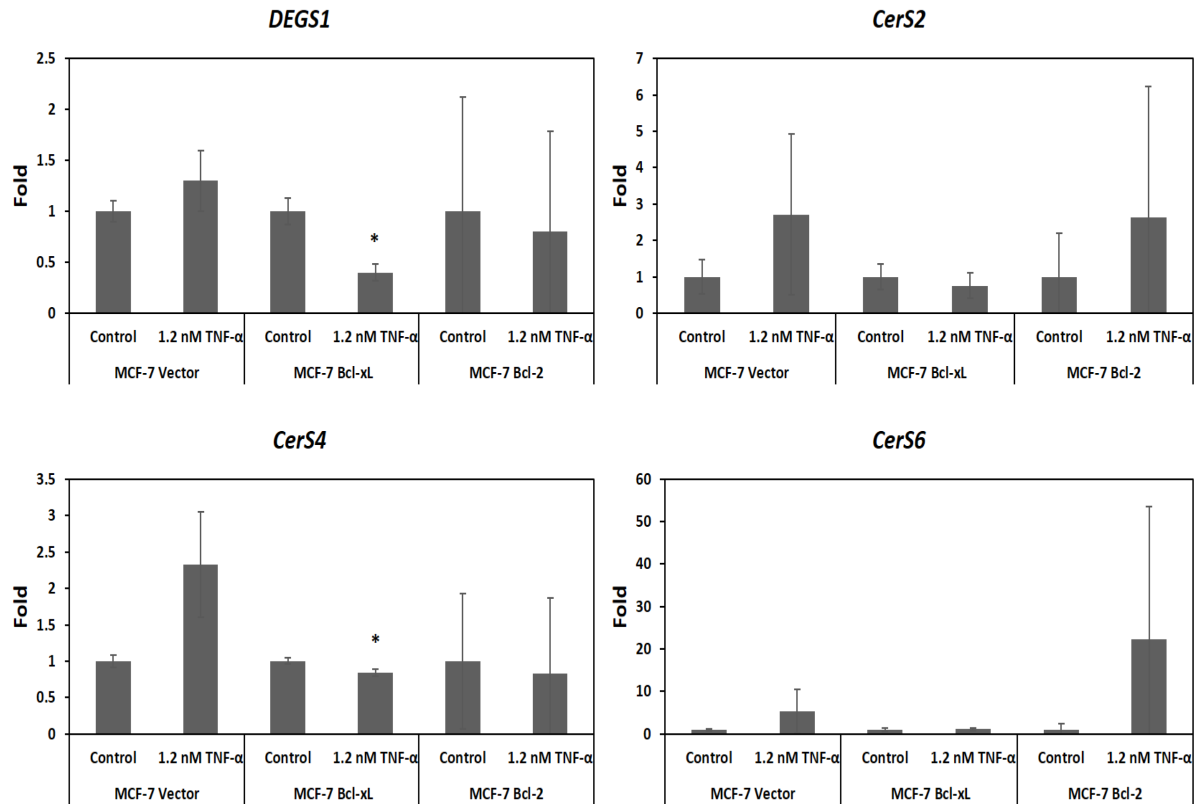

Figure 16. Evaluation of Bcl-xL and Bcl-2 effect on the mRNA expression of *de novo* ceramide synthesis enzymes in MCF-7 cells in response to TNF-α. *DEGS1*, *CerS2*, *CerS4*, and *CerS6* mRNA expression was analyzed by qRT-PCR in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells following 8h of treatment with 1.2 nM of TNF-α. Values were normalized to the expression of *β-actin*. Values are represented by the fold change of treated cells over untreated cells. Each column represents the average of two independent experiments ± S.D done in duplicates. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

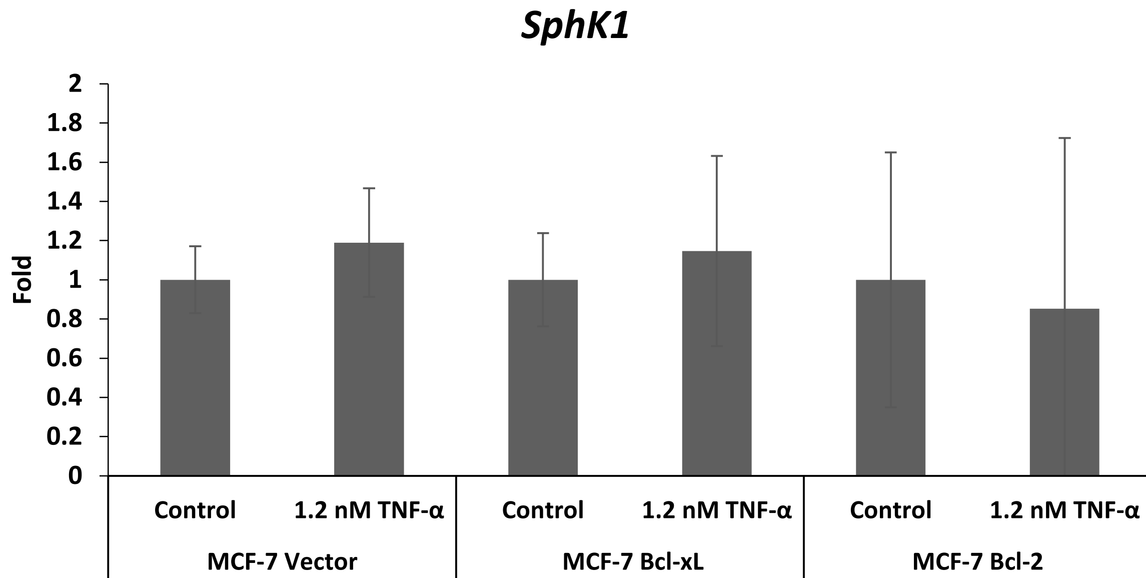

Figure 17. Evaluation of Bcl-xL and Bcl-2 effect on the mRNA expression of *SphK1*, an enzyme of the salvage metabolic pathway of ceramide, in MCF-7 cells in response to TNF-α. *SphK1* mRNA expression was analyzed by qRT-PCR in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells following 8h of treatment with 1.2 nM of TNF-α. Values were normalized to the expression of *β-actin*. Values are represented by the fold change of treated cells over untreated cells. Each column represents the average of two independent experiments ± S.D done in duplicates. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

D. Effect of SphK1 inhibition on the viability of MCF-7 cells overexpressing Bcl-2 or Bcl-xL in response to TNF-α

Given the previously discussed role of SphK1 and S1P in promoting cell survival, we inhibited SphK1, an enzyme of the salvage metabolic pathway of ceramide, by SK1-I to

investigate whether it will re-sensitize resistant cells to TNF-α. MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells were treated with a combination of SK1-I (5 μM) and TNF-α (1.2 nM) for 24h and 48h. Cell viability, analyzed by MTT, showed no effect of SK1-I in all of the three cell lines at 24h and 48h on TNF-α response **(Figure 18)**. These results demonstrate that SphK1 did not contribute to the resistance mediated by Bcl-2 or Bcl-xL in MCF-7 cells in response to TNF-α.

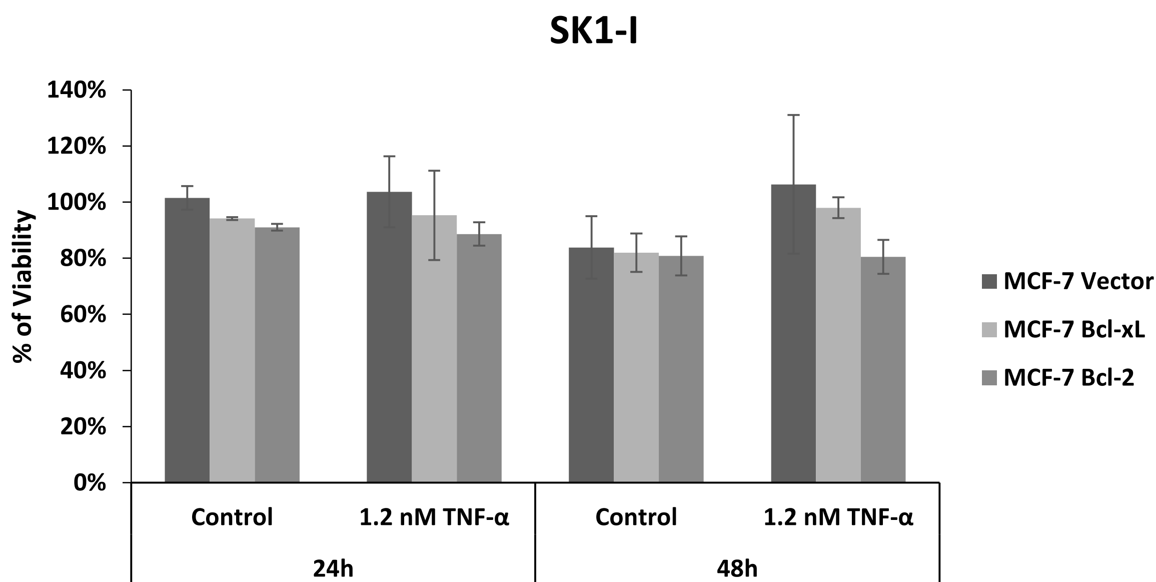

Figure 18. Evaluation of SK1-I effect on the response of MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells to TNF-α. Cell viability was analyzed by MTT and optical density (OD) was measured at 595 nm. Values are represented by the fold change of OD of SK1-I-treated cells (5 μM) over OD of DMSO-treated cells at 24 and 48 hours. The comparison in cell viability was done in absence of TNF-α (control) or in the presence of 1.2 nM TNF-α. Each column represents the mean ± S.D. of the ratios calculated from two independent experiments done triplicates.

Differences were considered significant when p value <0.05 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

E. Effect of enhancing *de novo* synthesis of ceramide and of DEGS1 inhibition by 4-HPR on the viability of MCF-7 cells overexpressing Bcl-2 or Bcl-xL in response to TNF-α

4-HPR has been previously shown to enhance the *de novo* synthesis pathway of ceramide and to inhibit DEGS1 activity, the terminal enzyme of this pathway, thereby accumulating dihydroceramide rather than ceramide [322, 323]. In order to study the potential role of DEGS1 and hence the *de novo* ceramide synthesis in response to TNF-α, cells were treated with a combination of 4-HPR (10 μM) and TNF-α (1.2 nM) for 24h and 48h. Cell viability analyzed by MTT showed no effect for 4-HPR in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells in the first 24h of TNF-α treatment. However, at 48h 4-HPR partially inhibited TNF-α-induced cell death in MCF-7 Vector, where cell viability was 74% upon TNF-α treatment compared to 68% for the control **(Figure 19)**. Further, 4-HPR did not show any cytotoxic or survival effect in MCF-7 Bcl-xL and MCF-7 Bcl-2 cells.

These results suggest that 4-HPR might be partially blocking cell death in MCF-7 Vector cells in response to TNF-α by interfering with proper *de novo* ceramide accumulation and this inhibition does not have an influence on the viability of MCF-7 Bcl-xL and MCF-7 Bcl-2 cells in which ceramide accumulation is already known to be inhibited [288].

Next, we examined whether the inhibition of *de novo* ceramide accumulation by 4-HPR might be compensated by other ceramide synthesis pathway and hence sensitize the cells to TNF-α. Therefore, MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells were treated with a

combination of 4-HPR (10 μM), SK1-I (5 μM), and TNF-α (1.2 nM). The addition of SK1-I was aimed to prevent any further metabolism of ceramide generated by other pathways other than the *de novo* pathway, into the anti-apoptotic S1P. As shown in **(Figure 20)**, MTT assay indicated that inhibiting DEGS1 and SphK1 did not demonstrate any effect on the viability of all three cell lines at 24h and 48h. Therefore, blocking the *de novo* synthesis pathway at the level of DEGS1 by 4-HPR results in a decrease in the mortality of MCF-7 cells which supports a role of the *de novo* synthesis in mediating TNF-α induced cell death.

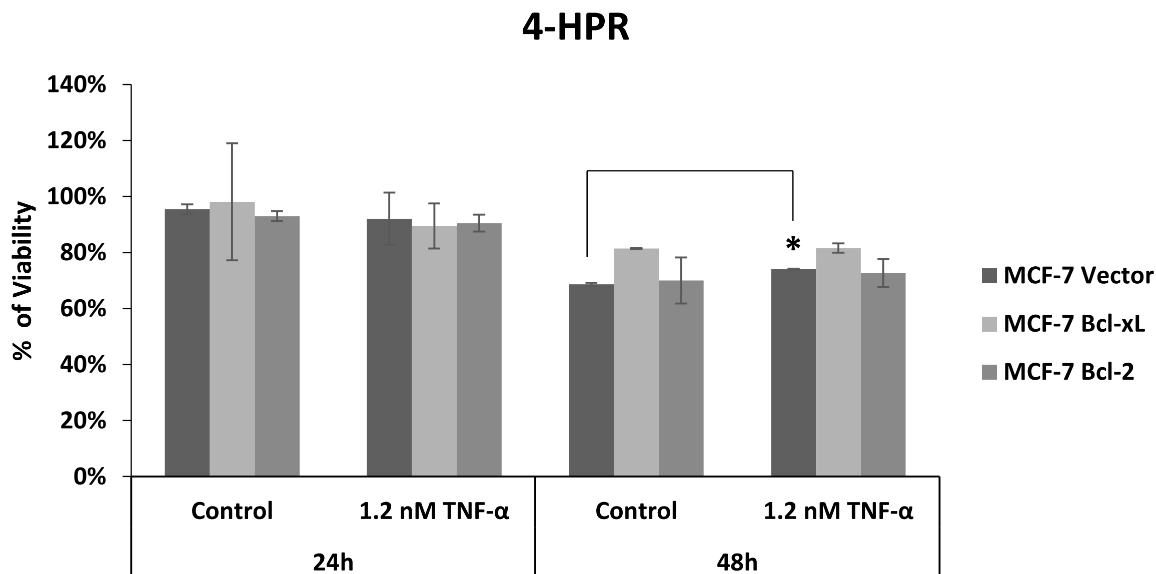

Figure 19. Evaluation of 4-HPR effect on the response of MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 to TNF-α. Cell viability was analyzed by MTT and optical density (OD) was measured at 595 nm. Values are represented by the fold change of OD of 4-HPR-treated cells (10μM) over OD of DMSO-treated cells at 24 and 48 hours. The comparison in cell viability was done in absence of TNF-α (control) or in the presence of 1.2 nM TNF-α. Each column represents

the mean ± S.D. of the ratios calculated from two independent experiments done in triplicates. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

