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RESTORATION OF CERAMIDE DE NOVO SYNTHESIS BY THE SYNTHETIC RETINOID ST1926 AS IT INDUCES ADULT T CELL LEUKEMIA CELL DEATH

by
BOTHEINA KHALIL GHANDOUR

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AN ABSTRACT OF THE DISSERTATION OF

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Title: Restoration of ceramide *de novo* synthesis by the synthetic retinoid ST1926 as it induces adult T-cell leukemia cell death

Ceramide is a potently bioactive cellular lipid with compartmentalized and tightly regulated levels. Distinct metabolic pathways lead to the generation of several ceramide species with distinguishable roles in oncogenesis, cancer therapy, and the stress response. Deregulation of ceramide pathways has emerged as an important mechanism for acquired chemotherapeutic resistance and disease progression. In fact, decreased cellular ceramide seems to be a hallmark of innate chemotherapy resistance. Adult T cell leukemia/lymphoma (ATL) is an aggressive neoplasm that develops following infection with Human T cell Lymphotropic Virus-1 (HTLV-1) where the viral oncogene Tax largely contributes to the pathogenesis and progression of the disease. Given the fact that ATL, similar to most blood cancers, displays a genetically heterogeneous clonal profile with extensive chemotherapy resistance, alternative therapies that could restore lethal levels of ceramide might have potential promise. In this context, retinoids could be used alone or as an adjuvant treatment to trigger a ceramide-centered approach to cancer therapy. We have previously reported that the synthetic retinoid ST1926 is a potent inducer of growth inhibition and massive apoptosis in malignant T cells and that ATL cells are defective in ceramide synthesis. The perturbation of ceramide pathways through which Tax exerts its tumor promoting functions in ATL remains poorly defined. At the mitochondrial level, the intrinsic pathway of apoptosis is tightly regulated by the Bcl-2 family members and orchestrated by the bioactive sphingolipid ceramide.

We investigated the effect of ST1926 on ceramide accumulation in malignant T cells, modulation of ceramide and dihydroceramide species, mechanisms of ceramide production, ceramide synthases (CerS) activity, gene, and protein expression levels, and cell death and ceramide response in a Bcl-2 overexpression leukemia model. HTLV-1 positive and negative cell lines were treated with ST1926 to determine the dose-response and kinetics of ceramide accumulation. Total cellular ceramide was measured using the diacylglycerol kinase assay. *De novo* ceramide synthesis was determined by measuring [^{3}H] labelled palmitic acid incorporation into ceramide. Sphingolipidomics analysis was performed using liquid chromatography-mass spectrometry (LC-MS). The activities of CerS(s) and dihydroceramide desaturase enzymes were indirectly measured by labeling cells with the unnatural 17-carbon sphinganine followed by LC-MS measurement of the resulting 17C-backbone products.

ST1926 treatment results in early Tax oncoprotein degradation in HTLV-1 treated cells. ST1926 induces cell death and a dose- and time-dependent accumulation of ceramide in HTLV-1 positive and negative malignant T cells. The kinetics and degree of ceramide production shows an early unique response upon ST1926 treatment *versus* previous synthetic retinoids such as HPR and CD437. Similarly, Tax degradation is unique to ST1926. Compared to HPR, which leads to the preferential accumulation of dihydroceramide, ST1926 enhances *de novo* ceramide synthesis via activation of CerS without inhibiting dihydroceramide desaturase, thereby accumulating ceramide rather than the less bioactive dihydroceramide. We showed that ST1926 preferentially induces the activities of a distinct set of CerS(s). Treatment with ST1926 increases gene expression of CerS(s) more prominently in HTLV-1 positive cells. Since ceramide synthesis follows ST1926-induced degradation of Tax, this raises the possibility that Tax is responsible for the ceramide synthesis defect in ATL cells. We detected a delay in cell death response and interruption of ceramide generation in response to ST1926 in Molt-4 cells overexpressing Bcl-2. These results highlight the potential role of ST1926 in inducing ceramide levels, thus lowering the threshold for cell death in malignant T cells.

CONTENTS

ILLUSTRATIONS

TABLES

ABBREVIATIONS

AF1q	ALL1-fused from chromosome 1q
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATL	adult T-cell leukemia/lymphoma
ATO	arsenic trioxide
ATRA	all-*trans* retinoic acid
BAK	Bcl‑2 homologous antagonist/killer
CAPP	ceramide-activated protein phosphatases
CDase	ceramidase
CD437	6-[3-(1-adamantyl)-4-hydroxyphenyl] -2-naphthalene carboxylic acid
CDK	cyclin dependent kinase
CerS	ceramide synthase
CERK	ceramide kinase
CERT	ceramide transfer protein
CLN	ceroid lipofuscinoses
CML	chronic myelogenous leukemia
COUP-TFI	chicken ovalbumin upstream promoter transcription factor I
C1P	ceramide-1-phosphate
SMS	sphingomyelin synthase
DEGS1	dihydroceramide desaturase
DGK	diacylglycerol kinase
DM-102	(2R,3Z)-N-(1-hydroxyoctadec-3-en-2-yl) pivaloylamide
DMS	dimethylsphingosine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ERα	estrogen receptor-α
SEFT	Ewing's sarcoma family of tumor
FBS	fetal bovine serum
FB1	fumonisin B1

FTY720	fingolimod
GCase	glucosylceramidase
GCS	glucosylceramide synthase
HPR	N-(4-hydroxyphenyl) retinamide
HTLV-1	human T-cell lymphotropic virus type-1
IκB	inhibitor of kappa b
JNK	c-Jun N-terminal Kinase
LC-MS	liquid chromatography-mass spectrometry
LXR	liver X receptors
MDR	multi-drug resistant
MLC	medium long-chain
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOE	N-oleoyl-ethanol amine
PBS	phosphate-buffered saline
PDMP	1-phenyl-2-decanoylamino-3-morpholinopropanol
PLAB	PLAcental Bone morphogenetic protein
PML	promyelocytic leukemia
POLA1	DNA polymerase 1 alpha
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PPMP	1-phenyl-2 palmitoylamino-3-morpholino-1- propanol
c-RA	*cis*-retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RB	retinoblastoma
RNA	ribonucleic acid
ROS	reactive oxygen species
RRMs	retinoid related molecules
RXR	retinoid X receptor
SD	standard deviation
SDS	sodium dodecyl sulfoxide
SMase	sphingomyelinase
SK	sphingosine kinase

SMS	sphingomyelin synthase
S1P	sphingosine-1-phosphate
SPT	serine palmitoyltransferase
ST1926	E-4-(4’-hydroxy-3’-adamantyl biphenyl-4-yl) acrylic acid TGZ troglitazone
TLC	thin layer chromatography
TNF-1	tumor necrosis factor-1
UV	ultraviolet
VLC	very long-chain
XIAP	X-lined inhibitor of apoptosis

CHAPTER I

THE UNFOLDING ROLE OF CERAMIDE IN COORDINATING RETINOID-BASED CANCER THERAPY

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A. Abstract

Sphingolipid-mediated regulation in cancer development and treatment is largely ceramide-centered with the complex sphingolipid metabolic pathways unfolding as attractive targets for anticancer drug discovery. The dynamic interconversion of sphingolipids is tightly controlled at the level of enzymes and cellular compartments in response to endogenous or exogenous stimuli, such as anticancer drugs, including retinoids. Over the past two decades, evidence emerged that retinoids owe part of their potency in cancer therapy to modulation of sphingolipid metabolism and ceramide generation. Ceramide has been proposed as a "tumor-suppressor lipid" that orchestrates cell growth, cell cycle arrest, cell death, senescence, autophagy, and metastasis. There is accumulating evidence that cancer development is promoted by the dysregulation of tumor-promoting sphingolipids whereas cancer treatments can kill tumor cells by accumulating endogenous ceramide levels. Resistance to cancer therapy may develop due to a disrupted equilibrium between the opposing roles of tumor-suppressor and tumor-promoter sphingolipids. Despite the undulating effect and complexity of sphingolipid pathways, there are emerging opportunities for a plethora of enzyme-targeted therapeutic interventions that overcome resistance resulting from perturbed sphingolipid pathways. Here, we have revisited the interconnectivity of sphingolipid

metabolism and the instrumental role of ceramide-biosynthetic and degradative enzymes, including bioactive sphingolipid products, how they closely relate to cancer treatment and pathogenesis, and the interplay with retinoid signaling in cancer. We focused on retinoid targeting, alone or in combination, of sphingolipid metabolism nodes in cancer to enhance ceramide-based therapeutics. Retinoid and ceramide-based cancer therapy using novel strategies such as combination treatments, synthetic retinoids, ceramide modulators, and delivery formulations hold promise in the battle against cancer.

B. Introduction

The simple mention of sphingolipids sounds obscure for those who are not familiar with their chemistry and nomenclature. However, it has become difficult to find an area where this class of lipids does not crucially entail bioactivity in the context of normal physiology and diseases where they orchestrate key roles in signaling and regulation of cellular processes. The rapid growth of the sphingolipids field, marked by discoveries over the past three to four decades, has established evolutionary landmarks in the life of these lipids, namely ceramide. Once placed in a second-class position that was hardly appreciated at first partly due to its hydrophobicity, ceramide has magnificently gained the patronizing status of a regulator of cell fate. Besides being widely recognized as a lipid second messenger, it is this extreme hydrophobicity that adds up to the unique properties of ceramide dynamics as it allows this sphingolipid to alter the biophysical properties of membranes and subsequent signaling, best described as a “domino effect” (Morad and Cabot 2013). Ceramide is in fact a lipid tumor suppressor that rewires the ability enables anticancer agents to control the equilibrium

between cell survival and cell death that is otherwise compromised in tumors. A plethora of stress stimuli has defined potential roles of ceramide in apoptosis, autophagy, senescence, cell cycle arrest, metastasis, and inflammation (Ogretmen and Hannun 2004). However, Defects in ceramide generation and metabolism in cancer cells unwittingly counteract the antineoplastic functions of ceramide and contribute to tumor cell survival and resistance to therapy. Retinoids are potent compounds known for their tumor-suppressive roles as they regulate cell fate and differentiation in a variety of solid tumors and hematological malignancies. Retinoids, in turn, owe a multitude of their anti-tumor potential to modulation of sphingolipid metabolism and ceramide generation (Murate, Suzuki et al. 2002, Rehman, Shanmugasundaram et al. 2004, Dbaibo, Kfoury et al. 2007, Fazi, Bursch et al. 2008, Liu, Su et al. 2010, Mao, Sun et al. 2010, Huynh, Sultan et al. 2019, Noguera, Catalano et al. 2019). Retinoids can influence one or more of the ceramide-metabolizing enzymes, including serine palmitoyltransferase (SPT), sphingomyelinase (SMase), ceramidase (CDase), and glucosylceramide synthase (GCS), which are the most important enzymes in the metabolic pathways of sphingolipids and cancer. Although all-*trans* retinoic acid (ATRA), the active metabolite of vitamin A, is used for the treatment of certain leukemia types, particularly acute promyelocytic leukemia (APL), the use of natural retinoids is hindered by acquired resistance and side effect (Huynh, Sultan et al. 2019). The retinoid related molecules (RRMs), such as N-(4-hydroxyphenyl)retinamide (HPR), 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437), and (2E)-3-[3'-(1-adamantyl)-4'-hydroxy[1,1'-biphenyl]-4-yl]-2-propenoic acid (ST1926) show potent anti-tumor activities (Darwiche, El-Sabban et al. 2001, Cincinelli, Dallavalle et al. 2003, Darwiche, Hatoum et al. 2004, Garattini, Gianni et al. 2004,

Garattini, Parrella et al. 2004, El Hajj, Khalil et al. 2014, Abdel-Samad, Aouad et al. 2018). These synthetic retinoids are promising compounds with high therapeutic potential in hematological and solid malignancies. Here, the realm of ceramide-centered approaches in retinoid cancer therapeutics is discussed. It also draws attention to targeting specific metabolic points that fortify the tumor-suppressive functions of ceramide and correct these defects in ceramide metabolism to overcome therapy resistance via retinoid signaling.

C. Ceramide: the Building Block of Sphingolipids

1. Overview on Sphingolipids

Sphingolipids are a class of lipids with which have a backbone of sphingoid base in common, which have been named after the Greek Sphinx of Thebes, because of their enigmatic properties (Futerman and Hannun 2004). It had been long thought that sphingolipids merely play structural roles in the regulation of plasma membrane fluidity and lipid rafts (Ogretmen and Hannun 2004). This changed with the emerging molecular and biochemical studies that added up over the past two to three decades describing sphingolipid myriad cellular functions. The sphingolipids ceramide, ceramide-1-phosphate, glucosylceramide, lactosylceramide, galactosylceramide, sphingosine, sphingosylphosphocholine, psychosine and sphingosine-1-phosphate (S1P) and others, exert crucial functions in several biological processes. Ceramide is at the center of sphingolipid metabolism and a crucial second messenger for cellular homeostasis, regulating cellular proliferation and death, stress response, cell cycle, senescence, and inflammation (Ogretmen and Hannun 2004, Mullen, Hannun et al.

2012, Castro, Prieto et al. 2014, Dany and Ogretmen 2015, Coant, Sakamoto et al. 2017).

2. Ceramide Biosynthesis and Degradation

Ceramide or N-acylsphingosine consists of a C18-sphingoid base backbone with an amino group to which a fatty acid chain made up of 14 to 26 carbons is bound (Reynolds, Maurer et al. 2004, Hartmann, Wegner et al. 2013). Ceramides can have mono-unsaturated or saturated fatty acid chains with various lengths that dictate their different physical properties (Hannun and Obeid 2011). Indeed, ceramide is no longer considered a single entity, but a family of over 200 structurally related sphingolipids that belong to the second largest class of membrane lipids (Hannun and Obeid 2011, Hannun and Obeid 2018). The most commonly found ceramides in mammalian cell membranes contain C16-C24 fatty acyl chains (Morad and Cabot 2013, Wegner, Schiffmann et al. 2016, Ghandour, Pisano et al. 2020). Numerous specialized enzymes in different compartment localizations regulate the levels of ceramide. Based upon the cell type and stimulus, endogenous levels of ceramide can be induced via three main metabolic pathways (Figure 1.1). First, ceramide can be generated *de novo* in the endoplasmic reticulum (ER) and on ER-associated membranes, such as the perinuclear membrane and mitochondria-associated membranes, and in mitochondria (Hannun and Obeid 2008, Bartke and Hannun 2009, Hernandez-Corbacho, Salama et al. 2017). The *de novo* ceramide-generation pathway is initiated by the condensation of L-serine and palmitoyl-CoA, catalyzed by SPT to form 3-ketosphinganine, which is then reduced into dihydrosphingosine (sphinganine) by the action of 3-ketosphinganine reductase. Then, dihydroceramide synthase enzyme, also called ceramide synthase (CerS), of

which there are six isoforms, acylates dihydrosphingosine to produce dihydroceramide (Taha, Mullen et al. 2006, Hannun and Obeid 2011, Wegner, Schiffmann et al. 2016). Different CerS(s) preferentially attach a relatively specific range of fatty acyl chain lengths to form dihydroceramide (s), supporting the idea of "many ceramides" that are metabolized by more than 28 enzymes (Hannun and Obeid 2011) . Although these isozymes seem to localize primarily in the ER, specific CerS(s) reside in the perinuclear region and mitochondria. Others might translocate to Golgi. In a final step, dihydroceramide is converted to ceramide by an oxidation reaction catalyzed by the enzyme dihydroceramide desaturase (DEGS1). Subsequently, a double bond is introduced between C4 and C5 of the sphingosine backbone, which is essential for its activity (Ogretmen and Hannun 2004, Hannun and Obeid 2018). ER-generated ceramide is then transported to the Golgi via the ceramide transfer protein (CERT), or through vesicular trafficking, and can be further metabolized into complex sphingolipids (Hanada, Kumagai et al. 2003).

Alternatively, ceramide can be produced from the hydrolysis of sphingomyelin at the cell membrane by the action of acidic, neutral, or alkaline SMase (Ogretmen and Hannun 2004). Sphingomyelinase is also active in lysosomes and mitochondria (Hannun and Obeid 2011). Finally, ceramide can be generated through a more complex mechanism, the salvage pathway, which accounts for 50% to 60% of sphingolipid biosynthesis (Ogretmen and Hannun 2004, Hannun and Obeid 2011). This latter pathway occurs in acidic compartments of the cell, such as late endosomes and lysosomes, where complex sphingolipids are degraded into sphingosine. In addition, ceramide is hydrolyzed by the action of lysosomal CDase into sphingosine and a free fatty acid, which unlike ceramide, can be released from the lysosome (Heinrich,

Neumeyer et al. 2004, Kitatani, Idkowiak-Baldys et al. 2008) and is probably trapped by CerS at the surface (Mullen, Jenkins et al. 2011). Ceramide is the hub of biosynthesis and degradation of other sphingolipids and controls homeostasis of sphingolipid metabolism (Huang, Chen et al. 2011). Ceramide can be further metabolized into bioactive sphingolipids such as glucosylceramide and sphingomyelin, produced by the enzymatic actions of GCS and sphingomyelin synthase (SMS), respectively (Ogretmen and Hannun 2004, Hannun and Obeid 2018). Ceramide could be further phosphorylated by ceramide kinase (CERK) to produce ceramide 1-phosphate (C1P) or hydrolyzed by CDases to generate sphingosine. The latter is acted upon by sphingosine kinase (SK) to produce S1P. Alternatively, ceramide could be generated by dephosphorylation of C1P or S1P via derivative- specific phosphatases, such as C1P phosphatase and S1P phosphatase. The fluidity of enzymatic activities is depicted in one of the very interconnected metabolic systems in cancer regulation, the "sphingolipid rheostat", whereby ceramide acts as a tumor-suppressor lipid and S1P acts as a tumor-promoting lipid (Cuvillier, Pirianov et al. 1996, Shaw, Costa-Pinheiro et al. 2018).

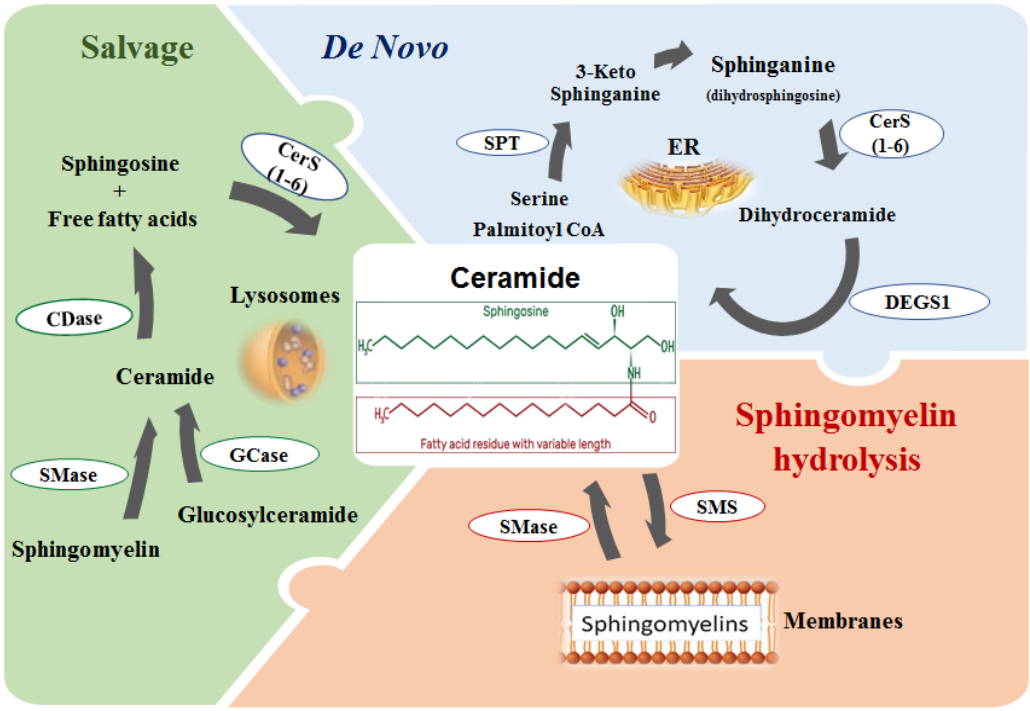

Figure 1.1. The Anabolic and Catabolic Pathways for Ceramide Generation. In the endoplasmic reticulum, ceramide can be synthesized via the *de novo* pathway from the initial condensation reaction of palmitoyl Co-A and serine by SPT, followed by the multistep reduction, addition, and oxidation reactions that lead to ceramide production. Alternatively, ceramide can be generated from the hydrolysis of sphingomyelin by different isoforms of sphingomyelinase, namely in the cytoplasmic membrane. In acidic compartments such as lysosomes, complex sphingolipids can be indirectly recycled via degradation into ceramide. Ceramide synthases (1-6), which are important enzymes in the *de novo* and salvage pathways, produce ceramide by attaching sphingosine to free fatty acids with different chain lengths.

CDase, ceramidase; **CerS**, ceramide synthase; **DEGS1**, dihydroceramide desaturase; **ER**, endoplasmic reticulum, **GCase**, glucosyceramidase; **SMase**, sphingomyelinase; **SMS**, sphingomyelin synthase; **SPT**, serine palmitoyltransferase.

3. Ceramide Cellular Mechanisms and Downstream Targets

Ceramide initiates anti-proliferative responses through several mechanisms that comprise cell cycle arrest, apoptosis, necroptosis, mitophagy, senescence, and/or differentiation (Ogretmen and Hannun 2004, Ogretmen 2018). Central to the multiplicity of ceramide tumor-suppressive functions is its extensively studied role in

apoptosis over the past three to four decades (Ogretmen and Hannun 2004). Multiple apoptotic stimuli including chemotherapeutic drugs, ultraviolet radiation (UV), hypoxia, CD95, tumor necrosis factor-1 (TNF-1), and DNA damage increase endogenous levels of ceramide supporting the role of ceramide in apoptosis (Pettus, Chalfant et al. 2002). Studies have shown that endogenous levels of ceramide controlled by specific enzymes are accumulated prior to activation of the apoptotic cascade (Dbaibo, Pushkareva et al. 1998). Ceramide accumulation seems to be highly compartmentalized and is based on the type and dosage of stimuli and cell type which dictate the subsequent response of its transduced functional effects (Ogretmen and Hannun 2004, Huang, Chen et al. 2011). In the ER, *de novo* ceramide is elevated in response to chemotherapeutic agents such as etoposide, daunorubicin, gemcitabine, cannabinoids, Fas ligand, and HPR (Chalfant, Ogretmen et al. 2001, Wang, Maurer et al. 2001, Chalfant, Rathman et al. 2002). Moreover, HPR enhances *de novo* ceramide synthesis via the activation of SPT and/or CerS, with simultaneous inhibition of DEGS1 (Valsecchi, Aureli et al. 2010).

The simple design of ceramide-mediated stimulus-response coupling is dictated by its interaction with distinct compartmentalized targets and regulation of membrane dynamics. Ceramide regulates a set of cellular phosphoproteins by activating protein phosphatases of PP1 and protein phosphatase 2A (PP2A) families (Hannun and Obeid 2018). At the plasma membrane, sphingomyelin hydrolysis generates ceramide that recruits protein phosphatases PP1Cα to dephosphorylate its substrates, such as ezrin (Canals, Roddy et al. 2012). Ezrin acts as a linker between plasma membrane and the actin cytoskeleton, which adds to the important roles of ceramide, such as cell adhesion, migration, and the organization of cell surface structures (Lamb, Ozanne et al. 1997, Adada, Canals et al. 2015). Ceramide formed by the action of acid SMase (aSMase)

regulates endocytosis, receptor signaling, and TNF superfamily member 6 (FAS) signaling via formation of specialized membrane sub-compartments known as lipid rafts (Hannun and Obeid 2018). Meanwhile, neutral SMase2 (nSMase2) is important for exocytosis, and SK1 and S1P control endocytosis. In the context of apoptosis, *de novo*-formed ceramide can activate the tumor suppressor serine/threonine-PP2A that results in dephosphorylation and inactivation of AKT. Similarly, the pro-apoptotic protein Bcl-2 homologous antagonist/killer (BAK) acts upstream of ceramide leading to activation of CerS, with the subsequent activation of PP2A, dephosphorylation, and inactivation of anti-apoptotic Bcl-2 proteins. Also, at the level of mitochondria, ceramide induces activity of the pro-apoptotic protein Bax. Meanwhile, Bax activates CerS in the outer mitochondrial membrane (Siskind 2005). Accumulating evidence illustrates the integral role of ceramide in the intrinsic mitochondrial pathway and the extrinsic pathway of cell death (Kroemer, Galluzzi et al. 2007). ER-generated ceramide trans-locates to the mitochondria along with another pool generated in mitochondria, where it activates PP2A (Siskind 2005). In the mitochondria, ceramide has various effects, including the release of mitochondrial intermembrane space proteins, cytochrome c, generation of reactive oxygen species (ROS) and alteration of calcium homeostasis of mitochondria and the ER (Pinton, Ferrari et al. 2001, Siskind, Kolesnick et al. 2002). In the intrinsic pathway, mitochondria can be targeted directly or through signaling transduction pathways via Bcl-2 proapoptotic members, Bax and Bak, which release cytochrome c and lead to caspase activation and apoptosis (Amaral, Xavier et al. 2010). Mitochondrial ceramides form large stable barrel-like channels either alone or with Bax (Colombini 2010). These channels allow cytochrome c release into the cytosol and activation of executioner caspases, such as caspase 3 (Birbes, El Bawab et al.

2001). Ardail *et al* have shown that mitochondrial outer membrane contains at least three-fold ceramide levels than the inner membrane (Ardail, Popa et al. 2001). In fact, enzymes involved in ceramide biosynthesis and hydrolysis, such as CerS and CDase, are present in the mitochondria (Bionda, Portoukalian et al. 2004), in addition to nSMase (Ogretmen and Hannun 2004). Ceramide generated here can activate the ceramide-activated protein phosphatases, PP1 and PP2A. PP2A can then dephosphorylate and inactivate anti-apoptotic proteins such as Bcl-2 and AKT, leading to apoptosis. PP1 also acts on the pro-apoptotic protein BID to potentiate cell death (Ogretmen and Hannun 2004). Bcl-2 or Bcl-xl overexpression prevents ceramide channel formation in the mitochondria and cytochrome c release (Quintans, Bonn et al. 1994, Wiesner, Kilkus et al. 1997). It has been reported that Bcl-2 inhibits ceramide-induced apoptosis without blocking ceramide generation (Zhang, Alter et al. 1996, Allouche, Bettaieb et al. 1997, Birbes, El Bawab et al. 2001), which indicates that ceramide acts upstream of Bcl-2. However, conflicting studies have shown that Bcl-2 prevents ceramide-induced apoptosis by inhibiting ceramide accumulation (Yoshimura, Banno et al. 1998, Tepper, de Vries et al. 1999, Kawatani, Uchi et al. 2003). Ceramide also affects vascular endothelial growth factor signaling (Mehra, Jackson et al. 2014) and immunosuppressive functions of regulatory T cells (Apostolidis, Rodriguez-Rodriguez et al. 2016) via activated phosphatases. In addition, ceramide generated at the level of lysosomes initiates the cathepsin B-activated apoptotic pathway that leads to degradation of X-lined inhibitor of apoptosis (XIAP) (Taniguchi, Ogiso et al. 2015). In the same compartment, aSMase links the functions of lysosomes to the apoptotic pathway in mitochondria by the subsequent activation of cathepsin D, followed by BID-mediated caspase-9 and caspase-3 activation (Heinrich, Neumeyer et al. 2004).

Remarkably, the development in the use of liquid chromatography-mass spectrometry (LC-MS) revealed C16-ceramide as a possible marker of the *de novo* pathway that plays a pivotal role in apoptosis (Chalfant, Ogretmen et al. 2001, Kroesen, Pettus et al. 2001, Grosch, Schiffmann et al. 2012). In lymphoma cells, apoptosis can be specifically induced by *de novo* C16-ceramide via activation of the B cell receptor (Kroesen, Pettus et al. 2001). Eto *et al*. showed that *de novo* accumulated C16-ceramide in LNCaP prostate cancer cells leads to apoptosis preceded by G_0/G_1 cell cycle arrest, which is prevented by inhibiting the *de novo* pathway by fumonisin B1 (FB1) (Eto, Bennouna et al. 2003). Specifically, this pathway induces the dephosphorylation of SR proteins, a family of serine/arginine-domain proteins and known modulators of mRNA splicing in the ER/nucleus, which cause the alternative splicing of the genes encoding Bcl-x and caspase 9 by activation of PP1.

4. Ceramide Metabolism in Cancer Biology and Therapeutics

Bioactive sphingolipids are substantially involved in cancer pathogenesis and treatment which is validated by the accumulating evidence of the altered levels and metabolizing enzymes of these sphingolipids in cancer (Ogretmen and Hannun 2004, Ogretmen 2018). In principle, the accumulation of endogenous ceramide levels is a combined outcome of the activities and/or expression of enzymes that metabolize ceramide, called “drains”, and the enzymes that generate ceramide, known as “faucets” (Figure 1.2) (Shaw, Costa-Pinheiro et al. 2018). As described earlier, the contribution of one or more pathways to ceramide occurs via the enzymatic “faucets” responsible for sphingomyelin hydrolysis (SMase), *do novo* sphingolipid synthesis (SPT, CerS, DEGS1), and the salvage pathway (glucosylceramidase-GCDase, CerS), mediating cell

death. For instance, treatment with retinoic acid derivatives and other chemotherapeutic agents stimulate SPT activity (Pettus, Chalfant et al. 2002, Gault, Obeid et al. 2010), a key rate-limiting step in the regulation of ceramide generation and its subsequent responses to anticancer drugs and stress stimuli to determine cell fate in cancer cells (Perry 2002, Morad and Cabot 2013). DEGS1, the enzyme responsible for converting dihydroceramide to ceramide, is directly inhibited by HPR leading to the accumulation of endogenous dihydroceramides rather than ceramides (Saddoughi and Ogretmen 2013). The emerging role of dihydroceramide in cancer abandons the previously long held knowledge that it is biologically inactive and suggests that dihydroceramide inhibits cancer cell growth without inducing apoptosis (Ogretmen 2018). The propensity of cancer cells to overcome drug sensitivity is depicted through altered sphingomyelinase pathway. Although activation of aSMase or the *de novo* pathway might occur independently upon induction by specific stimuli, these two pathways can be part of the salvage pathway by the coordinated action of aSMase and CerS. The first mammalian nSMase to be identified was nSMase 2 (Marchesini, Luberto et al. 2003) for its central role in the regulation of cell cycle progression and proliferation, but not of cell death, which backs up the concept of differential ceramide-mediated pathways in growth inhibition (Marchesini, Osta et al. 2004).

The increased activity of metabolizing or enzymatic "drains" (CDase, GCS, SMS, CERK, and/or SK, leads to increased levels of sphingolipids with anti-apoptotic functions (Pyne and Pyne 2010, Ogretmen 2018, Shaw, Costa-Pinheiro et al. 2018). Ceramidase is a hydrolase enzyme that catalyzes the breakdown of ceramide into sphingosine and fatty acids (Lai, Realini et al. 2017). Not only does hydrolysis culminate the pro-apoptotic ceramide pool and protect from ceramide-mediated cell

death, but also provide sphingosine, the direct substrate for S1P production by SK, which induces cells into a mitogenic state, unless being transformed into ceramide by CerS (Park and Schuchman 2006). In particular, acid CDase (aCDase), which is restricted to acidic compartments, has been the subject of extensive research in cancer development and therapeutics (Mahdy, Cheng et al. 2009, Frohbergh, He et al. 2015, Hannun and Obeid 2018), as it impacts tumor cell survival *versus* death (Ogretmen 2018). The focus of ceramide-based cancer therapeutics, therefore, has been engaged in the rigorous development of inhibitors for aCDase to overcome cancer-mediated blocking of ceramide generation, some of which showed re-sensitization of tumors to chemotherapy and radiotherapy (Mahdy, Cheng et al. 2009, Realini, Solorzano et al. 2013, Realini, Palese et al. 2016). In addition to anticancer agents such as etoposide, tamoxifen, and dacarbazine, there are also endogenous CDase inhibitors, including TNF, IL-1, platelet-derived growth factor that drive ceramide-mediated apoptosis (Morad, Levin et al. 2013).

SK1 activity is correlated with drug resistance in cancer cells, concomitant with attenuation of ceramide accumulation that favors induction of apoptosis (Bonhoure, Pchejetski et al. 2006, Baran, Salas et al. 2007, Bonhoure, Lauret et al. 2008). The anti-proliferative effects of ceramide in cancer are counteracted by S1P which mediates proliferation, inflammation, metastasis and resistance to therapy through S1PR-dependent or receptor independent mechanisms as a second messenger (Ogretmen 2018, Shaw, Costa-Pinheiro et al. 2018). Interestingly, S1P signaling pathway not only acts on cancer cells directly, but also indirectly by promoting angiogenesis and invasiveness (Heffernan-Stroud and Obeid 2013, Nagahashi, Hait et al. 2014, Galvani, Sanson et al. 2015). Consistently, inhibiting SK1 can re-sensitize chemoresistant cells

by hindering the production of S1P from sphingosine and restoring the pool of ceramide to promote apoptosis (Baran, Salas et al. 2007). Unlike ceramide which is hydrophobic, S1P acts extracellularly by binding to G-protein-coupled receptors (Sanchez and Hla 2004). Inside the cell, nuclear S1P can act as an epigenetic modulator controlling gene transcription that promotes tumorigenesis (Hait, Allegood et al. 2009, Hait and Maiti 2017). In mitochondria, S1P disables apoptotic pathways (Li, Li et al. 2014). S1P provokes pro-survival signaling pathways, including RAS and ERK, in a positive feedback mechanism (Pitson, Moretti et al. 2003, Li, Li et al. 2014).

Another well-known "sink" for ceramide is GCS, which drives the first step for complex glycosphingolipid biosynthesis (Lewis, Wallington-Beddoe et al. 2018). It is anticipated that cancer cells upregulate metabolic enzymes, such as GCS, which interfere with the chemotherapeutic potential of drugs and efficacy by neutralizing the death response initiated by ceramide (Ogretmen and Hannun 2001, Ogretmen and Hannun 2004). Inhibiting GCS reestablishes ceramide levels and intensifies the anti-tumor responses of ceramide which are inversely correlated with the degree of malignancy and prognosis. The well-known GCS inhibitors, 1-phenyl-2 palmitoylamino-3-morpholino-1- propanol (PPMP) and 1-phenyl-2-decanoylamino-3-morpholinopropanol (PDMP), synergize with anticancer agents to overcome resistance (Morad and Cabot 2013). Consequently, the anti-tumor responses of these latter inhibitors in combination with chemotherapies or retinoids, such as apoptosis, cell cycle arrest, and autophagy, are magnified in a variety of solid and hematological malignancies.