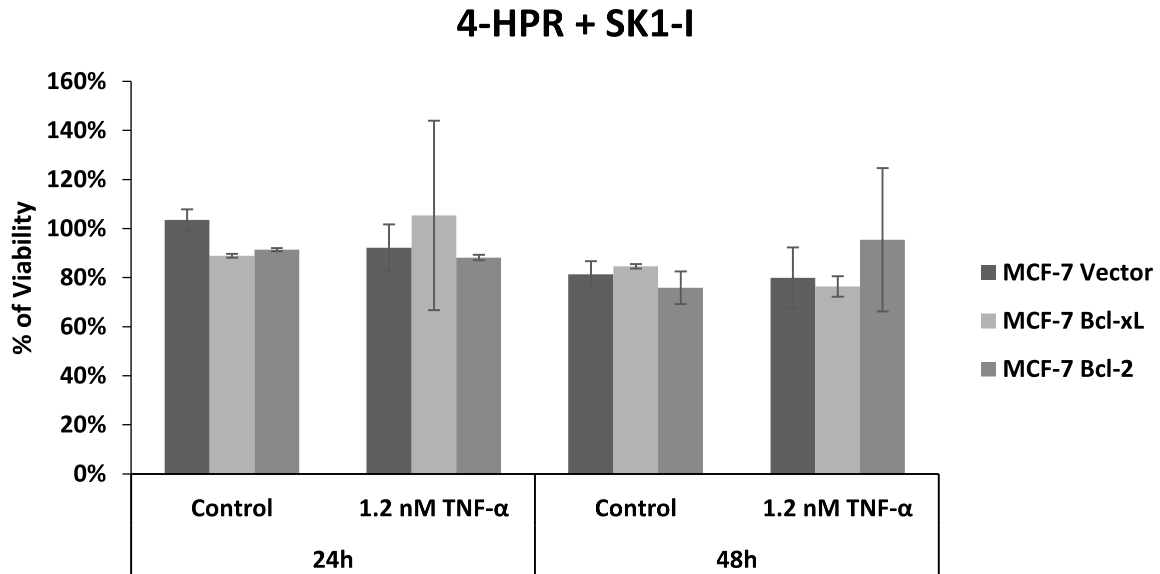

Figure 20. Evaluation of 4-HPR and SK1-I effect on the response of MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells to TNF-α. Cell viability was analyzed by MTT and optical density (OD) was measured at 595 nm. Values are represented by the fold change of OD of 4-HPR (10 μM) + SK-1 I (5 μM)-treated cells over OD of DMSO-treated cells at 24 and 48 hours. The comparison in cell viability was done in absence of TNF-α (control) or in the presence of 1.2 nM TNF-α. Each column represents the mean ± S.D. of the ratios calculated from two independent experiments done in triplicates. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

F. Effect of ST1926 on the viability and PARP-1 cleavage in MCF-7 cells overexpressing Bcl-2 or Bcl-xL in response to TNF-α

Previous research examined the effect of ST1926 on the *de novo* ceramide synthesis pathway and showed that it was activated leading to the accumulation of ceramide without inhibiting DEGS1 [325]. Therefore, we aimed to re-sensitize MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells to TNF-α by pre-treatment with the synthetic retinoid ST1926. MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells were treated with a combination of ST1926 (112.5 nM) and TNF-α (1.2 nM) for 48h. Results analyzed by MTT showed a synergistic activity between TNF-α and ST1926 on MCF-7 Vector cells with a significant 10% decrease in viability compared to the untreated control **(Figure 21)**. Similarly, pre-treatment with ST1926 significantly sensitized MCF-7 Bcl-xL to TNF-α by decreasing the viability by 10% relative to the untreated control. However, MCF-7 Bcl-2 displayed resistance to sensitization by this retinoid treatment **(Figure 21)**.

We next attempted to study PARP cleavage, a hallmark of apoptosis, in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells in the presence or absence of ST1926 and TNF-α at two time points (24h and 48h). Western blot analysis revealed prominent PARP-1 cleavage in MCF-7 Vector treated with TNF-α at 24h and 48h where it was significantly increased with difference changes 1.02 and 34.09, respectively compared to DMSO control. No significant change was detected in MCF-7 Bcl-xL and MCF-7 Bcl-2 cells at 24h; however, at 48h, only MCF-7 Bcl-xL displayed a minimal but significant increase in PARP-1 cleavage with a difference change 5.35 compared to DMSO control but lower than that observed in MCF-7 Vector cells **(Figure 22)**.

These data confirm that MCF-7 Bcl-xL and MCF-7 Bcl-2, in contrast to MCF-7 Vector, cells are resistant to TNF-α induced PARP cleavage, which is considered as a hallmark of apoptosis [67].

However, the expression of cleaved PARP-1 was significantly increased with a difference change 174.67 in ST1926 pre-treated MCF-7-Vector at 24h combined to TNF-α treatment compared to ST1926 only. This increase was highly significant at 48h with a difference change 47.91. Cleaved PARP-1 expression was slightly higher in treatment with both TNF-α and ST1926 compared to TNF-α-only. Importantly, PARP-1 was not cleaved in MCF-7 Vector cells upon treatment with ST1926 alone.

More interestingly, an enhanced expression of cleaved PARP-1 was also significantly recorded in ST1926 pre-treated MCF-7 Bcl-xL cells at 48h combined with TNF-α treatment and displayed a difference change around 7.16 upregulation compared to ST1926 only. Cleaved PARP-1 was slightly higher in treatment with both TNF-α and ST1926 compared to TNF-α only **(Figure 22)**. The latter result needs to be confirmed to show significance, but it was accompanied by a decrease in cell viability in MTT as shown earlier. However, MCF-7 Bcl-2 cells were shown to be resistant to ST1926 at both time points.

These results suggest that the retinoid ST1926 in response to TNF-α was able to slightly induce apoptosis in MCF-7 cells overexpressing Bcl-xL and not in MCF-7 cells overexpressing Bcl-2.

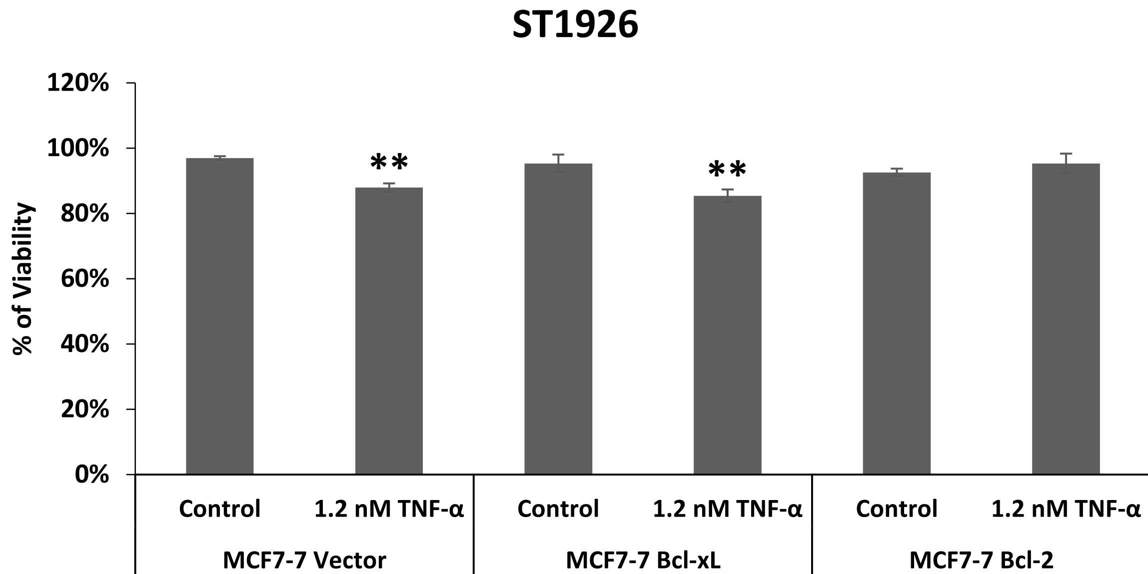

Figure 21. Evaluation of ST1926 effect on the response of MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells to TNF-α. Cell viability was analyzed by MTT and optical density (OD) was measured at 595 nm. Values are represented by the fold change of OD of ST1926 (112.5 nM)-treated cells over OD of DMSO-treated cells at 48 hours. The comparison in cell viability was done in absence of TNF-α (control) or in the presence of 1.2 nM TNF-α. Each column represents the mean ± S.D. of the ratios calculated from three independent experiments done in triplicates. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

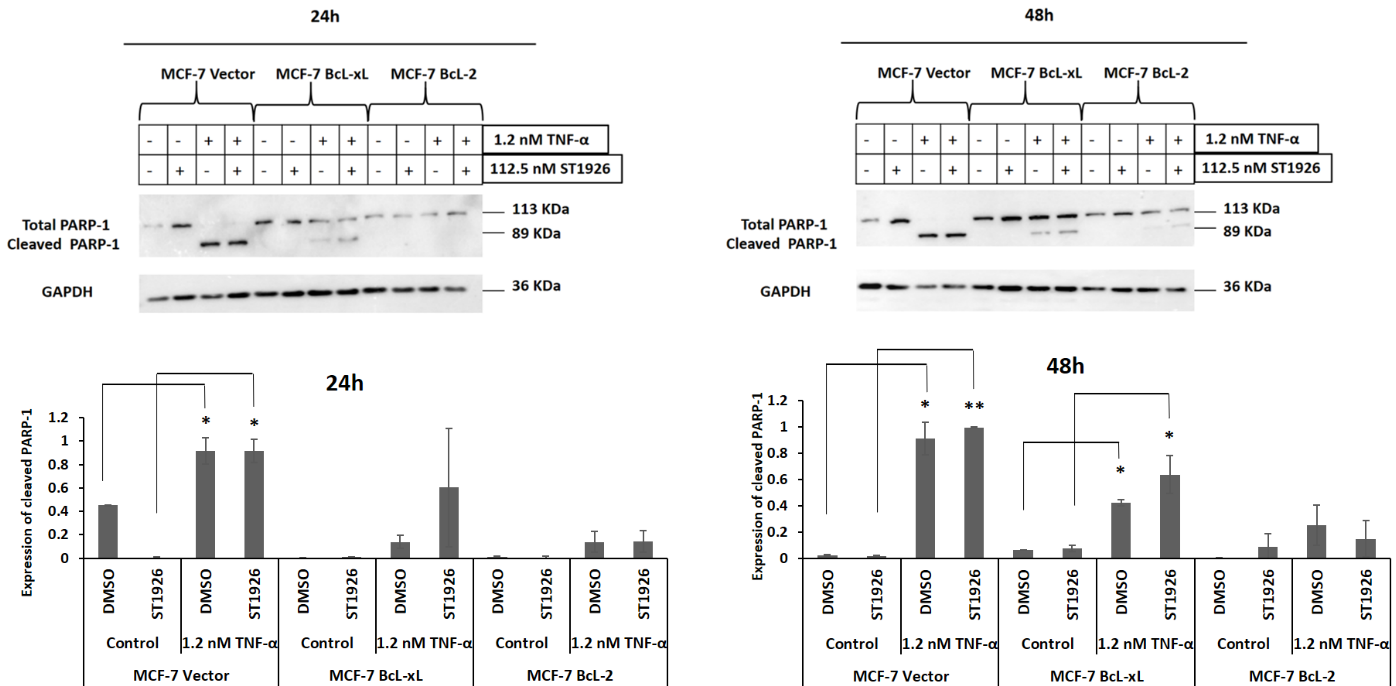

Figure 22. Western blot analysis illustrating the effect of TNF-α or/and of ST1926 on the protein expression of cleaved PARP-1 in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2 cells. Cells were treated with ST1926 (112.5 nM) or/and TNF-α (1.2 nM) for 24h and 48h, while the control was DMSO. Bands were quantified using ImageJ software. Values are represented by the ratio of cleaved PARP-1/ Total PARP-1. GAPDH expression was used as reference control. Each column represents the mean ± S.D. of two independent experiments. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

G. Effect of ST1926 on p53 expression in MCF-7 cells overexpressing Bcl-2 or Bcl-xL in response to TNF-α

p53 was previosuly illustrated to mediate anti-mitogenic role of TNF-α [342]. In this section, we aimed to study the status of p53 in TNF-α response, and the interference of ST1926 on its expression in the three MCF-7 cell lines. Western blot analysis was performed

and showed no significant change in p53 protein expression upon TNF-α treatment at 24h in all of the three cell lines **(Figure 23)**. However, MCF-7 Vector cells displayed a significant upregulation with 6.22 difference change in p53 protein expression upon TNF-α treatment at 48h compared to DMSO, while MCF-7 Bcl-xL cells did not show a significant change. However, p53 protein expression in TNF-α treated MCF-7 Bcl-2 cells was significantly increased by 15.43 difference change compared to the DMSO **(Figure 23)**.

This result can suggest that the increase in the protein expression of p53 upon TNF-α treatment in MCF-7 Bcl-2 may play a role in the slight decrease in viability of these cells which might not be related to apoptosis (absence of PARP cleavage) and might be due to cell cycle arrest. This was not observed in the case of MCF-7 Bcl-xL.

Interestingly, pre-treatment with retinoid did not affect the expression of p53 in all of the three cell lines at 24h and 48h, which demonstrates that ST1926 effect is not likely to be mediated through p53.