In addition to CDase, GCS and SK1/2 represent powerful targets to overcome drug resistance in cancer therapeutics by lowering the amplified accumulation of their

sphingolipid products, glucosylceramide and S1P, respectively, which emphasizes the role of these molecules as predictive biomarkers for resistance to cancer therapy. Sphingolipid enzyme-based investigations identify SK1, alkaline CDase 2 (alkCDase2), nSMase2, and CerSs as specific players in the DNA damage response (Hannun and Obeid 2018), which implicate that rewiring of sphingolipid metabolism to different enzymes in the metabolic pathway, such as inhibiting the triads SKs, CDases, and GCS, would provide numerous therapeutic approaches to induce ceramide accumulation (Ogretmen and Hannun 2004, Hannun and Obeid 2018) which may play a role in retinoid-based therapy.

Yet, the system is far more complicated with multiple enzymes performing the "same" reaction (Hannun and Obeid 2011). For instance, the six CerS(s) (Pewzner-Jung, Park et al. 2010), five CDase(s) (Mao, Sun et al. 2010), and at least four or five SMase(s) (Clarke, Guthrie et al. 2008, Ledesma, Prinetti et al. 2011, Zhang, Cheng et al. 2011) are all independent gene products. To understand the ceramide-orchestrated actions in cancer cells, one must give tribute to the distinct isoforms of CerS enzymes that generate a family of dihydroceramide and ceramide species (Hannun and Obeid 2011) and the isoenzymes that catalyze the conversion of sphingosine into ceramide in the salvage pathway (Ogretmen 2018). There is accumulating evidence that different fatty acyl chains and their corresponding CerS(s) play crucial roles in inducing cancer cell death and/or survival, depending on cell type, downstream targets, and stress stimuli (Abboushi, El-Hed et al. 2004, Bonhoure, Pchejetski et al. 2006, Chumanevich, Poudyal et al. 2010, Stiban, Tidhar et al. 2010, Ju, Gao et al. 2016, Ogretmen 2018). In fact, this represents a new form of "sphingolipid rheostat" determined by discrete

ceramide species that may take part in a compensatory regulatory mechanism (Cuvillier, Pirianov et al. 1996, Newton, Lima et al. 2015).

Consequently, opening these metabolic "faucets" and/or shutting some of the enzymatic "drains" to maintain lethal levels of ceramide offers a surplus of opportunities for chemotherapeutic regulation in increasing potency of anticancer drugs and overcoming resistance (Shaw, Costa-Pinheiro et al. 2018). Recent experiments have shown that exogenous sphingolipid mimetic delivery platforms and/or metabolic precursors of ceramide may also augment endogenous levels of ceramide via *de novo* synthesis or hydrolysis of sphingomyelin (Ogretmen and Hannun 2004, Grosch, Schiffmann et al. 2012, Shaw, Costa-Pinheiro et al. 2018). Here, we represent findings that involve the roles of these enzymes and their products in specific cancer types.

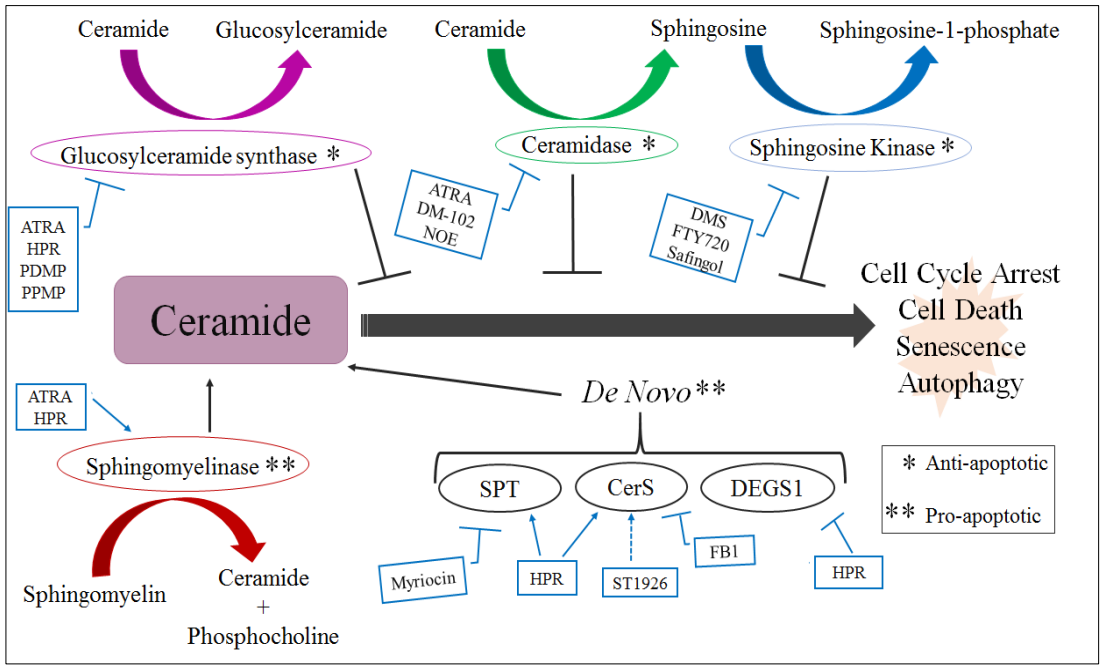

Figure 1.2. Ceramide Metabolism as a Route to Overcome Resistance to Cancer Therapy and Used Potential Nodes of Retinoid Therapeutic Interventions. The accumulation of ceramide is a pooled result of increasing the faucet flow through *de novo* or sphingomyelinase pathways and/or blocking the drain to prevent ceramide metabolism by the enzymes glucosylceramide synthase, ceramidase, and ceramide kinase. Activating these enzymes leads to tumorigenesis and chemoresistance, while

their inhibition counteracts resistance to cancer therapy. The equilibrium between total sphingosine-1-phosphate and ceramide levels is one of the determining factors for cell survival or cell death. The generated pool of ceramide induces selective toxicity to cancer cells, triggering cellular events such as cell cycle arrest, cell death, senescence, and autophagy. Retinoids can be potentially used as inhibitors and activators of specific enzymes in these pathways to overcome resistance and trigger cancer cell death.

ATRA, all-*trans*-retinoic acid; **CerS**, ceramide synthase; **DEGS1**, dihydroceramide desaturase; **DMS**, dimethyl sphingosine; **DM-102**, (2R,3Z)-N-(1-hydroxyoctadec-3-en-2-yl)pivaloylamide; **FB1**, fumonisin B1; **FTY720**, fingolimod, **HPR**, fenretinide; **NOE**, N-oleoyl-ethanol amine; **PDMP**, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; **PPMP**, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; **SPT**, serine palmitoyl CoA transferase; **ST1926**, E-4-(4'-hydroxy-3'-adamantyl biphenyl-4-yl) acrylic acid.

D. Retinoids

1. Overview

Vitamin A is found in animal and plant food and is a key component in human biology and disease (Connolly, Nguyen et al. 2013). The term "retinoids" refers to natural and synthetic vitamin A derivatives that exhibit a well-established role in anticancer therapy (Dobrotkova, Chlapek et al. 2018). During embryogenesis, retinoids are central regulators of development and organogenesis. Later in adult life, as in reproduction, they are potent players in growth arrest, apoptosis, differentiation, and the immune response (Freemantle, Spinella et al. 2003, Chambon 2005). Natural derivatives of vitamin A that are biologically active include ATRA, 9-cis retinoic acid (9-cis-RA), and 13-cis retinoic acid (13-cis-RA). They have been studied for their potential treatment and prevention of cancer (Sakashita, Kizaki et al. 1993, Khan, Villablanca et al. 1996, Reynolds 2000, Germain, Chambon et al. 2006). Synthetic retinoids include bexarotene and HPR, CD437 and ST1926, among others (Fontana and Rishi 2002, Valli, Paroni et al. 2008). In clinical practice, retinoids are used in the treatment of a wide range of dermatological disorders (Connolly, Nguyen et al. 2013).

Natural retinoids affect hematopoietic differentiation, an underlying mechanism targeted for treating several leukemias and lymphomas (Collins 2008). The best recognized approach for anticancer treatment of retinoids is ATRA-induced differentiation, a smart substitute for tumor cell killing that reverses cancer by abolishing the tumor phenotype (Dobrotkova, Chlapek et al. 2018). The Food and Drug Administration (FDA, USA) approved the use of bexarotene and ATRA for the treatment of cutaneous T cell lymphoma (Duvic, Hymes et al. 2001) and APL, respectively (Tallman, Andersen et al. 1997, Lin, Egan et al. 1999, Degos and Wang 2001, Bushue and Wan 2010). 13-cis-RA is useful for treating high-risk neuroblastoma after bone marrow transplantation (Villablanca, Khan et al. 1995). HPR is beneficial in cancer due to its inhibitory role in angiogenesis and pro-apoptotic effects, even in ATRA-resistant tumor cells, but exhibits minor side effects (Mittal, Malpani et al. 2014). HPR and CD437 induce growth arrest and cell death in several types of cancers, including those that are ATRA-resistant (Darwiche, El-Sabban et al. 2001, Darwiche, Hatoum et al. 2004). The more potent anti-tumor adamantyl retinoid ST1926 requires lower doses and has minimal toxicity, increased specificity and a broad spectrum of activity in solid and hematological malignancies *in vivo* and *in vitro*, including many cancers that are ATRA-resistant cancers (Cincinelli, Dallavalle et al. 2003, Garattini, Gianni et al. 2004, Garattini and Terao 2004, El Hajj, Khalil et al. 2014, Abdel-Samad, Aouad et al. 2018). These synthetic retinoids show potent anti-tumor activities with high therapeutic potential possibly functioning through RAR-dependent/independent mechanisms of action.

Retinoids have shown limited success in the treatment of solid cancers, including colorectal, breast, lung and prostate cancers (Abdel-Samad, Aouad et al.

2018, Ferreira, Napoli et al. 2020), but not in dermatological conditions (Khalil, Bardawil et al. 2017). ATRA is in clinical trials for treatment of melanoma, lung cancer, cervical cancer, kidney cancer, neuroblastoma, besides lymphoma and leukemia (Alizadeh, Bolhassani et al. 2014, Uray, Dmitrovsky et al. 2016). Exploring retinoid antitumor mechanisms may offer comprehension and are warranted for development of novel targeted cancer therapies. The medical use of retinoids has faced challenges due to their pharmacodynamic profile, toxicity, and evolved resistance which mitigates potential use in several neoplasms. To bypass the resistance to retinoids, synthetic retinoids were further developed in addition to combination treatments. In order to overcome the pharmacological and physiochemical limitations of retinoids, drug delivery systems such as nanoparticles are being formulated (Ferreira, Napoli et al. 2020).

2. Retinoids Mechanism of Action

Retinoic acids exert their functions through the classical genomic pathway or multiple non-classical or non-genomic pathways (Connolly, Nguyen et al. 2013). Retinoids act through binding and activating the retinoic acid (RARs) and retinoic X receptors (RXRs), which are members of the steroid/thyroid nuclear receptors, each having three subtypes (α, β, and γ) (Alvarez, Germain et al. 2007). ATRA and HPR binds specifically to RAR, and the rexinoid bexarotene interacts only with RXRs, and 9-cis RA and 13-cis RA bind to RAR or RXR (Bushue and Wan 2010, Theodosiou, Laudet et al. 2010). RARs and RXRs act as ligand-dependent transcription factors via heterodimerization, required for their activation and binding to the retinoic acid response elements (RAREs) located in the 5'-region of RA downstream genes. The

latter promotes cell differentiation, growth arrest, and apoptosis (Bushue and Wan 2010, Tang and Gudas 2011). Moreover, RARs and RXRs can dimerize with vitamin D receptor, thyroid hormone receptor, estrogen receptor-α (ERα), AP-1 transcription factors, peroxisome proliferator-activated receptor (PPARs), and liver X receptors (LXRs) (Connolly, Nguyen et al. 2013). Heterodimerization might simultaneously regulate processes that favor pro-survival responses, triggered by RAR or RXR partner receptors and development of cancer resistance to RA therapy. The use of natural retinoids has been hindered by acquired resistance and side effects (de The 1996, Fontana and Rishi 2002, Dobrotkova, Chlapek et al. 2018). For these reasons, modification of several functional groups has produced new synthetic retinoids with enhanced efficacy and minimal toxicity.

Synthetic retinoids (RRMs) demonstrate potential promise in cancer treatment (Fontana and Rishi 2002). Although exact mechanism of action of these retinoids is not fully elucidated, anti-proliferative and proapoptotic activities are attributed to ability to mediate both RAR-dependent and -independent mechanisms of action (Cincinelli, Dallavalle et al. 2003). The ability of RRMs to cause p53-independent apoptosis is pertinent. Mutations in p53 is a recurrent modification of tumor cells and the basis for resistance to DNA damaging agents (Fontana and Rishi 2002). HPR was synthesized by modifying the carboxyl end of ATRA with an amide-linked 4-hydroxyphenyl group, producing a more potent chemopreventive agent with less side effects (Formelli, Barua et al. 1996). HPR recruits a multitude of pathways to induce apoptosis.
These are RAR-independent in most studied cell types or might involve activation of certain RARs (Delia, Aiello et al. 1993, Cincinelli, Dallavalle et al. 2003). HPR can inhibit the growth of ATRA-resistant HL-60 (HL-60R) and NB306 myeloid malignant

cells defective for RARα signaling. Cellular events following HPR treatment seem cell type specific and include downregulation of c-Myc expression, telomerase activity, and crucial cell cycle regulator proteins as cyclin D1 and p34/cdc2 (Delia, Aiello et al. 1993, Bednarek, Shilkaitis et al. 1999). A major event in HPR-induced apoptosis is the surge in reactive oxygen species (ROS) (Delia, Aiello et al. 1997, Oridate, Suzuki et al. 1997, Sun, Li et al. 1999, Darwiche, Abou-Lteif et al. 2007). Other HPR-triggered apoptotic signals include activation of caspase 3 (DiPietrantonio, Hsieh et al. 1998, Lovat, Ranalli et al. 2000) and c-Jun N-terminal Kinase (JNK) (Chen, Zhou et al. 1999) reducing transcript levels of bcl-2/bax (Das, Banik et al. 2008) and elevating endogenous ceramide levels (Wang, Maurer et al. 2001, O'Donnell, Guo et al. 2002).

The adamantyl retinoid CD437 selectively binds RARγ (Bernard, Bernardon et al. 1992, Chao, Hobbs et al. 1997) and is active in retinoid-resistant T-cells. Retinoid antagonists cannot block this activity (Fontana and Rishi 2002). CD437 triggers cell cycle arrest in which the stage is dependent on cell type (Shao, Dawson et al. 1995). CD437-induced apoptosis could be mediated by p53 and the mitogen-activated protein kinase pathways (Sun, Li et al. 1999). ST1926, an analog of CD437, shows increased potency and bioavailability and less toxicity inducing apoptosis primarily via genotoxic stress in tumor cells that could be p53-independent (Cincinelli, Dallavalle et al. 2003). CD437 and ST1926 induce DNA damage, S-phase arrest, and can work through RAR-dependent/independent mechanisms (Valli, Paroni et al. 2008). They both target DNA polymerase 1 alpha (POLA1) (Han, Goralski et al. 2016, Abdel-Samad, Aouad et al. 2018). POLA1 levels are elevated in several tumors (Abdel-Samad, Aouad et al. 2018).

E. Ceramide and Retinoids, Along with Combination Treatments

1. ATRA in Cancer

a. Ceramide in ATRA Induced Differentiation

Sphingolipid-based cancer therapy has been an attractive area of research due to promising anti-tumor effects by partially inducing and lowering the threshold of apoptosis and/or reversing cancer drug resistance. It is necessary to emphasize the critical role of ceramide in retinoid-based cancer therapy and resistance, considering novel developments regarding behavior of key sphingolipids and their metabolism in the context of cancer biology and therapeutics. Clinical applications of ATRA has been hampered by acquired resistance of cancer cells (Giuli, Hanieh et al. 2020). One of the best-known effects of ATRA is differentiation in APL treatment denoted by induced myeloid differentiation of NB4 APL cells and accompanied by a robust enhancement of aSMase activity, gene transcription, and ceramide accumulation, without effect on nSMase (Murate, Suzuki et al. 2002) (Table 1.1). ATRA-resistant NB4 subclone NB4/RA, which lacks ATRA-binding capacity, fails to respond to ATRA-induced differentiation and change the activity/transcription of aSMase, nSMase and ceramide levels. Similar differentiating effects are observed with 9-*cis*-RA and 13-*cis*-RA, which concomitantly activate RARα (Kitamura, Kiyoi et al. 1997) and aSMase activity of NB4 but not NB4/RA (Murate, Suzuki et al. 2002). Altogether, this suggests that aSMase-generated ceramide plays a functional and crucial role in ATRA-mediated differentiation of APL cells and is dependent on RARα. Earlier reports describe that accumulation of ceramide and catalytically active protein kinase C-ς in nuclei of HL-60 cells leads to granulocytic differentiation after treatment with ATRA (Bertolaso, Gibellini et al. 1998).

Ceramide mediates differentiation of neuroblastoma Neuro2A cells by ATRA via contribution of two ceramide-generating pathways: *de novo* ceramide biosynthesis and, sphingomyelin degradation by nSMase (Riboni, Prinetti et al. 1995, Prinetti, Bassi et al. 1997). A well-described role of ATRA-induced differentiation is via ceramide acting as an upstream inhibitory regulator of telomerase activity, inducer of morphologic differentiation and cell growth arrest in SK-N-SH and SK-N-AS neuroblastoma cells (Kraveka, Li et al. 2003). These studies confirm previous reports on role of ceramide in senescence and differentiation responses of multiple tumor cell lines (Flores, Martinez et al. 1998, Ragg, Kaga et al. 1998, Lambeng, Michel et al. 1999). Simultaneous treatment with ATRA and the SPT inhibitor, myriocin, hampers ceramide generation and partially revives telomerase activity, suggesting the involvement of *de novo* ceramide synthesis in suppressing telomerase activity in response to ATRA in human neuroblastoma cells. Further analysis uncovered that ATRA treatment led to accumulation of very long chain C24:0 and C24:1 ceramide. The latter may be vital for the ATRA-mediated telomerase inhibition in neuroblastoma cells. This is not the case for A549 lung adenocarcinoma cells, where C16 ceramide in response to exogenous C6- ceramide is necessary for inhibition of telomerase (Ogretmen, Pettus et al. 2002). The effect of ATRA on ceramide-producing pathways, therefore, seems to be cell-type and context specific. ATRA treatment recapitulates the neuronal differentiation phenotype and increases generation of ceramide in SH-SY5Y human neuroblastoma cells (Tanaka, Tamiya-Koizumi et al. 2012). The increase in ceramide is attributed to downregulation of nCDase via GATA-2 acting as a transcription factor of nCDase gene expression in these cells. These results are specific to downregulation of nCDase as neither the expression nor the activities of other

ceramide metabolizing enzymes, such as SPT, CerS, aSMase, nSMase, GCS and aCDase, were modified with ATRA treatment. Downregulation of nSMase at the mRNA, protein, and activity levels does not alter levels of sphingosine and S1P in these cells further confirming the key role of nSMase in ATRA-induced accumulation of endogenous ceramide.

b. ATRA as a Transcriptional Regulator of Ceramide Metabolizing Enzymes

The ceramide-1-phosphate/ceramide ratio represents a determinant shift between survival and cell death when the balance tilts towards C1P production compared to ceramide by the action of CERK (Gomez-Munoz, Kong et al. 2005, Gomez-Munoz 2006). This is well-depicted in a separate study in SH-SY5Y cells, where over-expression of CERK offsets ATRA-triggered differentiation, while siRNA silencing of CERK augments ATRA growth inhibitory effects (Murakami, Ito et al. 2010). ATRA treatment reduces mRNA and protein levels of CERK, but treatment of SH-SY5Y cells with ATRA does not affect SK1 transcription. It is, thereofore, possible that CERK and SK1 do not perform overlapping functions, but rather compensatory roles in neuronal differentiation. The authors detected a diminished CERK promotor activity, essentially regulated by an ATRA-inducible transcription factor identified as chicken ovalbumin upstream promoter transcription factor I (COUP-TFI) and as the suppressive transcription factor of CERK. In a different differentiation setting of HL-60 cells induced by ATRA, CERK mRNA levels increase after treatment with ATRA, suggesting that presence and relative ratios of RAR/RXR and COUP-TFI/RXR or RAR in various cell lines might be determinative factors for the level of CERK transcription. It is noteworthy to mention that other differentiating agents of HL60 decrease CERK

mRNA, which confirms the cell type and stimuli-dependent responses on ceramide metabolizing enzymes that are also dictated by the cellular milieu (Date, Mitsutake et al. 2007).

c. Sphingosine Kinase/Sphingosine-1-Phosphate During Response to ATRA

The regulation of sphingolipids observed in the differential status of SK/S1P pathway holds some discrepancy when it comes to application of retinoids in various models of cancer (Liu, Sugiura et al. 2000, Kohno, Momoi et al. 2006, Dayon, Brizuela et al. 2009, Sukocheva, Wang et al. 2009). Increased expression of SK1 may act as an oncogene in several cancer types. Earlier observations document that S1P enhances the transcriptional activity of ATRA in osteoblast cells (Takeshita, Watanabe et al. 2000). Also, there is a correlation of SK1 and HPR resistance in ovarian cancer cells (Illuzzi, Bernacchioni et al. 2010). This implicates the involvement of SK/S1P pathway in modulating retinoid-induced sensitivity of tumor cells. In a retinoid responsive HT-29 colon carcinoma cell line, S1P obviates ATRA's inhibitory effect on growth suppression and G1 arrest. Co-transfection of HT-29 cells with an SK2 expression vector downregulates RARβ promotor activity induced by ATRA. By enhancing S1P levels, SKs can provoke G_1/S transition and induce cell growth when overexpressed in cancer cells (Dennis, Haq et al. 2001, French, Schrecengost et al. 2003, Pchejetski, Golzio et al. 2005). The activation of RARE by ATRA is also downregulated by SK2 overexpression. Subsequently, this has prevented the activation of the RXR/RAR heterodimer by ATRA. SK2 is primarily found in the nucleus or transferred between the cytosol and the nucleus (Igarashi, Okada et al. 2003), producing S1P from sphingosine. S1P downregulates RARβ expression and produces a plethora of effects on cell growth,

survival, and angiogenesis (Dennis, Haq et al. 2001, French, Schrecengost et al. 2003, Pchejetski, Golzio et al. 2005). These results highlight the protective role of S1P in cancer therapeutics via control of gene transcription. This creates an impetus for retinoid-based chemoprevention and treatment in cancer by decreasing S1P production or inhibiting SK activity as a target to overcome tumor resistance.

d. ATRA in Combination Treatment Amplifies the Ceramide Response

ATRA might concomitantly activate transcription of genes that favor cancer survival and growth in addition to activating non-genomic pathways. This is one mechanism of resistance. When ATRA is used in combination with other treatments, such as arsenic trioxide (ATO) in NB4 cells, it leads to synergistically induced differentiation and demethylation of APL target genes (Huynh, Sultan et al. 2019). Treatment of NB4 cells with ATRA and ATO triggers promyelocytic leukemia (PML)-RARα degradation and is more effective at maintaining high transcript levels of TGM2, RARβ, CCL2, and ASB2. In an effort to determine involvement of ceramide in the mechanism of action of ATRA alone or in combination with ATO in APL and Adult T cell leukemia\lymphoma (ATL), we investigated the effect of such treatment on *de novo* ceramide synthesis, GCS and SMase pathways (Dbaibo, Kfoury et al. 2007). Treatment with either ATO or ATRA gradually induces ceramide production in NB4 cells, which correlate with growth inhibition. Treatment with ATRA/ATO amplifies the rise in ceramide levels in these cells. Conceivably, the massive rise in ceramide levels is majorly attributed to activation of the *de novo* pathway and inhibition of GCS, with minimal contribution from sphingomyelin hydrolysis. The sustained ceramide accumulation in this model underscores the relevance of collaborative incorporation of

ceramide-generating/degrading pathways in cancer cell responses to therapy. ATO alone or in combination with ATRA suppresses nSMase activity, which seems to contradict previous studies by Murate *et al*. showing an increase in nSMase activity following ATRA treatment in NB4 cells (Murate, Suzuki et al. 2002, Dbaibo, Kfoury et al. 2007). Similar effects were observed in MCF-7 breast cancer cells, where ATRA provoked G_0/G_1 growth arrest by suppressing phosphorylation of ribosomal S6 kinase and its downstream targets S6 and eIF4B mediated by nSMase (Clarke, Mediwala et al. 2011, Clarke, Shamseddine et al. 2016). ATRA transcriptionally activates nSMase2 induction through epigenetic modulation via CBP/p300 histone acetylation in these cells (Clarke, Shamseddine et al. 2016). In addition to ATRA-mediated histone deacetylation, nSMase 2 is transcriptionally regulated by p53 in the context of the DNA damage response (Guillas, Kirchman et al. 2001, Clarke, Shamseddine et al. 2016).

Table 1.1. Effect of Natural Retinoids on Ceramide in Cancer

Cancer Model	Effect on tumor growth	Ceramide metabolism	Reference
Human neuroblastoma cells/ SH-SY5Y	- Neuronal differentiation, growth suppression, increase in RARα and COUP-TFI, decrease in RXRα	- Decrease of ceramide kinase mRNA, reduction of ceramide-1-phosphate levels - Transcriptional inhibition of neutral ceramidase	(Murakami, Ito et al. 2010, Tanaka, Tamiya-Koizumi et al. 2012)
Human neuroblastoma cells/ Neuro2A	- Ceramide-dependent differentiation	- *De novo* ceramide biosynthesis, activation of nSMase	(Riboni, Prinetti et al. 1995, Prinetti, Bassi et al. 1997, Wang, Maurer et al. 2008)
Human neuroblastoma cells/SK-N-SH and SK-N-AS	- Morphologic differentiation, inhibition of telomerase by endogenous ceramide	- Induction of *de novo* ceramide synthesis	(Kraveka, Li et al. 2003)
Colorectal cancer cells/HT29	- G_1 cell cycle arrest, apoptosis, RAR β expression	- Sphingosine kinase 2 overexpression or sphingosine-1-phosphate treatment antagonize effect of ATRA by modulating RARβ expression	(Sun, Gao et al. 2012)
Breast cancer cells/MCF7	- G_0/G_1 growth arrest, suppression of ribosomal S6 kinase phosphorylation and its downstream targets S6 and eIF4B	- nSMase2 induction through epigenetic modulation via CBP/p300 histone acetylation	(Clarke, Mediwala et al. 2011, Clarke, Shamseddine et al. 2016)

APL/NB4	- Differentiation, restoration of RARα target genes, PML-RARα degradation	- Increase in nSMase and aSMase activity, increase in ceramide levels, activation of *de novo* ceramide synthesis, inhibition of glucosylceramide synthase activity	(Murate, Suzuki et al. 2002, Dbaibo, Kfoury et al. 2007, Noguera, Catalano et al. 2019)
APL/NB4	- Synergistic effect with arsenic trioxide, PML-RARα degradation, more effective at maintaining high transcript levels of TGM2, RARβ, CCL2 and ASB2	- Suppression of nSMase activity	(Dbaibo, Kfoury et al. 2007, Huynh, Sultan et al. 2019)
AML/HL60	- Activation and nuclear localization of PKC-z, granulocytic differentiation	- Induction of nuclear ceramide levels	(Bertolaso, Gibellini et al. 1998)

ATRA, all-*trans*-retinoic acid; **SMase**, sphingomyelinase; **COUP-TFI**, chicken ovalbumin upstream promoter transcription factor I; **PML-RARα**, promyelocytic leukemia/retinoic acid receptor alpha; **AML**, acute myeloid leukemia; **APL**, acute promyelocytic leukemia.

2. Synthetic Retinoids in Cancer

a. Synthetic Retinoids in Solid Tumors

i. HPR at the Core of Ceramide-Based Cancer Therapy

HPR has proven efficacious in preclinical and clinical settings, owing to concurrent activation of the rate-limiting enzyme of *de novo* ceramide synthesis in addition to CerS (Zheng, Kollmeyer et al. 2006, Kraveka, Li et al. 2007, Wang, Maurer et al. 2008) (Table 1.2). HPR is a notorious inhibitor of DEGS1, the final enzyme in the *de novo* pathway. HPR leads to accumulation of dihydroceramide instead of ceramide in cell-free enzymatic assays as well as in intact cells (Zheng, Kollmeyer et al. 2006, Kraveka, Li et al. 2007). HPR is currently under clinical investigations for its antineoplastic potential in solid and hematological malignancies, including neuroblastoma, ovarian cancer, peripheral T cell lymphoma and non-Hodgkin lymphoma, alone or in combination treatment (Cooper, Reynolds et al. 2017, Mohrbacher, Yang et al. 2017, Shaw, Costa-Pinheiro et al. 2018). In neuroblastoma and prostate cancer cells, the activities of SPT and CerS are enhanced by treatment with HPR (Wang, Maurer et al. 2001, Wang, Charles et al. 2003). In both cancer types, this activation is accompanied with inhibition of DEGS1 enzyme, which increases dihydroceramide levels (Valsecchi, Aureli et al. 2010). The elevation in dihydroceramide is also detected in ovarian and colon carcinoma and leukemia cells (Wang, Maurer et al. 2008). In addition to direct inhibition by HPR, DEGS1 activity is negatively regulated by ROS (Rahmaniyan, Curley et al. 2011). One of the hallmarks of HPR-governed growth inhibitory effects is via oxidative stress (Sun, Li et al. 1999, Darwiche, Hatoum et al. 2004, Rehman, Shanmugasundaram et al. 2004) and ceramide production (DiPietrantonio, Hsieh et al. 1998). This bypasses resistance to ATRA as it

mediates its cytotoxic effects through alternative RAR-independent and RAR-dependent mechanisms. It is, therefore, crucial to investigate the mechanism of HPR-induced ROS signaling and its relation to ceramide metabolism for further development of HPR use in chemoprevention and cancer therapy. In the breast cancer cells T47D and MCF-7, treatment with HPR but not ATRA, elevates gene expression and protein levels of prosaposin, the precursor of saposins, that acts as a cofactor for sphingolipids hydrolysis and can be partly secreted (Panigone, Bergomas et al. 2001). Saposins stimulate sphingolipid hydrolases to degrade sphingomyelin into phosphorylcholine and ceramide. HPR triggers saposin secretion in both cells. Expression of prosaposin and integrin receptors are conversely correlated. Prosaposin negatively regulates cell anchorage and growth while integrins do the opposite. Prosaposin overexpression and HPR augment ceramide levels. Treatment of these cells with ceramide upregulates prosaposin and downregulates integrin receptors. Ceramide and prosaposin may act via a positive feedback loop to mediate HPR's chemo-preventive and detrimental effects on proliferation and metastasis of breast cancer cells by antagonizing integrin receptors through prosaposin. In MCF-7 cells, HPR obstructs cell survival and exhibits a stronger generation of ceramide than that detected in ATRA-treated cells, which is inversely associated with inhibition of HPR-induced cell survival (Rehman, Shanmugasundaram et al. 2004). The combination of ATRA and a PPAR-γ synthetic agonist, troglitazone (TGZ), synergistically enhances the proliferation-suppressive effects and cell death of MCF-7 cells (Elstner, Muller et al. 1998). These are completely blocked in the presence of TGZ and HPR (Rehman, Shanmugasundaram et al. 2004). TGZ is also known as a free radical scavenger and counteracts ceramide production in these cells, suggesting that the change in cellular oxidation state is a prerequisite for ceramide production,

adverse cytotoxic morphology, and anti-proliferation of HPR-treated MCF-7 cells. The oxidative response can act as an upstream regulator of sphingomyelinase (Andrieu-Abadie, Gouaze et al. 2001). Sphingomyelin hydrolysis, however, was not responsible for the increase in ceramide in these cells following treatment with HPR as no change in sphingomyelin levels occurred (Rehman, Shanmugasundaram et al. 2004). Blocking GCS with PDMP decreases levels of glucosylceramide, maintains the HPR-induced *de novo* ceramide pool and enhances the decline in survival of HPR-treated MCF-7 cells. The use of the *de novo* ceramide pathway inhibitors, myriocin and FB1, perturbs the HPR-induced ceramide response. Yet, the production of ceramide seems to be dissociated from HPR-mediated apoptosis, as the use of FB1 incompletely reverses the anti-tumor phenotype of HPR in MCF-7 and MDA MB 231 breast cancer cells. This might seem contradictory, but accumulation of cytotoxic sphingosine after inhibition with FB1 could also confer growth inhibitory effects (Schmelz and Merrill 1998). Further investigations in MCF-7 cells reveal that ceramide production in response to HPR is upstream or independent of caspase activation. In opposition to the HPR caspase-dependent mechanism of action during MCF-7 reduced survival (Rehman, Shanmugasundaram et al. 2004), HPR can also induce non-apoptotic tumor elimination via lethal autophagy (Fazi, Bursch et al. 2008, Messner and Cabot 2011) in line with ceramide-dependent non-apoptotic cell death in glioma cells (Kim, Choi et al. 2005). Autophagic cell death represents yet another mechanism by which HPR might circumvent resistance to cancer treatment in tumors that have deregulated apoptotic pathways, such as in MCF-7 cancer cells that are defective in caspase 3 function (Fazi, Bursch et al. 2008). HPR results in enhanced Beclin 1 expression, conversion of soluble LC-3 to autophagic LC3-II and the increase in lysosomes/autophagosomes in these

cells. Beclin 1 promotor contains an SP1-binding site and shows affinity for E2F, both classically regulated by ceramide (Weinmann, Bartley et al. 2001). This may explain the contribution of ceramide to HPR-induced Beclin 1 expression and autophagy. Meanwhile, caspase activation of HPR-induced cytotoxicity in MDA MB 231 cells is not necessary. These findings demonstrate that HPR triggers cell death by a complex mechanism of assorted apoptosis/necrosis by both caspase-dependent and independent pathways, as observed in neuroblastoma cells (Maurer, Metelitsa et al. 1999). This study highlights HPR at the core of ceramide-based chemotherapy, characterizing potentially successful applications in p53 and caspase independent manners.