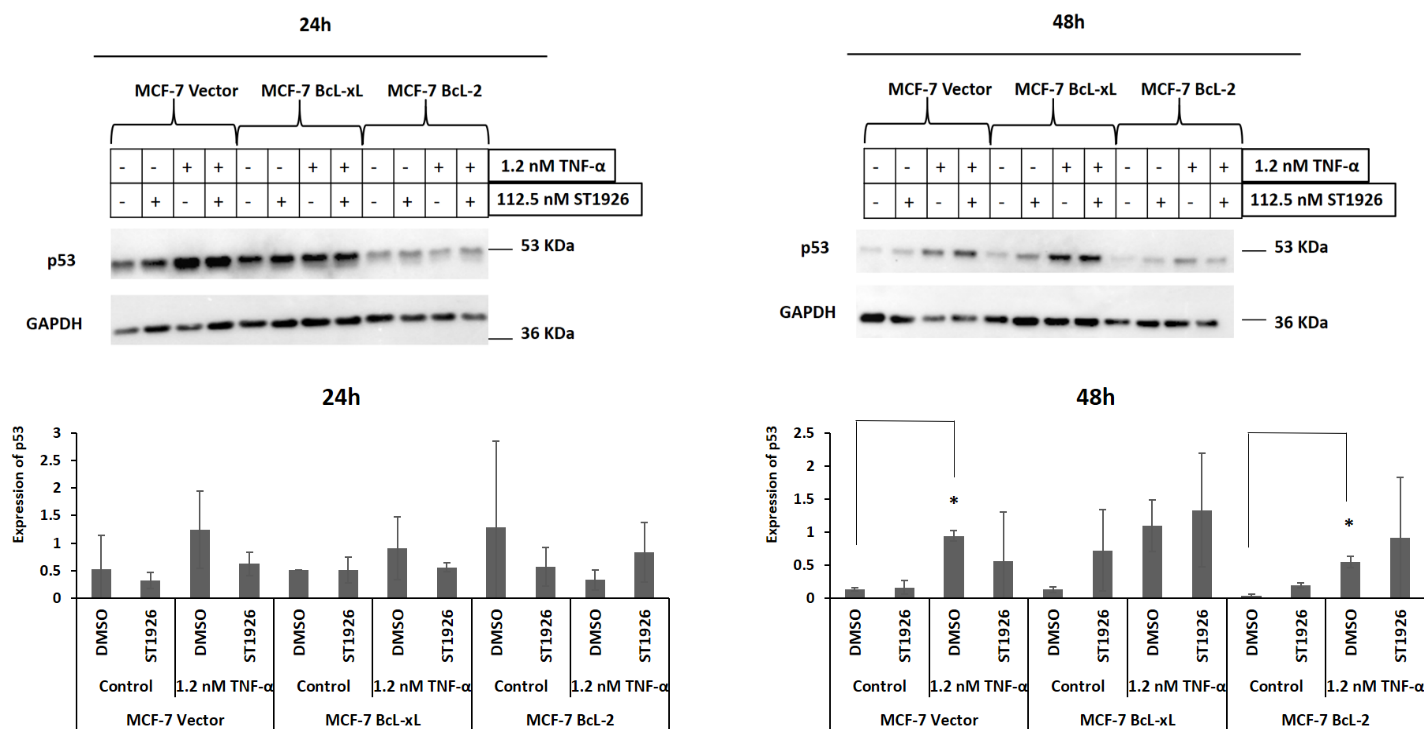

Figure 23. Western blot analysis illustrating the effect of TNF-α or/and ST1926 on p53 protein expression in MCF-7 Vector, MCF-7 Bcl-xL, and MCF-7 Bcl-2. Cells were treated with ST1926 (112.5 nM) or/and TNF-α (1.2 nM) for 24h and 48h, while the control was DMSO. Bands were quantified using ImageJ software. GAPDH expression was used as reference control. Values are represented by the ratio of p53/GAPDH. Each column represents the mean ± S.D. of two independent experiments. Differences were considered significant when p value <0.05, (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

CHAPTER V

DISCUSSION

Several challenges are still being faced in the treatment of breast cancer, the world's most prevalent cancer in women and its resistance to drugs has been associated with the upregulation in the expression of Bcl-2 and Bcl-xL in several models [113, 114, 116, 118, 124-128]. Understanding how they mediate their resistance remains crucial to find alternative therapeutic approaches. Their overexpression was shown to render cells resistant to TNF-α-induced apoptosis. Thus, the interaction of Bcl-2 and Bcl-xL with apoptotic ceramide was previously studied in response to TNF-α in MCF-7 breast cancer cell line. Although they were shown by previous literature to interfere with ceramide generation following TNF-α treatment in MCF-7 cells [288, 289], disclosing regulatory mechanisms controlling this interaction remains to be essential.

The *de novo* ceramide synthesis pathway was found to be one of the mechanisms by which it mediates TNF-α induced cell death in MCF-7 cells [172, 242]. In this study, we aim to characterize the involvement of Bcl-xL and Bcl-2 with *de novo* ceramide synthesis and with ceramide catabolism pathways in MCF-7 breast cancer cell line upon treatment with TNF-α.

We first demonstrated that MCF-7 cells overexpressing Bcl-xL and Bcl-2 are resistant to TNF-α-induced cytotoxicity associated with a minimal decrease in cell viability in both cell lines. While only 20% reduction in cell viability was achieved in MCF-7 Vector cells at 24h, a more prominent and highly significant decrease of 60% was recorded at 48h, with slightly higher selectivity to TNF-α on the viability of MCF-7 Bcl-2 cells compared to MCF-7 Bcl-xL which is

consistent with the previous study conducted by El-Assaad et al. [288]. In fact, the overexpression of Bcl-2 and Bcl-xL was shown to display an inhibitory effect against TNF-α-induced cell death in several cellular models [87, 88, 288, 339-341]. TNF-α has been shown to induce cell death in the MCF-7 breast carcinoma cell line by molecular and morphological mechanisms that are typical of apoptotic processes [288, 289, 343] that can be blocked by the overexpression of Bcl-2 and Bcl-xL [288]. TNF-α mediates its effects through TNFR1, which activates a signaling cascade involving caspase-8 resulting in Bid cleavage and BAX activation to mediate apoptosis by cytochrome c release from the mitochondria followed by effector caspases induction [344-346]. In this regard, it has been demonstrated that the fate of cell is influenced by the interaction of anti-apoptotic Bcl-2 family members (e.g. Bcl-2 and Bcl-xL) with the pro-apoptotic ones (e.g BAX and BAK). The ratio of Bcl-2/BAX and the interaction of Bcl-xL with BAX have been particularly important in dictating resistance to anti-apoptotic stimulus [347, 348].

Ceramide mediates extrinsic apoptotic processes in response to TNF-α [170-174] and FAS [175-177]. The resistance of Bcl-2 and Bcl-xL to TNF-α induced cell death in relation with ceramide accumulation in MCF-7 cell line has been previously studied [288]. Although, both anti-apoptotic proteins display resistance to TNF-α, they appeared to interfere differently with ceramide generation. In this study, we investigated the interference of Bcl-xL and Bcl-2 with ceramide metabolic pathways. Since ceramide was shown to start accumulating at around 12h in MCF-7 cells following TNF-α treatment [288], we investigated the effect of Bcl-2 and Bcl-xL on the transcription of *de novo* enzymes (DEGS1, CerS2, CerS4, and CerS6) of ceramide synthesis pathway at 8h (that is before early ceramide accumulation). MCF-7 Vector cells did not display a significant increase in the mRNA expression of *DEGS1*, and in that of the indicated *CerS*s after

8h of TNF-α (1.2 nM) treatment. This might be explained by the inability of cyclohexamide to affect ceramide generation upon TNF-α treatment of MCF-7 cells, and hence was not dependent on new protein synthesis but on enzyme activation as studied previously [289]. However, we showed that the overexpression of Bcl-xL in MCF-7 cells was able to downregulate the mRNA expression of *DEGS1* while no significant downregulation was detected in MCF-7 Bcl-2 cells. This enzyme has been illustrated to be a molecular target of 4-HPR [322], however, 4-HPR enhances the *de novo* pathway through the upregulation of SPT and/or CerSs and accumulating dihydroceramide rather than ceramide [323]. In this regard, we treated the three MCF-7 cellular models with a combination of 4-HPR and TNF-α. Interestingly, 4-HPR pre-treated MCF-7 Vector cells were resistant against TNF-α by inhibiting *de novo* accumulation of ceramide while no effect was recorded in MCF-7 Bcl-xL cells probably due to already downregulated mRNA expression of *DEGS1* and due to prior inhibition of ceramide accumulation in these cells. We also investigated whether the inhibition of *de novo* ceramide accumulation by 4-HPR might be compensated by another ceramide synthesis pathway and hence sensitize the cells to TNF-α. However, this hypothesis was not validated, since the pre-treatment with a combination of 4-HPR and SK1-I did not have any effect on TNF-α response in all three cell lines. 4-HPR, in fact, was shown to promote the polyubiquitination of DEGS1, and when these forms predominate prosurvival pathways might get activated accompanied by accumulation of dihydroceramide [349, 350]. It has also been reported that *DEGS1* knockout in embryonic cells displayed an accumulation of dihydroceramide accompanied by a protective effect against etoposide which is a chemotherapeutic agent and an inducer of sphingolipid synthesis [351]. While dihydroceramide has been generally described as ineffective or biologically inert [170, 257, 258], several reports have implicated it in several physiological processes, such as hypoxia [223, 224, 259], cell

proliferation [260, 261], autophagy [261-263], and in its ability to prevent mitochondrial ceramide channel formation [264]. One important question has emanated from this finding: is it the accumulation of dihydroceramide or the downregulation of ceramide contributing to the 4-HPR inhibitory effect against TNF-α in MCF-7 Vector cells? The decrease in ceramide accumulation can be confirmed by Liquid chromatography–mass spectrometry (LC-MS) and we can determine if the survival effect is related to dihydroceramide or not by pre-treating the cells with dihydroceramide before TNF-α which is something we aim to study in the future. The interference of Bcl-xL with the *de novo* pathway has been further investigated by analyzing its effect on the transcription of *CerS2*, *CerS4*, and *CerS6* after 8h of TNF-α treatment. Furthermore, MCF-7 Bcl-xL displayed a downregulation in the mRNA expression of *CerS4* only while the mRNA expression of *CerS2* and *CerS6* was not affected. CerS2 and CerS4 preferably synthesize C20-C26 Cer and C18-C20 Cer, respectively while CerS6 is specific for C14- and C16- Cer [230]. It has been previously demonstrated that the overexpression of CerS4 and CerS6 promoted the inhibition of cell proliferation and induction of apoptosis in MCF-7 cells while CerS2 showed a pro-growth effect [352]. In addition, their overexpression was accompanied by the accumulation of their corresponding ceramide species C18-20:0-Cer and C16:0-Cer for CerS4 and CerS6, respectively. Further, a high expression of ceramide synthases (CerS2/4/6), associated with increased levels of C16:0-Cer, C24:1-Cer and C24:0-Cer, was reported in malignant tissues compared to normal ones [256]. More importantly, C16 and C18 were found to be the first ceramide species to accumulate in the first stages of apoptosis in other studies [353, 354] while C24-Cer were predominantly induced in later stages [354]. While ceramide synthases also contribute to ceramide turnover from the salvage pathway, the downregulation in the transcription of *CerS4*, in addition to *DEGS1*, can further support the involvement of Bcl-xL

with the *de novo* pathway of ceramide that might be contributing to the resistance of MCF-7 Bcl-xL cells to TNF-α.

In contrast to Bcl-xL, Bcl-2 did not show any interference with the transcription of *DEGS*1 and ceramide synthases (*CerS2-4-6*) at 8h. This is consistent with the previous study where Bcl-2 was shown to inhibit ceramide-induced death, but not ceramide generation particularly at early time points [288, 289, 291]. These findings, however, oppose other conflicting studies stating that Bcl-2 prevents both ceramide accumulation and ceramide-induced cell death probably due to the involvement of different stimuli signaling in different cell lines [187, 293, 294]. In response to TNF-α, Bcl-2 overexpression protected MCF-7 cells from ceramide-induced apoptosis, and inhibition of ceramide accumulation was only observed at late time points (24h) while Bcl-xL prevented a sustainable ceramide accumulation and did not protect from ceramide-induced apoptosis [288, 289]. Further, this has led to the conclusion that Bcl-xL acts upstream of ceramide accumulation while Bcl-2 function at a downstream target; The higher selectivity of TNF-α in cells overexpressing Bcl-2 compared to Bcl-xL was compatible with the inability of Bcl-2 to completely inhibit caspase-8 which is believed to function at the apex of TNF-α response [288]. In addition, 4-HPR pre-treated MCF-7 Bcl-2 cells did not show any effect on its cell viability which might be explained by either inability of Bcl-2 to interfere with the *de novo* ceramide accumulation or by its late inhibitory effect on ceramide accumulation as previously shown in [288].

Given the role of SphK1/S1P in promoting resistance to stress agents in several cancers [335], we also investigated the involvement of SphK1 in promoting TNF-α resistance in MCF-7 Bcl-xL and MCF-7 Bcl-2 cells and we did not detect any significant effect on the transcriptional level of this enzyme in both MCF-7 models. Since the site of regulation might not be involving

the mRNA level, we pre-treated MCF-7 Bcl-xL and MCF-7 Bcl-2 with a selective inhibitor for SphK1 (SK1-I) to analyze whether it will be able to resensitize them to TNF-α-induced cell cytotoxicity. Consistent with the results at the transcriptional level of SphK1, no effect was detected in all three cell lines after SphK1 inhibition. This may suggest that the salvage pathway is unlikely to contribute to the insensitivity of MCF-7 Bcl-xL and MCF-7 Bcl-2 to TNF-α.

We later aimed to re-sensitize resistant cells to TNF-α by pre-treating them with ST1926 synthetic retinoid. While we already showed that the mRNA expression of some enzymes of *de novo* pathway were downregulated in MCF-7 Bcl-xL in response to TNF-α, we hypothesized that ST1926 might re-sensitize the cells to TNF-α by reactivating this pathway. Interestingly, ST1926 pre-treated MCF-7 Vector cells displayed a synergistic activity with TNF-α at 48h. Similarly, ST1926 was significantly able to re-sensitize MCF-7 Bcl-xL cells to TNF-α after 48h by 10%. We later analyzed PARP cleavage, a hallmark of apoptosis [67], in response to TNF-α alone or with a combination of TNF-α + ST1926 at 24h and 48h. Significant PARP-1 cleavage was recorded at 24h and 48h in MCF-7 Vector cells upon TNF-α treatment. While MCF-7 Bcl-2 displayed nearly total inhibition of PARP-1 cleavage, a slight significant increase was recorded in MCF-7 Bcl-xL at 48h but was still minimal compared to MCF-7 Vector. PARP cleavage further confirms the effect of TNF-α in activating the apoptotic mode of cell death, as also shown in previous studies [288, 289, 343], while the MCF-7 cells overexpressing Bcl-2 and Bcl-xL were resistant to it [288, 289]. Interestingly, ST1926 treatment did not display a cytotoxic activity on cells or an effect on PARP-1 cleavage. However, ST1926-pre-treated MCF-7 Vector cells displayed a significant PARP-1 cleavage in response to TNF-α at 24h, and highly significant cleavage at 48h compared to ST1926-control. This increase was even higher than the expression level of cleaved PARP-1 by TNF-α alone. While no significant PARP-1 cleavage was

recorded in MCF-7 Bcl-xL cells at 24h, pre-treatment with ST1926 displayed a significant synergistic activity with TNF-α on PARP cleavage compared to the ST1926-control at 48h, and, similar to MCF-7 Vector, this increase was higher than in those treated with TNF-α. Although these latter results need to be confirmed to show significance in comparison with TNF-α-treated cells, this enhanced PARP-1 cleavage was accompanied by a significant decrease in cell viability in MCF-7 Vector and MCF-7 Bcl-xL cells. In fact, ST1926 was previously shown to sensitize ATL resistant leukemia cells by restoring the *de novo* ceramide synthesis pathway by the upregulation of ceramide synthases without inhibiting DEGS1, unlike 4-HPR [325]. We cannot, however, conclude the re-activation of the *de novo* synthesis pathway in cells overexpressing Bcl-xL unless we treat the cells with [^{3}H]-palmitic acid and quantify its signals through its accumulation in ceramide lipids in response to TNF-α + ST1926. MCF-7 Bcl-2 cells, however, displayed resistance towards PARP-1 cleavage at both 24h and 48h when pre-treated with ST1926 which was compatible with our MTT results. These findings were consistent with the fact that Molt-4 cells overexpressing Bcl-2 were resistant to ST1926 growth inhibitory effect and ceramide accumulation through the *de novo* pathway [325]. Hence, the resistance of Bcl-2 to ST1926 can be explained by the fact that Bcl-2 is downstream of ceramide, but further research is needed to confirm this.