ii. HPR and Ceramide in Hypoxia

In SMS-LHN neuroblastoma cells, the anti-survival effect of HPR treatment is independent of p53, and in line with HPR-mediated cytotoxic responses in HL-60 (Delia, Aiello et al. 1995), SCLC (Kalemkerian, Slusher et al. 1995), and NSCLC (Zou, Wang et al. 1998) cells. ROS is not the only mechanism of HPR-mediated growth inhibition. High concentrations of HPR can still induce cytotoxicity in SMS-LHN and multi-drug resistant (MDR) CHLA-90 neuroblastoma cells after exposure to hypoxia and the antioxidant, NAC (Maurer, Metelitsa et al. 1999). One speculated mechanism may be through accumulation of sustained levels of cytotoxic ceramide, evident in the SMS-LHN and MDR CHLA-90 neuroblastoma cells concomitant with a decline in cell survival. HPR treatment of MDR CHLA-90 cells, which are resistant to ATRA and 13-*cis*-RA, elicits ROS and mixed cytotoxicity manifested by necrosis and apoptosis and is confirmed by using caspase inhibitors. HPR treatment suggests a major role for ceramide in apoptosis/necrosis in neuroblastoma resulting from synchronized activation

of SPT and CerS in the *de novo* pathway, with no documented change in sphingomyelin metabolism in MDR CHLA-90 cells and in cell-free assays (Wang, Maurer et al. 2001). HPR effectiveness via ceramide under low oxygen conditions may be particularly crucial in clinical settings where low oxygen tension in neuroblastoma tissues might affect the potency of chemotherapeutics. In cervical cancer HeLa cells, HPR triggers apoptosis during normoxia and induces protective autophagy under low oxygen settings, mediated by HIF-1α and independent of ROS production (Liu, Su et al. 2010). These findings provide insights into potentiating autophagy inhibition in order to antagonize hypoxia-induced resistance to HPR and, hence, enhance its anti-tumor functions. In a similar study, it was reported that HPR induces cytotoxicity of Ewing's sarcoma family of tumor (ESFT) cell lines, which is reduced in hypoxic conditions and synergistically increased by ceramide modulators (Batra, Reynolds et al. 2004). Use of the SK inhibitor, safingol (L-threo-dihydrosphingosine) with HPR causes synergistic sensitivity in these cells, compared to HPR alone, whether treatment occurs in low or normal oxygen conditions. HPR accumulates ceramide species predominantly by *de novo* synthesis in SK-N-MC, 5838, and TC-71 cell lines and is counteracted by hypoxia. Moreover, hypoxia enhances aCDase gene transcription and activity in SK-N-MC cells observed during HPR reduced growth suppression and decreased ceramide species induction. This suggests the potential restoration of HPR-mediated cytotoxicity which is partly dependent on ceramide production in these cells by using the aCDase inhibitor, NOE. HPR depolarizes the mitochondrial membrane and increases Bax protein expression and ROS levels, effects of which are prevented during hypoxia-governed HPR treatment in SK-N-MC cells (Batra, Reynolds et al. 2004). Increases of

ROS and ceramide species, however, are independent events during HPR treatment of EFST cell lines.

iii. HPR and Ceramide in Drug Resistance

Treatment of LNCaP prostate cancer cells *versus* PC-3 cells with HPR shows increased sensitivity. The latter demonstrated only two-fold accumulation of ceramide compared to the ten-fold increase in LNCaP cells (Wang, Charles et al. 2003). Abrogating HPR-mediated ceramide generation decreases the intensity of apoptosis. Inhibiting GCS by tamoxifen restores ceramide production nine-fold and reverses HPR resistance in PC-3 cells more than PPMP does. In multiple prostate cancer cell lines, using the aCDase inhibitor, DM-102, with HPR synergistically reduces cell survival, increases caspase activity, leads to a 30-fold rise in ROS levels and causes a surge in dihydroceramide levels (Shaw, Costa-Pinheiro et al. 2018). Analogous to neuroblastoma cells, in prostate cancer cells HPR influences ceramide metabolism through activation of SPT, a *de novo* pathway rate-limiting step (Wang, Maurer et al. 2001, Wang, Charles et al. 2003). HPR coordinates with ceramide via activation of two crucial enzymes of the *de novo* ceramide pathway, SPT and CerS, to trigger apoptosis in drug sensitive A2780 ovarian carcinoma cells (Prinetti, Basso et al. 2003). HPR anti-tumor activity strictly associates with the prominent upregulation of the apoptotic gene, PLAcental Bone morphogenetic protein (PLAB) (Appierto, Villani et al. 2007). Interestingly, A2780/HPR cells which are resistant to HPR show only slight upregulation of PLAB expression and impaired ceramide accumulation (Prinetti, Basso et al. 2003). Enhanced PLAB expression also occurs *in vivo* and in patient tumor cells treated with HPR. Consistent with its pro-apoptotic potential, PLAB is also upregulated

in HPR-sensitive breast cancer cells, MCF-7 and T47D. Protein expression analysis also identified another RA target gene, ALL1-fused from chromosome 1q (AF1q), to be differentially regulated in HPR treated ovarian, breast, neuroblastoma, and cervical cancer cells, showing differential sensitivity to HPR (Tiberio, Cavadini et al. 2012). This suggests that AF1q upregulation might be a shared molecular event in the HPR mechanism of action in cancer. It is also notable to mention that 4-oxo-HPR, an oxidized metabolite of HPR with modification at position 4 of the cyclohexene ring (Rahmaniyan, Curley et al. 2011), is a more potent inducer of RAR-independent tumor growth suppression than HPR in most cell lines of neuroblastoma, ovarian, and breast cancer. 4-oxo-HPR treatment, unlike HPR, results in apoptosis in sensitive A2780 and resistant A2780/HPR cells with activation of caspase-3 and caspase-9, but not caspase-8, exhibiting synergistic effects when combined with HPR. In the context of HPR resistance, 4-oxo-HPR induces G_2-M cell arrest while HPR minimally affects cell cycle phases. Both 4-oxo-HPR and HPR, induce ROS production and ceramide accumulation by *de novo* synthesis. Notably, 4-oxo-HPR regenerates *de novo* ceramide synthesis in the A2780/HPR resistant cells to HPR (Villani, Appierto et al. 2006, Appierto, Villani et al. 2007). 4-oxo-HPR, therefore, might be a promising therapeutic agent, either alone or in combination with HPR in overcoming drug resistance.

iv. Sphingolipid Profile of HPR Sensitive and Resistant Cells

One of the main functions of HPR is to activate *de novo* ceramide synthesis. Some data shows that it results in ceramide accumulation via aSMase pathway in neuroblastoma adding to the complexity of the drug-induced apoptotic response (Lovat, Corazzari et al. 2005, Valsecchi, Aureli et al. 2010). HPR-induced ceramide generation

is partly through concomitant activation of aSMase and GCS (Lovat, Corazzari et al. 2005). Performance of high throughput analysis of A2780 ovarian cancer cells is crucial to understand whether other ceramide pathways are involved in HPR or in 4-oxo-HPR mediated actions. A2780/HPR cells, sensitive to 4-oxo-HPR, have a different sphingolipid profile than HPR and 4-oxo-HPR sensitive A2780 cells (Prinetti, Basso et al. 2003). HPR treatment of A2780 cells activates SPT, CerS, but concomitantly inhibits DEGS1, which results in a massive increase of dihydroceramide species (Valsecchi, Aureli et al. 2010). 4-oxo-HPR also significantly increased dihydroceramides, but only led to slight increase in ceramide species levels (Valsecchi, Aureli et al. 2010). Treatment of A2780 with either drug results in a differential increase of dihydroceramide with specific fatty acyl chains, reminiscent HPR and 4-oxo-HPR diversely acting on earlier steps of the *de novo* pathway with different potencies on SPT and CerS as confirmed by changes of total ceramide cell content. While 4-oxo-HPR effect on SPT enzymatic activity overlaps with that of HPR, it activates CerS to a lower extent than HPR (Valsecchi, Aureli et al. 2010) and produces a higher inhibitory effect on DEGS1 (Rahmaniyan, Curley et al. 2011). Based on the analysis of the corresponding ceramide and dihydroceramide species accumulated, one may suggest that HPR is a relatively more potent activator of CerS-5/6 that have preference for producing C16-dihydrocermaide. 4-oxo-HPR more efficiently modifies CerS-2/3 that favor utilizing C24 fatty acyl-CoA. In turn, this will affect the downstream profile by modifying synthesis of complex sphingolipids, although total levels may not be altered. This might explain the different sensitivities of various cancer cell lines to the two drugs, in addition to the observed effectiveness of A2780/HPR that are also sensitive to 4-oxo-HPR alone (Villani, Appierto et al. 2006). A2780/HPR cells display altered

sphingolipid metabolism compared to HPR-sensitive A2780 cells (Prinetti, Basso et al. 2003). Glucosylceramide levels are comparable in sensitive and resistant cells. In contrast, resistant cells show lower lactosylceramide and higher ganglioside levels than HPR-sensitive cells, both of which are downstream of glucosylceramide.

v. Dihydrosphingosine During HPR Response

The accumulation in dihydroceramides but not ceramides, is in parallel with evidence in colon HT-29 carcinoma and leukemia HL-60 cells and MDR ovarian carcinoma NCI/ADR-RES cells (Wang, Maurer et al. 2008), where the 16- and 18-carbon chain species are elevated and C16-ceramide is decreased. HPR dramatically increases incorporation of the dihydroceramide backbone in sphingomyelin and monohexosyl-glycosphingolipids and decreases ceramide-containing sphingolipids, including sphingomyelin with the ceramide backbone in HT-29, HL-60, and NCI/ADR-RES cells. Similarly, HPR elevates dihydroceramide in MDR DU-145 prostate cancer (Zheng, Kollmeyer et al. 2006) and in Molt-4 ALL leukemia cells (Kong, Wang et al. 2008), which implicates a common ceramide response in cancer. This is accompanied with autophagy induction in DU-145 cells. In NCI/ADR-RES cells, HPR also stimulates a substantial increase in sphinganine and sphinganine 1-phosphate, but not sphingosine or S1P (Wang, Maurer et al. 2008). An additional finding assigns additional roles to HPR-induced cytotoxicity through increasing levels of both pro-apoptotic, sphinganine and anti-apoptotic sphinganine1-phosphate mediators (Yu, Lee et al. 2001, Hait, Oskeritzian et al. 2006). Blocking sphinganine 1-phosohate production using dimethyl-sphingosine (DMS) combined with HPR treatment triggers a synergistic apoptotic response in HT-29, HL-60, and NCI/ADR-RES cells, possibly by increasing

levels of cytotoxic sphinganine (Wang, Maurer et al. 2008). Thus, it is critical to understand the mechanism of HPR-produced dihydrosphingosine (sphinganine) in cancer cells. Mao *et al*. demonstrated that in cervical tumor HeLa cells HPR increases expression of alkCDase 2 in a caspase-dependent manner and through RAR-independent pathways (Mao, Sun et al. 2010) ,although ATRA also induces expression of alkCDase through RAR (Sun, Hu et al. 2009). Like nSMase induction by ATRA, alkCDase 2 is also a target of p53‑mediated activation in the context of the DNA damage response, where it is responsible for the generation of sphingosine (Wang, Zhang et al. 2017). The simultaneous activation of alkCDase 2 and inhibition of DEGS1 by HPR or GT11 markedly increases dihydrosphingosine levels essential for conferring massive cytotoxic effects in these cells. The upregulation of alkCDase 2, which drives the hydrolysis of dihydroceramides into dihydrosphingosine, plays a vital role in mediating HPR accumulation of dihydrosphingosine and subsequent growth suppression, as confirmed by alkCDase 2 overexpression and knock-down assays in HeLa cells. AlkCDase 2 activation and/or expression sensitize tumor cells to cytotoxic levels of dihydrosphingosine and cell death and might enhance efficacy of anticancer agents including HPR.

vi. HPR and Ceramide in Lethal and Survival Autophagy

As a prominent producer of dihydroceramide, HPR in the "double-edged sword effects" in autophagy has been defined as lethal autophagy in breast and pancreatic cancer (Fazi, Bursch et al. 2008, Messner and Cabot 2011) and survival autophagy in cervical cancer (Liu, Su et al. 2010). Although dihydroceramide is not considered to have apoptotic functions, its role in autophagic cell death has been explicitly studied

(Zheng, Kollmeyer et al. 2006). Lethal autophagy parallels with the accumulation of long-chain dihydroceramides and ceramides in pancreatic tumor cells (Messner and Cabot 2011). HPR results in ceramide accumulation via *de novo* synthesis and not the SMase salvage pathway in Panc-1 and MIA PaCa-2 pancreatic cancer cells, although 50% of the generated ceramide is converted to the non-toxic sphingolipids, glucosylceramide and sphingomyelin. The combination of GCS inhibitors, such as PPMP, with HPR synergistically represses the growth of numerous cancer types, such as neuroblastoma and melanoma and prostate, lung, colon, breast and pancreatic tumor cells by augmenting sustained ceramide levels (Maurer, Melton et al. 2000, Weiss, Hettmer et al. 2003). HPR detected sensitivity seems to be dependent on ROS generation and not ceramide in Panc-1 and MIA PaCa-2 cells (Messner and Cabot 2011), while inhibiting ROS does not alter ceramide synthesis. HPR treatment induces autophagy marked by increased LC3 II expression and formation of acidic vesicles, enhanced JNK and p38 phosphorylation and decreased ERK phosphorylation. Dihydroceramides likely play key roles in toxic or protective autophagy depending on various factors, including cell type and stimuli. HPR can drive survival autophagy in cervical cancer (Liu, Su et al. 2010), in addition to its role in apoptosis and lethal autophagy in other cancer types. Production of S1P by SK is an important feature of survival autophagy (Lavieu, Scarlatti et al. 2006, Lavieu, Scarlatti et al. 2007). Inhibition of HPR–induced sphinganine 1-phosphate might shift the balance towards lethal autophagy, plausibly mediating the synergistic effect of HPR and DMS drug combination. The study by Rahmaniyan *et al*. shows that DEGS1, which introduces the 4–5 double bond into ceramide, is a direct target for HPR in SMS-KCNR neuroblastoma cells and in cell-free settings (Rahmaniyan, Curley et al. 2011). The

accumulated dihydroceramide drives G_1/S phase cell cycle arrest in these cells. As an inhibitor of DEGS1, HPR is implicated in *de novo* ceramide pathway of SCC19 cells during Foscan-mediated photodynamic therapy (PDT) (Boppana, DeLor et al. 2016). HPR combination with Foscan enhances C16-dihydroceramide production without changing total and individual ceramide levels. The combination also augments mitochondrial apoptosis, CerS activation, and caspases mediation of cell death. HPR enhances PDT-driven tumor cures in a mouse model of SCCVII squamous cell carcinoma cells. These findings highlight the key role of HPR in sphingolipid metabolism and might prove efficacious when administered in combination with other cancer treatments and drugs.

b. Synthetic Retinoids in Hematological Cancers

i. Ceramide Signaling During HPR-Triggered Response, Role for Bcl-2 and ROS

In hematological malignancies, HPR anti-tumor activities include potentiation of ceramide-generating pathways and/or ROS production. A deeper understanding of ceramide signaling in HPR treated ALL cells elucidates the sequential molecular events during HPR-induced cell death in CEM T leukemia/lymphoma cells (Morales, Perez-Yarza et al. 2007). HPR initiates ceramide generation via early sphingomyelinase activation and later from *de novo* ceramide synthesis in CEM cells. The resultant accumulation of ceramide acts upstream of ROS-mediated intrinsic apoptosis, leading to conformational activation of Bak and Bax, loss of mitochondrial membrane potential, mitochondrial membrane permeabilization, and cell death. Conversely, HPR fails to induce these mitochondrial changes and apoptosis, but not oxidative stress in CEM cells overexpressing Bcl-2. The latter delineates a protective role of Bcl-2 by altering events

downstream of ROS generation. Generation of ROS and ceramide are key events in HPR-induced cell death of HL-60, but not in NB4 and U937 cells (Jiang, Pan et al. 2011). In fact, blocking ROS generation in HL-60/VCR, that are multi-drug resistant and KG-1 AML cells protects from HPR-induced cell death, indicating the crucial input of ROS to the anti-proliferative capacity of HPR in AML cells (Morad, Davis et al. 2015).

ii. HPR with Ceramide Modulators to Antagonize Resistance

A wider evidence for HPR-induced cytotoxicity in ALL shows that treatment with this synthetic retinoid induces *de novo* ceramide synthesis in a dose and time-dependent manner (O'Donnell, Guo et al. 2002, Morad, Davis et al. 2015). The addition of PPMP, an inhibitor of GCS, and HPR had synergistic effects on growth inhibition and increased ceramide production compared to treatment with HPR alone in Molt-3, Molt-4, NALL-1, and NALM-6 ALL cell lines. The positive effects of PPMP on HPR-induced ceramide can be recognized in Molt-3, Molt-4, and NALL-1 cells, as it blunts glucosylceramide levels induced by HPR alone in tested ALL cells and might explain the increased sensitivity to HPR. In contrast, in non-malignant lymphoid cells, it confers minimal sensitivity and negligible increase in ceramide levels, implicating the neoplastic-specific regulation of *de novo* ceramide synthesis. The metabolism of ceramide has distinct signatures in different types of leukemia: acute myeloid leukemia (AML), chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL) (Morad, Davis et al. 2015). This is well-depicted in studies on CML showing that HPR fails to induce cell death and ceramide accumulation in K-562 cells, while cells remain sensitive to treatment with exogenous C6-ceramide. This suggests that ceramide-

mediated signaling is intact in these cells. Accordingly, targeting ceramide metabolism might enhance sensitivity to HPR, such as combining PPMP and HPR in ALL cells (O'Donnell, Guo et al. 2002) and, PPMP with imatinib in CML cells (Baran, Bielawski et al. 2011). Likewise, combination of HPR with imatinib intensifies apoptosis in K-562 CML cells (Du, Xia et al. 2014). In imatinib-resistant cells, HPR might potentiate efficacy of imatinib. These studies highlight the effective use of modulators of ceramide metabolism to fortify HPR-based therapies and antagonize drug resistance.

iii. Ceramide Response During HPR Treatment Is Cell Type Specific

The activation of HPR-driven stress signaling pathways occurs differently in a variety of cancer types which adds to the complexity of treatment and the heterogeneity of leukemia models. The origins of robust endogenous levels of generated ceramide govern the cytotoxic effects of HPR, not only in solid tumors compared to hematological malignancies, but also in leukemia types. This may be directed by the rates of turnover of glucosylceramide and sphingomyelin in different cell types. Molt-3 ALL cells accumulate ceramide, which is actively shunted into synthesis of glucosylceramide and sphingomyelin, thus contributing to the relative resistance of these cells. Like shown in ALL (Morales, Perez-Yarza et al. 2007), HPR triggers increased ceramide levels by contribution of SMase and the *de novo* pathway in HL-60/VCR and KG-1 AML cells (Morad, Davis et al. 2015). This is not recapitulated in other models such as neuroblastoma, which accumulates ceramide via concordant activation of SPT and CerS in the *de novo* pathway (Wang, Maurer et al. 2001). HL-60/VCR cells show an increase of glucosylceramide levels, however, these cells exhibit vigorous activation of SMase pathway in all tested AML, ALL, and CML cells. HL-

60/VCR cells are more sensitive to HPR compared to their wild-type counterpart HL-60 cells. The latter display only two-fold increase in ceramide levels compared to a 20-fold increase seen in HL-60/VCR cells. The vulnerability of HL-60/VCR cells to HPR may be associated with molecular species of ceramide that are produced by sphingomyelin hydrolysis compared to *de novo*-derived ceramide molecular species with little amounts shifted to glucosylceramide synthesis. ROS might also be a critical factor for HPR-induced sensitivity of HL-60/VCR cells strongly suggesting the effectiveness of HPR application in this drug-resistant situation.

We have studied the role of HPR on ceramide generation in virally driven hematological malignancies such as the HTLV-1 transformed or ATL cells. In HTLV-1 negative malignant cells, but not in ATL cells, HPR generates significant ceramide levels. Furthermore, ATL cells have lower sensitivity than HTLV-1 negative cells to treatment with exogenous C2- and C6- ceramide (Darwiche, Abou-Lteif et al. 2005). This was attributed to a defect in *de novo* ceramide synthesis in HTLV-1 positive cells treated with HPR and might be explained by the fact that viral oncoprotein Tax-induced activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway may protect HTLV-1 transformed cells from caspase-dependent apoptosis and ceramide accumulation (Darwiche, Hatoum et al. 2004, Darwiche, Abou-Lteif et al. 2005). We have shown that suppressing ceramide glucosylation by PDMP raises cellular ceramide levels and increases the sensitivity of HTLV-1 positive T cells to HPR indicating that this approach may be therapeutically feasible [13]. The detailed ceramide analysis in our study shows CerS(s) as candidate enzymes might be inhibited by Tax oncoprotein to lower ceramide levels and confer less sensitivity in HTLV-1 positive cells, in addition to abrogating other secondary cellular events, including ROS

generation, otherwise detected in HTLV-1 negative cells leading to growth inhibition by HPR (Darwiche, Hatoum et al. 2004, Darwiche, Abou-Lteif et al. 2007). HPR treatment in HLTV-1 negative cells results in significant loss of mitochondrial membrane potential, cytochrome c release, ceramide accumulation, cell cycle arrest, activation of the caspase cascade, including caspase-3, 8 and 9, caspase-dependent apoptosis and PARP cleavage (Darwiche, Hatoum et al. 2004). In ATL cells, HPR does not induce ceramide accumulation nor caspase activation. Likewise, CD437 elicits ceramide accumulation in HTLV-1 negative cells only (unpublished data).

iv. ST1926, a Potential Regulator of Ceramide Metabolism

We studied the effects of ST1926 on ceramide pathways in ATL cells, since we previously identified a defect in ceramide synthesis in these cells (Darwiche, Abou-Lteif et al. 2005). ST1926 provokes similar ceramide responses, in both HTLV-1 positive and negative malignant T cells not observed with previously tested RRMs such as HPR and CD437 (Ghandour, Pisano et al. 2020). ST1926, but not HPR or CD437, causes early Tax degradation. Compared with HPR, which leads to accumulated dihydroceramide, ST1926 enhances *de novo* ceramide synthesis via activation of specific CerS(s) without inhibiting DEGS1, thereby leading to accumulation of ceramide rather than dihydroceramide. Labeling experiments with the unnatural 17-carbon sphinganine and measuring the generated ceramide species showed that ST1926 preferentially induces the activities of a distinct set of CerS(s), specifically CerS2, CeS4, CerS5 and Cers6. At the mitochondrial level, the intrinsic pathway of apoptosis is tightly regulated by Bcl-2 family members and orchestrated by the bioactive sphingolipid, ceramide. Pro-apoptotic ceramide might antagonize the effects of Bcl-2

oncoprotein in a multitude of pathways, promoting dissection of this interplay. We observed that Bcl-2 attenuates the ST1926 growth-inhibitory response and interrupts *de novo* ceramide accumulation and cell death in Molt-4 cells (Ghandour, Pisano et al. 2020). These results highlight the potential role of ST1926 in increasing ceramide levels and lowering the threshold for cell death in ATL cells and overcoming resistance with a role for mitochondrial apoptosis. HTLV-1 positive cells, resistant to ATRA and defective in ceramide generation, accumulate ceramide with treatment by ATO or ST1926 (Darwiche, Hatoum et al. 2004, Darwiche, Abou-Lteif et al. 2005, Dbaibo, Kfoury et al. 2007). In ATL-derived cells ATO induces ceramide accumulation in a manner comparable to that observed in APL cells by concomitant inhibition of the ceramide metabolizing enzyme, GCS, and stimulation of the *de novo* ceramide pathway (Dbaibo, Kfoury et al. 2007). Indeed, blocking ceramide glucosylation by GCS with PDMP acts in synergy with HPR treatment to induce cell death in HTLV-1 positive T-cells (Darwiche, Abou-Lteif et al. 2005). ATO or ST1926, but not HPR, CD437, or ATRA stimulate Tax degradation. One probable explanation is that ST1926 degrades Tax and relieves its suppressive effect on ceramide-generating pathway(s) in ATL cells. The expression of Tax alone in transfected Hela cells is potent enough to partially prevent *de novo* ceramide synthesis by inhibiting CerS activity (Darwiche, Abou-Lteif et al. 2005). Tax deregulates the NF-κB pathway (Xiao, Cvijic et al. 2001, Shirinian, Kfoury et al. 2013) by inducing expression of genes that block apoptosis and might lead to chemoresistance, such as Bcl-2 (Nakashima, Kawakami et al. 2003, Fu, Qu et al. 2011, Alibek, Irving et al. 2014). Tax inhibits p53 function which can act upstream of ceramide accumulation and cell death (Dbaibo, Pushkareva et al. 1998). Indeed, ATL cells are sensitive to ceramide-induced cell killing, and this was confirmed after

treatment of these cells with exogenous ceramides (Dbaibo, Kfoury et al. 2007, Ghandour, Pisano et al. 2020).

v. HPR and Ceramide, Synergistic Cytotoxicity

Perturbations in ceramide metabolism render cancer cells more resistant to elimination by retinoids and elevation of endogenous ceramide levels lowers the threshold for apoptosis induction by these agents. One of the important studies in this regard suggests a common mechanism for HPR and ceramide modulators for replenishing cytotoxic ceramide levels and mediating growth inhibition of cancer cells. Using safingol in combination with HPR synergistically induces cytotoxicity in neuroblastoma, melanoma, lung, colon, prostate and pancreatic cancer cells in a p53- and caspase-independent manner, and preserves sensitivity under hypoxic physiological conditions (Maurer, Melton et al. 2000). Inhibitors of GCS, such as tamoxifen and PPMP, enhance HPR growth suppressive effects, which are clearly observed with HPR/safingol in combination and show ceramide shunting pathways in these cells are decidedly functional. In fact, overexpression of GCS (Liu, Han et al. 1999) decreases cytotoxicity of HPR and HPR/safingol. HPR/safingol is also effective against myelogenous and lymphocytic leukemia cell lines. Use of inhibitors of the *de novo* pathway highlights the major contribution of ceramide synthesis in tested cells in the indicated combination treatment settings. We have previously used the same concept to inhibit ceramide glucosylation with PDMP, which produces synergistic cell killing with HPR in ATL cells (Darwiche, Abou-Lteif et al. 2005). In another report, combining HPR and tamoxifen suppresses growth of MDA-MB-231 cells, which is associated with a significant increase in ganglioside GM3 (Aoyama 2002). Likewise, treatment of

prostate cancer cells with HPR and an inhibitor of aCDase, DM-102, strengthens HPR-induced apoptosis (Gouaze-Andersson, Flowers et al. 2011).

3. Retinoids and Ceramide in Nanoliposomes

As discussed earlier, the "domino effect" of ceramide anticancer function is exerted partly due to its hydrophobicity. This, however, is the same reason that renders ceramide less bioavailable. Thus, researchers have tried to formulate an effective ceramide delivery platform by developing short chain ceramide analogues in nanoliposomes that confer less lipophilic properties and increase bioavailability, allowing utilizing of ceramide itself as an anticancer agent. These nanoliposomes show more selectivity in tumor cells, probably due to the perturbed ceramide metabolism that is a hallmark of cancer (Barth, Cabot et al. 2011). An extra and beneficial characteristic of nanoliposomal preparation is the prospect of simultaneously introducing adjuvants, such as HPR, that impact ceramide metabolism, enhancing selectivity of apoptotic tumor responses, counteracting resistance to therapy, and possibly bypassing side effects. Such delivery platforms can be particularly efficacious when combined with retinoids that generate long chain ceramides increasing their growth suppressive functions in tumors (Tran, Smith et al. 2008, Morad, Levin et al. 2012). Hence, construction of ceramide nanoliposome-based formulas, which pair hydrophobic chemotherapies with short chain ceramides, might establish a treatment option worth exploring, given the inherent hydrophobic feature and promising anti-neoplastic efficacies of both ceramide and retinoids.

Table 1.2. Effect of Synthetic Retinoids on Ceramide Metabolism in Cancer

Synthetic Retinoids	Cancer Model	Effect on tumor growth	Ceramide metabolism	Reference
N-(4 hydroxyphenyl) retinamide HPR	Breast cancer cells/MCF7, MDA MB 231	- Apoptosis, retinoid receptor independent mechanism - Redox-sensitive ceramide production - Autophagy, increase of Beclin 1 expression, conversion of soluble LC-3 to autophagic LC3-II, autophagosome formation	- Early *de novo* ceramide synthesis, reduction of ceramide levels with CerS inhibitor FB1 and SPT inhibitor, myriocin -No effect on glucosylceramide levels, no effect on ceramide accumulation with PDMP, inhibitor of GCS	(Rehman, Shanmugasundaram et al. 2004, Fazi, Bursch et al. 2008)
HPR	Breast cancer cells/ T47D, MCF7	- Growth inhibition, upregulation of oncogene AF1q, a retinoic acid target gene, upregulation of prosaposin - ROS generation - Upregulation of apoptotic PLAB	- Elevation in total ceramide levels	(Panigone, Bergomas et al. 2001, Appierto, Villani et al. 2007, Tiberio, Cavadini et al. 2012)
HPR	Ovarian cancer cells/A2780, OVCA432, and SKOV-3	- Apoptosis, increase in AF1q protein levels, increase in dihydroceramide levels. - ROS generation, ER stress response, JNK activation, and induction of PLAB - Increase in PLAB expression *in vivo* and in tumor cells of patients	- *De novo* ceramide biosynthesis: activation of SPT and CerS, inhibition of DEGS1	(Appierto, Villani et al. 2007, Valsecchi, Aureli et al. 2010, Tiberio, Cavadini et al. 2012)

HPR/4-oxo-HPR	Ovarian cancer cells/A2780 and resistant to HPR A2780/HPR	- Synergistic cell killing and ROS generation in both cell types - Induction of ceramide by HPR only in A2780 sensitive cells, accumulation of ceramide by 4-oxo-HPR in both cell types	- Activation of *de novo* ceramide synthesis by both retinoids - Different potencies on CerS and SPT	(Villani, Appierto et al. 2006, Valsecchi, Aureli et al. 2010)
HPR	MDR ovarian cancer cells/ NCI/ADR-RES	- Synergistic cell killing with DMS	- Increase in dihydroceramide	(Wang, Maurer et al. 2008)
HPR	MDR neuroblastoma cells/CHLA-90	- ROS generation, mixed cytotoxicity (necrosis/ apoptosis, early ceramide elevation; biphasic increase. - Ceramide accumulation in a time- and dose-dependent manner	- Activation of SPT and CerS *in vitro* and in cell-free conditions - No sphingomyelin degradation	(Maurer, Metelitsa et al. 1999, Wang, Maurer et al. 2001)
HPR	Neuroblastoma cells/ SMS-KCNR	- Accumulation of dihydroceramide - G_1/S phase cell cycle arrest	- Inhibition of DEGS1 activity	(Kraveka, Li et al. 2007, Rahmaniyan, Curley et al. 2011)
HPR	Neuroblastoma cells/ SMS-LHN	- Cytotoxicity	- Early time-dependent increase in ceramide; biphasic increase	(Maurer, Metelitsa et al. 1999)
HPR	Colorectal cancer cells/HT29	-Synergistic cell killing with DMS	- Increase in dihydrosphingolipids, increase in C16-dihydroceramide, no change in ceramide	(Wang, Maurer et al. 2008)
HPR	Prostate cancer cells/LNCaP, PC-3	- Apoptosis, increase in ceramide levels	- SPT activation	(Tanaka, Tamiya-Koizumi et al. 2012)

HPR	MDR prostate cancer cells/ DU-145	- Autophagy, autophagosomal vesicles formation -Increase in dihydroceramide	-Activation of *de novo* synthesis and inhibition of DEGS1	(Zheng, Kollmeyer et al. 2006)
HPR	Pancreatic cancer cells /Panc-1, MIA PaCa-2	- Apoptosis, ROS-dependent autophagy, increase in JNK and p38 phosphorylation, decrease in ERK phosphorylation - LC3 II expression and formation of acidic vesicles	- Early *de novo* ceramide synthesis - Increase glucosylceramide and sphingomyelin - No effect on SMase pathway	(Messner and Cabot 2011)
HPR	Cervical cancer cells/HeLa	- Cell death, increase in dihydrosphingosine, ROS generation - Induction of HIF-1α mediated autophagy in hypoxia, independent of ROS - Apoptosis in normoxia	- Increase in alkaline ceramidase 2 activity and protein levels, independent on RAR, but dependent on caspase activation - Inhibition of DEGS1	(Liu, Su et al. 2010, Mao, Sun et al. 2010)
HPR/ Safingol	ESFT/ SK-N-MC, 5838, and TC-71	- Synergistic cytotoxicity - Accumulation of ceramide, antagonized by hypoxia via acid ceramidase - Mitochondrial membrane depolarization - Increase in Bax protein and ROS	- *de novo* ceramide synthesis	(Batra, Reynolds et al. 2004)
HPR	AML/HL60	- Apoptosis, synergistic cell killing with DMS	- Increase in dihydrosphingolipids, increase in C16-dihydroceramide - No change in ceramide	(Wang, Maurer et al. 2008)
HPR	AML/HL60	- Apoptosis, reduction of Cyclin D1, cdk4 expression, and Rb phosphorylation, PARP cleavage, transient increase in ceramide - Reduction of ceramide levels, extent of PARP cleavage, and apoptosis upon simultaneous treatment with FB1	- Increase in ceramide, small increments glucosylceramide, No effect on sphingomyelin levels	(DiPietrantonio, Hsieh et al. 1998, Morad, Davis et al. 2015)

HPR	MDR AML/HL60-VCR	-Reduction in cell proliferation, ROS generation - Enhanced sensitivity with tamoxifen (the latter blocks GCS)	- Contribution of *de novo* ceramide synthesis and SMase, - Major increase in ceramide, less increase in glucosylceramide, decrease in sphingomyelin levels, robust GCS activity before treatment	(Morad, Davis et al. 2015)
HPR	AML/KG1	- Decrease in cell proliferation, ROS generation	- Contribution of *de novo* ceramide synthesis and SMase - Major increase in ceramide, less increase in glucosylceramide, decrease in sphingomyelin levels	(Morad, Davis et al. 2015)
HPR	CML/K-562	- Resistance to growth suppression, - Intensified apoptosis in combination with imatinib	- No ceramide, cells sensitive to C6-ceramide	(Du, Xia et al. 2014, Morad, Davis et al. 2015)
HPR	ALL/Molt-3	- Reduced proliferation	- Moderate ceramide production, strong increase in glucosylceramide and sphingomyelin	(Maurer, Metelitsa et al. 1999, Morad, Davis et al. 2015)
HPR	ALL/Molt-3, Molt-4, NALM-6	- Cytotoxicity, synergistic effect with PPMP, ROS generation	- Increase in ceramide via *de novo* synthesis - Increased induction of ceramide levels with PPMP, abrogation of glucosylceramide levels that are induced with HPR alone	(O'Donnell, Guo et al. 2002, Darwiche, Abou-Lteif et al. 2005)
HPR	Molt-4	- Growth inhibition, ROS generation - Increase in dihydroceramide and decrease in ceramide	- Stimulation of *de novo* pathway, inhibition of DEGS1	(Darwiche, Hatoum et al. 2004, Darwiche, Abou-Lteif et al. 2007, Kong, Wang et al. 2008)

HPR	ALL/CEM, SMS-SB	- ROS mediated ceramide accumulation, cell cycle arrest and apoptosis, additive effect with PPMP - Cytochrome c release, dissipation of mitochondrial membrane potential - Activation of caspases 3, 8 and 9, and PARP cleavage in CEM	- Increase in ceramide via *de novo* synthesis	(Maurer, Metelitsa et al. 1999, Darwiche, El-Sabban et al. 2001, O'Donnell, Guo et al. 2002, Darwiche, Abou-Lteif et al. 2005)
HPR	ALL/CEM, Jurkat	- ROS-independent cell death, dihydroceramide accumulation, both of which can occur independently	- Increase in dihydroceramide and not ceramide at sub-lethal concentrations, accumulation of dihydrosphingosine, and slight increase in sphingosine - DEGS1 inhibition in CEM	(Apraiz, Idkowiak-Baldys et al. 2012)
HPR	Head and neck cancer cells/SCC17B Mouse model of SCCVII squamous cell carcinoma	- Increase in cell death mediated by CerS and caspases in combination with PDT - Induction of DEGS1/ *de novo* sphingolipid synthesis after Foscan PDT+HPR treatment - Enhanced mitochondrial depolarization upon PDT+HPR treatment - Enhanced PDT-mediated tumor cures in mice with HPR	- *De novo* ceramide synthesis, enhanced accumulation of C16-dihydroceramide, no effect on ceramide levels upon combination - Increase in C16-dihydroceramide and decrease in total levels of ceramide with HPR alone - Foscan PDT alone induces C16-dihydroceramide, individual and total ceramide.	(Boppana, DeLor et al. 2016)
HPR	ALL/ CCRF-CEM cells, 10E1-CEM (overexpressing Bcl-2)	- Ceramide-mediated ROS generation, activation of Bak and Bax, loss of mitochondrial membrane potential leading to permeabilization and cell death. - Identifies sequential events in ceramide-mediated cell death	- Early activation of SMase, later *de novo* ceramide synthesis	(Morales, Perez-Yarza et al. 2007)

ST1926	ATL/HuT-102, MT-2	- Induces apoptosis of ATL cells and prolongs survival of ATL mice. - Causes early DNA damage, upregulates p53, and downregulates Tax expression. - Early accumulation of ceramide	- Activation of *de novo* ceramide pathway - Activation of CerS(s)	(El Hajj, Khalil et al. 2014, Ghandour, Pisano et al. 2020)
ST1926	ALL/Molt-4, Jurkat	- Induces apoptosis, early DNA damage	- Early accumulation of ceramide - Activation of *de novo* ceramide pathway - Activation of CerS(s)	(El Hajj, Khalil et al. 2014, Ghandour, Pisano et al. 2020)

CerS, ceramide synthase; **FB1**, fumonisin B1; **SPT**, serine palmitoyltransferase; **PDMP**, 1-phenyl-2-decanoylamino-3-morpholinopropanol; **GCS**, glucosylceramide synthase; **AF1q**, ALL1-fused from chromosome 1q; **ROS**, reactive oxygen species; **PLAB**, PLAcental Bone morphogenetic protein; **JNK**, c-Jun N-terminal Kinase; **DEGS1**, dihydroceramide desaturase; **DMS**, dimethylsphingosine; **MDR**, multidrug resistance; **cdk**, cyclin-dependent kinase; **Rb**, retinoblastoma; **PARP**, Poly (ADP-ribose) polymerase; **PDT**, photodynamic therap; **ATL**, adult T cell leukemia/lymphoma; **ALL**, acute lymphocytic leukemia; **AML**, acute myeloid leukemia; **CML**, chronic myelogenous leukemia; **ESFT**, Ewing's sarcoma family of tumor.