We further investigated TNF-α and TNF-α + ST1926 effect on the protein expression of p53 using western blot. All three cell lines did not show a significant increase in the levels of p53 at 24h and 48h when they were treated with a combination of TNF-α and ST1926. This can be supported by the fact that several studies have elucidated that the growth inhibitory function of ST1926 to be independent of the status of p53 in several cell lines [329, 355]. Interestingly, MCF-7 Vector has shown a significant upregulation of p53 protein at 48h upon TNF-α treatment

while MCF-7 Bcl-xL did not show a significant increase. Similar to other studies, TNF-α was found to mediate its growth inhibitory or apoptotic effect through p53 in MCF-7, LNCaP human prostatic carcinoma cell line, and HT29 colorectal cancer cell line and human glioma cell lines [173, 342, 356, 357]. In other systems, however, TNF-α was able to induce growth suppression in mechanisms that are independent of p53, such as through ceramide induction [209]. Moreover, multiple studies have revealed that ceramide induction can be downstream, upstream or even independent of p53 in several cellular models under different types of stimuli [210]. While we did not conclude the dependency of ceramide accumulation on p53 in response to TNF-α, the overexpression of Bcl-xL, which was previously shown to interfere with ceramide accumulation, did not show a significant change in the levels of p53 upon TNF-α treatment. Thus, it might be possible that ceramide accumulation is dependent on p53 in our model, and Bcl-xL is interfering with its expression, but this must be confirmed in future studies. Interestingly, the overexpression of Bcl-xL in some cell lines, such that in the case of squamous carcinoma, promoted multi-drug resistance in cells with wild type p53, while this resistance was not correlated with Bcl-2 [358]. Consistently with this, a significant increase in p53 was recorded in our study in MCF-7 Bcl-2 in response to TNF-α. Further, several studies have described the function of Bcl-2 in inhibiting p53-dependent apoptosis but not cell cycle arrest, so that Bcl-2 functions downstream of p53 [359, 360]. While Bcl-2 displayed nearly complete inhibition of PARP-1 cleavage in response to TNF-α in our study, we raise the possibility that the increase in p53 expression in MCF-7 Bcl-2 cells may induce growth arrest instead of apoptosis in response to TNF-α.

CHAPTER VI

CONCLUSION AND PERSPECTIVES

Bcl-2 and Bcl-xL are two anti-apoptotic proteins that are associated with drug resistance in cancer therapy and particularly in breast cancer [361]. Thus, understanding their signaling mechanisms remains essential to discover new therapeutic targets and approaches. In this study, we characterized the involvement of Bcl-xL and Bcl-2 with the *de novo* ceramide synthesis pathway using two synthetic retinoids 4-HPR and ST1926 in TNF-α response in MCF-7 breast cancer cell line, and illustrated their different function and targets which might play a role in their resistance or susceptibility to TNF-α. However, further research is needed to confirm our conclusions.

Based on these results and the limitations that we faced, in the future perspectives we aim to:

- Perform sphingolipid profiling for the studied MCF-7 cells upon TNF-α treatment through LC-MS to indicate which ceramide metabolites mediate the apoptotic response or more likely to contribute to the resistance or sensitivity to TNF-α response.
- Confirm the apoptotic response of MCF-7 cells upon TNF-α treatment through Annexin V-PI.
- Investigate the transcriptional regulation of *de novo* enzymes of ceramide synthesis pathway at late time points (16h, 18h, and 20h), especially in MCF-7 Bcl-2 cells, due to the ability of Bcl-2 to slightly inhibit ceramide accumulation at around 24h in the previous study [288].
- Determine the change in the protein expression of CerS2/4/6 and DEGS1 in response to TNF-α in the different MCF-7 cellular models by western blot.

- Confirm whether the accumulation of dihydroceramide or the downregulation of ceramide is contributing to the inhibitory effect of 4-HPR against TNF-α response in MCF-7 Vector. This can further be explained by studying the effect of dihydroceramide treatment on the viability of the cells prior to the exposure to TNF-α.
- Confirm the increase in the expression of cleaved PARP-1 with ST1926 + TNF-α in MCF-7 Vector and MCF-7 Bcl-xL in comparison to TNF-α.
- Confirm the ability of ST1926 to restore the *de novo* synthesis pathway of ceramide in TNF-α resistant MCF-7 Bcl-xL by pre-treating the cells with [^{3}H]-palmitic acid and track its signal through the accumulation of ceramide.
- Study whether ST1926 is able to re-induce caspase-8 cleavage in TNF-α response which was proven to be completely inhibited in MCF-7 Bcl-xL, being correlated with its resistance to TNF-α [288].
- Study p53 role in TNF-α induced ceramide accumulation, and cell cycle arrest upon TNF-α treatment especially to investigate the upregulation of p53 in MCF-7 Bcl-2.
- Characterize the effect of Bcl-xL and Bcl-2 on nSMase activation pathway in MCF-7 cells in response to TNF-α.

BIBLIOGRAPHY

1. WHO. *Breast cancer*. 2021 [cited 2022 February 19th]; Available from: https://www.who.int/news-room/fact-sheets/detail/breast-cancer#.
2. Fares, M.Y., et al., *Breast cancer epidemiology among Lebanese women: an 11-year analysis.* Medicina, 2019. **55**(8): p. 463.
3. Turnbull, C. and N. Rahman, *Genetic predisposition to breast cancer: past, present, and future.* Annu. Rev. Genomics Hum. Genet., 2008. **9**: p. 321-345.
4. Roy, R., J. Chun, and S.N. Powell, *BRCA1 and BRCA2: different roles in a common pathway of genome protection.* Nature Reviews Cancer, 2012. **12**(1): p. 68-78.
5. Mavaddat, N., et al., *Cancer risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE.* JNCI: Journal of the National Cancer Institute, 2013. **105**(11): p. 812-822.
6. Lalloo, F. and D. Evans, *Familial breast cancer.* Clinical genetics, 2012. **82**(2): p. 105-114.
7. Antoniou, A.C. and D. Easton, *Models of genetic susceptibility to breast cancer.* Oncogene, 2006. **25**(43): p. 5898-5905.
8. Walsh, T., et al., *Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer.* Jama, 2006. **295**(12): p. 1379-1388.
9. Momenimovahed, Z. and H. Salehiniya, *Epidemiological characteristics of and risk factors for breast cancer in the world.* Breast Cancer: Targets and Therapy, 2019. **11**: p. 151.
10. Sharma, G.N., et al., *Various types and management of breast cancer: an overview.* Journal of advanced pharmaceutical technology & research, 2010. **1**(2): p. 109.
11. Coopey, S.B. and K.S. Hughes, *Breast Cancer Risk Prediction in Women with Atypical Breast Lesions*, in *Atypical Breast Proliferative Lesions and Benign Breast Disease*. 2018, Springer. p. 103-113.
12. Hartmann, L.C., et al., *Atypical hyperplasia of the breast—risk assessment and management options.* New England Journal of Medicine, 2015. **372**(1): p. 78-89.
13. van Seijen, M., et al., *Ductal carcinoma in situ: to treat or not to treat, that is the question.* British journal of cancer, 2019. **121**(4): p. 285-292.
14. Cowell, C.F., et al., *Progression from ductal carcinoma in situ to invasive breast cancer: revisited.* Molecular oncology, 2013. **7**(5): p. 859-869.
15. Shehata, M., et al., *Ductal carcinoma in situ: current concepts in biology, imaging, and treatment.* Journal of breast imaging, 2019. **1**(3): p. 166-176.
16. Wen, H.Y. and E. Brogi, *Lobular carcinoma in situ.* Surgical pathology clinics, 2018. **11**(1): p. 123-145.
17. Makki, J., *Diversity of breast carcinoma: histological subtypes and clinical relevance.* Clinical medicine insights: Pathology, 2015. **8**: p. CPath. S31563.
18. Waks, A.G. and E.P. Winer, *Breast cancer treatment: a review.* Jama, 2019. **321**(3): p. 288-300.
19. Hance, K.W., et al., *Trends in inflammatory breast carcinoma incidence and survival: the surveillance, epidemiology, and end results program at the National Cancer Institute.* Journal of the National Cancer Institute, 2005. **97**(13): p. 966-975.

20. Cristofanilli, M., A.U. Buzdar, and G.N. Hortobagyi, *Update on the management of inflammatory breast cancer.* The oncologist, 2003. **8**(2): p. 141-148.
21. Mamouch, F., et al., *Inflammatory breast cancer: a literature review.* World journal of oncology, 2018. **9**(5-6): p. 129.
22. Karakas, C., *Paget's disease of the breast.* Journal of carcinogenesis, 2011. **10**.
23. Lopes Filho, L.L., et al., *Mammary and extramammary Paget's disease.* Anais brasileiros de dermatologia, 2015. **90**: p. 225-231.
24. Mishra, S.P., et al., *Phyllodes tumor of breast: a review article.* International Scholarly Research Notices, 2013. **2013**.
25. Zhang, Y. and C.G. Kleer, *Phyllodes tumor of the breast: histopathologic features, differential diagnosis, and molecular/genetic updates.* Archives of pathology & laboratory medicine, 2016. **140**(7): p. 665-671.
26. Soren, D.N., et al., *Primary mammary angiosarcoma: literature review.* Cureus, 2020. **12**(6).
27. Hodgson, N.C., et al., *Angiosarcomas of the breast: a review of 70 cases.* American journal of clinical oncology, 2007. **30**(6): p. 570-573.
28. Malhotra, G.K., et al., *Histological, molecular and functional subtypes of breast cancers.* Cancer biology & therapy, 2010. **10**(10): p. 955-960.
29. Akram, M., et al., *Awareness and current knowledge of breast cancer.* Biological research, 2017. **50**(1): p. 1-23.
30. Hilton, H.N., C.L. Clarke, and J.D. Graham, *Estrogen and progesterone signalling in the normal breast and its implications for cancer development.* Molecular and cellular endocrinology, 2018. **466**: p. 2-14.
31. Joshi, H. and M.F. Press, *Molecular oncology of breast cancer*, in *The Breast*. 2018, Elsevier. p. 282-307. e5.
32. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* science, 1987. **235**(4785): p. 177-182.
33. Callahan, R. and S. Hurvitz, *HER2-positive breast cancer: current management of early, advanced, and recurrent disease.* Current opinion in obstetrics & gynecology, 2011. **23**(1): p. 37.
34. Iancu, G., et al., *Triple positive" breast cancer-a novel category.* Rom J Morphol Embryol, 2017. **58**(1): p. 21-26.
35. Foulkes, W.D., I.E. Smith, and J.S. Reis-Filho, *Triple-negative breast cancer.* New England journal of medicine, 2010. **363**(20): p. 1938-1948.
36. Yin, L., et al., *Triple-negative breast cancer molecular subtyping and treatment progress.* Breast Cancer Research, 2020. **22**(1): p. 1-13.
37. Denkert, C., et al., *Molecular alterations in triple-negative breast cancer—the road to new treatment strategies.* The Lancet, 2017. **389**(10087): p. 2430-2442.
38. Hudis, C.A., *Trastuzumab—mechanism of action and use in clinical practice.* New England journal of medicine, 2007. **357**(1): p. 39-51.
39. Anampa, J., D. Makower, and J.A. Sparano, *Progress in adjuvant chemotherapy for breast cancer: an overview.* BMC medicine, 2015. **13**(1): p. 1-13.
40. Mina, L.A., et al., *Immunotherapy for the treatment of breast cancer: emerging new data.* Breast Cancer: Targets and Therapy, 2019. **11**: p. 321.
41. Arora, S., et al., *US FDA Drug Approvals for Breast Cancer-A Decade in Review.* Clinical Cancer Research, 2021.

42. Kwok, G., et al., *Pembrolizumab (keytruda).* Human vaccines & immunotherapeutics, 2016. **12**(11): p. 2777-2789.
43. Sledge, G.W., et al., *Past, present, and future challenges in breast cancer treatment.* Journal of clinical oncology, 2014. **32**(19): p. 1979.
44. Brill, A., et al., *The role of apoptosis in normal and abnormal embryonic development.* Journal of assisted reproduction and genetics, 1999. **16**(10): p. 512-519.
45. Renehan, A.G., C. Booth, and C.S. Potten, *What is apoptosis, and why is it important? Education and debate.* Bmj, 2001. **322**(7301): p. 1536-1538.
46. Tower, J., *Programmed cell death in aging.* Ageing research reviews, 2015. **23**: p. 90-100.
47. Su, J.H., G. Deng, and C.W. Cotman, *Bax protein expression is increased in Alzheimer's brain: correlations with DNA damage, Bcl-2 expression, and brain pathology.* Journal of Neuropathology & Experimental Neurology, 1997. **56**(1): p. 86-93.
48. Jenner, P. and C.W. Olanow, *Understanding cell death in Parkinson's disease.* Annals of neurology, 1998. **44**(S1 1): p. S72-S84.
49. Mattson, M.P., *Apoptosis in neurodegenerative disorders.* Nature reviews Molecular cell biology, 2000. **1**(2): p. 120-130.
50. Olivetti, G., et al., *Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart.* Journal of molecular and cellular cardiology, 1996. **28**(9): p. 2005-2016.
51. Teringova, E. and P. Tousek, *Apoptosis in ischemic heart disease.* Journal of translational medicine, 2017. **15**(1): p. 1-7.
52. Worth, A., A.J. Thrasher, and H. Bobby Gaspar, *Autoimmune lymphoproliferative syndrome: molecular basis of disease and clinical phenotype.* British journal of haematology, 2006. **133**(2): p. 124-140.
53. Mahajan, A., et al., *Autoimmune diseases and apoptosis: Targets, challenges, and innovations*, in *Clinical Perspectives and Targeted Therapies in Apoptosis*. 2021, Elsevier. p. 285-327.
54. Roulston, A., R.C. Marcellus, and P.E. Branton, *Viruses and apoptosis.* Annual Reviews in Microbiology, 1999. **53**(1): p. 577-628.
55. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* cell, 2000. **100**(1): p. 57-70.
56. Mohammad, R.M., et al. *Broad targeting of resistance to apoptosis in cancer*. in *Seminars in cancer biology*. 2015. Elsevier.
57. Deveraux, Q.L., et al., *IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases.* The EMBO journal, 1998. **17**(8): p. 2215-2223.
58. Berchem, G.J., et al., *Androgens induce resistance to bcl-2-mediated apoptosis in LNCaP prostate cancer cells.* Cancer research, 1995. **55**(4): p. 735-738.
59. Robertson, L., et al., *Bcl-2 expression in chronic lymphocytic leukemia and its correlation with the induction of apoptosis and clinical outcome.* Leukemia, 1996. **10**(3): p. 456-459.
60. Galluzzi, L., et al., *Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018.* Cell Death & Differentiation, 2018. **25**(3): p. 486-541.
61. Kale, J., E.J. Osterlund, and D.W. Andrews, *BCL-2 family proteins: changing partners in the dance towards death.* Cell Death & Differentiation, 2018. **25**(1): p. 65-80.

62. Singh, R., A. Letai, and K. Sarosiek, *Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins.* Nature Reviews Molecular Cell Biology, 2019. **20**(3): p. 175-193.
63. Chipuk, J., L. Bouchier-Hayes, and D. Green, *Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario.* Cell Death & Differentiation, 2006. **13**(8): p. 1396-1402.
64. Parrish, A.B., C.D. Freel, and S. Kornbluth, *Cellular mechanisms controlling caspase activation and function.* Cold Spring Harbor perspectives in biology, 2013. **5**(6): p. a008672.
65. Saraste, A. and K. Pulkki, *Morphologic and biochemical hallmarks of apoptosis.* Cardiovascular research, 2000. **45**(3): p. 528-537.
66. Chaitanya, G.V., J.S. Alexander, and P.P. Babu, *PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration.* Cell Communication and Signaling, 2010. **8**(1): p. 1-11.
67. Kaufmann, S.H., et al., *Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis.* Cancer research, 1993. **53**(17): p. 3976-3985.
68. Panduri, V., et al., *Mitochondrial-derived free radicals mediate asbestos-induced alveolar epithelial cell apoptosis.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2004. **286**(6): p. L1220-L1227.
69. Corazzari, M., et al., *Endoplasmic reticulum stress, unfolded protein response, and cancer cell fate.* Frontiers in oncology, 2017. **7**: p. 78.
70. Eriksson, D. and T. Stigbrand, *Radiation-induced cell death mechanisms.* Tumor Biology, 2010. **31**(4): p. 363-372.
71. Kaufmann, S.H. and W.C. Earnshaw, *Induction of apoptosis by cancer chemotherapy.* Experimental cell research, 2000. **256**(1): p. 42-49.
72. Greijer, A. and E. Van der Wall, *The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis.* Journal of clinical pathology, 2004. **57**(10): p. 1009-1014.
73. Collins, M.K., et al., *Growth factors as survival factors: regulation of apoptosis.* Bioessays, 1994. **16**(2): p. 133-138.
74. Mercille, S. and B. Massie, *Induction of apoptosis in nutrient-deprived cultures of hybridoma and myeloma cells.* Biotechnology and Bioengineering, 1994. **44**(9): p. 1140-1154.
75. Zhan, Q., et al., *Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis.* Oncogene, 1994. **9**(12): p. 3743-3751.
76. Nakano, K. and K.H. Vousden, *PUMA, a novel proapoptotic gene, is induced by p53.* Molecular cell, 2001. **7**(3): p. 683-694.
77. Oda, E., et al., *Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis.* Science, 2000. **288**(5468): p. 1053-1058.
78. Zhan, Q., et al., *Induction of BCL2 family member MCL1 as an early response to DNA damage.* Oncogene, 1997. **14**(9): p. 1031-1039.
79. Mihara, M., et al., *p53 has a direct apoptogenic role at the mitochondria.* Molecular cell, 2003. **11**(3): p. 577-590.
80. Deng, J., et al., *Proapoptotic BH3-only BCL-2 family protein BIM connects death signaling from epidermal growth factor receptor inhibition to the mitochondrion.* Cancer research, 2007. **67**(24): p. 11867-11875.

81. Winter, P.S., et al., *RAS signaling promotes resistance to JAK inhibitors by suppressing BAD-mediated apoptosis.* Science signaling, 2014. **7**(357): p. ra122-ra122.
82. Zhang, P., et al., *PROTACs are effective in addressing the platelet toxicity associated with BCL-XL inhibitors.* Exploration of targeted anti-tumor therapy, 2020. **1**: p. 259.
83. Locksley, R.M., N. Killeen, and M.J. Lenardo, *The TNF and TNF receptor superfamilies: integrating mammalian biology.* Cell, 2001. **104**(4): p. 487-501.
84. Varfolomeev, E.E. and A. Ashkenazi, *Tumor necrosis factor: an apoptosis JuNKie?* cell, 2004. **116**(4): p. 491-497.
85. Wang, X. and Y. Lin, *Tumor necrosis factor and cancer, buddies or foes? 1.* Acta Pharmacologica Sinica, 2008. **29**(11): p. 1275-1288.
86. Wajant, H., K. Pfizenmaier, and P. Scheurich, *Tumor necrosis factor signaling.* Cell Death & Differentiation, 2003. **10**(1): p. 45-65.
87. Hennet, T., et al., *Expression of BCL-2 protein enhances the survival of mouse fibrosarcoid cells in tumor necrosis factor-mediated cytotoxicity.* Cancer Research, 1993. **53**(6): p. 1456-1460.
88. Gozzelino, R., et al., *BCL-XL regulates TNF-α-mediated cell death independently of NF-κB, FLIP and IAPs.* Cell research, 2008. **18**(10): p. 1020-1036.
89. Malagarie-Cazenave, S., et al., *Sphingolipid signalling: molecular basis and role in TNF-α-induced cell death.* Expert Reviews in Molecular Medicine, 2002. **4**(28): p. 1-15.
90. Packham, G., *Mutation of BCL-2 family proteins in cancer.* Apoptosis, 1998. **3**(2): p. 75-82.
91. García-Aranda, M., E. Pérez-Ruiz, and M. Redondo, *Bcl-2 inhibition to overcome resistance to chemo-and immunotherapy.* International journal of molecular sciences, 2018. **19**(12): p. 3950.
92. Chen, L., Y. Zeng, and S.-F. Zhou, *Role of apoptosis in cancer resistance to chemotherapy.* Current understanding of apoptosis-programmed cell death, 2018.
93. Tsujimoto, Y., et al., *Cloning of the chromosome breakpoint of neoplastic B cells with the t (14; 18) chromosome translocation.* Science, 1984. **226**(4678): p. 1097-1099.
94. Tsujimoto, Y., et al., *Involvement of the bcl-2 gene in human follicular lymphoma.* Science, 1985. **228**(4706): p. 1440-1443.
95. Miyashita, T. and J.C. Reed, *Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line.* 1993.
96. Marin, M.C., et al., *Evidence that p53 and bcl-2 are regulators of a common cell death pathway important for in vivo lymphomagenesis.* Oncogene, 1994. **9**(11): p. 3107-3112.
97. Del Bufalo, D., et al., *Bcl-2 overexpression enhances the metastatic potential of a human breast cancer line.* The FASEB journal, 1997. **11**(12): p. 947-953.
98. Wick, W., et al., *BCL-2 promotes migration and invasiveness of human glioma cells.* FEBS letters, 1998. **440**(3): p. 419-424.
99. Koehler, B.C., et al., *Beyond cell death–antiapoptotic Bcl-2 proteins regulate migration and invasion of colorectal cancer cells in vitro.* PloS one, 2013. **8**(10): p. e76446.
100. Trisciuoglio, D., et al., *Bcl-2 overexpression in melanoma cells increases tumor progression-associated properties and in vivo tumor growth.* Journal of cellular physiology, 2005. **205**(3): p. 414-421.
101. Noujaim, D., et al., *N-Myc and Bcl-2 coexpression induces MMP-2 secretion and activation in human neuroblastoma cells.* Oncogene, 2002. **21**(29): p. 4549-4557.

102. Choi, J., et al., *Bcl-2 promotes invasion and lung metastasis by inducing matrix metalloproteinase-2.* Cancer research, 2005. **65**(13): p. 5554-5560.
103. Brambilla, E., et al., *p53 mutant immunophenotype and deregulation of p53 transcription pathway (Bcl2, Bax, and Waf1) in precursor bronchial lesions of lung cancer.* Clinical Cancer Research, 1998. **4**(7): p. 1609-1618.
104. Redondo, M., et al., *Expression of the antiapoptotic proteins clusterin and bcl-2 in laryngeal squamous cell carcinomas.* Tumor Biology, 2006. **27**(4): p. 195-200.
105. Ishijima, N., et al., *The immunohistochemical expression of BCL-2 oncoprotein in colorectal adenocarcinoma.* Surgery today, 1999. **29**(7): p. 682-684.
106. Wang, Z.B., et al., *Bcl-xL overexpression restricts γ-radiation-induced apoptosis.* Cell biology international, 2006. **30**(1): p. 15-20.
107. Liu, R., et al., *Overexpression of Bcl-xL promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model.* The American journal of pathology, 1999. **155**(6): p. 1861-1867.
108. Ho, J.N., et al., *Bcl-XL and STAT3 mediate malignant actions of γ-irradiation in lung cancer cells.* Cancer science, 2010. **101**(6): p. 1417-1423.
109. Weiler, M., et al., *BCL-xL: time-dependent dissociation between modulation of apoptosis and invasiveness in human malignant glioma cells.* Cell Death & Differentiation, 2006. **13**(7): p. 1156-1169.
110. Rubio, N., et al., *Metastatic behavior of human breast carcinomas overexpressing the Bcl-xL gene: a role in dormancy and organospecificity.* Laboratory investigation, 2001. **81**(5): p. 725-734.
111. Schulze-Bergkamen, H., et al., *BCL-xL and Myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells.* World journal of gastroenterology: WJG, 2008. **14**(24): p. 3829.
112. Watanabe, J., et al., *Prognostic significance of Bcl-xL in human hepatocellular carcinoma.* Surgery, 2004. **135**(6): p. 604-612.
113. Olopade, O.I., et al., *Overexpression of BCL-x protein in primary breast cancer is associated with high tumor grade and nodal metastases.* The cancer journal from Scientific American, 1997. **3**(4): p. 230-237.
114. Keitel, U., et al., *Bcl-xL mediates therapeutic resistance of a mesenchymal breast cancer cell subpopulation.* Oncotarget, 2014. **5**(23): p. 11778.
115. Zhang, K., et al., *Bcl-xL overexpression and its association with the progress of tongue carcinoma.* International Journal of Clinical and Experimental Pathology, 2014. **7**(11): p. 7360.
116. Fiebig, A.A., et al., *Bcl-XL is qualitatively different from and ten times more effective than Bcl-2 when expressed in a breast cancer cell line.* BMC cancer, 2006. **6**(1): p. 1-15.
117. Luo, D., et al., *Effects of Bcl-2 and Bcl-XL protein levels on chemoresistance of hepatoblastoma HepG2 cell line.* Biochemistry and Cell Biology, 2000. **78**(2): p. 119-126.
118. Real, P.J., et al., *Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells.* Oncogene, 2002. **21**(50): p. 7611-7618.
119. Teixeira, C., J.C. Reed, and M.C. Pratt, *Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells.* Cancer research, 1995. **55**(17): p. 3902-3907.

120. Leung, L. and T. Wang, *Paradoxical regulation of Bcl-2 family proteins by 17β-oestradiol in human breast cancer cells MCF-7.* British journal of cancer, 1999. **81**(3): p. 387-392.
121. Dawson, S.-J., et al., *BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received.* British journal of cancer, 2010. **103**(5): p. 668-675.
122. Oakes, S.R., et al., *Sensitization of BCL-2–expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737.* Proceedings of the National Academy of Sciences, 2012. **109**(8): p. 2766-2771.
123. Vaillant, F., et al., *Targeting BCL-2 with the BH3 mimetic ABT-199 in estrogen receptor-positive breast cancer.* Cancer cell, 2013. **24**(1): p. 120-129.
124. Musgrove, E.A. and R.L. Sutherland, *Biological determinants of endocrine resistance in breast cancer.* Nature Reviews Cancer, 2009. **9**(9): p. 631-643.
125. Hwang, K.T., et al., *Prognostic influence of BCL2 expression in breast cancer.* International journal of cancer, 2012. **131**(7): p. E1109-E1119.
126. Bessou, M., et al., *The apoptosis inhibitor Bcl-xL controls breast cancer cell migration through mitochondria-dependent reactive oxygen species production.* Oncogene, 2020. **39**(15): p. 3056-3074.
127. Martin, S.S., et al., *A cytoskeleton-based functional genetic screen identifies Bcl-xL as an enhancer of metastasis, but not primary tumor growth.* Oncogene, 2004. **23**(26): p. 4641-4645.
128. Fernandez, Y., et al., *Bcl-xL promotes metastasis of breast cancer cells by induction of cytokines resistance.* Cell Death & Differentiation, 2000. **7**(4): p. 350-359.
129. Sorenson, C.M., *Bcl-2 family members and disease.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2004. **1644**(2-3): p. 169-177.
130. Petros, A.M., et al., *Solution structure of the antiapoptotic protein bcl-2.* Proceedings of the National Academy of Sciences, 2001. **98**(6): p. 3012-3017.
131. Muchmore, S.W., et al., *X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death.* Nature, 1996. **381**(6580): p. 335-341.
132. Petros, A.M., E.T. Olejniczak, and S.W. Fesik, *Structural biology of the Bcl-2 family of proteins.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2004. **1644**(2-3): p. 83-94.
133. Popgeorgiev, N., L. Jabbour, and G. Gillet, *Subcellular localization and dynamics of the Bcl-2 family of proteins.* Frontiers in cell and developmental biology, 2018. **6**: p. 13.
134. Sattler, M., et al., *Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis.* Science, 1997. **275**(5302): p. 983-986.
135. Petros, A.M., et al., *Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies.* Protein Science, 2000. **9**(12): p. 2528-2534.
136. Bertini, I., et al., *The anti-apoptotic Bcl-xL protein, a new piece in the puzzle of cytochrome c interactome.* PLoS One, 2011. **6**(4): p. e18329.
137. Correia, C., et al., *Emerging understanding of Bcl-2 biology: Implications for neoplastic progression and treatment.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2015. **1853**(7): p. 1658-1671.
138. Breitschopf, K., et al., *Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway.* Molecular and cellular biology, 2000. **20**(5): p. 1886-1896.