F. Ceramide Role in (Patho)physiology

The study of sphingolipids, particularly ceramide, has reached the forefront in pathological conditions other than cancer, with a close connection to cardiovascular systems, metabolic disorders, such as fatty liver disease (Kasumov, Li et al. 2015), obesity (Boini, Zhang et al. 2010), insulin resistance (Chaurasia and Summers 2015, Choi and Snider 2015). The cellular functions of sphingolipids encompass other diseases, such as arthritis and inflammation, diabetes, immune function, and neurodegenerative disorders (Boini, Zhang et al. 2010, Choi and Snider 2015, Kasumov, Li et al. 2015). As sphingolipids control brain development and neurodegeneration, pathways of sphingolipid metabolism have become imperative targets for therapy. The best-established sphingolipid modulator is FTY720, an analogue of sphingoid bases, which has been used in clinical medicine for the treatment of the autoimmune disorder multiple sclerosis, which is a neuronal disease characterized by demyelination of neurons (Boustany 2014). Patients of neuronal ceroid lipofuscinoses (CLN) show perturbed sphingolipid metabolism, characterized by increased or decreased ceramide levels. This relies on the type of CLN that is genetically divided. Aberrations of CLN5, CLN8, CerS1, and CerS2 proteins all lead to progressive myoclonus encephalopathies with reduced levels of C16, C18, or C18:1, C24 or C24:1 ceramide and dihydroceramide species (Boustany 2014). CLN results from mutations of lysosomal proteins, of which types are identified by abnormal lipofuscin accumulation (Kollmann, Uusi-Rauva et al. 2013). CerS1 is identified as a CLN-related protein, but the exact mechanisms of CLN remain undefined. Overexpression of CerS1 recovered C18:0-ceramide levels in CLN9-deficient cells, resulting in normal growth (Schulz, Mousallem et al. 2006). CerS1 is implicated in

central nervous system development and diseases such as Parkinson's disease, with specific role for CerS1 in apoptotic processes (Abbott, Li et al. 2014). Ceramide species in the skin, including other sphingolipids, contain ultra-long fatty acyl chains that maintain impermeability and protection. Therefore, disrupted sphingolipid metabolism can result in the loss of these unusually long chain ceramides and lead to various forms of skin and hair defects that are often fatal (reviewed in (Hannun and Obeid 2018)). Tissue-specific metabolism of sphingolipids also suggests a strong role for neutral SMase2 in bone and cartilage development (Stoffel, Jenke et al. 2005). Interestingly, sphingolipid enzymes with no human counterparts exist in certain parasites, including fungi and protozoa, which might serve as targets for the development of anti-parasitic drugs (Rollin-Pinheiro, Singh et al. 2016).

G. Conclusions and Future Perspectives

The growing appreciation of sphingolipids has reached the tip of the iceberg stage, as we speculate that there is no area in cell biology where this class of lipids does not play fundamental roles in health and disease. The examples presented emphasize on the role and regulation of specific sphingolipid enzymes studied in the context of tumorigenesis and cancer treatment, such as SMases, CDases, CerS(s), SK, GCS, CERT, S1P phosphatase, and S1P lyase. All have key roles in survival, apoptosis, autophagy, cell cycle, and angiogenesis. All this has encouraged further exploration in cancer therapeutics and understanding of other physiological and pathophysiological conditions. Some of these impediments have their roots in intrinsic complexities of working with sphingolipids, their enzymes, and their targets. Former generations of lipid chemists were skeptical about the bioactivity of sphingolipids, exclusively

assigning roles of lipids in maintaining membrane structure and contributing to energy metabolism, with no significant cell regulatory roles. The current understanding in the field is the product of evolution and provides lessons whereby sphingolipids no longer exist as innate entities but act as vital bioeffector molecules. Difficulties in sphingolipid discoveries highlight the power of biochemical tools that shaped our understanding of the sphingolipid pathways through synergistic approaches of implementing knockout enzyme models, mass spectrometry-based lipidomic analysis, confocal microscopy, chemical biology, mathematical modelling, and systems biology approaches.

Due to their potent antineoplastic profile and relatively low toxicity, retinoids represent an attractive option for chemoprevention and treatment of malignancies. The resistance of some tumors has limited their clinical applications. Identification of molecular mechanism of tumor resistance will improve the efficiency of retinoid therapy. In this review, we have explored well-established models of retinoid-induced activation or inhibition of ceramide metabolizing enzymes that rationally explain altered ceramide levels in cancer cells and how they confer drug sensitivity or resistance. Such retinoid regulations can occur via transcriptional induction or repression of sphingolipid genes and/or through receptor-independent mechanisms. Clearly, more research is required to dissect retinoid-regulated pathways of ceramide metabolism and to define the mechanisms of regulation of enzymes. Since sphingolipid-mediated pathways operate at the level of individual organelles, they should be studied as such. This would bring us a step further in selective targeting of enzymes which avoids off target effects, whereby modern analytical and drug formulation techniques and the mathematical approaches of systems biology will lead to advancements in cancer therapy.

CHAPTER II

ADULT T CELL LEUKEMIA/LYMPHOMA

HTLV-1 associated ATL is an aggressive neoplasm of CD4+ T lymphocytes (Baydoun, Bellon et al. 2008). First described in 1977 by Uchiyama and Takatsuki as a progressive peripheral T lymphocytic malignancy, ATL emerged as unusual clusters in specified areas of Japan suggesting a transmissible mediator of the disease (Baydoun, Bellon et al. 2008, Tsukasaki and Tobinai 2013). HTLV-1 was consequently recognized as the first retrovirus associated with human disease (Matsuoka and Jeang 2007). HTLV-1 is also the causative agent of a neurological disease called HTLV-1 associated myelopathy/tropical spastic paraplegia (Bazarbachi, Ghez et al. 2004). This disis characterized by the continuous demyelination of long motor neurons of the spinal cord that leads to a debilitating inflammatory disease of the central nervous system (Nasr, El Hajj et al. 2011). HTLV-1 can be mainly transmitted by three routes which are mother-to-child *via* breast-feeding, blood transfusions, and sexual intercourse (Tsukasaki and Tobinai 2013).

HTLV-1 causes T cell transformation and development of ATL after a mean latency period of over 50 years in 3-5% of the approximate 10-20 million HTLV-1 asymptomatic carriers (Gallo 2011). The long latency period which precedes the onset of ATL suggests a multistep process in the development and oncogenesis of the disease where HTLV-1 infection is responsible for the initial events that drives carcinogenesis of ATL. A clear elucidation of the cellular and molecular mechanisms, however, still lags behind. It is proposed that multiple factors, such as the involvement of viral proteins in the genetic and epigenetic alterations of host genome, and immune status of

the hosts, could be implicated in leukemogenesis of ATL (Yasunaga and Matsuoka 2007). One of these viral regulatory proteins, Tax, can induce transformation of HTLV-1 infected cells and regulate cell cycle progression, cell death, and angiogenesis (Kfoury, Nasr et al. 2005). Once cell-to-cell transmission occurs, HTLV-1 virus increases its copy number by the oligoclonal expansion of infected cells.

The viral trans-activator Tax plays a crucial role in this initial stage of infection by enhancing proliferation and inhibiting apoptosis (Barnard, Igakura et al. 2005). Tax is at the root of numerous cellular-transforming events, as it promotes cell cycle progression, initiates cellular transcription factor pathways and resists apoptosis (Yoshida 2001). Tax activates a set of critical transcription factors such as NF-κB, cyclic AMP response element-binding protein, serum responsive factor, and AP-1 (Nasr, El Hajj et al. 2011). NF-κB activation, mediated by Tax, induces expression of genes that block apoptosis and might lead to chemoresistance, such as Bcl-2 and Bcl-xl (Nakashima, Kawakami et al. 2003, Fu, Qu et al. 2011), as well as genes involved in cell cycle regulation (Wang, Han et al. 2014, Bangham and Matsuoka 2017, Karimi, Mohammadi et al. 2017). Therefore, in the context of ATL, novel approaches for effective treatment aim to overcome resistance to apoptosis by repressing NF-κB activity, which is crucial to Tax-mediated oncogenesis. Tax blocks the transcription of pro-apoptotic Bax gene and inhibits the caspase cascade via the NF-κB pathway which induces the expression of the inhibitors of apoptosis: X-IAP, cIAP-1, and c-IAP-2 (Kawakami, Nakashima et al. 1999, Nakashima, Kawakami et al. 2003). Tax also interrupts apoptosis by suppressing the expression levels of Bim and Bid, members of pro-apoptotic Bcl-2 family proteins (Muhleisen, Giaisi et al. 2014). The expression of p53 is elevated in HTLV-1 transformed cell lines (Yamato, Oka et al. 1993). However,

it is mutated and/or functionally inactive because of the inhibitory role of Tax (Pise-Masison, Mahieux et al. 2000). One of the cellular effects of Tax is activation of the NF-κB pathway which plays a critical role in the inhibition of p53 transactivation functions. Tax alone represses the transcriptional activity of p53 and abrogates G_1 arrest and apoptosis induced by p53. Tax also inhibits p53 through the phosphorylation of p53 on Ser 15 and Ser 392.

Treatment options for ATL are suggested based on the clinical subtype classification and prognostic features (Bazarbachi, Suarez et al. 2011, Tsukasaki and Tobinai 2013). These include "watch and wait policy", conventional combined chemotherapy, antiretroviral therapy such as interferon alfa (IFN), zidovudine (AZT), arsenic trioxide (AsO3), allogeneic hematopoietic stem cell transplantation (allo-HSCT), monoclonal antibody. Other approaches include kinase inhibitors used to impede angiogenesis by targeting the interaction of ATL cells with endothelial cells (El-Sabban, Merhi et al. 2002). The use of both natural and synthetic retinoids in several hematological malignancies is documented. HTLV-1 transformed cells show resistance due to their lack of RAR α, through which ATRA exerts its antitumor effects (Darwiche, El-Sabban et al. 2001). Use of synthetic retinoids, however, bypasses this limitation by inducing apoptosis in HTLV-1 infected cells *via* both receptor-dependent and -independent pathways (Darwiche, Hatoum et al. 2004, El Hajj, Khalil et al. 2014).

CHAPTER III

AIMS

ATL is an aggressive hematological neoplasm caused by infection with HTLV-1 virus. Tax, an HTLV-1 protein is a potent viral transactivator oncoprotein that deregulates several cellular processes and promotes disease maintenance and progression (Barbeau, Peloponese et al. 2013, Yasuma, Matsuzaki et al. 2016, Bangham and Matsuoka 2017). Ceramide, a well-known lipid tumor suppressor, induces cell differentiation, cell cycle arrest and apoptosis in several types of tumor cells. Depending on different inducers of stress response, ceramide accumulation occurs as a contribution of catabolic and anabolic pathways, such as sphingomyelin breakdown or by *de novo* synthesis. Our purpose is to determine the role of ceramide as it relates to cancer pathogenesis and treatment, and the interplay with retinoid signaling, particularly ST1926, in virally driven hematological malignancies such as the HTLV-1 transformed or ATL cells. The alteration of ceramide pathways through which Tax exerts its tumor promoting functions in ATL remains poorly defined. Interestingly, we showed that ST1926 but not HPR or CD437 causes early Tax degradation (El Hajj, Khalil et al. 2014). The blunted ceramide response was significant in HTLV-1 transformed cells, which was attributed to a Tax-induced defect in *de novo* ceramide synthesis in HTLV-1 positive cells, with likely inhibition of CerS activity (Darwiche, Abou-Lteif et al. 2005).

The synthetic retinoid, HPR, is a direct inhibitor of DEGS1 in the final critical step of *de novo* ceramide pathway. It accumulates dihydroceramide rather than ceramide (Rahmaniyan, Curley et al. 2011). The classical DGK assay used in our previous research on HPR does not distinguish individual ceramide and

dihydroceramide species, nor the accumulation of either molecule. Understanding the effects of ST1926 on these sphingolipids is an important step in deciphering the mechanisms of its action. Moreover, the comparison between HTLV-1 positive and negative cells will assist to identify the hypothesized ceramide differential response that could be mediated by Tax. Fortunately, the utilization of new instrumentation with improved sensitivity or LC-MS will provide insights about the variable effects of these species in the cell death or survival response of both cell types, in addition to involvement of distinct CerS(s). In our ATL model, we showed earlier that *de novo* ceramide synthesis is compromised in response to HPR, probably due to Tax inhibitory effect on CerS activity (Darwiche, Abou-Lteif et al. 2005). Using labeling experiments with the unnatural 17-carbon sphinganine, in combination with LC-MS, to measure generated ceramide species, and the induction of distinct set of CerS(s) activities will bring us a step further to clarifying whether CerS activity is specifically targeted by Tax in HTLV-1 infected cells. To clarify the role of ceramide, it is important to investigate possible impact of ST1926 on relevant CerS(s) in HTLV-1 positive and negative T cells. We will, therefore, assess CerS(s) status at mRNA and protein levels in response to ST1926 treatment, in addition to activity levels.

Determining expression and/or activity of CerS(s) will in turn provide insights into deciphering the ultimate cell fate determined by the equilibrium between medium, long and very long chain ceramides inducing apoptosis *versus* survival (Hartmann, Wegner et al. 2013). The epigenetic, transcriptional, translational, and post-translational regulation of individual CerS(s) have been poorly investigated (Wegner, Schiffmann et al. 2016), which might impede our understanding of the effects of the corresponding species with different fatty acyl Co-A and their spatiotemporal regulation.

Although many downstream effector targets of the ceramide apoptotic response are known, the way ceramide triggers these pathways still needs to be unveiled (Zhang, Alter et al. 1996, Guenther, Peralta et al. 2008). CerS(s) can be differentially found in the mitochondrial outer membrane, which contributes to the development of ceramide channels prior to the induction process of apoptosis and facilitates the release of pro-apoptotic proteins from the intermembrane space into the cytoplasm (Siskind 2005, Skommer, Wlodkowic et al. 2007). An inhibitor of apoptosis is the proto-oncogene Bcl-2, which also functions at the mitochondrial membrane (Reed, Haldar et al. 1989, Hockenbery, Nunez et al. 1990, Reed, Cuddy et al. 1990, Reed, Meister et al. 1991). Based on cell type and injury, the accumulated ceramide can either induce cell cycle arrest by activation of the retinoblastoma protein or induce apoptosis by a Bcl-2-regulated mechanism (Dbaibo, Pushkareva et al. 1995, Dbaibo 1997, El-Assaad, El-Sabban et al. 1998). It is noteworthy to delineate the possibility that ceramide antagonizes Bcl2-inhibitory pathways of cell death. The literature reports conflicting data in describing the interplay between ceramide and Bcl-2 in the context of cell death. Molt-4 cells overexpressing Bcl-2 will be used as a model to investigate the role of Bcl-2 during the induction of cell death and ceramide accumulation during the ST1926-mediated response. While Tax participates in tumorigenesis as a viral oncoprotein, Bcl-2 is a cellular oncoprotein that contributes to cancer development and resistance. Both collaborate in a common pathway that contributes to ATL pathogenesis and resistance (Nakashima, Kawakami et al. 2003, Fu, Qu et al. 2011, Alibek, Irving et al. 2014, Warren, Wong-Brown et al. 2019). It is, therefore, imperative to investigate their mutual relationships in the ceramide and cell death responses to ST1926 treatment. Targeting ceramide metabolism might alter drug resistance in a variety of solid tumors

and hematological malignancies by unfolding key knots or nodes in retinoid regulation of ceramide-based metabolism, helping foster cancer therapeutics (Huang, Chen et al. 2011). In this study previous findings will be extended to elucidate the role of ST1926 in alleviating Tax-inhibitory effects on ceramide generation.

Aim1: Determine the ceramide dose and kinetic response to ST1926 treatment in HTLV-1 positive and negative T cells.

Aim2: Characterize ceramide and dihydroceramide species modulated in response to ST1926 action on HTLV-1 positive and negative T cells.

Aim 3: Delineate ST1926-mediated regulation of the *de novo* ceramide pathway in HTLV-1 positive and negative malignant T cells.

Aim 4: Clarify ST1926-induced crosstalk between ceramide and the Bcl-2 oncoprotein and the ensuing pathway of cell death in Molt-4 T cells.

CHAPTER IV

RESTORATION OF DE NOVO CERAMIDE SYNTHESIS BY THE SYNTHETIC RETINOID ST1926 AS IT INDUCES ADULT T CELL LEUKEMIA CELL DEATH

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Ceramide (Cer) is a bioactive cellular lipid with compartmentalized and tightly regulated levels. Distinct metabolic pathways lead to the generation of Cer species with distinguishable roles in oncogenesis. Deregulation of Cer pathways has emerged as an important mechanism for acquired chemotherapeutic resistance. Adult T-cell leukemia (ATL) cells are defective in Cer synthesis. ATL is an aggressive neoplasm that develops following infection with human T cell lymphotropic virus-1 (HTLV-1) where the viral oncogene Tax contributes to the pathogenesis of the disease. ATL cells, resistant to all-*trans*-retinoic acid, are sensitive to pharmacologically achievable concentrations of the synthetic retinoid ST1926. We studied the effects of ST1926 on Cer pathways in ATL cells. ST1926 treatment resulted in early Tax oncoprotein degradation in HTLV-1 treated cells. ST1926 induced cell death and a dose- and time-dependent accumulation of Cer in malignant T cells. The kinetics and degree of Cer production showed an early response upon ST1926 treatment. ST1926 enhanced *de novo* Cer synthesis via activation of ceramide synthase CerS(s) without inhibiting dihydroceramide desaturase, thereby accumulating Cer rather than the less bioactive dihydroceramide. Using labeling experiments with the unnatural 17-C sphinganine and measuring the generated Cer species, we showed that ST1926 preferentially induces the activities of a distinct set

of CerS(s). We detected a delay in cell death response and interruption of Cer generation in response to ST1926 in Molt-4 cells overexpressing Bcl-2. These results highlight the potential role of ST1926 in inducing Cer levels, thus lowering the threshold for cell death in ATL cells.

A. Introduction

Retinoids are powerful compounds known for their tumor-suppressive roles as they regulate hematopoietic cell proliferation and differentiation (Altucci and Gronemeyer 2001, El Hajj, Khalil et al. 2014, Han, Goralski et al. 2016, Uray, Dmitrovsky et al. 2016) . Although all-*trans* retinoic acid (ATRA), the active metabolite of vitamin A, is used for the treatment of certain leukemia types particularly acute promyelocytic leukemia (APL), the use of natural retinoids is hindered by acquired resistance and side effects (de The 1996, Fontana and Rishi 2002). The retinoid related molecules (RRMs), such as N-(4-hydroxyphenyl) retinamide (HPR), 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437), and (2E)-3-[3'-(1-adamantyl)-4'-hydroxy[1,1'-biphenyl]-4-yl]-2-propenoic acid (ST1926) show potent anti-neoplastic activities (Fontana and Rishi 2002). In adult T-cell leukemia (ATL), both HPR and CD437 induce growth arrest and cell death in human T-cell lymphotropic virus-1 (HTLV-1) positive and negative T cells, including those that are ATRA-resistant (Darwiche, El-Sabban et al. 2001, Darwiche, Hatoum et al. 2004). However, treatment with ST1926 requires lower doses and shows potent anti-tumor effects with minimal toxicity, increased specificity, and broad spectrum of activity in solid and hematological malignancies *in vivo* and *in vitro*, including many that are ATRA-resistant (Cincinelli, Dallavalle et al. 2003, Garattini and Terao 2004). In

addition to inducing DNA damage and S-phase arrest, working through RAR-dependent and independent mechanisms (Valli, Paroni et al. 2008), both CD437 and ST1926 target DNA polymerase 1 alpha (POLA1) (Han, Goralski et al. 2016, Abdel-Samad, Aouad et al. 2018). We have previously reported that ST1926 is a more potent inducer of growth inhibition and massive apoptosis than HPR or CD437, especially in HTLV-1 positive T cells, probably due to the fact that only ST1926 causes early downregulation of Tax, the critical oncoprotein of HTLV-1 (Darwiche, Hatoum et al. 2004, El Hajj, Khalil et al. 2014). ST1926 also prolongs survival and reduces leukemic cell infiltration in ATL mice, decreases Tax mRNA and DNA, and induces apoptosis *in vivo* (El Hajj, Khalil et al. 2014). Meanwhile, Tax oncoprotein protected cells from ceramide (Cer) accumulation and apoptosis (Darwiche, Hatoum et al. 2004, Darwiche, Abou-Lteif et al. 2005). Given the fact that ATL, similar to most blood cancers, displays a genetically heterogeneous clonal profile and due to chemotherapy resistance, alternative therapies that could be mediated by the accumulation of lethal levels of Cer might have potential promise (Casson, Howell et al. 2013, Pitman, Powell et al. 2015, Tan, Liu et al. 2016, Lewis, Wallington-Beddoe et al. 2018).

Cer, a sphingolipid-tumor suppressor, acts as a second messenger that mediates cell death and growth suppression through several mechanisms such as apoptosis, necroptosis, senescence, cell cycle arrest, and autophagy (Hannun and Bell 1987, Perry, Obeid et al. 1996, Hannun and Obeid 2008, Ogretmen 2018). Cer pathways are highly conserved and act as coordinators of eukaryotic stress response (Hannun 1996). A variety of signals could trigger Cer production, including chemotherapeutic agents (Hannun and Luberto 2000, Pettus, Chalfant et al. 2002). Numerous studies have identified direct Cer targets, namely Cer-activated protein phosphatases (CAPPs),

which constitute serine/threonine protein phosphatases PP1 and PP2A (Dobrowsky, Kamibayashi et al. 1993). These phosphatases act on several substrates that promote changes in growth arrest, apoptosis, and/or senescence, such as retinoblastoma gene product RB, Bcl-2, AKT, and c-Jun (Hannun 1996, Chalfant, Ogretmen et al. 2001, Ogretmen and Hannun 2004). Cer production is highly compartmentalized and occurs via three main metabolic pathways, through *de novo* synthesis, sphingomyelin turnover, or the salvage pathway following the reacylation of sphingosine generated from hydrolyzed Cer that is in turn generated from the hydrolysis of more complex sphingolipids (Bankeu, Mustafa et al. 2010, Mullen, Spassieva et al. 2011, Ogretmen 2018). Alternatively, Cer could be also generated by the inhibition of its metabolism by glucosyl-ceramide synthase (GCS) and/or sphingomyelin synthase (SMS), or its clearance by ceramidase (CDase) (Itoh, Kitano et al. 2003, Bedia, Casas et al. 2011, Morad and Cabot 2015). Indeed, acid CDase was found to be overexpressed in acute myeloid leukemia (AML) patients (Tan, Liu et al. 2016), modulating Mcl-1 expression, while its inhibition sensitizes cells to chemotherapeutics (Bedia, Casas et al. 2011, Flowers, Fabrias et al. 2012). Moreover, using acid CDase inhibitor along with HPR treatment sensitizes human prostate cancer cells to apoptosis (Morad and Cabot 2013). GCS was found to be overexpressed in several types of leukemic cell lines including those that are chemotherapy resistant (Itoh, Kitano et al. 2003, Grazide, Terrisse et al. 2004, Kartal Yandim, Apohan et al. 2013), and maintains a potential role in lymphoma and myeloma tumor initiation (Pavlova, Archer et al. 2015). Indeed, inhibiting GCS by 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and the subsequent Cer accumulation sensitize imatinib-resistant chronic myeloid leukemia cells among other types of cancer cells (Baran, Bielawski et al. 2011, Kartal Yandim, Apohan et al. 2013).

Moreover, blocking Cer glucosylation with PDMP synergizes with HPR treatment to induce cell death in HTLV-1 positive human T-cells (Darwiche, Abou-Lteif et al. 2005). We have previously shown that HPR increases Cer levels in HTLV-1 negative leukemia cells, but not in HTLV-1 transformed cells, concomitant with lower sensitivity of HTLV-1 positive cells to treatment with exogenous C2- and C6-Cer. This was attributed to a defect in *de novo* Cer synthesis in HTLV-1 positive cells treated with HPR. Indeed, Tax protein transfected cells were less sensitive to HPR-induced cytotoxicity and generated lower levels of Cer. In fact, targeting Cer metabolism might alter drug resistance in a variety of solid tumors and hematological malignancies (Huang, Chen et al. 2011).

Also known as N-acylsphingosine, Cer consists of a C18-sphingoid base backbone to which fatty acid chains of variable lengths are added (Reynolds, Maurer et al. 2004, Ogretmen 2018), whereby the most commonly found Cer species in mammalian cell membranes are with C16-C24 fatty acyl chains (Morad and Cabot 2013). Based on the lengths of different fatty acyl chains they utilize in the *de novo* pathway, six different ceramide synthases (CerSs) have been discovered that are products of different genes (Riebeling, Muller et al. 2003, Hannun and Obeid 2011, Lewis, Wallington-Beddoe et al. 2018, Ogretmen 2018). The specificity of these enzymes includes substrate preference for different fatty acyl chain lengths generating the corresponding Cer species, which vary in their tissue and subcellular localization, context of stimulation, and availability of downstream targets that ultimately dictates distinct roles in cancer cell death and/or survival (Laviad, Albee et al. 2008, Levy and Futerman 2010, Ogretmen 2018). The chain length specific effects of Cer have gained more attention in tumorigenesis, as high-throughput, structural, and quantitative

analytical methods allowed for better understanding of the corresponding CerS(s) spatiotemporal regulation and role in cancer cell killing (Grosch, Schiffmann et al. 2012). As for the spatial regulation, it has been recently shown that CerS(s) can localize in the mitochondrial outer membrane, which leads to Cer channel formation prior to the induction phase of apoptosis and facilitates the release of pro-apoptotic proteins from the intermembrane space into cytoplasm (Siskind 2005, Skommer, Wlodkowic et al. 2007). Cancer lipidomics is emerging as a new cancer profiling method to monitor prognosis, diagnosis, and treatment (Shen, Yang et al. 2017). There is solid evidence for dysregulation of sphingolipid metabolism in hematological malignancies which often confer resistance to current treatment regimens (Lewis, Wallington-Beddoe et al. 2018). Thus, the promise of combining sphingolipid modulators with chemotherapeutics would provide novel approaches for treating several blood cancers.

In the current work, we demonstrate that clinically achievable concentrations of ST1926 provoke similar Cer responses, in both HTLV-1 positive and negative malignant T cells, that was not observed with previously tested RRMs such as HPR and CD437. ST1926 but not HPR or CD437 causes early Tax degradation. Compared to HPR, which accumulates dihydroceramide (dhCer), ST1926 enhances *de novo* Cer synthesis via activation of specific CerS(s) without inhibiting dihydroceramide desaturase (DEGS1), thereby accumulating Cer rather than dhCer. At the mitochondrial level, the intrinsic pathway of apoptosis is tightly regulated by the Bcl-2 family members and orchestrated by the bioactive sphingolipid ceramide. At this level, the pro-apoptotic Cer might antagonize the effects of Bcl-2 oncoprotein in a multitude of pathways. We dissected the interplay between the anti-apoptotic protein Bcl-2 and Cer. We observed that Bcl-2 attenuated ST1926 growth-inhibitory response and interrupted

both *de novo* Cer accumulation and cell death in Molt-4 cells. These results highlight the potential role of ST1926 in inducing Cer levels, thus lowering the threshold for cell death in ATL cells and overcoming resistance.

B. Materials and Methods

1. Cell lines, Drugs, and Culture Conditions

The HTLV-1 transformed CD4+ T cell-lines, HuT-102 and MT-2, and HTLV-1 negative CD4+ T- cell lines, Jurkat and Molt-4 were grown as previously described (Darwiche, El-Sabban et al. 2001). Molt-4 cells transfected with p-MEP4 with (Molt4-Bcl2) or without (Molt4-MEP4) full length murine *bcl-2* were clonally selected for once per week and before each experiment with Hygromycin B1 (Invitrogen) (500 μg/ml). ST1926 was prepared as stock solutions in dimethylsulfoxide (DMSO) at 1 x 10^{-2} M in amber tubes and stored at -80°C. All cells were grown in RPMI-1640 (Lonza) medium containing 10% (v/v) Fetal Bovine Serum (FBS) and antibiotics. All experiments were done under dim light. A seeding density of $3x10^5$ cells/mL was chosen for all experiments, unless specified otherwise. The synthetic retinoid, ST1926 was kindly provided by Sigma-Tau and Biogem (Ariano Irpino, Italy) and reconstituted in DMSO at a concentration of 10^{-2} M and stored at -80^{o}C. The final concentrations of DMSO never exceeded 0.1%, which showed no effect on the proliferation of all tested cells. The unnatural 17C-sphinganine was purchased from Avanti Polar Lipids and reconstituted in DMSO to be added at a concentration of 4 μM.

2. Ceramide Measurement

Lipids were collected by the method of Bligh and Dyer (Bligh and Dyer 1959). Cer was measured with a modified diacylglycerol kinase (DGK) assay using external standards as described previously (Bielawska, Perry et al. 2001). Briefly, 80% of the lipid sample was dried under N_2. The dried lipid was solubilized in 20 µl of an octyl β-D-glucoside/dioleoyl phosphatidylglycerol micellar solution (7.5% octyl β-D-glucoside, 25 mM dioleoyl phosphatidylglycerol) by several cycles of sonication for 30 mins. The reaction buffer was prepared as a 2X solution, containing 100 mM imidazole HCl (pH 6.6), 100 mM LiCl, 25 mM $MgCl_2$ and 2 mM EGTA. To the lipid micelles, 50 µl of 2X reaction buffer were added, 0.2 µl of 1M dithiothreitol, 5 µg of diglycerol kinase membranes and dilution buffer (10 mM imidazole, pH 6.6, 1 mM diethylenetriaminepenta-acetic acid, pH 7) to a final volume of 90 µl. The reaction was started by adding 10 µl of 2.5mM [γ -^{32}P] ATP solution (specific activity of 75000–200000 cpm/nmol). The reaction was allowed to proceed at 25°C for 30 min. Bligh and Dyer lipid extraction was performed and a 1.5 ml aliquot of the organic phase was dried under N_2. Lipids were then resuspended in 50 µl of methanol/chloroform (1:9, v/v) and 25 µl was spotted on to a 20 cm silica gel TLC plate. Plates were developed using chloroform/acetone/methanol/acetic acid/H_2O (50:20:15:10:5, by vol), air-dried and subjected to autoradiography. The radioactive spots corresponding to phosphatidic acid and ceramide-phosphate, the phosphorylated products of diacylglycerol and Cer, respectively, were identified by comparison with known standards. Spots were scraped into a scintillation vial containing 4 ml of scintillation fluid and counted in a scintillation counter. Linear curves of phosphorylation were produced over a concentration range of 0–960 pM of external standards (CIII-ceramide, Sigma). Cer

levels were routinely normalized to lipid phosphate levels. It is important to note that under these conditions, there was a total conversion of Cer and diacylglycerol into their phosphorylated products, and there was no change in the specific activity of the DGK enzyme. Cer levels were normalized to lipid phosphate levels.

3. De Novo Ceramide Synthesis

At initiation of treatment, [^{3}H]-palmitic acid (1 μCi/mL medium) purchased from Perkin Elmer (32.0 Ci/mmol) was added to treated and untreated samples. At the indicated time points, lipids were extracted according to Bligh and Dyer method (Bligh and Dyer 1959), N_2 dried, and resuspended in 60 μL chloroform:methanol (2:1); 40 μL were spotted on 20 cm silica gel TLC plates. Plates were developed with ethylacetate:isooctane:acetic acid (90:50:20, v/v), air dried, and sprayed lightly with En3hance® (Perkin Elmer) to enhance tritium readings. [^{3}H]-Cer spots were visualized by iodine vapor mark. Radioactivity was visualized by autoradiography after 96 h at -80˚C and the [^{3}H]-Cer spots were scraped into scintillation vials containing 4 ml of scintillation fluid and counted on a Packard scintillation counter. [^{3}H]-Cer counts were normalized to lipid phosphate levels.

4. In Vitro Ceramide Synthase and Dihydroceramide Desaturase Activity

Cells were treated with unnatural 17C-sphinganine and either 0.1% DMSO or 1 μM ST1926 for 24h. Activities of the mentioned enzymes were determined using lyophilized samples from ST1926 treated and untreated cells. The subsequently generated unnatural 17C-dihydroceramide (17dhCer) and 17C-ceramide (17Cer) species levels were identified and quantified using LC-MS and then normalized to total lipid

phosphates to reflect the activities of the corresponding CerS(s) and DEGS1, respectively.

5. Liquid Chromatography-Mass Spectrometry (LC-MS)

Cells were harvested at the indicated time-points, and cell pellets were washed twice with 1X PBS, and shipped as lyophilized samples to the Medical University of South Carolina (MUSC) for sphingolipidomics analysis (LC-MS) as described (Bielawski, Pierce et al. 2010). Samples were fortified with internal standards and lipids were extracted with ethyl acetate/isopropanol/water (60:30:10 by vol), evaporated to dryness and reconstituted in 100 µl of methanol. Analysis was performed using electrospray ionization MS/MS analysis on a Thermo Finnigan TSQ 7000 triple quadruple mass spectrometer, operating in multiple-reactions-monitoring positive-ionization mode, as previously described (Pettus, Bielawska et al. 2003).

6. Immunoblot Analysis

Total protein extracts from treated and untreated cells were prepared as previously described (Darwiche, Hatoum et al. 2004). GAPDH (MAB5476) (Abnova, Heidelberg, Germany) was used as control. Mouse monoclonal anti-Tax (168-A51) was obtained from the National Institutes of Health AIDS Research and Reagent Program. Rabbit polyclonal anti-CerS2 antibody (ab227501) and rabbit polyclonal anti-LASS6/CerS6 (ab115539) were obtained from abcam.

7. RNA Extraction

Cells were lysed and total RNA isolated using TRI Reagent (Sigma Aldrich) according to the manufacturer's protocol and quantified using DeNovix DS-11FX Spectrophotometer according to the manufacturer's protocol.

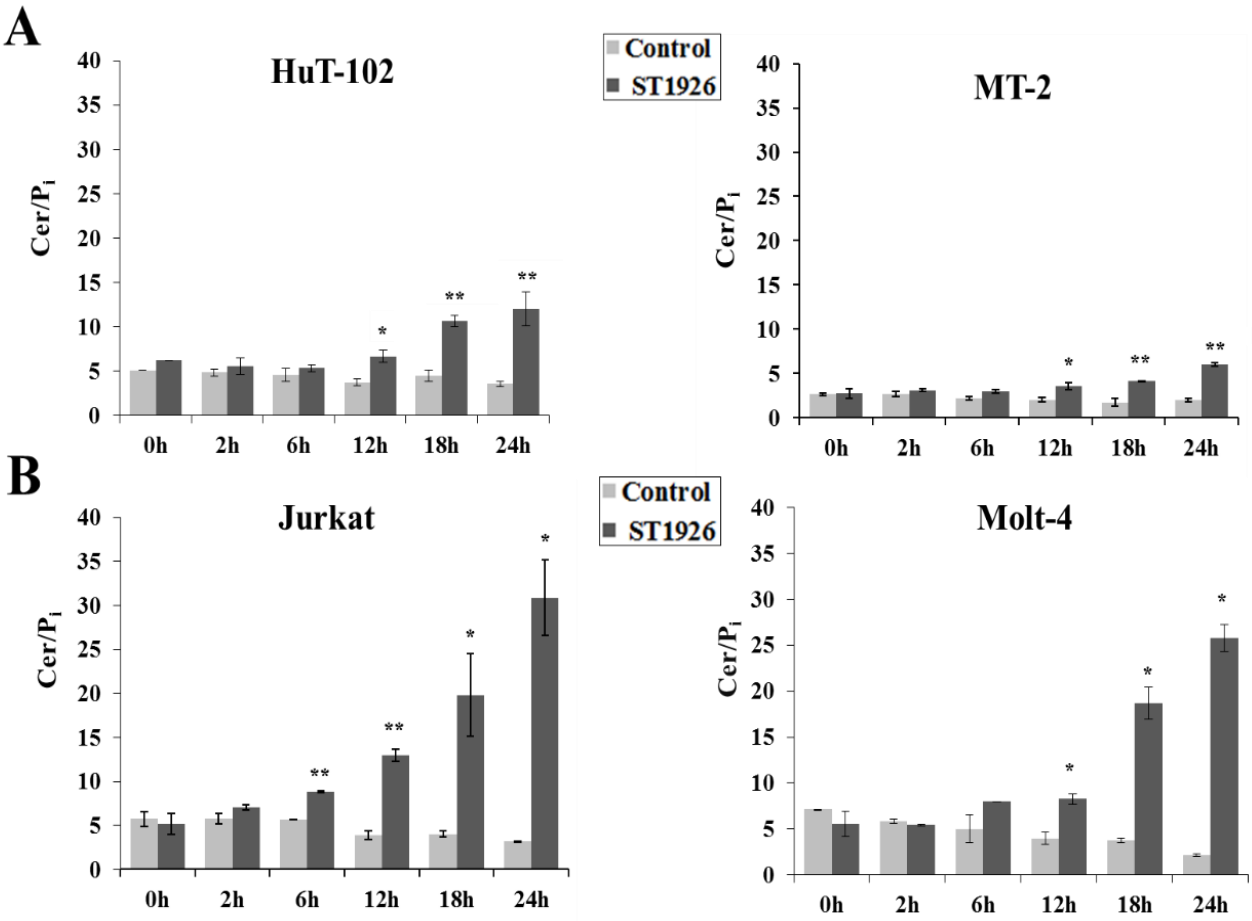
8. cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA samples were reverse-transcribed using QuantiTect Reverse Transcription Kit (QIAGEN, catalogue #205311) according to the manufacturer's instructions. Primer sequences for the three CerS genes and for the housekeeping gene β-actin are: **CerS2,** 5'-CCG ATT ACC TGC TGG AGT CAG-3' (Forward), and 5'-GGC GAA GAC GAT GAA GAT GTT G -3' (Reverse); **CerS4**, 5'-CTT CGT GGC GGT CAT CCT G-3' (Forward), and 5'-TGT AAC AGC AGC ACC AGA GAG-3' (Reverse); **CerS6**, 5'-GGG ATC TTA GCC TGG TTC TGG-3' (Forward), and 5'-GCC TCC TCC GTG TTC TTC AG-3' (Reverse); **β-actin**, 5'-ATT GGC AAT GAG CGG TTC C-3' (Forward), and 5'-GGT AGT TTC GTG GAT GCC ACA-3' (Reverse). Briefly, cDNA was amplified in a 20 µL mixture loaded in 96-well plates containing forward and reverse primers, SYBR® Green JumpStart Taq ReadyMix (Sigma-Aldrich, catalogue #S4438) and RNase-free water. The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing for 45 s and extension at 72 °C for 1 min. A melt curve was incorporated at the end of each reaction to ensure the specificity of the product. Relative expression analysis was performed using the $2^{-\Delta\Delta Ct}$ calculation method by normalization to the housekeeping gene β-actin.

C. Results

1. ST1926 Induces Early Ceramide Accumulation in Both HTLV- 1 Positive and Negative Malignant T cells

We have previously determined that HPR produces distinct Cer responses in HTLV-1 positive and HTLV-1 negative malignant T cells, whereby Tax-transformed T cells showed a defect in accumulating Cer (Darwiche, Abou-Lteif et al. 2005). Therefore, we tested the effect of ST1926 on the kinetics of Cer accumulation in HTLV-1 positive (HuT-102 and MT-2) and HTLV-1 negative (Molt-4 and Jurkat) malignant T cell lines. We selected an ST1926 concentration of 1 μM, as it results in more than 90% of growth inhibition at 48 h in all tested cells, with no effect on resting or activated peripheral blood mononuclear cells (El Hajj, Khalil et al. 2014). Treatment with this dose generated a time-dependent response in Cer in HTLV-1 positive cells (Figure 4.1A). Cer accumulation started at 12 h, reaching 3-fold of baseline by 24 h in HuT-102 and MT-2 cells. In HTLV-1 negative cells, treatment with 1 μM ST1926 resulted in a similar, but earlier and more pronounced accumulation of Cer (Figure 4.1B). The significant rise in Cer levels became apparent by 6 h in Jurkat and by 12 h in Molt-4 cells with 2-fold increase, reaching at least 10-fold by 24 h in both cell types (Figure 4.1B). Notably, the sustained increase in Cer levels preceded major ST1926-induced growth suppression and cell death in all tested HTLV-1 positive and negative cells at the indicated concentrations (El Hajj, Khalil et al. 2014). Furthermore, we observed a dose-dependent response of Cer accumulation in all tested cells using ST1926 concentrations ranging from 0.05 μM up to 5 μM for 24 h. Cer increase reached 2-fold of baseline levels with concentrations of ST1926 as low as 0.1 μM in HTLV-1 positive cells (Figure 4.1C). Meanwhile, Cer accumulation reached 5-fold and 3-fold of baseline in Molt-4 and Jurkat cells respectively when the concentration was increased to 1 μM (Figure 4.1D). Our results show a dose-dependent accumulation of Cer in both ST1926-treated HTLV-1 positive and negative T cells.


A
HuT-102
MT-2
Control
ST1926
Cer/Pi
0h
2h
6h
12h
18h
24h
B
Jurkat
Molt-4
Control
ST1926
Cer/Pi

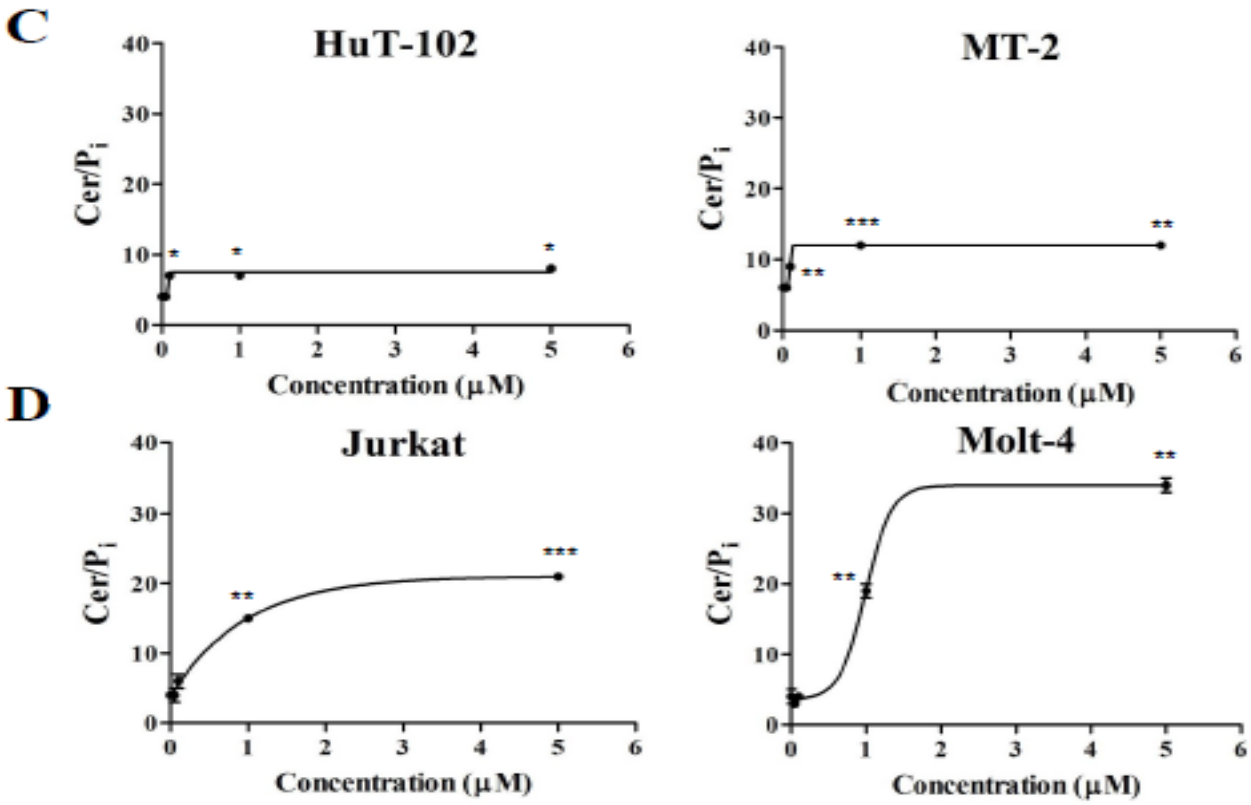


Figure 4.1. ST1926 Treatment Causes Early Time- and Dose-Dependent Accumulation of Ceramide in HTLV-1 Positive and Negative Malignant Human T cells.

(A) Ceramide (Cer) levels in HTLV-1 positive (HuT-102 and MT-2) and **(B)** HTLV-1 negative (Jurkat and Molt-4) human T-cell lines. Cells were seeded at a density of $3x10^5$ cells/ml and treated with 0.1% DMSO as control or with 1 μM ST1926 for the times indicated. Cer levels were determined in triplicates using the DGK assay as described in the Methods section and normalized to total cellular lipid phosphate levels. Data points represent the mean (± SD). Results are representative of two independent experiments. **(C)** Dose-response to ST1926 treatment in HTLV-1 positive (HuT-102 and MT-2) and **(D)** HTLV-1 negative (Jurkat and Molt-4). Cells were seeded at a density of 3.5×10^5 cells/ml and treated with 0.1% DMSO as a control, or the indicated concentrations of ST1926, for 24 h. Cer levels were determined as in **(A and B)**. Data points represent the mean (± SD). Results are representative of two independent experiments. The asterisks * and ** indicate statistically significant differences at $p\leq0.05$ or $p\leq0.01$, respectively, *versus* control using the *t*-test.

2. ST1926 Induces Early De Novo Ceramide Synthesis in HTLV-1 Positive and Negative Malignant T cells

We have previously shown that HTLV-1 positive cells have impaired Cer production in response to HPR, such that *de novo* Cer synthesis is only induced in treated HTLV-1 negative cells (Darwiche, Abou-Lteif et al. 2005). Moreover, the defect in Cer synthesis upon HPR treatment is Tax-dependent in HTLV-1 positive cells, as well as in Tax over-expressing Molt-4 and HeLa cells. Therefore, we examined the contribution of *de novo* Cer synthesis in HuT-102 and Molt-4 cells in response to ST1926 in order to elucidate the role of this pathway in the resulting Cer accumulation in HTLV-1 positive and HTLV-1 negative malignant T cells, respectively. HuT-102 and Molt-4 cells were treated with 1 μM ST1926 at the indicated time-points, and *de novo* synthesized Cer was measured by quantifying [^{3}H]-palmitate incorporation into newly synthesized Cer following treatment. ST1926 treatment caused an early time-dependent increase in [^{3}H]-Cer in both cell lines, as early as 12 h post treatment (Figure 4.2A and 4.2B). ST1926-induced *de novo* synthesis was more pronounced in Molt-4 cells reaching about 10-fold increase over baseline levels versus a 2-fold increase in HuT-102 cells at 24 h post treatment (Figure 4.2A and 4.2B). Interestingly, *de novo* Cer accumulation in HuT-102 started at 12 h and was preceded by Tax protein degradation in HuT102 that was demonstrable by 8 h (Figure 4.2C).

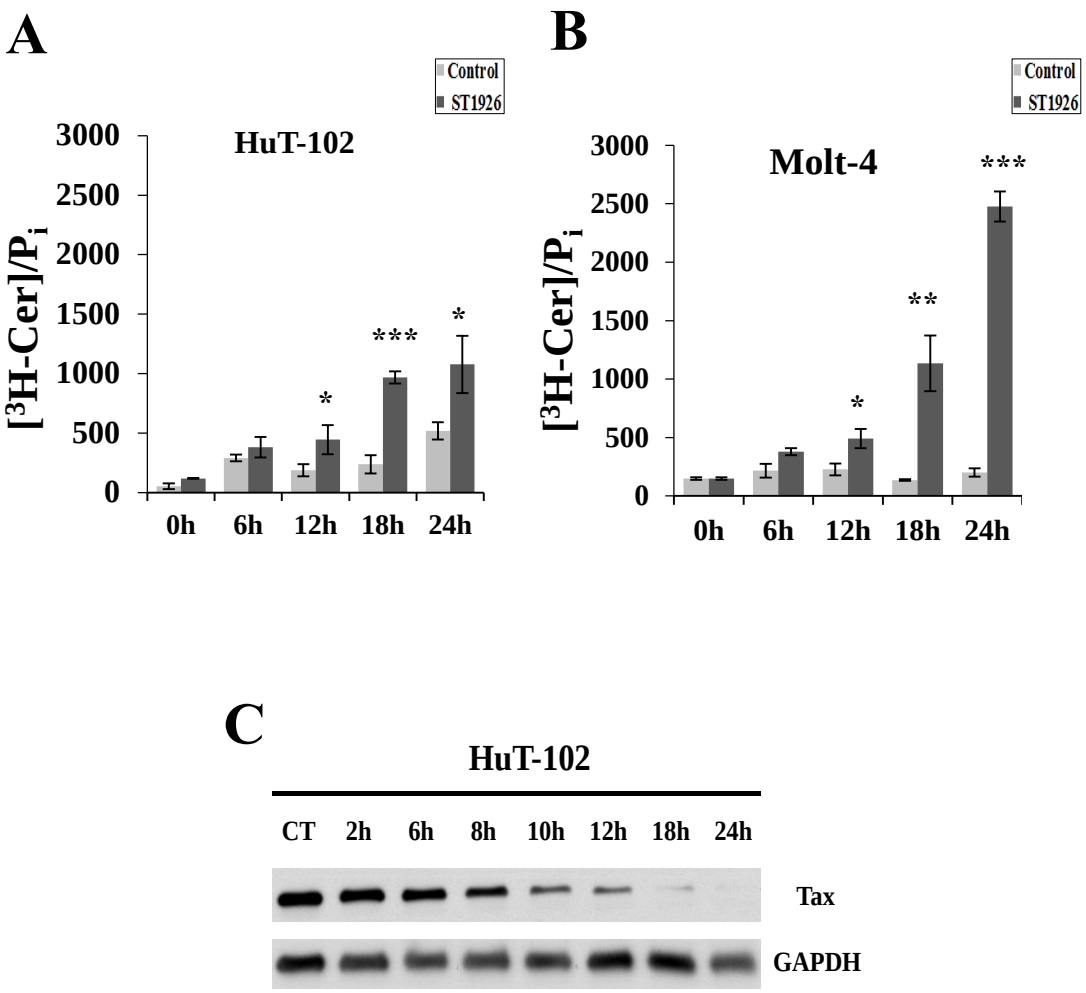


Figure 4.2. ST1926 Stimulates Early *De Novo* Ceramide Synthesis in HTLV-1 Positive and Negative Malignant Human T Cells.
(A) ST1926 induces *de novo* ceramide (Cer) production in HTLV-1 positive (HuT-102) and **(B)** HTLV-1 negative (Molt-4) malignant T-cells. HuT-102 and Molt-4 cells were seeded at a density of 3x 10^5 cells/ml and treated with 0.1% DMSO as control or 1 μM ST1926 for the times indicated. *De novo* Cer levels were determined in triplicates using the [^{3}H]-palmitate incorporation method as described in Methods and normalized to total cellular lipid phosphate levels. Data points represent the mean (± SD). Results are representative of two independent experiments. The asterisks *, **, and *** indicate statistically significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively, *versus* control using the *t*-test. **(C)** ST1926 causes early degradation of Tax oncoprotein levels in HuT-102 cells. Cells were seeded at a density of 2x10^5 cells/ml and treated with 0.1% DMSO as control or 1 μM ST1926 for the times indicated. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against Tax antibody. The blot was re-probed against GAPDH to ensure equal protein loading.

3. Ceramide, But Not Dihydroceramide, Species Predominantly Accumulate in Response to ST1926 Treatment in HTLV-1 Positive and Negative Malignant T Cells

We have previously shown that HTLV-1 positive and HTLV-1 negative cells showed distinct Cer responses upon treatment with HPR, whereby Tax overexpression alone in cells not infected with HTLV-1 was sufficient to suppress *de novo* Cer synthesis (Darwiche, Abou-Lteif et al. 2005). It was later reported that DEGS1, the enzyme responsible for converting dhCer to Cer, is directly inhibited by HPR leading to the accumulation of endogenous dhCers rather than Cers (Rahmaniyan, Curley et al. 2011). Although dhCer, unlike Cer, has been considered biologically inactive in the context of cell death induction (Saddoughi and Ogretmen 2013), there is evidence that it might rather have an inhibitory role (Stiban, Fistere et al. 2006). Stiban *et al.* showed that dhCer interferes with Cer channel formation in the mitochondria, consequently inhibiting permeabilization and transition to the apoptotic response. In fact, reports demonstrate opposing roles of DEGS1 in apoptosis (Siddique, Li et al. 2015), whereby its polyubiquitination results in "gain of function" with pro-survival effects (Alsanafi, Kelly et al. 2018). Indeed, inhibiting DEGS1 activity with drugs other than HPR, siRNA, or gene depletion causes resistance to apoptosis by various stimuli (Gagliostro, Casas et al. 2012, Siddique, Bikman et al. 2012, Breen, Joseph et al. 2013, Siddique, Li et al. 2013). Meanwhile, depending on cell types, dhCer might not have a role in HPR-triggered apoptosis (Uyama, Hong et al. 2005), suggesting that the apoptogenic potential depends on the ratio of dhCer to Cer, rather than dhCer alone (Uyama, Hong et al. 2005, Stiban, Fistere et al. 2006).

In order to determine whether the effects of ST1926 on the regulation of Cer metabolism were similar to, or distinguishable from, those of HPR, we measured various Cer and/or dhCer species that are generated upon ST1926 treatment in HTLV-1

positive and negative malignant T-cells as represented by HuT-102 and Molt-4 cells, respectively. Cer and/or dhCer species were identified based on the fatty acyl- chain length that is preferentially introduced by different CerS(s) on the sphingoid base backbone of sphinganine. In this regard, the resulting Cer species were categorized as medium long-chain (MLC: C14- C18), long-chain (LC: C20- C22), and very long-chain (VLC: C24-C26). In HuT-102 cells, Cer+dhCer levels increased from 738 pmol/µmol to 1923 pmol/µmol, which represents an increase by 1185 pmol/µmol upon treatment with 1 µM ST1926 for 24 hours (Table 4.1A). In Molt-4 treated cells, Cer+dhCer levels increased from 947 pmol/µmol to 3369 pmol/µmol, which represents an increase by 2422 pmol/µmol. Overall, MLC species accounted for most of the accumulation of the total dhCer and Cer levels in both cell lines, followed by VLC species, with the least contribution by the LC species (Table 4.1B). ST1926 treatment resulted in a major accumulation of Cer species relative to dhCer species in both HuT102 and Molt-4 cells. Importantly, Cer elevation was found to be consistent among most MLC (Figure 4.3A), LC (Figure 4.3B), and VLC (Figure 4.3C) Cer species. For instance, among MLC Cers, accumulation of C16-Cer represented the most prominent increase among other species in this category in both HuT-102 and Molt-4 treated cells (Figure 4.3A). Similarly, there was a total increase in LC Cers, majorly represented by C22 and C22:1-Cer species, among others in both cell lines (Figure 4.3B). Consistently, VLC Cers had similar trends of increasing levels in both HuT-102 and Molt-4 cells, with C24:1-Cer being the most dominant in this category (Figure 4.3C). This analysis supports the conclusion that ST1926 induces early *de novo* Cer synthesis in both cell lines without appreciable accumulation of dhCer suggesting a lack of inhibition of DEGS1 by ST1926.

Table 4.1. Percent Accumulation of Total and Specific Fatty-Acyl Chain Ceramide (Cer) and Dihydroceramide (dhCer) Species in HTLV-1 Positive and Negative Malignant T Cells upon Treatment with ST1926.

Percent increase in total Cer and dhCer species in HuT-102 and Molt-4 cells

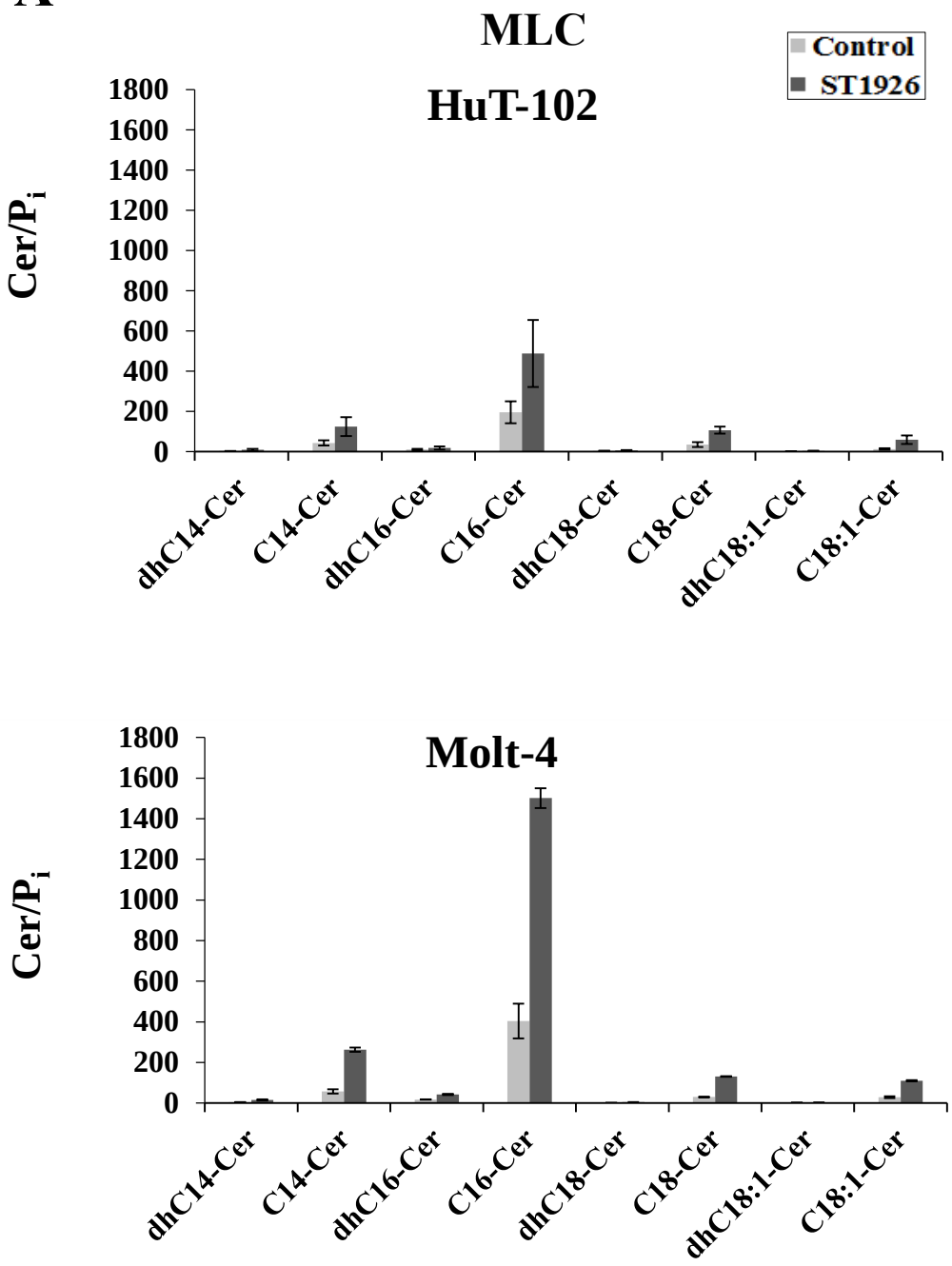
	Total (dhCer +Cer)	Ceramide (% Total Cer)	Dihydroceramide (% Total Cer)
HuT102	1185	98	2
Molt-4	2422	97	3

Percent increase of MLC, LC, and VLC Cer and dhCer species in HuT-102 and Molt-4 cells

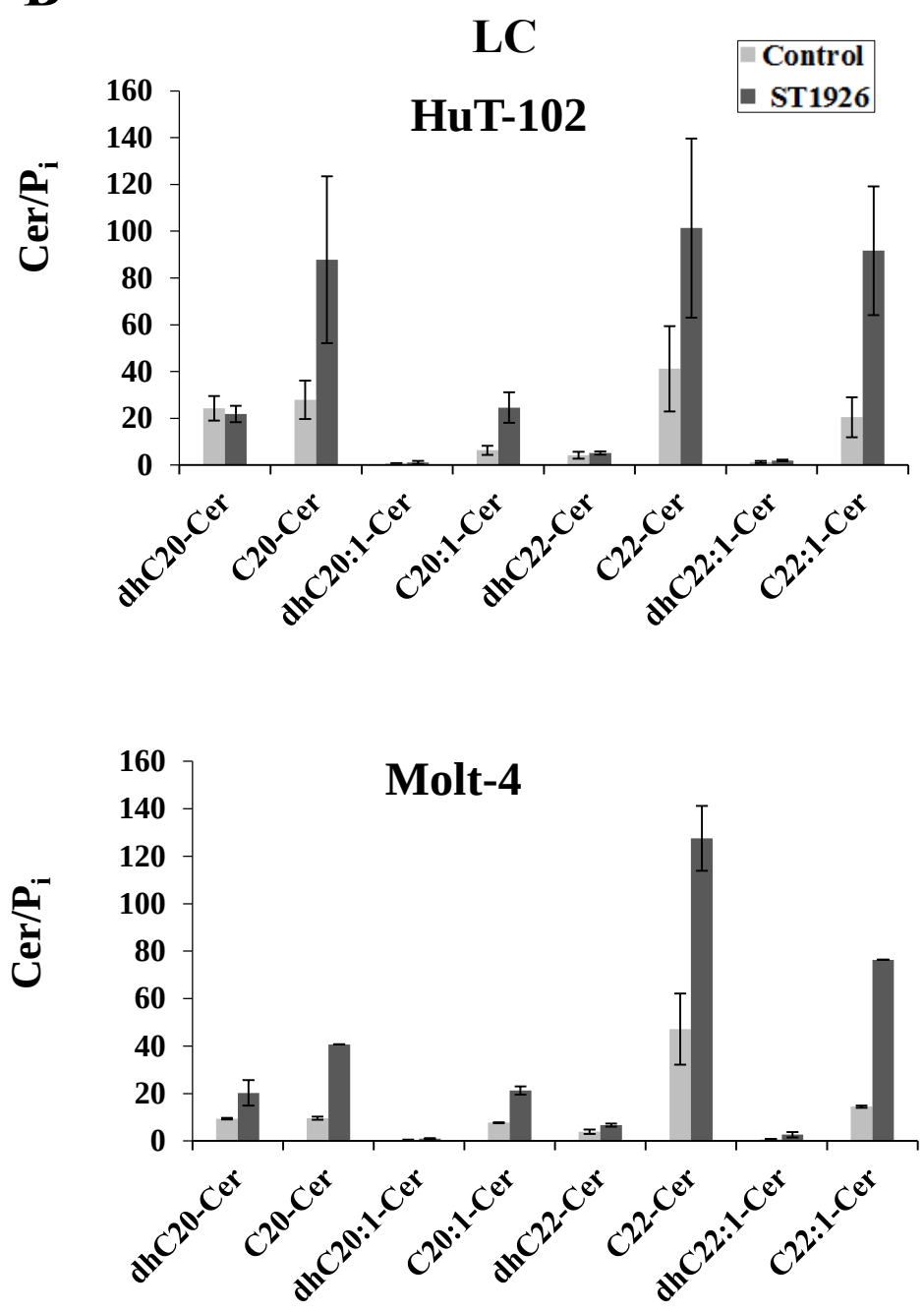
	Total (dhCer +Cer)	MLC Cer (% Total Cer)	LC Cer (% Total Cer)	VLC Cer (% Total Cer)
HuT102	1185	43	18	39
Molt-4	2422	63	8	29

(A) Percent increase in total ceramide (Cer) and dihydroceramide (dhCer) species in HTLV-1 positive (HuT-102) and negative (Molt-4) cells. **(B)** Percent increase of medium long chain (MLC), long chain (LC), and very long chain (VLC) Cer and dhCer species in HuT-102 and Molt-4 cells. Cells were seeded at a density of $3x10^5$ cells/ml and treated with 0.1% DMSO as control or 1µM ST1926 for 24h. DhCer/Cer species levels (pmol) were measured in lyophilized samples as duplicates by LC-MS as described in Methods and normalized to total cellular lipid phosphate levels (µmol). Data points represent the mean $\pm$ range (n=2). Results are representative of two independent experiments.