139. Wei, Y., et al., *JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy.* Molecular cell, 2008. **30**(6): p. 678-688.
140. Haldar, S., A. Basu, and C.M. Croce, *Serine-70 is one of the critical sites for drug-induced Bcl2 phosphorylation in cancer cells.* Cancer research, 1998. **58**(8): p. 1609-1615.
141. Furukawa, Y., et al., *Phosphorylation of Bcl-2 protein by CDC2 kinase during G2/M phases and its role in cell cycle regulation.* Journal of Biological Chemistry, 2000. **275**(28): p. 21661-21667.
142. De Chiara, G., et al., *Bcl-2 Phosphorylation by p38 MAPK: identification of target sites and biologic consequences.* Journal of Biological Chemistry, 2006. **281**(30): p. 21353-21361.
143. Asnaghi, L., et al., *Bcl-2 phosphorylation and apoptosis activated by damaged microtubules require mTOR and are regulated by Akt.* Oncogene, 2004. **23**(34): p. 5781-5791.
144. Blagosklonny, M.V., et al., *Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death.* Cancer research, 1997. **57**(1): p. 130-135.
145. Sakurikar, N., J.M. Eichhorn, and T.C. Chambers, *Cyclin-dependent kinase-1 (Cdk1)/cyclin B1 dictates cell fate after mitotic arrest via phosphoregulation of antiapoptotic Bcl-2 proteins.* Journal of Biological Chemistry, 2012. **287**(46): p. 39193-39204.
146. Terrano, D.T., M. Upreti, and T.C. Chambers, *Cyclin-dependent kinase 1-mediated Bcl-xL/Bcl-2 phosphorylation acts as a functional link coupling mitotic arrest and apoptosis.* Molecular and cellular biology, 2010. **30**(3): p. 640-656.
147. Srivastava, R.K., et al., *Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase.* Molecular and cellular biology, 1998. **18**(6): p. 3509-3517.
148. Maundrell, K., et al., *Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1.* Journal of Biological Chemistry, 1997. **272**(40): p. 25238-25242.
149. Srivastava, R.K., et al., *Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis.* Proceedings of the National Academy of Sciences, 1999. **96**(7): p. 3775-3780.
150. Ruvolo, P.P., et al., *A functional role for mitochondrial protein kinase Cα in Bcl2 phosphorylation and suppression of apoptosis.* Journal of Biological Chemistry, 1998. **273**(39): p. 25436-25442.
151. Scatena, C.D., et al., *Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and Taxol-induced growth arrest.* Journal of Biological Chemistry, 1998. **273**(46): p. 30777-30784.
152. Shitashige, M., et al., *Dissociation of Bax from a Bc1-2/Bax heterodimer triggered by phosphorylation of serine 70 of Bc1-2.* The Journal of Biochemistry, 2001. **130**(6): p. 741-748.
153. TAKAYAMA, S., et al., *Evolutionary conservation of function among mammalian, avian, and viral homologs of the Bcl-2 oncoprotein.* DNA and cell biology, 1994. **13**(7): p. 679-692.
154. Chang, B.S., et al., *Identification of a novel regulatory domain in Bcl-xL and Bcl-2.* The EMBO journal, 1997. **16**(5): p. 968-977.

155. Upreti, M., et al., *Identification of the major phosphorylation site in Bcl-xL induced by microtubule inhibitors and analysis of its functional significance.* Journal of Biological Chemistry, 2008. **283**(51): p. 35517-35525.
156. Kharbanda, S., et al., *Translocation of SAPK/JNK to mitochondria and interaction with Bcl-xL in response to DNA damage.* Journal of Biological Chemistry, 2000. **275**(1): p. 322-327.
157. Thudichum, J.L.W., *A Treatise on the Chemical Constitution of the Brain*. 1962: Archon Books.
158. Hannun, Y.A. and L.M. Obeid, *Sphingolipids and their metabolism in physiology and disease.* Nature reviews Molecular cell biology, 2018. **19**(3): p. 175-191.
159. Sunshine, H. and M.L. Iruela-Arispe, *Membrane lipids and cell signaling.* Current opinion in lipidology, 2017. **28**(5): p. 408.
160. Ogretmen, B., *Sphingolipid metabolism in cancer signalling and therapy.* Nature Reviews Cancer, 2018. **18**(1): p. 33-50.
161. Choi, R.H., et al., *Ceramides and other sphingolipids as drivers of cardiovascular disease.* Nature reviews Cardiology, 2021. **18**(10): p. 701-711.
162. Futerman, A.H. and Y.A. Hannun, *The complex life of simple sphingolipids.* EMBO reports, 2004. **5**(8): p. 777-782.
163. Cha, H.J., et al., *Intercellular and intracellular functions of ceramides and their metabolites in skin.* International Journal of Molecular Medicine, 2016. **38**(1): p. 16-22.
164. Wijesinghe, D.S., et al., *Ceramide kinase is required for a normal eicosanoid response and the subsequent orderly migration of fibroblasts [S].* Journal of lipid research, 2014. **55**(7): p. 1298-1309.
165. Rivera, I.-G., et al., *Ceramide 1-phosphate regulates cell migration and invasion of human pancreatic cancer cells.* Biochemical pharmacology, 2016. **102**: p. 107-119.
166. Bieberich, E., *Ceramide in stem cell differentiation and embryo development: novel functions of a topological cell-signaling lipid and the concept of ceramide compartments.* Journal of lipids, 2011. **2011**.
167. Jayadev, S., et al., *Role for Ceramide in Cell Cycle Arrest (∗).* Journal of Biological Chemistry, 1995. **270**(5): p. 2047-2052.
168. Mencarelli, C. and P. Martinez–Martinez, *Ceramide function in the brain: when a slight tilt is enough.* Cellular and Molecular Life Sciences, 2013. **70**(2): p. 181-203.
169. Wang, J., et al., *Functions of Sphingolipids in Pathogenesis During Host–Pathogen Interactions.* Frontiers in Microbiology, 2021: p. 1926.
170. Obeid, L.M., et al., *Programmed cell death induced by ceramide.* Science, 1993. **259**(5102): p. 1769-1771.
171. Kim, M.-Y., et al., *Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation.* Journal of Biological Chemistry, 1991. **266**(1): p. 484-489.
172. Dbaibo, G.S., et al., *Ceramide generation by two distinct pathways in tumor necrosis factor α-induced cell death.* FEBS letters, 2001. **503**(1): p. 7-12.
173. Sawada, M., et al., *Molecular mechanisms of TNF-α-induced ceramide formation in human glioma cells: P53-mediated oxidant stress-dependent and-independent pathways.* Cell Death & Differentiation, 2004. **11**(9): p. 997-1008.
174. Colell, A., et al., *Ceramide generated by acidic sphingomyelinase contributes to tumor necrosis factor-α-mediated apoptosis in human colon HT-29 cells through*

glycosphingolipids formation: Possible role of ganglioside GD3. FEBS letters, 2002. **526**(1-3): p. 135-141.
175. Cifone, M.G., et al., *Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase.* The Journal of experimental medicine, 1994. **180**(4): p. 1547-1552.
176. Tepper, C.G., et al., *Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity.* Proceedings of the National Academy of Sciences, 1995. **92**(18): p. 8443-8447.
177. Cuvillier, O., L. Edsall, and S. Spiegel, *Involvement of sphingosine in mitochondria-dependent Fas-induced apoptosis of type II Jurkat T cells.* Journal of Biological Chemistry, 2000. **275**(21): p. 15691-15700.
178. Bose, R., et al., *Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals.* Cell, 1995. **82**(3): p. 405-414.
179. Perry, D.K., et al., *Serine palmitoyltransferase regulates de novoceramide generation during etoposide-induced apoptosis.* Journal of Biological Chemistry, 2000. **275**(12): p. 9078-9084.
180. Boojar, M.M.A., et al., *Ceramide generation as a novel biological mechanism for chemo-preventive and cytotoxic effects of hesperidin on HT-144 melanoma cells.* Beni-Suef University journal of basic and applied sciences, 2018. **7**(4): p. 640-645.
181. Biswal, S.S., et al., *Changes in ceramide and sphingomyelin following fludarabine treatment of human chronic B-cell leukemia cells.* Toxicology, 2000. **154**(1-3): p. 45-53.
182. Charles, A.G., et al., *Taxol-induced ceramide generation and apoptosis in human breast cancer cells.* Cancer chemotherapy and pharmacology, 2001. **47**(5): p. 444-450.
183. Modrak, D.E., et al., *Ceramide regulates gemcitabine-induced senescence and apoptosis in human pancreatic cancer cell lines.* Molecular Cancer Research, 2009. **7**(6): p. 890-896.
184. Haimovitz-Friedman, A., et al., *Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis.* The Journal of experimental medicine, 1994. **180**(2): p. 525-535.
185. Liao, W.-C., et al., *Ataxia telangiectasia-mutated gene product inhibits DNA damage-induced apoptosis via ceramide synthase.* Journal of Biological Chemistry, 1999. **274**(25): p. 17908-17917.
186. Bitar, F.F., et al., *Ceramide Accumulation in the Heart of a Hypoxic Rat Model Mimicking Cyanotic Congenital Heart Disease.* Pediatric Research, 1999. **45**(7): p. 20-20.
187. Yoshimura, S.-i., et al., *Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death: inhibitory effects of Bcl-2 on ceramide formation and caspase-3 activation.* Journal of Biological Chemistry, 1998. **273**(12): p. 6921-6927.
188. Wells, G.B., R.C. Dickson, and R.L. Lester, *Heat-induced elevation of ceramide in Saccharomyces cerevisiae via de novo synthesis.* Journal of Biological Chemistry, 1998. **273**(13): p. 7235-7243.
189. Schaefer, J.T., W. Barthlen, and P. Schweizer, *Ceramide induces apoptosis in neuroblastoma cell cultures resistant to CD95 (Fas/APO-1)-mediated apoptosis.* Journal of pediatric surgery, 2000. **35**(3): p. 473-479.
190. Voelkel-Johnson, C., Y.A. Hannun, and A. El-Zawahry, *Resistance to TRAIL is associated with defects in ceramide signaling that can be overcome by exogenous C6-*

ceramide without requiring down-regulation of cellular FLICE inhibitory protein. Molecular cancer therapeutics, 2005. **4**(9): p. 1320-1327.

191. Zhang, Y., T. Yoshida, and B. Zhang, *TRAIL induces endocytosis of its death receptors in MDA-MB-231 breast cancer cells.* Cancer biology & therapy, 2009. **8**(10): p. 917-922.
192. Wright, S.C., H. Zheng, and J. Zhong, *Tumor cell resistance to apoptosis due to a defect in the activation of sphingomyelinase and the 24 kDa apoptotic protease (AP24).* The FASEB journal, 1996. **10**(2): p. 325-332.
193. Cai, Z., et al., *Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF7 cells to tumor necrosis factor-α-mediated cytotoxicity.* Journal of Biological Chemistry, 1997. **272**(11): p. 6918-6926.
194. Heinrich, M., et al., *Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and-3 activation.* Cell Death & Differentiation, 2004. **11**(5): p. 550-563.
195. Minarowska, A., et al., *Regulatory role of cathepsin D in apoptosis.* Folia histochemica et cytobiologica, 2007. **45**(3): p. 159-163.
196. Heinrich, M., et al., *Ceramide as an activator lipid of cathepsin D*, in *Cellular Peptidases in Immune Functions and Diseases 2*. 2002, Springer. p. 305-315.
197. Siskind, L.J., R.N. Kolesnick, and M. Colombini, *Ceramide channels increase the permeability of the mitochondrial outer membrane to small proteins.* Journal of Biological Chemistry, 2002. **277**(30): p. 26796-26803.
198. Siskind, L.J., R.N. Kolesnick, and M. Colombini, *Ceramide forms channels in mitochondrial outer membranes at physiologically relevant concentrations.* Mitochondrion, 2006. **6**(3): p. 118-125.
199. Siskind, L.J. and M. Colombini, *The lipids C2-and C16-ceramide form large stable channels: implications for apoptosis.* Journal of Biological Chemistry, 2000. **275**(49): p. 38640-38644.
200. Lee, H., et al., *Mitochondrial ceramide-rich macrodomains functionalize Bax upon irradiation.* PloS one, 2011. **6**(6): p. e19783.
201. von Haefen, C., et al., *Ceramide induces mitochondrial activation and apoptosis via a Bax-dependent pathway in human carcinoma cells.* Oncogene, 2002. **21**(25): p. 4009-4019.
202. Ganesan, V., et al., *Ceramide and activated Bax act synergistically to permeabilize the mitochondrial outer membrane.* Apoptosis, 2010. **15**(5): p. 553-562.
203. Dobrowsky, R.T., et al., *Ceramide activates heterotrimeric protein phosphatase 2A.* Journal of Biological Chemistry, 1993. **268**(21): p. 15523-15530.
204. Van Hoof, C. and J. Goris, *Phosphatases in apoptosis: to be or not to be, PP2A is in the heart of the question.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2003. **1640**(2-3): p. 97-104.
205. Ozaki, T. and A. Nakagawara, *Role of p53 in cell death and human cancers.* Cancers, 2011. **3**(1): p. 994-1013.
206. Carroll, B., J.C. Donaldson, and L. Obeid, *Sphingolipids in the DNA damage response.* Advances in biological regulation, 2015. **58**: p. 38-52.
207. Hage-Sleiman, R., et al., *p53 and ceramide as collaborators in the stress response.* International journal of molecular sciences, 2013. **14**(3): p. 4982-5012.
208. Heffernan-Stroud, L.A. and L.M. Obeid, *p53 and regulation of bioactive sphingolipids.* Advances in enzyme regulation, 2011. **51**(1): p. 219.