A


MLC
HuT-102
Control
ST1926
Cer/P$_i$
1800
1600
1400
1200
1000
800
600
400
200
0
dhC14-Cer
C14-Cer
dhC16-Cer
C16-Cer
dhC18-Cer
C18-Cer
dhC18:1-Cer
C18:1-Cer
Molt-4
Cer/P$_i$
1800
1600
1400
1200
1000
800
600
400
200
0
dhC14-Cer
C14-Cer
dhC16-Cer
C16-Cer
dhC18-Cer
C18-Cer
dhC18:1-Cer
C18:1-Cer

B


LC
HuT-102
Control
ST1926
Cer/Pi
160
140
120
100
80
60
40
20
0
dhC20-Cer
C20-Cer
dhC20:1-Cer
C20:1-Cer
dhC22-Cer
C22-Cer
dhC22:1-Cer
C22:1-Cer
Molt-4
Cer/Pi
160
140
120
100
80
60
40
20
0
dhC20-Cer
C20-Cer
dhC20:1-Cer
C20:1-Cer
dhC22-Cer
C22-Cer
dhC22:1-Cer
C22:1-Cer

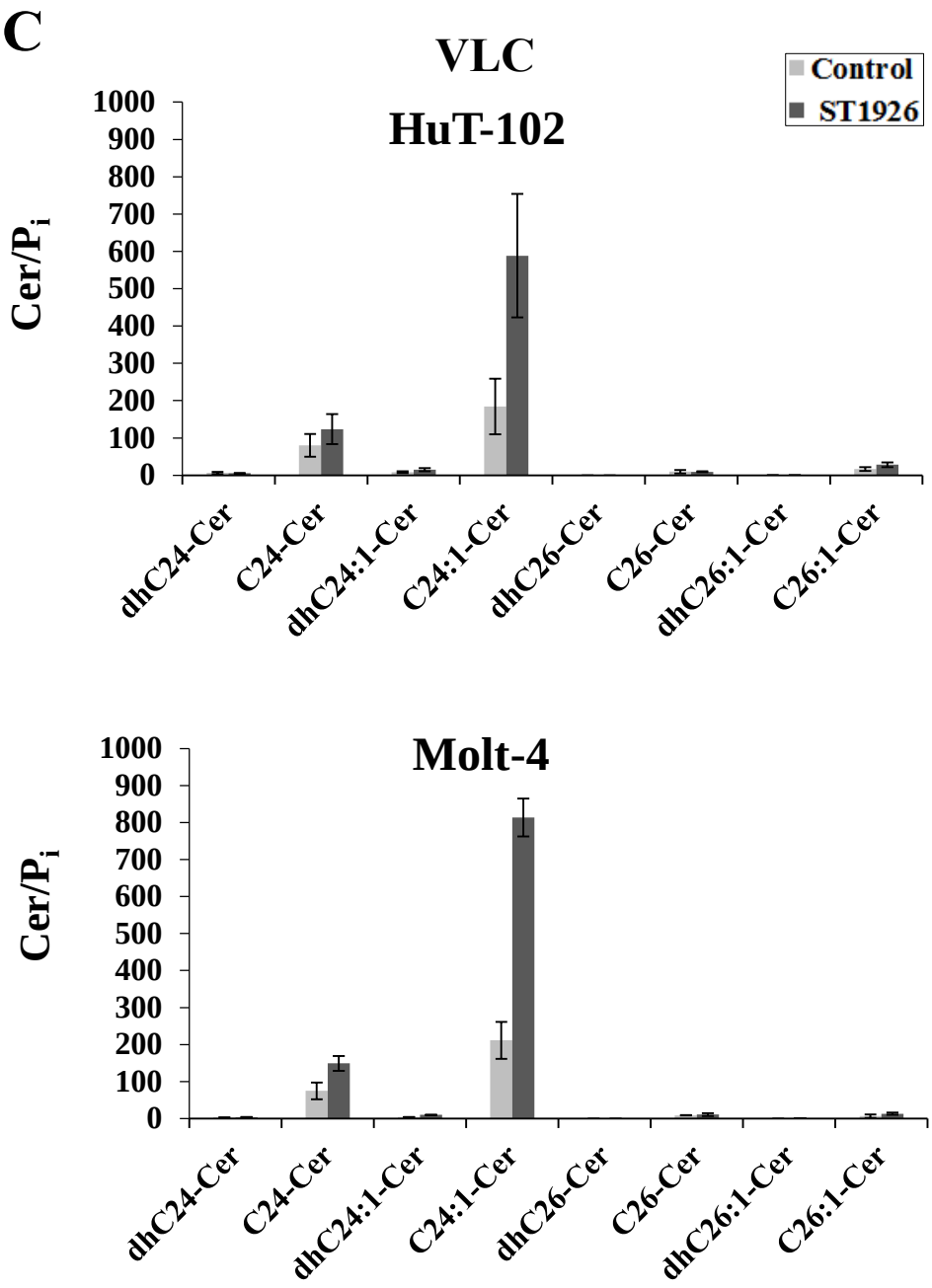


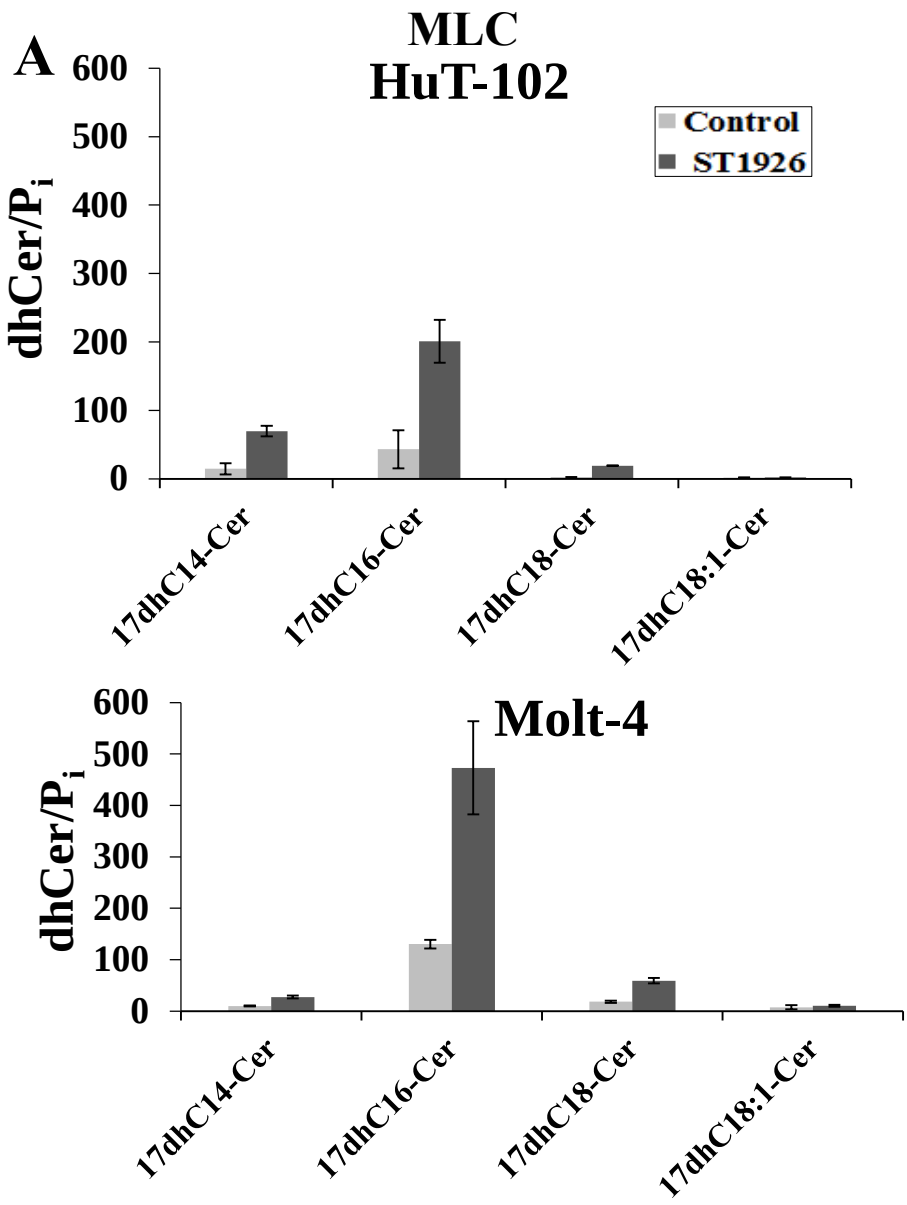
Figure 4.3. Accumulation of Ceramide, But Not Dihydroceramide Species in HTLV-1 Positive and Negative Malignant T Cells in Response to Treatment with ST1926.

(A) Medium long-chain (MLC), **(B)** Long-chain (LC), and **(C)** Very long-chain (VLC) dihydroceramide (dhCer) or ceramide (Cer) species levels in HTLV-1 positive (HuT-102) and HTLV-1 negative (Molt-4) cells. Cells were seeded at a density of $3x10^5$ cells/ml and treated with 0.1% DMSO as control or 1μM ST1926 for 24 h. DhCer/Cer species levels (pmol) were measured in lyophilized samples in duplicates by LC-MS as described in Methods and normalized to total cellular lipid phosphate levels (μmol). Data points represent the mean ± range (n=2). Results are representative of two independent experiments.

4. Treatment with ST1926 Activates Ceramide Synthases in Both HTLV-1 Positive and Negative Leukemic T cells

Previously, we have shown that Tax expression in cells not infected with HTLV-1 was sufficient to inhibit the generation of Cer in response to HPR and to suppress *de novo* Cer synthesis, probably due to Tax inhibitory effect on CerS activity (Darwiche, Abou-Lteif et al. 2005). To further characterize Tax-dependent regulation of *de novo* Cer synthesis and to identify the enzymes involved in HTLV-1 positive and negative leukemic T cells in response to ST1926, we treated cells labeled with the unnatural 17C-sphinganine to indirectly determine the activities of CerS(s) and DEGS1. Measurement of the resulting unnatural endogenous 17dhCer and 17Cer species, the products of the acylation followed by desaturation reactions of the 17C-sphinganine by CerS(s) and desaturase, respectively, were obtained using LC-MS analysis following treatment with ST1926 for 24 h in HuT-102 and Molt-4 cells. In HuT-102 cells, we observed a 5-fold increase above baseline in each of 17dhC16 (Figure 4.4A), 17dhC20 and 17dhC22, 10-fold increase in 17dhC22:1 (Figure 4.4B), and 5-fold increase in 17dhC24:1 (Figure 4.4C), among others. Likewise, treatment of Molt-4 cells with ST1926 resulted in 4-fold increase in 17dhC16 (Figure 4.4A), 2-fold increase in each of 17dhC20 and 17dhC22, 3-fold increase in 17dhC22:1 (Figure 4.4B), and 2-fold increase in 17dhC24:1 (Figure 4.4C), among others. The enhanced production of specific endogenous 17dhCer species indicated that ST1926 preferentially induces the activities of distinct CerS(s) in both tested cell lines, with the most likely candidates being CerS2, CerS4, CerS5, and CerS6 based on fatty acyl preferences of these enzymes (Tidhar, Zelnik et al. 2018). In total, treatment with ST1926 elicited 3-fold and 4-fold increase in 17dhCer species in Molt-4 and HuT-102 cells over baseline, respectively (Figure 4.5A). Meanwhile, there was no significant change in the ratio of most 17Cer/17dhCer species

upon ST1926 treatment in both cell lines (Figure 4.6), indicating that ST1926 activates CerS(s), but does not inhibit the DEGS1, thus maintaining the increase of 17dhCer species that are being synthesized by CerSs and then DEGS1. Moreover, HuT-102 cells displayed lower basal levels of 17dhCer species compared to Molt-4 cells (Figure 4.4, Figure 4.5A), while no difference was observed in the basal level ratios of 17Cer/17dhCer species between the two cell lines (Figure 4.5B). This is consistent with our previous results that HTLV-1 positive cells have a partial defect in Cer synthesis (Darwiche, Abou-Lteif et al. 2005). Indeed, these findings suggest that Tax oncoprotein has an inhibitory effect on CerS(s), and not the DEGS1.



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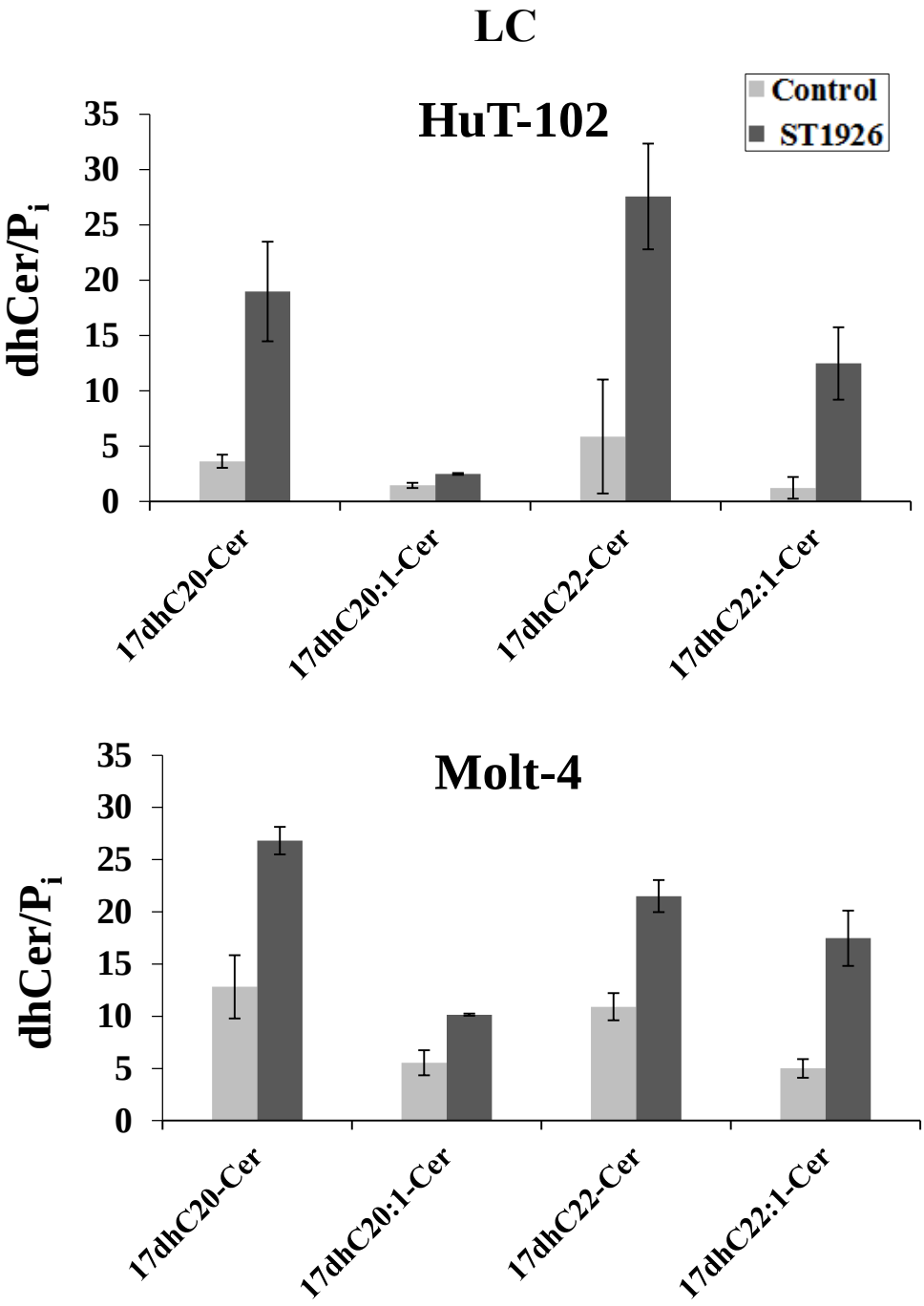

LC
HuT-102
Control
ST1926
dhCer/Pi
35
30
25
20
15
10
5
0
17dhC20-Cer
17dhC20:1-Cer
17dhC22-Cer
17dhC22:1-Cer
Molt-4
dhCer/Pi
35
30
25
20
15
10
5
0
17dhC20-Cer
17dhC20:1-Cer
17dhC22-Cer
17dhC22:1-Cer

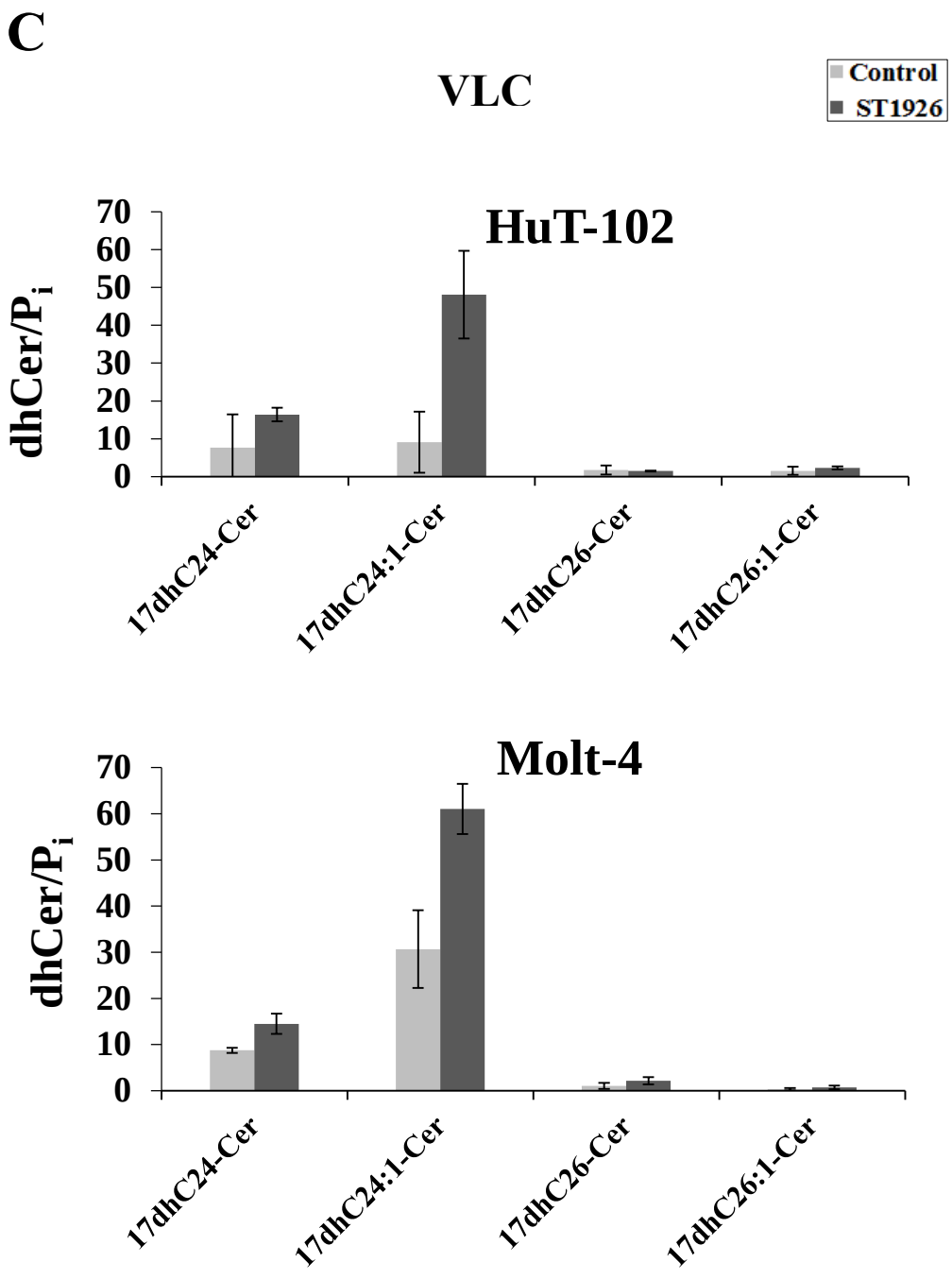


Figure 4.4. ST1926 Activates Ceramide Synthase in HTLV-1 Positive and Negative Malignant T Cells.
(A) Medium long-chain (MLC), **(B)** Long-chain (LC), and **(C)** Very long-chain (LC) 17C-dihydroceramide (17dhCer) species levels in HTLV-1 positive (HuT-102) and HTLV-1 negative (Molt-4) cells. Cells were seeded at a density of $3x10^5$ cells/ml, labeled with 4 µM of unnatural 17C-sphinganine and treated with either 0.1% DMSO as control or 1µM ST1926 for 24 h. 17dhCer species levels (pmol) were measured in lyophilized samples in duplicates by LC-MS as described in Methods and normalized to total cellular lipid phosphate levels (µmol). Data points represent the mean $\pm$ range (n=2). Results are representative of two independent experiments.

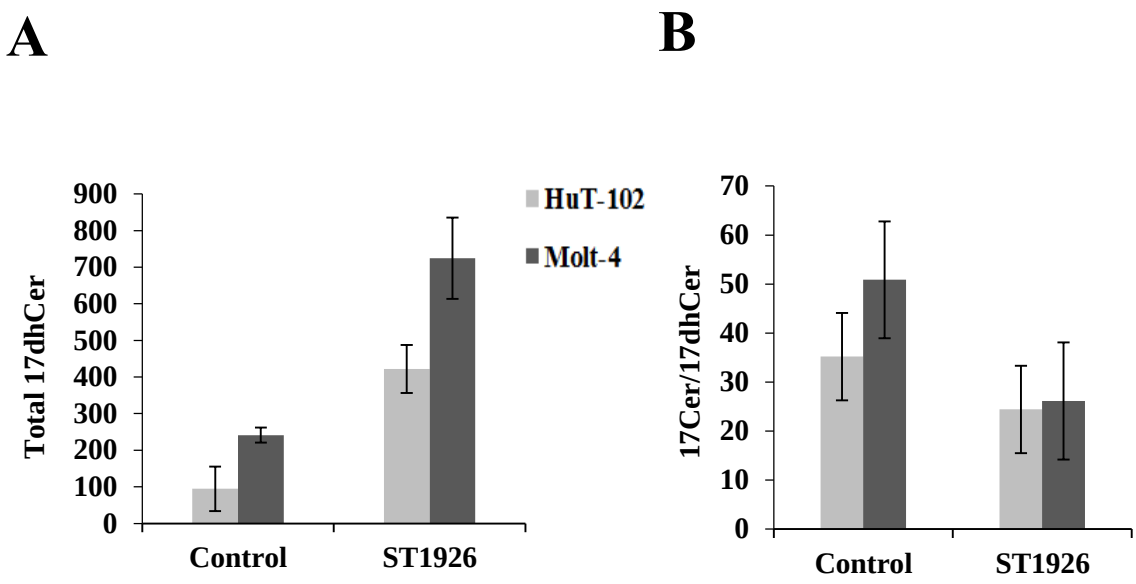
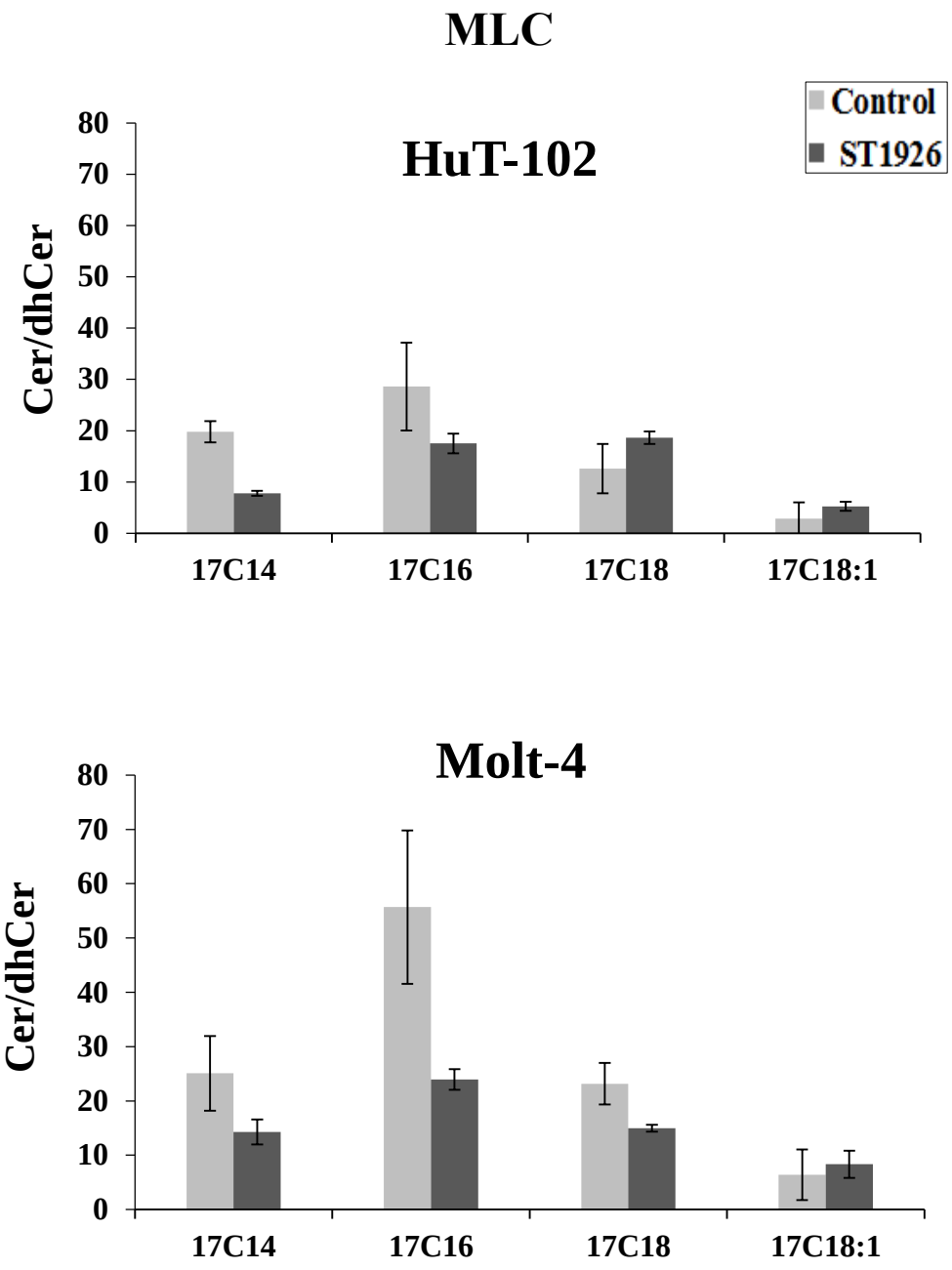
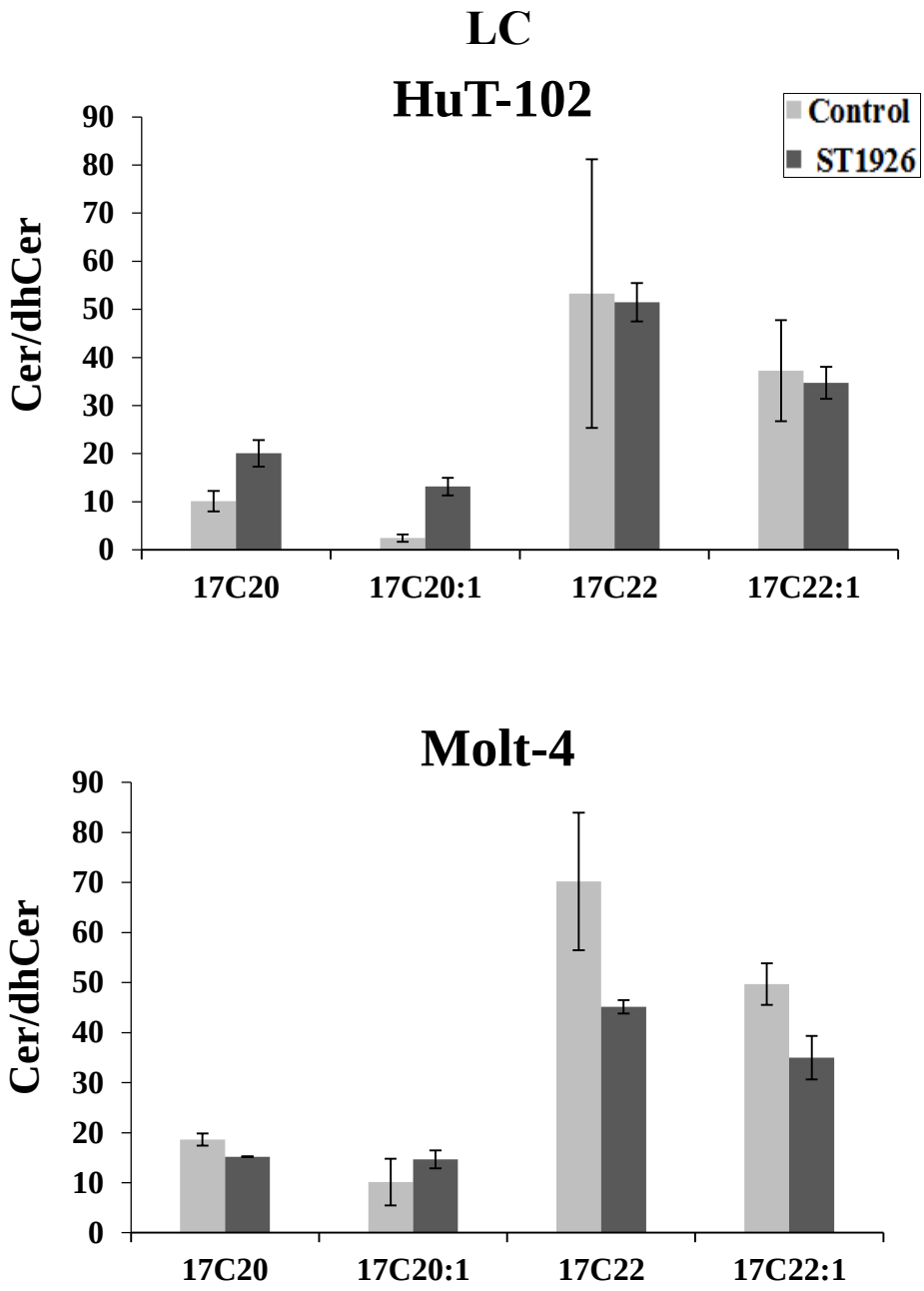


Figure 4.5. Effect of ST1926 on Total Levels of 17dhCer, and Ratio of Total 17Cer to 17dhCer Species in HTLV-1 Positive and Negative Malignant T Cells.
(A) HTLV-1 positive (HuT-102) cells have lower basal levels of 17C-dihydroceramide (17dhCer species) than Molt-4 cells, and treatment with ST1926 increased 17dhCer levels in both HuT-102 and Molt-4 cells, while (B) there was no significant change in the ratio of 17Cer/17dhCer species upon ST1926 treatment in both cell lines, or in the basal level ratios of 17Cer/17dhCer species between the two cell lines. Cells were seeded at a density of 3x105 cells/ml, labeled with 4 µM of unnatural 17C-sphinganine and treated with either 0.1% DMSO as control or 1µM ST1926 for 24h. 17dhCer species levels (pmol) were measured in lyophilized samples as duplicates by LC-MS as described in Methods and normalized to total cellular lipid phosphate levels (µmol). Data points represent the mean $\pm$ range (n=2). Results are representative of two independent experiments

A


MLC
Control
ST1926
HuT-102
Cer/dhCer
80
70
60
50
40
30
20
10
0
17C14
17C16
17C18
17C18:1
Molt-4
Cer/dhCer
80
70
60
50
40
30
20
10
0
17C14
17C16
17C18
17C18:1

B


LC
HuT-102
Control
ST1926
Cer/dhCer
90
80
70
60
50
40
30
20
10
0
17C20
17C20:1
17C22
17C22:1
Molt-4
Cer/dhCer
90
80
70
60
50
40
30
20
10
0
17C20
17C20:1
17C22
17C22:1

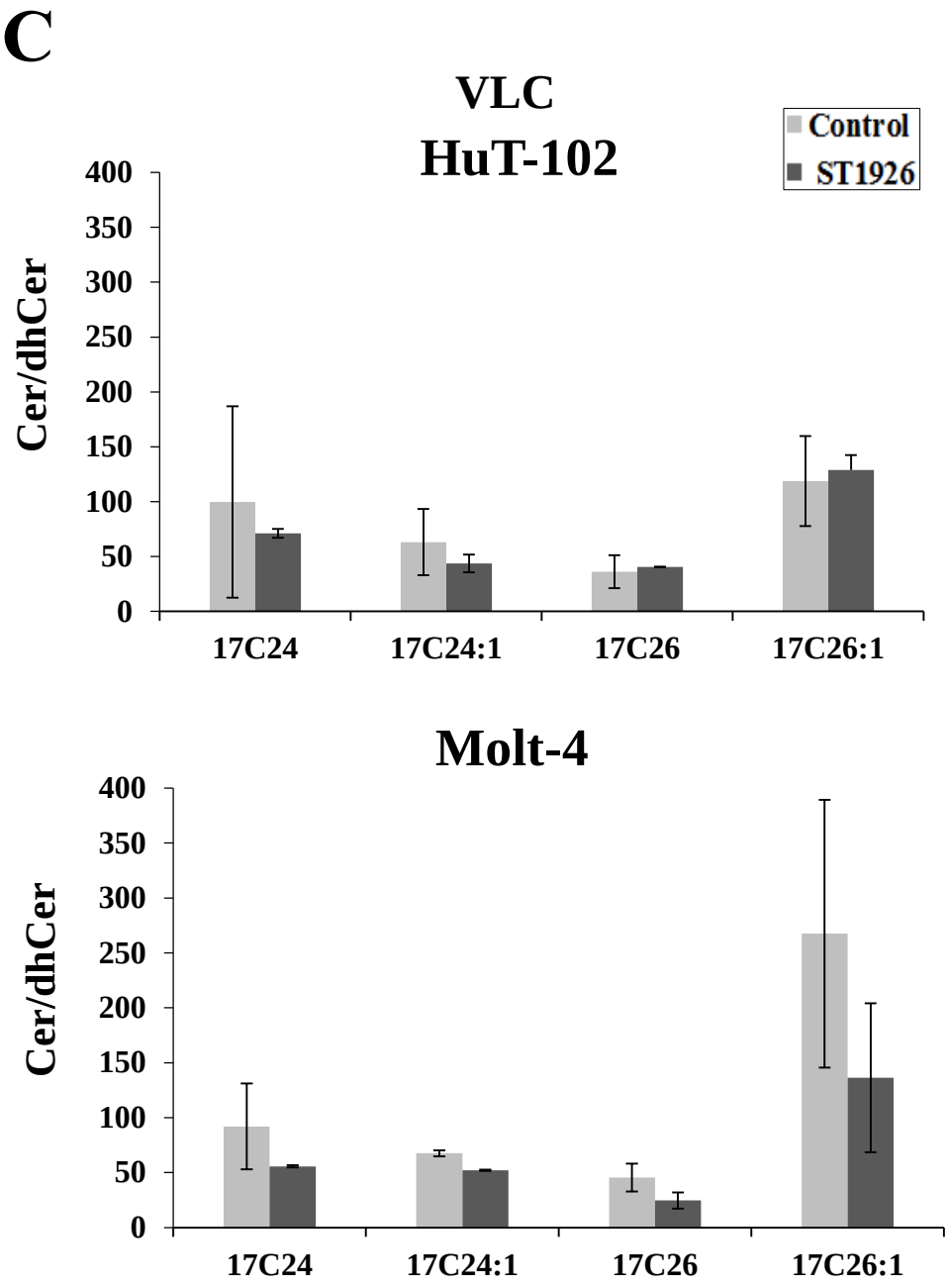
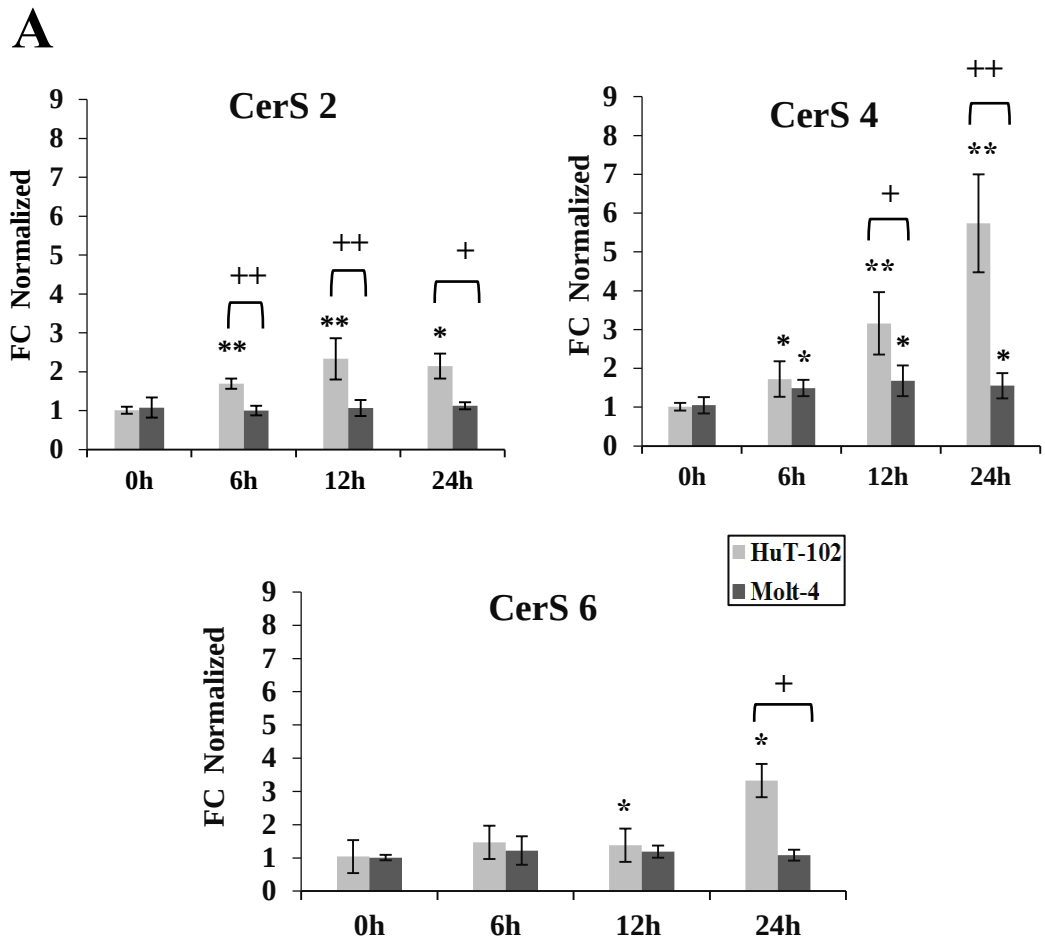


Figure 4.6. Effect of ST1926 on the Activity of Dihydroceramide Desaturase (DEGS1) in HTLV-1 Positive and Negative Malignant T Cells.
(A) Medium long-chain (MLC), **(B)** Long-chain (LC), and **(C)** Very long-chain (VLC) 17C-ceramide (17Cer) species levels in HTLV-1 positive (HuT-102) and HTLV-1 negative (Molt-4) cells. Cells were seeded at a density of $3x10^5$ cells/ml, labeled with 4 µM of unnatural 17C-sphinganine and treated with either 0.1% DMSO as control or 1µM ST1926 for 24h. 17Cer species levels (pmol) were measured in lyophilized samples as duplicates by LC-MS as described in Methods and the activity of DEGS1 was determined by the ratio of 17Cer to 17C-dihydroceramide (17dhCer) levels. Data points represent the mean $\pm$ range (n=2). Results are representative of two independent experiments.

5. Treatment with ST1926 Increases Gene Expression of Ceramide Synthases More Prominently in HTLV-1 Positive Cells

While advances in spectrometric analyses facilitated the identification of Cer species, much less is known about the regulation of CerS(s). A better understanding of the regulation of CerS(s) is central to identify prognostic markers for different diseases and for the implementation of new treatments. In addition to enzyme activity, CerS(s) could be transcriptionally and/or translationally regulated (Wegner, Schiffmann et al. 2016). HTLV-1 driven tumorigenesis is highly driven by Tax-mediated genomic instabilities that maintain pathogenesis and progression of the disease via pathways that regulate cell cycle, inhibit apoptosis, and favor survival (Giam and Semmes 2016, Fochi, Mutascio et al. 2018). Tax is required for malignant transformation of T cells and this is thought to be mediated through NFκB activity (Higuchi and Fujii 2009). We suspect that there is a crosstalk between NFκB and Cer during ST1926-induced degradation of Tax and cell death. Tax, perhaps through NFκB, may suppress the expression of some CerS(s) and once it is degraded the inhibition is released. Therefore, it was imperative to investigate the effect of ST1926 on the transcription of specific CerS(s) that are found to be highly expressed in leukocytes, namely CerS2, CerS4, and CerS6, in both HTLV-1 positive and negative malignant T cells (Levy and Futerman 2010). We assessed CerS(s) status at mRNA and protein levels in response to treatment with 1 μM ST1926. Interestingly, we found a distinct time-dependent profile in mRNA levels of CerS2, CerS4, and CerS6 in HuT-102 treated cells, and to a lesser extent, in Molt-4 treated cells. In HuT-102 cells, ST1926 prompted a significant fold change in gene expression of CerS2 at 6 h (fold change= 1.7), CerS4 at 6 h (fold change= 1.7), and CerS6 at 12 h (fold change= 1.4), reaching to a fold change of 2, 5.7, and 3.3 at 24 h, respectively (Figure 4.7A). However, treatment of Molt-4 cells with ST1926 resulted

in a less significant steady increase in gene expression of CerS4 than in HuT-102 (fold change= 1.7 at 12 h and fold change= 1.6 at 24 h), and no fold change in both CerS2 and CerS6 gene expression (Figure 4.7A). Meanwhile, treatment with ST1926 had no effect on the protein levels of CerS2 and CerS6 in both HuT-102 and Molt-4 cells (Figure 4.7B). Our reported data from LC-MS represent an indirect evidence that the activity of these CerS(s) is also increased by ST1926 in both HTLV-1 positive and negative malignant T cells. These results suggest that ST1926 plausibly regulates CerS(s) at the transcriptional and enzyme activity levels, without altering translational levels. Remarkably, the time-dependent increase in CerS(s) transcription levels in HuT-102, and not Molt-4, cells suggests that ST1926 relieves Tax-suppressive effects on Cer generation in HTLV-1 positive T cells.



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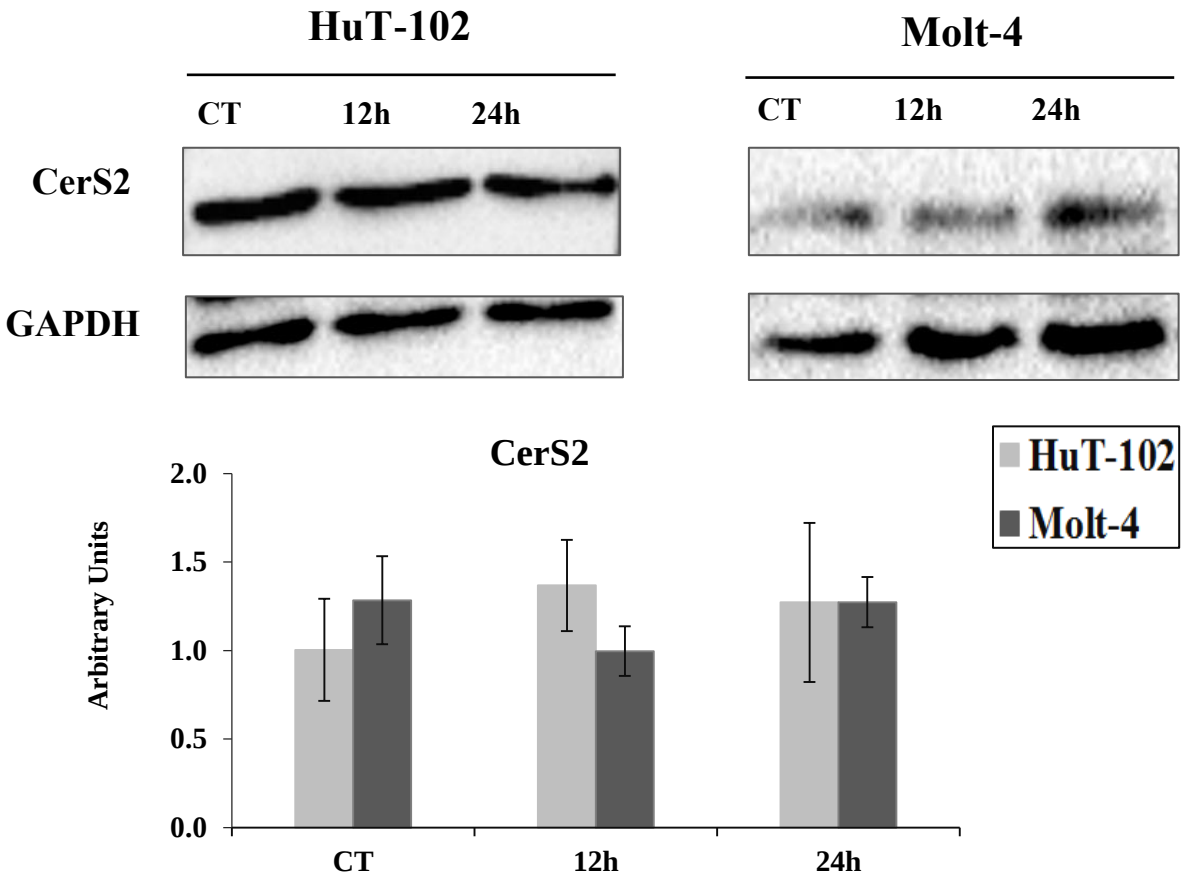

HuT-102
Molt-4
CT
12h
24h
CT
12h
24h
CerS2
GAPDH
CerS2
HuT-102
Molt-4
Arbitrary Units
2.0
1.5
1.0
0.5
0.0
CT
12h
24h

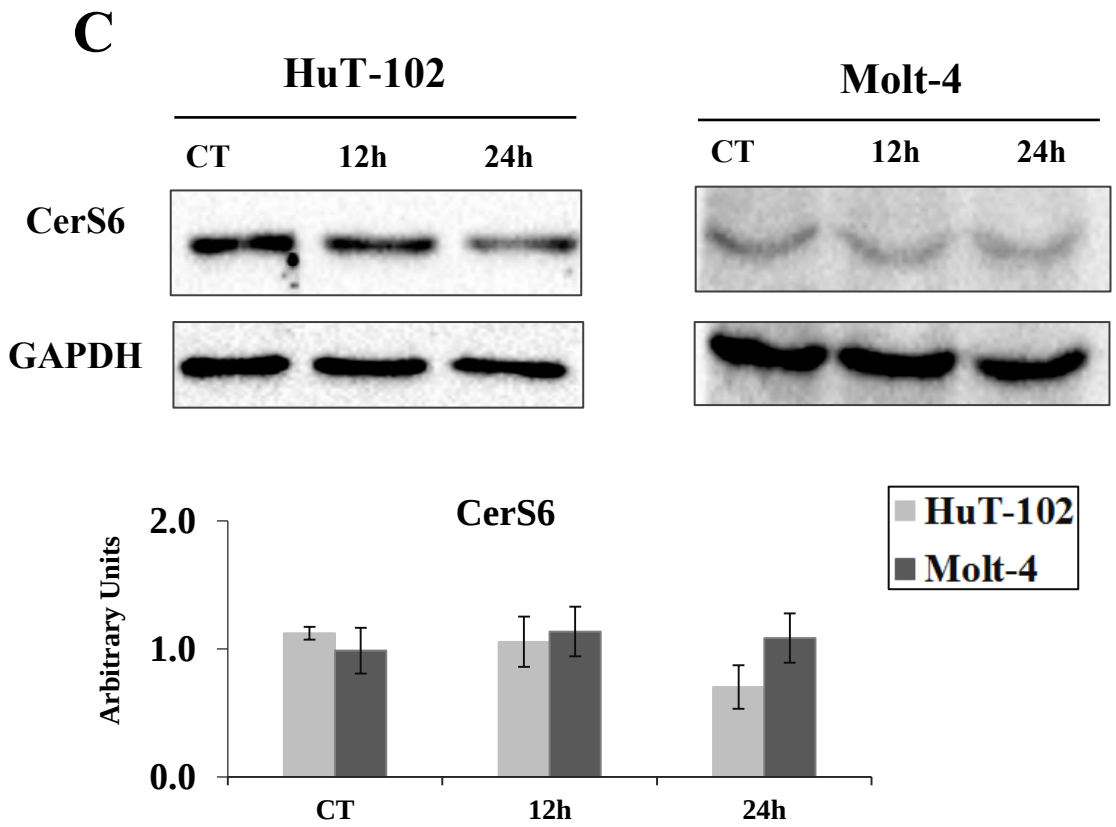
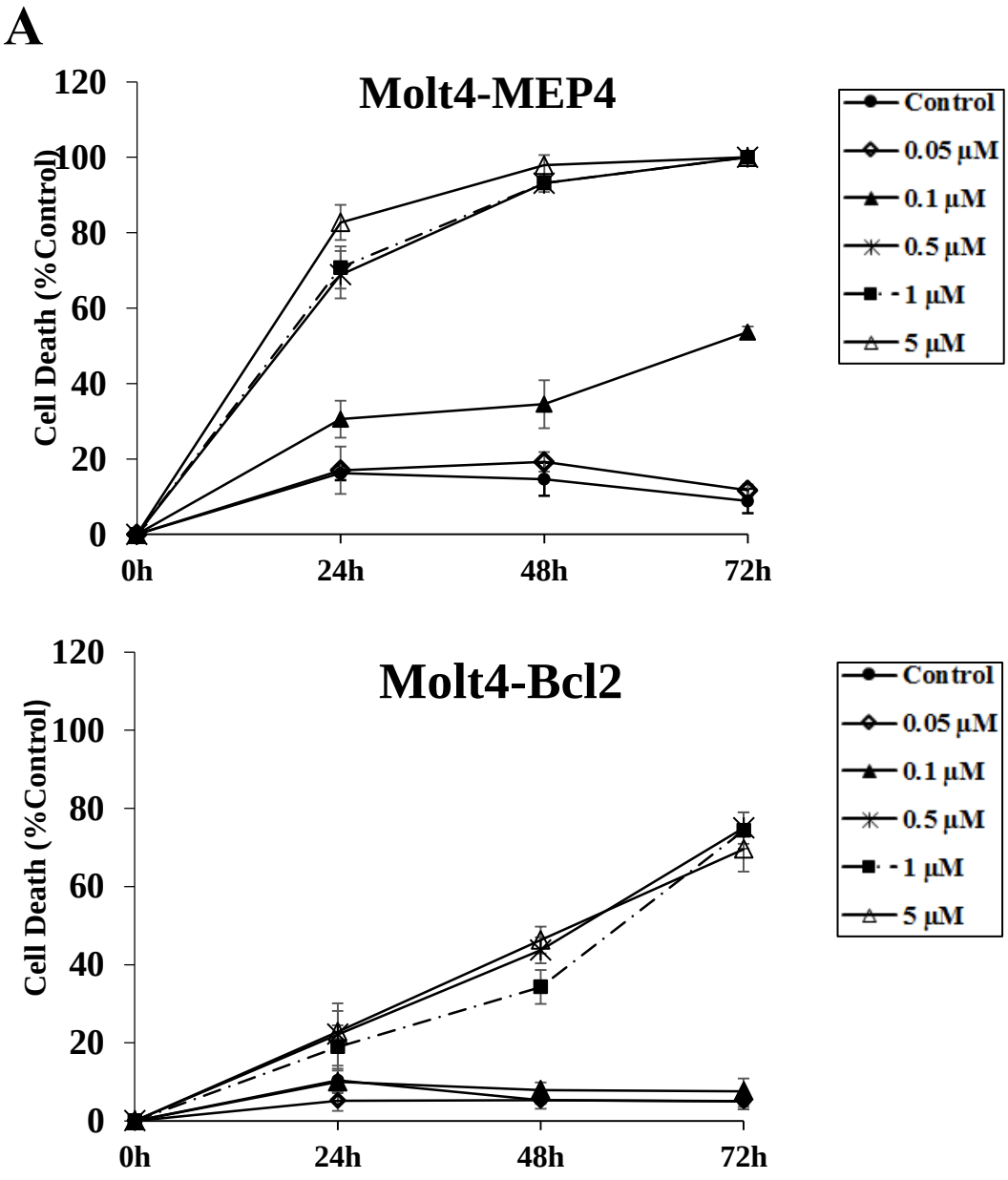


Figure 4.7. Effect of ST1926 on Transcript and Protein Levels of Ceramide Synthases in HTLV-1 Positive and Negative Malignant T Cells.
(A) ST1926 increased gene expression levels of CerS2, CerS4, and CerS6 in HTLV-1 positive (HuT-102) and to a lesser extent in HTLV-1 negative (Molt-4 cells). HuT-102 and Molt-4 cells were seeded at a density of 3x 10^5 cells/ml and treated with 0.1% DMSO as control or 1 µM ST1926 for the times indicated. mRNA levels of CerS2, CerS4, and CerS6 were measured using qRT-PCR for the times indicated. The levels of mRNA were normalized relative to host β-actin. Fold change (FC) was quantified relative to cells treated with 0.1% DMSO as control. Data points represent the mean $\pm$ SEM. Results are representative of three independent experiments. The asterisks *, **, and *** indicate statistically significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively, *versus* control using the *t*-test. Similarly, the plus sign $^{+}$, $^{++}$, and $^{+++}$ indicate statistically significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively, HuT-102 *versus* Molt-4 using the *t*-test. **(B)** ST1926 treatment had no effect on protein levels of CerS2 and CerS6 in HTLV-1 positive (HuT-102) and negative (Molt-4) cells. HuT-102 and Molt-4 cells were seeded at a density of 3x 10^5 cells/ml and treated with 1 µM ST1926 for the indicated timepoints. Whole SDS protein lysates (50 µg/lane) were prepared and immunoblotted against CerS2 and CerS6 antibodies. The blots were re-probed against GAPDH to ensure equal protein loading. Data points represent the mean $\pm$ SEM. Results are representative of three independent experiments.

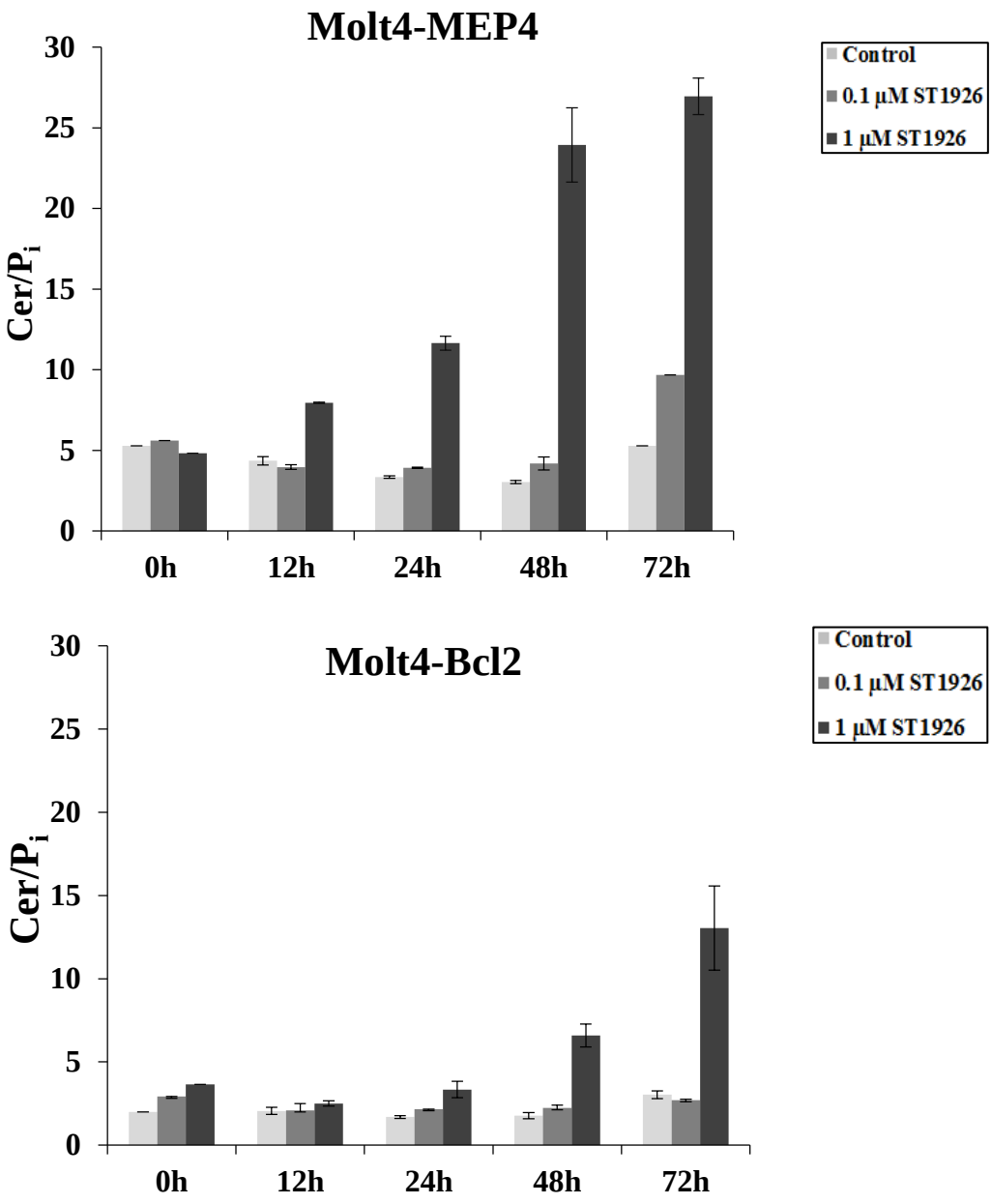
6. Bcl-2 Delays Cell Death Response and Interferes with De Novo Ceramide Synthesis Induced by ST1926 in Molt-4 Cells

Based on cell type and stimuli, Cer coordinates a plethora of apoptotic pathways. Some of these pathways have been identified in hematological malignancies, whereby Cer mediates its apoptotic effects via its inhibitory action on Bcl-2 phosphorylation (Morad and Cabot 2013). In other types of cancers, and based on the treatment used, Bcl-2 acts downstream of Cer, preventing Cer -mediated death in Molt-4 cells without interfering with its vincristine-induced accumulation (Zhang, Alter et al. 1996). Therefore, it became important to delineate the role of Bcl-2 in ST1926-initiated Cer production in cell death of malignant T cells. We treated Molt-4 cells overexpressing Bcl-2 (Molt4-Bcl2) and Molt-4 containing the empty vector p-MEP4 (Molt4-MEP4) with 0.1 μM or 1 μM ST1926 for 12, 24, 48, or 72 h. Cells were assayed for viability by trypan blue exclusion assay, while monitoring Cer accumulation upon treatment with the indicated concentrations and time-points. Bcl-2 attenuated the marked accumulation of Cer in Molt-4 starting at 24 h in response to ST1926 (Figure 4.8B). In particular, 0.1 μM ST1926 caused 50% cell death at 72 h, while no effect was observed in Bcl-2 overexpressing cells (Figure 4.8A). In addition, 1 μM ST1926 caused 70% and 20% cell death in Molt4-MEP4 and Molt4-Bcl2 cells, respectively (Figure 4.8A). Consistently, Bcl-2 interrupted early Cer accumulation at 12 h and blunted the level of accumulation up to 72 h (Figure 4.8B). One μM ST1926 caused a 2-fold increase in Cer as early as 12 h in Molt4-MEP4, while the same effect was delayed and observed in Molt4-Bcl-2 cells only after 48 h (Figure 4.8B). Moreover, treatment with 0.1 μM ST1926 showed no Cer accumulation up to 72 h in Molt4-Bcl2 cells, while causing a 2-fold increase in Molt4-MEP4 cells (Figure 4.8B). Our results indicate that Bcl-2 delays cell death-induced ST1926 treatment of malignant T cells and interferes

with Cer generation. Since we have shown that ST1926 activates *de novo* pathway of Cer production (Figure 4.2), and that Bcl-2 interrupts Cer accumulation upon ST1926 treatment (Figure 4.8B), we wanted to investigate whether Bcl-2 has an inhibitory action on *de novo* generated Cer in response to ST1926 treatment in Molt-4 cells. We treated Molt4-MEP4 and Molt4-Bcl2 cells with 1 μM ST1926 for 12, 24, and 48 h in the presence of [^{3}H] palmitate. Bcl-2 inhibited *de novo* Cer synthesis in response to ST1926 resulting in Cer levels that were several folds lower compared to control starting from 12 h post treatment (Figure 4.8C).



B


Molt4-MEP4
Control
0.1 µM ST1926
1 µM ST1926
Cer/P$_i$
30
25
20
15
10
5
0
0h
12h
24h
48h
72h
Molt4-Bcl2
Control
0.1 µM ST1926
1 µM ST1926
Cer/P$_i$
30
25
20
15
10
5
0
0h
12h
24h
48h
72h

C

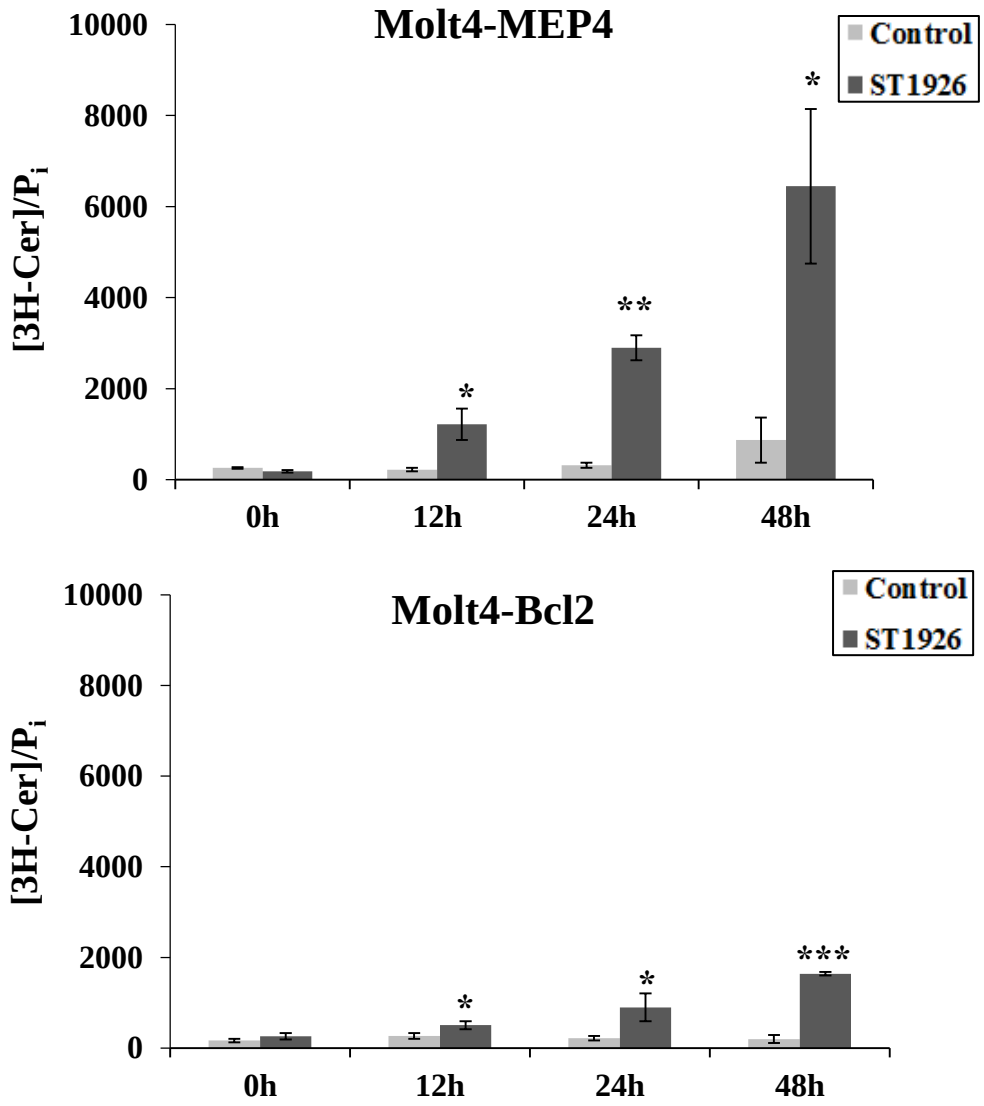


Figure 4.8. Bcl-2 Interrupts ST1926-Induced Cell Death and *De Novo* Ceramide Accumulation in Molt-4 Cells.
(A) Molt4-MEP4 and Molt4-Bcl2 were seeded at a density of $2x10^5$ cells/ml, treated with 0.1% DMSO or with increasing concentrations of ST1926 ranging from $5x10^{-8}$ to $5x10^{-6}$ M up to three days. Cells were assayed for death in quadruplicates using trypan blue exclusion assay. **(B)** Total ceramide (Cer) levels in Molt4-MEP4 and Molt4-Bcl2 at the indicated timepoints treated with 0.1 μM and 1 μM ST1926. Cer levels were determined using DGK assay as described in Methods and normalized to total cellular lipid phosphate levels. Data points represent the mean (± SD). Results are representative of two independent experiments. **(C)** Bcl-2 inhibits ST1926-induced *de novo* synthesis of Cer in Molt-4 cells. Cells were seeded at a density of $3x10^5$ cells/ml. *De novo* Cer levels were determined in triplicates using the [^{3}H]-palmitate incorporation method as described in Methods and normalized to total cellular lipid phosphate levels. Data points represent the mean (± SD). The asterisks *, **, and *** indicate statistically significant differences at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$, respectively, *versus* control using the *t*-test.

Table 4.2. Percent Accumulation of 17dhC16, 17dhC22, and 17dhC24:1 in HTLV-1 positive (HuT-102) and Negative (Molt-4) Malignant T Cells, Representing Most Prominent Increase in Their Respective Categories of Medium Long Chain (MLC), Long Chain (LC), and Very Long Chain (VLC) 17dhCer.

Percent increase in individual 17dhCer species in HuT-102 and Molt-4 cells

	Total 17dhCer	17dhC16 (% Total 17dhCer)	17dhC22 (% Total 17dhCer)	17dhC24:1 (% Total dhCer)
HuT-102	327	48	7	12
Molt-4	483	71	2	6

Cells were seeded at a density of $3x10^5$ cells/ml and treated with 0.1% DMSO as control or 1µM ST1926 for 24h. 17dhCer species levels (pmol) were measured in lyophilized samples as duplicates by LC-MS as described in Methods and normalized to total cellular lipid phosphate levels (µmol). Data points represent the mean ± range (n=2). Results are representative of two independent experiments.

D. Discussion

We report that ST1926 elicits early Cer accumulation in both HTLV-1 positive and negative malignant T cells. The kinetics and degree of Cer production show a unique response upon ST1926 treatment *versus* previously tested synthetic retinoids such as HPR, whereby Cer was only produced in HTLV-1 negative cells (Darwiche, Abou-Lteif et al. 2005). We have previously demonstrated that *de novo* Cer production is compromised in HTLV-1 positive cells probably due to the inhibitory action of Tax on CerS(s) activity in response to HPR (Darwiche, Abou-Lteif et al. 2005). Our results show that ST1926 concentrations as low as 0.1 µM increase Cer production and concomitantly reduce cell viability by 20% in HuT-102 cells (El Hajj, Khalil et al. 2014). ST1926 is a more potent inducer of cell cycle arrest and apoptosis than HPR or CD437, especially in HTLV-1 positive T cells, probably due to the fact that only

ST1926 causes early reduction of Tax oncoprotein levels (Darwiche, Hatoum et al. 2004, El Hajj, Khalil et al. 2014). Among the previously mentioned synthetic retinoids, only ST1926 activates *de novo* Cer pathway in both HTLV-1 transformed and HTLV-1 negative malignant T cells. Interestingly, we have shown that early downregulation of Tax oncoprotein by ST1926, but not HPR (Darwiche, Abou-Lteif et al. 2005), precedes *de novo* Cer generation, which might be relieving the inhibitory effect of Tax on CerS(s). Therefore, it would be of interest to characterize the mechanisms by which Tax deregulates Cer pathways. Indeed, Tax disrupts major cellular processes, such as cell cycle, apoptosis, and cell proliferation (Fochi, Mutascio et al. 2018). Tax deregulates the NF-κB pathways (Xiao, Cvijic et al. 2001, Shirinian, Kfoury et al. 2013), inducing the expression of genes that block apoptosis and might lead to chemoresistance, such as Bcl-2 (Nakashima, Kawakami et al. 2003, Fu, Qu et al. 2011, Alibek, Irving et al. 2014). Meanwhile, depending on stimuli and cell context, Bcl-2 is required for the inhibition of Cer synthesis (Warren, Wong-Brown et al. 2019) and resistance to permeabilization of mitochondrial membrane by Cer (Ganesan and Colombini 2010). There is accumulating evidence that describes the relationship between Cer and NF-κB. Because Tax-mediated activation of NF-κB is central to transformation in HTLV-1-infected cells, we suspect that it may play a role in the inhibition of Cer. Alternatively, in HTLV-1 positive cells, Cer may exert some of its effects by inhibiting Tax-induced, NF-κB-mediated, anti-apoptotic pathways via activating various CAPPs such that, once Tax is degraded, the balance tips in favor of Cer and apoptosis proceeds. One probable explanation is that ST1926 degrades Tax and relieves its suppressive effect on Cer -generating pathway(s) in HTLV-1 positive malignant T cells. These inhibitory effects are in line with the established pro-apoptotic activities of Cer and the pro-survival, pro-

proliferation, and pro-inflammatory effects of NF-κB. Interestingly, prediction studies using USCS Genome Browser suggest NF-κB transcription factor binding sites are present in the promotor regions of specific CerS(s) that generate Cer (Wegner, Schiffmann et al. 2016). Therefore, the Tax-mediated activation of NF-κB and inhibition of Cer that promote transformation make it imperative to investigate their mutual relationships in this context and in response to ST1926 treatment as this may uncover novel approaches in the treatment of this highly malignant tumor.