209. Dbaibo, G.S., et al., *p53-dependent ceramide response to genotoxic stress.* The Journal of clinical investigation, 1998. **102**(2): p. 329-339.
210. Jeffries, K.A. and N.I. Krupenko, *Ceramide signaling and p53 pathways.* Advances in cancer research, 2018. **140**: p. 191-215.
211. Fekry, B., et al., *CerS6 is a novel transcriptional target of p53 protein activated by non-genotoxic stress.* Journal of Biological Chemistry, 2016. **291**(32): p. 16586-16596.
212. Shamseddine, A., et al., *P53-dependent upregulation of neutral sphingomyelinase-2: role in doxorubicin-induced growth arrest.* Cell death & disease, 2015. **6**(10): p. e1947-e1947.
213. Stancevic, B. and R. Kolesnick, *Ceramide-rich platforms in transmembrane signaling.* FEBS letters, 2010. **584**(9): p. 1728-1740.
214. Sasset, L., et al., *Sphingolipid de novo biosynthesis: a rheostat of cardiovascular homeostasis.* Trends in Endocrinology & Metabolism, 2016. **27**(11): p. 807-819.
215. Mashima, R., T. Okuyama, and M. Ohira, *Biosynthesis of long chain base in sphingolipids in animals, plants and fungi.* Future Science OA, 2020. **6**(1): p. FSO434.
216. Levy, M. and A.H. Futerman, *Mammalian ceramide synthases.* IUBMB life, 2010. **62**(5): p. 347-356.
217. Lachkar, F., et al., *Dihydroceramides: Their emerging physiological roles and functions in cancer and metabolic diseases.* American Journal of Physiology-Endocrinology and Metabolism, 2021. **320**(1): p. E122-E130.
218. Tan-Chen, S., et al., *Sphingolipid metabolism and signaling in skeletal muscle: From physiology to physiopathology.* Frontiers in Endocrinology, 2020. **11**: p. 491.
219. Kang, M.S., et al., *Hypoxia-induced neuronal apoptosis is mediated by de novo synthesis of ceramide through activation of serine palmitoyltransferase.* Cellular signalling, 2010. **22**(4): p. 610-618.
220. Panjarian, S., et al., *De novo N-palmitoylsphingosine synthesis is the major biochemical mechanism of ceramide accumulation following p53 up-regulation.* Prostaglandins & other lipid mediators, 2008. **86**(1-4): p. 41-48.
221. Rath, G., et al., *De novo ceramide synthesis is responsible for the anti-tumor properties of camptothecin and doxorubicin in follicular thyroid carcinoma.* The international journal of biochemistry & cell biology, 2009. **41**(5): p. 1165-1172.
222. Siddique, M.M., et al., *Dihydroceramides: from bit players to lead actors.* Journal of Biological Chemistry, 2015. **290**(25): p. 15371-15379.
223. Azzam, R., et al., *Regulation of de novo ceramide synthesis: the role of dihydroceramide desaturase and transcriptional factors NFATC and Hand2 in the hypoxic mouse heart.* DNA and cell biology, 2013. **32**(6): p. 310-319.
224. Devlin, C.M., et al., *Dihydroceramide-based response to hypoxia.* Journal of Biological Chemistry, 2011. **286**(44): p. 38069-38078.
225. Pewzner-Jung, Y., S. Ben-Dor, and A.H. Futerman, *When do Lasses (longevity assurance genes) become CerS (ceramide synthases)?: Insights into the regulation of ceramide synthesis.* Journal of Biological Chemistry, 2006. **281**(35): p. 25001-25005.
226. Mullen, T.D., Y.A. Hannun, and L.M. Obeid, *Ceramide synthases at the centre of sphingolipid metabolism and biology.* Biochemical Journal, 2012. **441**(3): p. 789-802.
227. Hernández-Corbacho, M.J., et al., *Tumor necrosis factor-α (TNFα)-induced ceramide generation via ceramide synthases regulates loss of focal adhesion kinase (FAK) and programmed cell death.* Journal of Biological Chemistry, 2015. **290**(42): p. 25356-25373.

228. Ruckhäberle, E., et al., *Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer.* Breast cancer research and treatment, 2008. **112**(1): p. 41-52.
229. Wigger, D., et al., *Monitoring the sphingolipid de novo synthesis by stable-isotope labeling and liquid chromatography-mass spectrometry.* Frontiers in Cell and Developmental Biology, 2019: p. 210.
230. Sokolowska, E. and A. Blachnio-Zabielska, *The role of ceramides in insulin resistance.* Frontiers in Endocrinology, 2019: p. 577.
231. Gillard, B.K., R.G. Clement, and D.M. Marcus, *Variations among cell lines in the synthesis of sphingolipids in de novo and recycling pathways.* Glycobiology, 1998. **8**(9): p. 885-890.
232. Tettamanti, G., et al., *Salvage pathways in glycosphingolipid metabolism.* Biochimie, 2003. **85**(3-4): p. 423-437.
233. Kitatani, K., J. Idkowiak-Baldys, and Y.A. Hannun, *The sphingolipid salvage pathway in ceramide metabolism and signaling.* Cellular signalling, 2008. **20**(6): p. 1010-1018.
234. Hussain, M.M., W. Jin, and X.-C. Jiang, *Mechanisms involved in cellular ceramide homeostasis.* Nutrition & metabolism, 2012. **9**(1): p. 1-7.
235. Chakraborty, M. and X.-C. Jiang, *Sphingomyelin and its role in cellular signaling.* Lipid-mediated Protein Signaling, 2013: p. 1-14.
236. Andrieu-Abadie, N. and T. Levade, *Sphingomyelin hydrolysis during apoptosis.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2002. **1585**(2-3): p. 126-134.
237. Zhang, Y., et al., *Involvement of the acid sphingomyelinase pathway in uva-induced apoptosis.* Journal of Biological Chemistry, 2001. **276**(15): p. 11775-11782.
238. De Palma, C., et al., *Endothelial nitric oxide synthase activation by tumor necrosis factor α through neutral sphingomyelinase 2, sphingosine kinase 1, and sphingosine 1 phosphate receptors: a novel pathway relevant to the pathophysiology of endothelium.* Arteriosclerosis, thrombosis, and vascular biology, 2006. **26**(1): p. 99-105.
239. Nikolova-Karakashian, M.N. and M.B. Reid, *Sphingolipid metabolism, oxidant signaling, and contractile function of skeletal muscle.* Antioxidants & redox signaling, 2011. **15**(9): p. 2501-2517.
240. Karakashian, A.A., et al., *Expression of neutral sphingomyelinase-2 (NSMase-2) in primary rat hepatocytes modulates IL-β-induced JNK activation.* The FASEB journal, 2004. **18**(9): p. 968-970.
241. Marchesini, N., et al., *Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells.* Journal of Biological Chemistry, 2004. **279**(24): p. 25101-25111.
242. Luberto, C., et al., *Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase.* Journal of Biological Chemistry, 2002. **277**(43): p. 41128-41139.
243. Hait, N.C. and A. Maiti, *The role of sphingosine-1-phosphate and ceramide-1-phosphate in inflammation and cancer.* Mediators of inflammation, 2017. **2017**.
244. Pyne, N.J. and S. Pyne, *Sphingosine 1-phosphate and cancer.* Nature Reviews Cancer, 2010. **10**(7): p. 489-503.
245. Diaz Escarcega, R., L.D. McCullough, and A.S. Tsvetkov, *The functional role of sphingosine kinase 2.* Frontiers in Molecular Biosciences, 2021. **8**: p. 428.

246. Arana, L., et al., *Ceramide and ceramide 1-phosphate in health and disease.* Lipids in health and disease, 2010. **9**(1): p. 1-12.
247. Ruckhäberle, E., et al., *Gene expression of ceramide kinase, galactosyl ceramide synthase and ganglioside GD3 synthase is associated with prognosis in breast cancer.* Journal of cancer research and clinical oncology, 2009. **135**(8): p. 1005-1013.
248. Zhu, S., et al., *Ceramide kinase mediates intrinsic resistance and inferior response to chemotherapy in triple-negative breast cancer by upregulating Ras/ERK and PI3K/Akt pathways.* Cancer cell international, 2021. **21**(1): p. 1-11.
249. Kroll, A., H.E. Cho, and M.H. Kang, *Antineoplastic agents targeting sphingolipid pathways.* Frontiers in Oncology, 2020. **10**: p. 833.
250. Wattenberg, B.W., *The long and the short of ceramides.* Journal of Biological Chemistry, 2018. **293**(25): p. 9922-9923.
251. Koybasi, S., et al., *Defects in cell growth regulation by C18: 0-ceramide and longevity assurance gene 1 in human head and neck squamous cell carcinomas.* Journal of Biological Chemistry, 2004. **279**(43): p. 44311-44319.
252. Senkal, C.E., et al., *Antiapoptotic roles of ceramide-synthase-6-generated C16-ceramide via selective regulation of the ATF6/CHOP arm of ER-stress-response pathways.* The FASEB Journal, 2010. **24**(1): p. 296-308.
253. Mesicek, J., et al., *Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells.* Cellular signalling, 2010. **22**(9): p. 1300-1307.
254. Sassa, T., et al., *A shift in sphingolipid composition from C24 to C16 increases susceptibility to apoptosis in HeLa cells.* Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 2012. **1821**(7): p. 1031-1037.
255. Law, B.A., et al., *Lipotoxic very-long-chain ceramides cause mitochondrial dysfunction, oxidative stress, and cell death in cardiomyocytes.* The FASEB Journal, 2018. **32**(3): p. 1403-1416.
256. Schiffmann, S., et al., *Ceramide synthases and ceramide levels are increased in breast cancer tissue.* Carcinogenesis, 2009. **30**(5): p. 745-752.
257. Bielawska, A., et al., *Selectivity of ceramide-mediated biology. Lack of activity of erythro-dihydroceramide.* Journal of Biological Chemistry, 1993. **268**(35): p. 26226-26232.
258. Ueda, N., et al., *Role of enhanced ceramide generation in DNA damage and cell death in chemical hypoxic injury to LLC-PK1 cells.* Kidney international, 1998. **54**(2): p. 399-406.
259. Noureddine, L., et al., *Modulation of total ceramide and constituent ceramide species in the acutely and chronically hypoxic mouse heart at different ages.* Prostaglandins & other lipid mediators, 2008. **86**(1-4): p. 49-55.
260. Kraveka, J.M., et al., *Involvement of dihydroceramide desaturase in cell cycle progression in human neuroblastoma cells.* Journal of Biological Chemistry, 2007. **282**(23): p. 16718-16728.
261. Gagliostro, V., et al., *Dihydroceramide delays cell cycle G1/S transition via activation of ER stress and induction of autophagy.* The international journal of biochemistry & cell biology, 2012. **44**(12): p. 2135-2143.
262. Hernández-Tiedra, S., et al., *Dihydroceramide accumulation mediates cytotoxic autophagy of cancer cells via autolysosome destabilization.* Autophagy, 2016. **12**(11): p. 2213-2229.

263. Siddique, M.M., et al., *Ablation of dihydroceramide desaturase 1, a therapeutic target for the treatment of metabolic diseases, simultaneously stimulates anabolic and catabolic signaling.* Molecular and cellular biology, 2013. **33**(11): p. 2353-2369.
264. Stiban, J., D. Fistere, and M. Colombini, *Dihydroceramide hinders ceramide channel formation: Implications on apoptosis.* Apoptosis, 2006. **11**(5): p. 773-780.
265. Li, L.-y., et al., *DNA Repair Pathways in Cancer Therapy and Resistance.* Frontiers in Pharmacology, 2021. **11**: p. 2520.
266. Matthews, H.K., C. Bertoli, and R.A. de Bruin, *Cell cycle control in cancer.* Nature Reviews Molecular Cell Biology, 2021: p. 1-15.
267. Wang, S., K. Xie, and T. Liu, *Cancer Immunotherapies: From Efficacy to Resistance Mechanisms–Not Only Checkpoint Matters.* Frontiers in Immunology, 2021: p. 2904.
268. Kolesnick, R. and Z. Fuks, *Radiation and ceramide-induced apoptosis.* Oncogene, 2003. **22**(37): p. 5897-5906.
269. Dubois, N., et al., *Plasma ceramide, a real-time predictive marker of pulmonary and hepatic metastases response to stereotactic body radiation therapy combined with irinotecan.* Radiotherapy and Oncology, 2016. **119**(2): p. 229-235.
270. Abdul Aziz, N.A., et al., *A 19-Gene expression signature as a predictor of survival in colorectal cancer.* BMC medical genomics, 2016. **9**(1): p. 1-13.
271. Sheridan, M. and B. Ogretmen, *The Role of Ceramide Metabolism and Signaling in the Regulation of Mitophagy and Cancer Therapy.* Cancers, 2021. **13**(10): p. 2475.
272. Rosa, R., et al., *Sphingosine kinase 1 overexpression contributes to cetuximab resistance in human colorectal cancer models.* Clinical cancer research, 2013. **19**(1): p. 138-147.
273. Bektas, M., et al., *A sphingosine kinase inhibitor induces cell death in temozolomide resistant glioblastoma cells.* Cancer chemotherapy and pharmacology, 2009. **64**(5): p. 1053-1058.
274. Zhang, R., et al., *Network model of survival signaling in large granular lymphocyte leukemia.* Proceedings of the National Academy of Sciences, 2008. **105**(42): p. 16308-16313.
275. Beljanski, V., C.S. Lewis, and C.D. Smith, *Antitumor activity of sphingosine kinase 2 inhibitor ABC294640 and sorafenib in hepatocellular carcinoma xenografts.* Cancer biology & therapy, 2011. **11**(5): p. 524-534.
276. Tran, M.A., et al., *Combining nanoliposomal ceramide with sorafenib synergistically inhibits melanoma and breast cancer cell survival to decrease tumor development.* Clinical Cancer Research, 2008. **14**(11): p. 3571-3581.
277. Morad, S.A., et al., *Ceramide–antiestrogen nanoliposomal combinations—novel impact of hormonal therapy in hormone-insensitive breast cancer.* Molecular cancer therapeutics, 2012. **11**(11): p. 2352-2361.
278. Jiang, Y., et al., *Combinatorial therapies improve the therapeutic efficacy of nanoliposomal ceramide for pancreatic cancer.* Cancer biology & therapy, 2011. **12**(7): p. 574-585.
279. Li, G., et al., *Nanoliposome C6-ceramide increases the anti-tumor immune response and slows growth of liver tumors in mice.* Gastroenterology, 2018. **154**(4): p. 1024-1036. e9.
280. Liu, X., et al., *Targeting of survivin by nanoliposomal ceramide induces complete remission in a rat model of NK-LGL leukemia.* Blood, The Journal of the American Society of Hematology, 2010. **116**(20): p. 4192-4201.

281. Jiang, F., et al., *Liposomal C6 ceramide activates protein phosphatase 1 to inhibit melanoma cells.* PLoS One, 2016. **11**(9): p. e0159849.
282. Ma, Y.-Y., et al., *Delivery systems of ceramide in targeted cancer therapy: ceramide alone or in combination with other anti-tumor agents.* Expert opinion on drug delivery, 2016. **13**(10): p. 1397-1406.
283. Morad, S.A. and M.C. Cabot, *Ceramide-orchestrated signalling in cancer cells.* Nature Reviews Cancer, 2013. **13**(1): p. 51-65.
284. Henry, B., et al., *Targeting the ceramide system in cancer.* Cancer letters, 2013. **332**(2): p. 286-294.
285. Nagahashi, M., et al., *High levels of sphingolipids in human breast cancer.* journal of surgical research, 2016. **204**(2): p. 435-444.
286. Sarkar, S., et al., *Sphingosine kinase 1 is required for migration, proliferation and survival of MCF-7 human breast cancer cells.* FEBS letters, 2005. **579**(24): p. 5313-5317.
287. Sankala, H.M., et al., *Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin.* Cancer research, 2007. **67**(21): p. 10466-10474.
288. EL-ASSAAD, W., et al., *Distinct sites of action of Bcl-2 and Bcl-xL in the ceramide pathway of apoptosis.* Biochemical Journal, 1998. **336**(3): p. 735-741.
289. Dbaibo, G.S., et al., *Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF)-α: CrmA and Bcl-2 target distinct components in the apoptotic pathway.* The Journal of experimental medicine, 1997. **185**(3): p. 481-490.
290. Zhang, J., et al., *Bcl-2 interrupts the ceramide-mediated pathway of cell death.* Proceedings of the National Academy of Sciences, 1996. **93**(11): p. 5325-5328.
291. Allouche, M., et al., *Influence of Bcl-2 overexpression on the ceramide pathway in daunorubicin-induced apoptosis of leukemic cells.* Oncogene, 1997. **14**(15): p. 1837-1845.
292. Birbes, H., et al., *Selective hydrolysis of a mitochondrial pool of sphingomyelin induces apoptosis.* The FASEB Journal, 2001. **15**(14): p. 2669-2679.
293. Tepper, A.D., et al., *Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95-and DNA damage–induced apoptosis.* The Journal of clinical investigation, 1999. **103**(7): p. 971-978.
294. Kawatani, M., et al., *Transmembrane domain of Bcl-2 is required for inhibition of ceramide synthesis, but not cytochrome c release in the pathway of inostamycin-induced apoptosis.* Experimental cell research, 2003. **286**(1): p. 57-66.
295. Chang, K.-T., et al., *Ceramide channels: destabilization by Bcl-xL and role in apoptosis.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2015. **1848**(10): p. 2374-2384.
296. Perera, M.N., et al., *Bax and Bcl-xL exert their regulation on different sites of the ceramide channel.* Biochemical Journal, 2012. **445**(1): p. 81-91.
297. Siskind, L.J., et al., *Anti-apoptotic Bcl-2 family proteins disassemble ceramide channels.* Journal of Biological Chemistry, 2008. **283**(11): p. 6622-6630.
298. Laychock, S.G. and S.M. Sessanna, *Sphingosine 1-phosphate modulates apoptosis in cytokine treated pancreatic islet B-cells*. 2006, Wiley Online Library.