It is well-established that HPR activates the rate-limiting enzyme, serine palmitoyltransferase (Zheng, Kollmeyer et al. 2006, Kraveka, Li et al. 2007, Wang, Maurer et al. 2008). Concurrently, HPR activates CerS, but inhibits DEGS1 in cell-free enzymatic assays as well as in intact cells, therefore, accumulating dhCer, but not Cer (Zheng, Kollmeyer et al. 2006, Kraveka, Li et al. 2007). The effects of altered Cer levels have been widely studied in physiology and disease, with inadequate knowledge about the distinct Cer species. This might impede our understanding of the role of Cer, as most Cer species could be generated by at least two CerS(s). The epigenetic, transcriptional, translational, and post-translational regulation of the individual CerS(s) has been poorly investigated (Wegner, Schiffmann et al. 2016). What is established is that CerS(s) regulate their activities by forming homo and heterodimers (Laviad, Kelly et al. 2012). The activities of CerS(s) could also be regulated by phosphorylation, glycosylation, and acetylation to maintain protein stability (Mizutani, Kihara et al. 2005, Olsen, Blagoev et al. 2006, Wegner, Schiffmann et al. 2016). Using LC-MS, we found that both HTLV-1 positive and negative T cell types markedly favor the accumulation of Cer *versus* dhCer molecular species upon treatment with ST1926. Specific Cer species, mainly MLC and VLC, preferentially accumulate in response to

ST1926 treatment of HTLV-1 positive and negative T cells, indicating activity of the corresponding CerS(s), particularly CerS2, CerS4, CerS5, and CerS6. It has been shown that CerS5 mostly uses C16-CoA, and CerS6 mostly utilizes C14, C16, and C18-CoA, while CerS2 and CerS4 preferably use C20-C26 and C18-C20 fatty acyl CoAs, respectively (Lahiri and Futerman 2005, Laviad, Albee et al. 2008, Ogretmen 2018, Wattenberg 2018). The activation of these enzymes would explain our finding of increased 17dhC16, 17dhC20, 17dhC22, 17dhC22:1, and 17dhC24:1, among others, following treatment in the presence of 17C-sphinganine labeling. One possible explanation for specific species accumulation might be related to temporal regulation of CerS(s) activities, such that apoptotic cells mainly produce C16 and C18 Cers at the beginning of the apoptotic program (Kroesen, Jacobs et al. 2003, Siskind, Mullen et al. 2010), while C24-Cer predominantly accumulate during the final stages of apoptosis (Kroesen, Jacobs et al. 2003). Interestingly, we found that HuT-102 and Molt-4 apoptotic cells mainly accumulate 17dhC16, that represents 48% and 71% of total 17dhCer species increase in HuT-102 and Molt-4 treated cells, respectively (Table 4.2). Based on the accumulated Cer species in this work, our results suggest that specific CerS(s) might be involved in the Cer response to ST1926, plausibly through modulation of the CerS(s) enzymatic activity, without altering CerS(s) protein levels. Future experiments that perform *in vitro* CerS assays using different acyl-CoAs in cells treated with or without ST1926 would further validate our claim. We did find a differential CerS(s) gene expression response in the resistant and sensitive cells, whereby the expression of these specific CerS(s) genes was increased in Tax-positive cells (HuT-102) and, to a significantly lesser extent, in Tax-negative cells (Molt-4). This might be explained by the inhibitory effect of Tax on ceramide production. One of the

deregulated mechanisms by Tax is the NF-κB pathway (Fochi, Mutascio et al. 2018). NF-κB might function upstream of ceramide, and this might be a plausible axis of regulation. This highlights the importance of future mechanistic experiments that shall explain the interplay between Tax, NF-κB, and Cer, and verify the suggested inhibitory effect of Tax on CerS(s).

There is accumulating evidence that different fatty acyl chains generated by different CerS(s) play crucial roles in inducing cancer cell death and/or survival, depending on cell types and context (Stiban, Tidhar et al. 2010, Ogretmen 2018). Mecisek *et al*. reported that induction of *de novo* Cer synthesis is mediated by the activation of CerS 2, 5, and 6 isoforms in irradiated HeLa cells, and their subsequent interplay determines the balance between opposing anti- and pro-apoptotic Cer species (Mesicek, Lee et al. 2010). The authors showed that while overexpression of CerS2 partially protected from irradiation (IR)-induced apoptosis, overexpression of CerS5 augmented the apoptotic effect in HeLa cells. The increase of C16-Cer in IR-induced Jurkat and HeLa cells has a pro-apoptotic role, while overexpression of CerS2, which generates C24-Cer, inhibits IR-induced apoptosis in these cells (Deng, Gao et al. 2009, Mesicek, Lee et al. 2010, Grosch, Schiffmann et al. 2012). CerS expression is reduced by Fms-like tyrosine kinase 3 (FLT3) signaling in AML which confers resistance, while inhibition of FLT3 activity restores Cer synthase-induced mitophagy (Dany, Gencer et al. 2016). Interestingly, the pro-apoptotic effects of C16 and C18 Cers have been described in human leukemia cells and hematopoetic cells, respectively (Grosch, Schiffmann et al. 2012). Moreover, CerS6 expression is increased in breast tumors, induces cell death in lung cancer cells, is elevated in breast tumors, and protects from graft-versus-host disease in a mouse model of leukemia (Schiffmann, Sandner et al.

2009, Fekry, Esmaeilniakooshkghazi et al. 2016, Suzuki, Cao et al. 2016, Sofi, Heinrichs et al. 2017). However, it seems that the mere increase in a specific Cer species does not determine the cell fate (Hartmann, Wegner et al. 2013). Indeed, CerS co-transfection experiments in HCT116 cells and their effect on different Cer species production revealed that the equilibrium of medium, long, and very long ceramides committed cells to survival or death. Consistently, results have shown that VLCs, products of CerS2, inhibit mitochondrial C16-channel formation *ex vivo* and vice versa, which could also be regulated by Bcl-2 family proteins (Taha, Mullen et al. 2006, Stiban and Perera 2015). Whether alone or in combination treatment, such as with Bcl-2 selective inhibitors, ST1926 could be used to stimulate mitochondrial pathway of cell death. We have shown that Bcl-2 attenuates ST1926-induced cell growth arrest in Molt-4 cells. In addition, exogenous C6-Cer induced endogenous Cer-dependent cell death in these cells (Zhang, Alter et al. 1996). We observed early accumulation of Cer levels in Molt-4 cells by 12 h, which precedes cell death in these cells. However, Bcl-2 rescued Molt-4 cells from early Cer accumulation and cell death. Our results suggest a Cer-dependent mechanism of programmed cell death in response to ST1926 treatment, in which Bcl-2 acts upstream to prevent both Cer *de novo* synthesis and consequently, cell death in Molt-4 cells. This suggests that Bcl-2 and Cer act in a common pathway of the cell death mechanism, most probably with Cer acting in the "sensing" phase rather in the "execution" phase of apoptosis (Zhang, Alter et al. 1996). Therefore, this data might define a mitochondrial pathway of ST1926 action, with a critical inhibitory role of Bcl-2 in Cer production and cell death. Indeed, in some cancer systems, early Cer accumulation occurs downstream of Bcl-2 action and mitochondrial pathway, whereas in others Cer production occurs upstream of these actions. It is established that *de novo*

Cer synthesis occurs in the endoplasmic reticulum as well as in the mitochondria and mitochondria associated membranes (Bionda, Portoukalian et al. 2004), wherein CerS(s) localize in both the outer and inner mitochondrial membranes. Therefore, drugs that would exclusively target a specific CerS and control apoptosis are potentially useful in chemoresistance. Given the diversity in regulating homo- or hetero-dimerization of different CerS(s) and the results presented in this research, it may be attractive to develop agents that allow the manipulation of essential apoptotic Cer acyl chain composition *in vivo* (Laviad, Kelly et al. 2012). Further gene knock-down assays of the corresponding enzymes might help dissect these pathways in our and other systems. Interestingly, we observed that, compared to Molt-4, HuT-102 cells display lower basal levels of 17dhCer species, indicating a lower baseline activity of CerS, which elevate similarly in both cell lines upon ST1926 treatment. However, no difference is observed in the basal level ratios of 17Cer/17dhCer species between the two cell lines. This suggests that, under baseline conditions, Tax inhibitory effect is at the level of CerS and not the desaturase. Remarkably, these findings add to the novelty of the mechanism of action of ST1926 compared to the previously tested synthetic retinoids, HPR and CD437, whereby ST1926 relieves Tax inhibitory effect on CerS(s) and increases Cer levels.

One of the hurdles in cancer therapeutics is chemoresistance due to dysregulations in Cer metabolism (Morad and Cabot 2013). In fact, decreased cellular Cer dictated by oncogene activation tilts sphingolipid metabolism to the production of pro-survival sphingolipids and correlates with tumorigenesis and drug resistance (Lewis, Wallington-Beddoe et al. 2018). We have previously shown that the viral oncogene Tax could target the Cer pathway by inhibiting CerS activity, which might

explain the resistance of HTLV-1 transformed cells to therapy (Darwiche, Abou-Lteif et al. 2005). We have also shown that Cer glucosylation by PDMP raises cellular Cer levels and increases the sensitivity of HTLV-1 positive T cells to HPR indicating that this approach may be therapeutically feasible (Darwiche, Abou-Lteif et al. 2005). Similarly to the inhibition of GCS, increasing endogenous Cer could be achieved by inhibiting its clearance by CDase, activation of sphingomyelin breakdown, and/or increasing *de novo* synthesis (Lewis, Wallington-Beddoe et al. 2018). The detailed Cer analysis in this study shows CerS as candidate enzyme that might be inhibited by Tax oncoprotein to particularly lower Cer levels in HTLV-1 positive cells, that is otherwise restored upon ST1926 treatment.

Therefore, novel approaches that target Cer metabolism might overcome drug resistance, plausibly by combining sphingolipid modulators with retinoids in order to provide novel approaches for treating cancer.

E. Abbreviations

ATRA, all-*trans* retinoic acid; **APL**, acute promyelocytic leukemia; **RRMs**, retinoid related molecules; **HPR**, as N-(4-hydroxyphenyl; **CD437**, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; **ST1926**, (2E)-3-[3'-(1-adamantyl)-4'-hydroxy[1,1'-biphenyl]-4-yl]-2-propenoic acid; **ATL**, adult T-cell leukemia; **HTLV-1**, human T-cell lymphotropic virus-1; **POLA**1, DNA polymerase 1 alpha; **Cer**, ceramide **CAPP**, ceramide-activated protein phosphatases; **PP1/PP2A**, serine/threonine protein phosphatases; **GCS**, glucosylceramide synthase; **SMS**, sphingomyelin synthase; **CDase**, ceramidase; **AML**, acute myeloid leukemia; **PDMP**, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; **CerS**, ceramide synthase; **dhCer**, dihydroceramide; **DEGS1**,

dihydroceramide synthase; **DMSO**, dimethylsulfoxide; **DGK**, diacylglycerol kinase; **17Cer**, 17C-ceramide; **17dhCer**, 17dihydroceramide; **MLC**, medium long-chain; **LC**, long-chain; **VLC**, very long-chain; **NF-κB**, nuclear factor kappa-light-chain-enhancer of activated B cells; **FLT3**, Fms-like tyrosine kinase 3.

CHAPTER V

CONCLUSIONS

The synthetic retinoid ST1926 is a strong inducer of cell death and apoptosis in several types of human cancers, including those that are ATRA-resistant. Likewise, in ATL, ST1926 treatment results in massive apoptosis and increases survival of ATL mice (El Hajj, Khalil et al. 2014). In our viral oncogene-driven leukemia model, we have determined that ST1926 exerts a comparable ceramide response in ATL and T cell lymphoma. The kinetics and dose of ceramide production shows a unique pattern upon ST1926 treatment *versus* previously tested synthetic retinoids. We have previously shown that HPR or CD437 treatment results in ceramide production only in HTLV-1 negative cells (Darwiche, Abou-Lteif et al. 2005). This may be due to the fact that only ST1926 triggers early downregulation of Tax protein (Darwiche, Hatoum et al. 2004, Darwiche, Abou-Lteif et al. 2005). Therefore, it is pertinent to decipher the mechanism by which ST1926 induces Tax downregulation in future research. For instance, the proteasome was implicated in ST1926 induced apoptotic response in AML (Fratelli, Fisher et al. 2013). We have previously shown that HTLV-1 positive cells have a defect in ceramide synthesis in response to HPR (Darwiche, Abou-Lteif et al. 2005). LC-MS analysis of sphingolipids from HPR-treated cells have shown that dihydroceramide, and not ceramide, is accumulated upon HPR treatment, accompanied by simultaneous activation of SPT and/or CerS, with concurrent inhibition of DEGS1 (Wang, Maurer et al. 2001, Zheng, Kollmeyer et al. 2006, Kraveka, Li et al. 2007). In fact, the latter enzyme is inhibited by HPR in cell-free enzymatic assays as well as in intact cells. Since ST1926 reduces the viral oncoprotein Tax and activates *de novo* ceramide

synthesis, and knowing that HPR significantly inhibits DEGS1, we have performed a high throughput sphingolipidomics analysis using LC-MS to identify the ceramide and/or dihydroceramide molecular species that are modulated in response to ST1926 in HTLV-1 positive and HTLV-1 negative cells. Here we report a marked accumulation of ceramide *versus* dihydroceramide molecular species in both cell types. This adds to the novelty of the mechanism of action of ST1926 compared to our previously tested synthetic retinoids HPR and CD437.

In a previous work, we have demonstrated that the expression of Tax alone in cells not infected with HTLV-1 is sufficient and responsible for maintaining the impaired *de novo* ceramide synthetic pathway in response to HPR or exogenous C6-ceramide (Darwiche, Abou-Lteif et al. 2005). There is accumulating evidence that cell permeant analogues of short-chain ceramides such as C2- and C6- could be used to investigate apoptosis mediated by accumulation of endogenous cellular ceramide in response to extracellular signals including chemotherapeutic drugs (Bielawska, Crane et al. 1993, Mathias and Kolesnick 1993, Cifone, De Maria et al. 1994, Tepper, Jayadev et al. 1995). Furthermore, exogenous C6-ceramide was shown to induce *de novo* ceramide synthesis in A549 lung carcinoma cells, which is dependent on CerS to produce endogenous ceramides (Ogretmen, Pettus et al. 2002). Ceramide's backbone sphingosine is recycled after deacylation/reacylation into endogenous long-chain ceramides. In a previous work, we proved that both ceramide analogues induce *de novo* ceramide synthesis in HTLV-1 positive and negative T cells, which is substantially lower in the former ones (Darwiche, Abou-Lteif et al. 2005). This suggests that CerS is inhibited in these cells. In addition, CerS is one of the crucial enzymes of this pathway that we previously found to be suppressed by Tax, which confers chemoresistance of

HTLV-1 transformed cells (Darwiche, Hatoum et al. 2004, Darwiche, Abou-Lteif et al. 2005). Since ST1926 restores *de novo* ceramide synthesis, it was noteworthy to investigate the effect of this treatment on CerS activity in our ST1926-treated ATL model. As discussed earlier, each CerS is specific for the addition of relatively distinct lengths of fatty acyl-CoAs to produce ceramide species with different chain lengths (Stiban, Tidhar et al. 2010). Specific ceramide species, mainly MLC and VLC, preferentially accumulate in response to ST1926 treatment of HTLV-1 positive and negative T cells, which represents indirect evidence of the activity of corresponding CerS(s), particularly CerS2, CerS4, CerS5, and CerS6. The activation of these enzymes might explain our finding of increased C16, C20, C22, C22:1, and C24:1 d17-dihydroceramides, among others, following treatment in the presence of 17-carbon sphinganine labeling. One possible explanation for the specific species accumulation might be related to temporal regulation of CerS(s) activities, such that apoptotic cells mainly produce C16 and C18 ceramides at the beginning of the cell death program (Kroesen, Jacobs et al. 2003, Siskind, Mullen et al. 2010), while C24-ceramide predominantly accumulate during the final stages of apoptosis (Kroesen, Jacobs et al. 2003). Interestingly, we found that both types of apoptotic cells mainly accumulate C16 d17-dihydroceramide, a signature of activated *de novo* ceramide pathway in apoptosis (Ogretmen and Hannun 2004). Moreover, accumulating evidence suggests the role of the different CerS(s) in regulating cell death (Stiban, Tidhar et al. 2010). Indeed, certain ceramide species such as C16 and C18 ceramides have proapoptotic effects in human leukemia cells and hematopoetic cells, respectively (Grosch, Schiffmann et al. 2012).

Based on the accumulated ceramide species in this work, our results suggest that specific CerS(s) might be involved in the ceramide response to ST1926, plausibly

through modulation of the CerS(s) enzymatic activity, without altering CerS(s) protein levels. Future experiments using *in vitro* CerS assays of different acyl-CoAs in cells treated with or without ST1926 would further validate our claim. We did find a differential CerS(s) gene expression response in the relatively resistant and sensitive cells, whereby the expression of these specific CerS(s) genes was increased in Tax-positive cells and, to a significantly lesser extent, in Tax-negative cells. Therefore, it is intriguing to further investigate the role of these CerS subtypes in mediating the growth-suppressive effects upon ST1926 in HTLV-1 positive and HTLV-1 negative malignant T cells. In this regard, further gene knock-out assays of the corresponding enzymes and/or overexpression models would help unveil the suggested roles of the specific CerS(s) and their corresponding species in malignant T cells. Also, future studies that use *in vitro* enzymatic activity assays, whether CerS(s) or other *de novo* enzymes, such as SPT and DEGS1, are modulated in response to ST1926 in HTLV-1 positive and negative cells. These investigations are crucial knowing that resistance to chemotherapeutic drugs could be attributed to a certain defect in ceramide synthesis and/or metabolism. For instance, Tamoxifen blocks the conversion of ceramide to glucosylceramide, thus, bypassing drug resistance by increasing endogenous ceramide levels (Pandey, Murphy et al. 2007). In addition, HPR increases the levels of intracellular ceramide in drug-resistant neuroblastoma cell lines (Maurer, Metelitsa et al. 1999).

The observed defect in the *de novo* ceramide pathway might be explained by the inhibitory effect of Tax on ceramide production. We found that ceramide synthesis follows ST1926-induced degradation of Tax (Figure 5.1). This raises the possibility that Tax is responsible for the ceramide synthesis defect in ATL cells, and ST1926 might be

relieving this inhibitory effect on CerS(s). Therefore, it would be of interest to characterize the mechanisms by which Tax deregulates ceramide pathways. One of the deregulated mechanisms by Tax is the NF-κB pathway (Fochi, Mutascio et al. 2018). NF-κB might function upstream of ceramide, and this might be a plausible axis of regulation (Figure 5.1). There is accumulating evidence that describes the relationship between ceramide and NF-κB. Because Tax-mediated activation of NF-κB is central to transformation in HTLV-1-infected cells, we suspect that it may play a role in the inhibition of ceramide generation. Alternatively, in HTLV-1 positive cells, ceramide may exert some of its effects by inhibiting Tax-induced NF-κB-mediated anti-apoptotic pathways via activating various CAPPs such that, once Tax is degraded, the balance tips in favor of ceramide and apoptosis proceeds (Figure 5.1). One probable explanation is that ST1926 degrades Tax and relieves its suppressive effect on ceramide-generating pathway(s) in HTLV-1 positive T cells. These inhibitory effects are in line with the established pro-apoptotic activities of ceramide and the pro-survival, pro-proliferation, and pro-inflammatory effects of NF-κB. Our results showed that treatment with ST1926 increases gene expression of ceramide synthases more prominently in HTLV-1 positive cells. We suspect that there is a crosstalk between NF-κB and ceramide during ST1926-induced degradation of Tax and cell death. Therefore, it was imperative to investigate the effect of ST1926 on the transcription of specific CerS(s) that are found to be highly expressed in leukocytes, namely CerS2, CerS4, and CerS6, in both HTLV-1 positive and negative malignant T cells. Remarkably, the time-dependent increase in CerS(s) transcription levels in HuT-102, and not Molt-4, cells suggests that ST1926 relieves Tax-suppressive effects on ceramide generation in HTLV-1 positive T cells. These results suggest that ST1926 plausibly regulates CerS(s) at the transcriptional and

enzyme activity levels, without altering translational levels. Therefore, future mechanistic experiments that will explain the interplay between Tax, NF-κB, and ceramide, and verify the suggested inhibitory effect of Tax on CerS(s). Interestingly, prediction studies using USCS Genome Browser suggest NF-κB transcription factor binding sites are present in the promotor regions of specific CerS(s) that generate ceramide (Wegner, Schiffmann et al. 2016). Tax oncoprotein represses the transcriptional activity of the tumor suppressor protein p53 through the activation of the NF-*κ*B pathway, which is also disrupted by Tax (Pise-Masison, Mahieux et al. 2000). Interestingly, p53 can regulate the generation of ceramide in response to chemotherapeutic agents or *γ* -irradiation (Dbaibo, Pushkareva et al. 1998). One future aim, therefore, would be to examine whether NF-*κ*B and p53 mediate the inhibitory effects of Tax on the *de novo* synthesis of ceramide.

Tax disrupts major cellular processes, such as cell proliferation, cell cycle, and apoptosis (Fochi, Mutascio et al. 2018), partly by deregulating the NF-κB pathways (Xiao, Cvijic et al. 2001, Shirinian, Kfoury et al. 2013), inducing the expression of genes that block apoptosis and might lead to chemoresistance, such as Bcl-2 (Nakashima, Kawakami et al. 2003, Fu, Qu et al. 2011, Alibek, Irving et al. 2014, Warren, Wong-Brown et al. 2019). Depending on stimuli and cell context, Bcl-2 is required for the inhibition of ceramide synthesis (Warren, Wong-Brown et al. 2019) and resistance to permeabilization of mitochondrial membrane by ceramide (Ganesan and Colombini 2010). One of the apoptotic pathways that interplay with ceramide are those driven by members of Bcl-2 family of proteins (Taha, Mullen et al. 2006). We have shown that Bcl-2 attenuates ST1926-induced growth suppression in Molt-4 cells (Figure 5.1). In addition, exogenous C6-ceramide induces ceramide-dependent cell death in

these cells (Zhang, Alter et al. 1996). We have observed an early accumulation of ceramide levels in Molt-4 cells, which precedes apoptosis, and Bcl-2 rescues these cells from early ceramide accumulation and demise. Our results suggest a ceramide-dependent mechanism of cell death in response to ST1926 treatment, in which Bcl-2 acts upstream to prevent both ceramide accumulation and consequently, cell death in Molt-4 cells. This implicates that Bcl-2 and ceramide act in a common pathway, most probably with ceramide acting in the "sensing" phase rather in the "execution" phase of apoptosis (Zhang, Alter et al. 1996). Our results also shed light into a mitochondrial pathway of cell death, which is fine-tuned by ceramide, but antagonized by Bcl-2 inhibition of ST1926 induced ceramide and apoptosis. Indeed, in some tumor systems, early ceramide accumulation occurs downstream of Bcl-2 action and mitochondrial pathway, whereas in others ceramide production occurs upstream of these actions. The mechanism by which ceramide exerts its proapoptotic functions is not fully unveiled. However, one of the proposed pathways involves the generation of mitochondrial ceramide prior to the induction phase of apoptosis and forming ceramide channels in the outer mitochondrial membrane that facilitate the release of proapoptotic proteins from the intermembrane space into the cytoplasm (Siskind 2005). *De novo* ceramide synthesis occurs in the ER and in the mitochondria (Bionda, Portoukalian et al. 2004). This underscores the relevance of *de novo* ceramide pathway in the mitochondria and suggests the involvement of Bcl-2 family members. In our study, we showed that Bcl-2 interrupts early *de novo* ceramide synthesis upon ST1926 treatment in Molt-4 cells. Indeed, it has been recently shown that enzymes responsible for *de novo* ceramide synthesis, specifically CerS, localize in the outer mitochondrial membrane (Bionda, Portoukalian et al. 2004). Meanwhile, two of the proposed mechanisms by which Bcl-2

inhibitory actions could be mediated are through direct binding to ceramide channels and/or altering the activity of the enzymes responsible for ceramide synthesis (Siskind 2005). Thus, it is imperative to further investigate how Bcl-2 protects from *de novo* ceramide-induced cell death in the context of mitochondrial ceramide-induced apoptosis, as this would add to the novelty of RAR-independent mechanisms of action of synthetic retinoids, such as ST1926. Altogether, this suggests the involvement and localization of certain CerS(s) to the mitochondria and the potential participation of specific species in ST1926-induced apoptosis. Yet, more experiments with pure mitochondrial extracts need to be done combined with enzymatic activity assays and LC-MS analyses in order to hold for a solid conclusion. Finally, it would be crucial to identify the mechanisms of ceramide-mediated action upon ST1926 treatment *in vivo*, eventually, identifying the enzyme (s) that might be also involved in this retinoid treatment. Therefore, drugs that would exclusively target a specific CerS and control apoptosis are potentially useful to counteract chemoresistance. Given the diversity in regulating homo- or hetero-dimerization of different CerS(s) and the results presented in this research, it may be attractive to develop agents that allow the manipulation of essential apoptotic ceramide acyl chain composition *in vivo* (Laviad, Kelly et al. 2012). We observed that, compared with Molt-4, HuT-102 cells display lower basal levels of d17-dihydroceramide species, indicating a lower baseline activity of CerS, which elevate similarly in both cell lines upon ST1926 treatment. However, no difference is observed in the basal level ratios of d17-ceramide/d17-dihydroceramide species between the two cell lines (Figure 3.5). This suggests that, under baseline conditions, Tax inhibitory effect is at the level of CerS and not the desaturase. These findings add to the novelty of the mechanism of action of ST1926 compared to previously tested

synthetic retinoids, whereby ST1926 relieves Tax inhibitory effect on CerS(s) and increases ceramide levels.

Chemoresistance is commonly observed in cancer therapeutics and is a complex process. Sphingolipids metabolism may play a crucial role in cancer sensitivity or resistance to several commonly used anticancer drugs. Our results provide evidence that ST1926 is a strong inducer of ceramide in malignant T cells and support for the use of this drug in chemoresistant cancer cells whether alone or in combination treatment. Cancer lipidomics is emerging as a new cancer profiling method to inform prognosis, diagnosis, and treatment (Shen, Yang et al. 2017). There is ample evidence for dysregulation of sphingolipid metabolism in hematological malignancies that often develop resistance to current treatment regimens (Lewis, Wallington-Beddoe et al. 2018). Ceramide metabolism is at the central hub of a larger network of sphingolipid turnover and synthesis that seem to be tightly regulated both at the spatial and temporal levels. The typical methods used to investigate ceramide metabolism include thin layer chromatography, high performance thin-layer chromatography, and diglycerol kinase assay as adapted for ceramide. Although such sphingolipid analyses provide insights of changes in mass levels using total cell lysates or activity of individual enzymes, they fail to dissect major dynamic changes that occur throughout complex sphingolipid network. Therefore, there is a critical need for more comprehensive approaches that track and map the metabolism of ceramide in response to specific conditions. The incorporation and metabolism of the synthetic and unnatural 17-carbon sphinganine (dihydrosphingosine) precursor into metabolites with 17C sphingoid bases that can be measured using LC-MS has been recently suggested to provide the needed dynamic and topological information about sphingolipid changes in response to stimuli (Valsecchi,

Aureli et al. 2010, Snider, Snider et al. 2018, Snider, Luberto et al. 2019). This one-step *in situ* assay, along with utilization of specific inhibitors, provides a practical tool to identify *de novo* sphingolipid flux, thus determining different spatio-temporal phases of the individual enzyme activities involved and plausible levels of regulation. Pulse labeling and analysis of precursor metabolism will clarify sequential, well-defined stages of sphingolipid synthesis, corresponding to the activity of different enzymes in the pathway, further confirmed using specific inhibitors and modulators of sphingolipid metabolism to confirm the validity of the assay. Thus, understanding the dynamics and topology of ceramide generation holds the promise of translating this information by combining sphingolipid modulators with retinoids in order to provide novel targeted approaches for treating, not only ATL, but also several other blood cancers.

Determining the metabolic fate of d17 sphingoid-based precursor elucidates the activities of the relevant sphingolipid metabolizing enzymes. This will help us draw a map of sphingolipid modulation in response to ST1926. Moreover, the comparison between the HTLV-1 positive and negative cells will assist us in identifying the hypothesized Tax-disabled or inhibited pathways. Recently, a comprehensive approach using mass spectrometry was described by Snider *et al*. to monitor the dynamic incorporation of d17 sphinganine pulse into d17-dihydroceramide, d17-ceramide, d17-sphingomyelin, d17-hexosylceramide, d17-sphingosine, and d17-sphingosine-1-phosphate, thus determining *in situ* enzymatic activities for CerS, DEGS, SMS, GCS, CDases, and SK, respectively (Snider, Luberto et al. 2019). The integration of the obtained results from our research and this recent approach would allow to distinguish between *de novo* synthesized and hydrolytically generated ceramide, which would be further verified through use of enzyme inhibitors (Snider, Luberto et al. 2019). In the

future, using probes as labeling tools along with genetic manipulation of enzymes would help determine the pools of their sphingolipid products and the localization of their enzymes, thus clarifying their distinct roles in cancer and allowing for more efficient targeted treatments. Clearly, more research is required to dissect retinoid-regulated pathways of ceramide metabolism and define the mechanisms of regulation of enzymes. Since sphingolipid-mediated pathways operate at the level of individual organelles, they should be studied as such. This would bring us a step further in selective targeting of enzymes which avoids offset targets, whereby modern analytical and drug formulation techniques and the mathematical approaches of systems biology will convey the foremost advancements in cancer therapy.

It is intriguing for our future perspectives to revolve around the emerging hypothesis from this report that ST1926 degrades Tax and relieves its suppressive effect on ceramide-generating pathway(s) in HTLV-1 positive malignant T cells, which might be mediated by the effect of Tax on NF-κB. We will be examining the crosstalk between NF-κB and ceramide during ST1926-induced degradation of Tax. Using gene knock-down and overexpression assays would describe the role of Tax and NF-κB in ST1926-induced accumulation of ceramide and cell death, which might be mediated through Bcl-2. We will also test for the effect of ST1926 or exogenous ceramide treatment on NF-κB pathways in HTLV-1 positive and negative malignant T cells, using inhibitors of CAPP to clarify whether ceramide is acting upstream of NF-κB during the induction of ST1926-induced cell death in ATL. Alternatively, ST1926 effects on ceramide and/or cell death might be NF-κB-driven irrespective of Tax degradation, and this is one area worth investigating in future studies using NF-κB overexpressing and knock-down models. Novel approaches for effective treatment aim

to overcome resistance to apoptosis by repressing NF-κB activity, which is crucial to Tax-mediated oncogenesis.

In the future, high throughput analysis would allow better characterization of our model by characterizing, quantifying, and comparing distinct bioactive sphingolipids that might be involved in ST1926-mediated response in HTLV-1 positive and negative malignant T cells. We will compare the sphingolipid metabolic flux using d17 sphingoid-based precursor to identify the activities of the relevant sphingolipid metabolizing enzymes in Tax-positive and negative cells. The integration between these two approaches using lipidomics analysis will help us determine the sphingolipid profiles of both cell types. Experiments that use these comprehensive approaches in Tax knock-down systems would validate our claim that Tax-inhibited ceramide production is restored by treatment with ST1926. It is also encouraging to generate ST1926 resistant and sensitive cells by combining these methods to delineate their sphingolipid profile and flux in future work and highlight the altered ceramide metabolizing enzymes in the context of ST1926 resistance. This would help us understand how targeting ceramide metabolism might overcome resistance to cancer therapy, plausibly by combining sphingolipid modulators with retinoids to provide novel approaches for treating cancer.

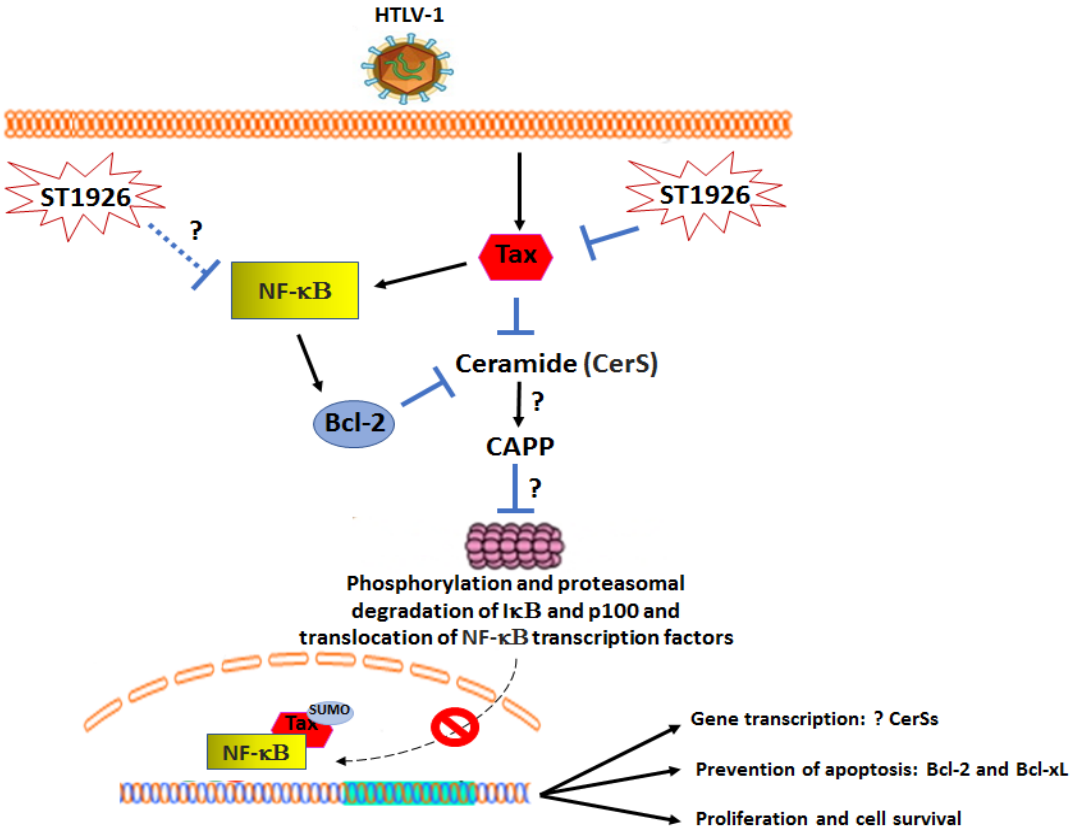


Figure 5.1. Model for the Interplay Between Tax and Ceramide with Proposed Role Played by NF-κB and Suggested ST1926 Sites of Action. ST1926 degrades Tax and might relieve its inhibitory effect on ceramide accumulation, which could be dissociated from or mediated by NF-κB. Ceramide might suppress NF-κB activity via CAPP inhibition of phosphorylation and degradation of IκB and p100 and subsequent translocation of NF-κB transcription factors that regulate proliferation and cell death genes. Both pathways might simultaneously occur such that when Tax is degraded, ceramide completes the inhibition of NF-κB. Bcl-2, which might be regulated by NF-κB family of transcription factors, acts upstream of ceramide accumulation by ST1926.

IkB: inhibitor of κB; **CAPP:** Ceramide-activated protein phosphatases; **CerS**: ceramide synthase; **HTLV-1**, Human T cell Lymphotropic Virus Type I; **NF-κB:** nuclear factor kappa-light-chain-enhancer of activated B cells.

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