299. Sauer, B., et al., *Sphingosine 1-phosphate is involved in cytoprotective actions of calcitriol in human fibroblasts and enhances the intracellular Bcl-2/Bax rheostat.* Die Pharmazie-An International Journal of Pharmaceutical Sciences, 2005. **60**(4): p. 298-304.
300. Gómez-Muñoz, A., et al., *Ceramide-1-phosphate promotes cell survival through activation of the phosphatidylinositol 3-kinase/protein kinase B pathway.* FEBS letters, 2005. **579**(17): p. 3744-3750.
301. Altucci, L. and H. Gronemeyer, *The promise of retinoids to fight against cancer.* Nature Reviews Cancer, 2001. **1**(3): p. 181-193.
302. Rosenthal, D., et al., *The epidermal response to retinoic acid in cell culture and in vivo.* Alternative Methods in Toxicology, 1993. **1**(9): p. 73-73.
303. De Luca, L.M., K. Kosa, and F. Andreola, *The role of vitamin A in differentiation and skin carcinogenesis.* The Journal of Nutritional Biochemistry, 1997. **8**(8): p. 426-437.
304. Zasada, M. and E. Budzisz, *Retinoids: Active molecules influencing skin structure formation in cosmetic and dermatological treatments.* Advances in Dermatology and Allergology/Postępy Dermatologii i Alergologii, 2019. **36**(4): p. 392.
305. Gumireddy, K., et al., *All-trans-retinoic acid-induced apoptosis in human medulloblastoma: activation of caspase-3/poly (ADP-ribose) polymerase 1 pathway.* Clinical cancer research, 2003. **9**(11): p. 4052-4059.
306. Zhang, H., et al., *All-trans retinoic acid (atRA) differentially induces apoptosis in matched primary and metastatic melanoma cells–a speculation on damage effect of atRA via mitochondrial dysfunction and cell cycle redistribution.* Carcinogenesis, 2003. **24**(2): p. 185-191.
307. Hatoum, A., et al., *Overexpression of retinoic acid receptors alpha and gamma into neoplastic epidermal cells causes retinoic acid-induced growth arrest and apoptosis.* Carcinogenesis, 2001. **22**(12): p. 1955-1963.
308. Koay, D.C., et al., *Anti-tumor effects of retinoids combined with trastuzumab or tamoxifen in breast cancer cells: induction of apoptosis by retinoid/trastuzumab combinations.* Breast Cancer Research, 2010. **12**(4): p. 1-19.
309. Ortiz, M.A., et al., *Retinoids in combination therapies for the treatment of cancer: mechanisms and perspectives.* Drug resistance updates, 2002. **5**(3-4): p. 162-175.
310. Dobrotkova, V., et al., *Traffic lights for retinoids in oncology: molecular markers of retinoid resistance and sensitivity and their use in the management of cancer differentiation therapy.* BMC cancer, 2018. **18**(1): p. 1-13.
311. Fontana, J. and A. Rishi, *Classical and novel retinoids: their targets in cancer therapy.* Leukemia, 2002. **16**(4): p. 463-472.
312. Cooper, J.P., et al., *Clinical development of fenretinide as an antineoplastic drug: Pharmacology perspectives.* Experimental Biology and Medicine, 2017. **242**(11): p. 1178-1184.
313. Hail, N., H.a. Kim, and R. Lotan, *Mechanisms of fenretinide-induced apoptosis.* Apoptosis, 2006. **11**(10): p. 1677-1694.
314. Oridate, N., et al., *Involvement of reactive oxygen species in N-(4-hydroxyphenyl) retinamide-induced apoptosis in cervical carcinoma cells.* JNCI: Journal of the National Cancer Institute, 1997. **89**(16): p. 1191-1198.
315. Maurer, B.J., et al., *Increase of ceramide and induction of mixed apoptosis/necrosis by N-(4-hydroxyphenyl)-retinamide in neuroblastoma cell lines.* Journal of the National Cancer Institute, 1999. **91**(13): p. 1138-1146.

316. Delia, D., et al., *Role of antioxidants and intracellular free radicals in retinamide-induced cell death.* Carcinogenesis, 1997. **18**(5): p. 943-948.
317. Boya, P., et al., *The chemopreventive agent N-(4-hydroxyphenyl) retinamide induces apoptosis through a mitochondrial pathway regulated by proteins from the Bcl-2 family.* Oncogene, 2003. **22**(40): p. 6220-6230.
318. Kalli, K.R., et al., *Heterogeneous role of caspase-8 in fenretinide-induced apoptosis in epithelial ovarian carcinoma cell lines.* Molecular pharmacology, 2003. **64**(6): p. 1434-1443.
319. Darwiche, N., et al., *Retinoic acid dramatically enhances the arsenic trioxide-induced cell cycle arrest and apoptosis in retinoic acid receptor alpha-positive human T-cell lymphotropic virus type-I-transformed cells.* The Hematology Journal, 2001. **2**(2): p. 127-135.
320. Darwiche, N., et al., *N-(4-hydroxyphenyl) retinamide induces growth arrest and apoptosis in HTLV-I-transformed cells.* Leukemia, 2004. **18**(3): p. 607-615.
321. Darwiche, N., G. Abou-Lteif, and A. Bazarbachi, *Reactive oxygen species mediate N-(4-hydroxyphenyl) retinamide-induced cell death in malignant T cells and are inhibited by the HTLV-I oncoprotein Tax.* Leukemia, 2007. **21**(2): p. 261-269.
322. Rahmaniyan, M., et al., *Identification of dihydroceramide desaturase as a direct in vitro target for fenretinide.* Journal of Biological Chemistry, 2011. **286**(28): p. 24754-24764.
323. Valsecchi, M., et al., *Sphingolipidomics of A2780 human ovarian carcinoma cells treated with synthetic retinoids.* Journal of lipid research, 2010. **51**(7): p. 1832-1840.
324. Han, H.S., et al., *Potent effect of 5-HPBR, a butanoate derivative of 4-HPR, on cell growth and apoptosis in cancer cells.* International journal of cancer, 2004. **109**(1): p. 58-64.
325. Ghandour, B., et al., *Restoration of ceramide de novo synthesis by the synthetic retinoid ST1926 as it induces adult T-cell leukemia cell death.* Bioscience reports, 2020. **40**(10): p. BSR20200050.
326. Karam, L., et al., *Antitumor activity of the synthetic retinoid ST1926 on primary effusion lymphoma in vitro and in vivo models.* Oncology Reports, 2018. **39**(2): p. 721-730.
327. Aouad, P., et al., *Antitumor activities of the synthetic retinoid ST1926 in two-dimensional and three-dimensional human breast cancer models.* Anti-Cancer Drugs, 2017. **28**(7): p. 757-770.
328. Abdel-Samad, R., et al., *Mechanism of action of the atypical retinoid ST1926 in colorectal cancer: DNA damage and DNA polymerase α.* American journal of cancer research, 2018. **8**(1): p. 39.
329. Cincinelli, R., et al., *A novel atypical retinoid endowed with proapoptotic and antitumor activity.* Journal of medicinal chemistry, 2003. **46**(6): p. 909-912.
330. Papamichos-Chronakis, M., et al., *Global regulation of H2A. Z localization by the INO80 chromatin-remodeling enzyme is essential for genome integrity.* Cell, 2011. **144**(2): p. 200-213.
331. Giaimo, B.D., et al., *The histone variant H2A. Z in gene regulation.* Epigenetics & chromatin, 2019. **12**(1): p. 1-22.
332. Fratelli, M., et al., *New insights into the molecular mechanisms underlying sensitivity/resistance to the atypical retinoid ST1926 in acute myeloid leukaemia cells: The role of histone H2A. Z, cAMP-dependent protein kinase A and the proteasome.* European Journal of Cancer, 2013. **49**(6): p. 1491-1500.

333. Hałubiec, P., et al., *Synthetic Retinoids as Potential Therapeutics in Prostate Cancer—An Update of the Last Decade of Research: A Review.* International Journal of Molecular Sciences, 2021. **22**(19): p. 10537.
334. Bu, Y., et al., *Therapeutic Potential of SphK1 Inhibitors Based on Abnormal Expression of SphK1 in Inflammatory Immune Related-Diseases.* Frontiers in Pharmacology, 2021: p. 2872.
335. Heffernan-Stroud, L.A. and L.M. Obeid, *Sphingosine kinase 1 in cancer.* Advances in cancer research, 2013. **117**: p. 201-235.
336. Nagahashi, M., et al., *Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis.* Cancer research, 2012. **72**(3): p. 726-735.
337. Sauer, L., et al., *Sphingosine kinase 1 inhibition sensitizes hormone-resistant prostate cancer to docetaxel.* International journal of cancer, 2009. **125**(11): p. 2728-2736.
338. French, K.J., et al., *Discovery and evaluation of inhibitors of human sphingosine kinase.* Cancer research, 2003. **63**(18): p. 5962-5969.
339. Jäättelä, M., et al., *Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells.* Oncogene, 1995. **10**(12): p. 2297-2305.
340. Herrmann, J.L., et al., *Bcl-2 suppresses apoptosis resulting from disruption of the NF-κB survival pathway.* Experimental cell research, 1997. **237**(1): p. 101-109.
341. Gottlieb, E., M.G. Vander Heiden, and C.B. Thompson, *Bcl-xL prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis.* Molecular and cellular biology, 2000. **20**(15): p. 5680-5689.
342. Jeoung, D.-i., B. Tang, and M. Sonenberg, *Effects of tumor necrosis factor-α on antimitogenicity and cell cycle-related proteins in MCF-7 cells.* Journal of Biological Chemistry, 1995. **270**(31): p. 18367-18373.
343. Tewari, M. and V.M. Dixit, *Fas-and Tumor Necrosis Factor-induced Apoptosis Is Inhibited by the Poxvirus crmA Gene Product (∗).* Journal of Biological Chemistry, 1995. **270**(7): p. 3255-3260.
344. Luo, X., et al., *Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors.* Cell, 1998. **94**(4): p. 481-490.
345. Roucou, X., et al., *Bid induces cytochrome c-impermeable Bax channels in liposomes.* Biochemical Journal, 2002. **363**(3): p. 547-552.
346. Pei, Y., et al., *Real-time monitoring full length bid interacting with Bax during TNF-α-induced apoptosis.* Apoptosis, 2007. **12**(9): p. 1681-1690.
347. Sedlak, T.W., et al., *Multiple Bcl-2 family members demonstrate selective dimerizations with Bax.* Proceedings of the National Academy of Sciences, 1995. **92**(17): p. 7834-7838.
348. Yang, E., et al., *Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death.* Cell, 1995. **80**(2): p. 285-291.
349. Alsanafi, M., et al., *Dihydroceramide desaturase functions as an inducer and rectifier of apoptosis: effect of retinol derivatives, antioxidants and phenolic compounds.* Cell Biochemistry and Biophysics, 2021. **79**(3): p. 461-475.
350. Alsanafi, M., et al., *Native and polyubiquitinated forms of dihydroceramide desaturase are differentially linked to human embryonic kidney cell survival.* Molecular and cellular biology, 2018. **38**(23): p. e00222-18.

351. Siddique, M.M., et al., *Ablation of dihydroceramide desaturase confers resistance to etoposide-induced apoptosis in vitro.* 2012.
352. Hartmann, D., et al., *Long chain ceramides and very long chain ceramides have opposite effects on human breast and colon cancer cell growth.* The international journal of biochemistry & cell biology, 2012. **44**(4): p. 620-628.
353. Siskind, L.J., et al., *The BCL-2 protein BAK is required for long-chain ceramide generation during apoptosis.* Journal of Biological Chemistry, 2010. **285**(16): p. 11818-11826.
354. Kroesen, B.-J., et al., *BcR-induced apoptosis involves differential regulation of C16 and C24-ceramide formation and sphingolipid-dependent activation of the proteasome.* Journal of Biological Chemistry, 2003. **278**(17): p. 14723-14731.
355. Basma, H., et al., *The synthetic retinoid ST 1926 as a novel therapeutic agent in rhabdomyosarcoma.* International journal of cancer, 2016. **138**(6): p. 1528-1537.
356. Rokhlin, O.W., et al., *p53 is involved in tumor necrosis factor-α-induced apoptosis in the human prostatic carcinoma cell line LNCaP.* Oncogene, 2000. **19**(15): p. 1959-1968.
357. Pastor, D.M., R.B. Irby, and L.S. Poritz, *Tumor Necrosis Factor α Induces p53 Up-regulated Modulator of Apoptosis Expression in Colorectal Cancer Cell Lines.* Diseases of the colon & rectum, 2010. **53**(3): p. 257-263.
358. Noutomi, T., et al., *Bcl-xL confers multi-drug resistance in several squamous cell carcinoma cell lines.* Oral oncology, 2002. **38**(1): p. 41-48.
359. Chiou, S.-K., L. Rao, and E. White, *Bcl-2 blocks p53-dependent apoptosis.* Molecular and cellular biology, 1994. **14**(4): p. 2556-2563.
360. Wang, Y., et al., *bcl-2 Inhibits Wild-Type p53-triggered Apoptosis but not G~ 1 Cell Cycle Arrest and Transactivation of WAF1 and bax.* Cell Growth and Differentiation-Publication American Association for Cancer Research, 1995. **6**(9): p. 1071-1076.
361. Thomas, S., et al., *Targeting the Bcl-2 family for cancer therapy.* Expert opinion on therapeutic targets, 2013. **17**(1): p. 61-75.