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Sensitizing Pancreatic Cancer to Gemcitabine Through Sphingosine Kinase 2 Inhibition

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by

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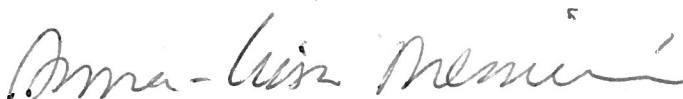
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To my labmates, thank you for the friendships, laughs, and edifying scientific discussions.

To Dr. Peterson, your friendship and mentorship have been priceless. Your influence continually challenges me to become a better scientist.

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Abstract

Pancreatic cancer is extraordinarily resistant to chemotherapy. With current treatment options on average extending life by less than a year, advancements in sensitizing tumors to chemotherapy are a must. The combination of gemcitabine and nab-paclitaxel is the current standard of care for treating pancreatic cancer. Resistance to both of these drugs can occur through deregulation of the NF-κB pathway, MAPK pathway, Bcl-2 family members, as well as the balance of sphingolipids. More importantly, resistance to gemcitabine occurs through aberrations in its metabolism – mainly overexpression of the M2 subunit of ribonucleotide reductase. Additionally, overexpression of the transcription factor c-Myc has been shown to contribute to gemcitabine resistance. The specific sphingosine kinase 2 inhibitor ABC294640 has previously been shown to diminish the activation of ERK1/2 and NF-κB as well as alter the balance of Bcl-2 family members, thereby promoting cell death. ABC294640 can also diminish c-Myc protein levels. Preliminary studies revealed that ABC294640 synergized with both gemcitabine and paclitaxel *in vitro* in three pancreatic cancer cell lines. This led me to propose the central hypothesis that inhibition of nuclear SphK2 by ABC294640 synergizes with gemcitabine in different cell types by alteration of one or more common pathways that can be traceable to a common S1P mediated signaling event. Specific Aim 1 was to determine whether ABC294640 synergizes with gemcitabine through inhibition of the growth pathways known to promote gemcitabine resistance or alteration of the expression ratios of Bcl-2 family. Specific Aim 2 was to determine whether ABC294640 synergizes with gemcitabine by altering

gemcitabine metabolism. In tracing the mechanism of the synergistic cell killing produced by the combination of ABC294640 and gemcitabine it was discovered that ABC294640 had no consistent impact on the NF-κB and MAPK pathways nor did it alter the ratio of Bcl-2 family members. However, treatment with ABC294640 decreased *RRM2* and *MYC* transcript as well as their protein expression in three pancreatic cancer cell lines (BxPC-3, MiaPaCa-2, and Panc-1). Inhibition of RRM2 and c-Myc was shown to occur as a result of increased histone acetylation, which lead to increased expression of the cyclin-dependent kinase inhibitor p21. p21 prevents cyclinD/cdk4/6 mediated phosphorylation of Rb preventing release of the E2F transcription factors, thereby diminishing the transcription of its target genes two of which are *RRM2* and *MYC*. ABC294640 caused a reduction in transcription of these genes. Due to the requirement of E2F in cell cycle progression, ABC294640 also caused G1 arrest in three pancreatic cancer cell lines. These data led to a model in which inhibition of SphK2 by ABC294640 in the nucleus leads to inhibition of histone deacetylase activity because of loss of nuclear S1P and/or elevation of nuclear sphingosine levels. The rise in histone acetylation leads to an increase in p21, which binds CDK/Cyclin-D1 complexes preventing the phosphorylation of Rb. Hypophosphorylated Rb sequesters the transcription factor E2F suppressing the expression of E2F target genes such as *MYC* and *RRM2*. This results in a decrease in proliferative signaling and an inhibition of cancer growth.

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Chapter One

Review of Literature and Significance

1.1   Introduction to Pancreatic Cancer

Pancreatic cancer is the fourth leading cause of cancer related deaths in the United States. The American Cancer Society estimates that nearly 49,000 people will be diagnosed with PC this year resulting in greater than 40,000 deaths[1]. The one-year survival rate is 27% and the five-year a dismal 5%[2]. These figures are disheartening, and they have been steady for nearly 40 years. The lethality of this disease is due, in large part, to the delay in the onset of symptoms. When most tumors are discovered, they are no longer localized, and therefore, more difficult to treat. There is no known cause of pancreatic cancer, however, smokers, heavy drinkers, and those with a history of diabetes mellitus or pancreatitis or a family history of pancreatic cancer are at higher risk of developing the disease[3]. Though there are multiple cancers of the pancreas both endocrine and exocrine, the focus of this project will be on the exocrine pancreatic adenocarcinomas, which account for the vast majority of all pancreatic cancer related deaths.

The development of pancreatic cancer begins with mutations in cells of the ductal epithelium forming an intraepithelial neoplasm. Further mutations result in greater dysplasia and the accumulation of mutations leads to invasive carcinoma. For pancreatic cancer, the same cast of mutations has been observed with regularity. 90% of tumors have a mutation in the *KRAS* gene, leading to a continuously activated protein product that promotes growth. 95% of pancreatic cancers have an inactivating mutation in the cell cycle regulatory gene *CDKN2A*, which encodes p16, an inhibitor of cyclin dependent kinases 4 and 6. *SMAD4* is inactivated in 50% of

pancreatic cancers resulting in disruption of the inhibitory effect of TGF-β

signaling[4]. Inactivating mutations of the tumor suppressor gene encoding p53,

TP53, are observed in 50-75% of pancreatic cancers[5]. Additionally, activating

mutations of c-Myc have also been frequently observed (20-70%)[6] as well as

constitutive activation of NF-κB (70%)[7] and overexpression of Bcl-2 (23%)[8].

The treatment of this disease is largely dependent on the stage at which it is

discovered. Early detection leads to surgical resection followed by chemotherapy

and possibly radiation therapy depending on the circumstances. For non-resectable

tumors, either chemotherapy alone or in combination with radiation therapy have

been proven to be the most advantageous way of managing the disease. However,

treatment is mostly palliative[5]. Resistance to these chemotherapeutics remains

difficult to overcome. This body of work represents efforts aimed at overcoming

modes of resistance to gemcitabine.

1.2   Introduction to Gemcitabine and Gemcitabine Resistance

1.2.1   General Introduction to Gemcitabine

Gemcitabine is a nucleoside analog of deoxycytidine featuring fluorine atoms

on the 2′ carbon. It was created in the lab of Larry Hertel at Eli Lilly in the 1980's[9]

and was originally patented as an antiviral drug[10], but its anti-tumor activity was

quickly recognized leading to its development as a chemotherapeutic agent. Its anti-

proliferative activity was first disclosed at the 1986 proceedings of the American

Association of Cancer Researchers[11] and subsequently described in the literature

in 1988[12]. Its anti-tumor effects were first shown *in vivo* in 1990, and this effect

was confirmed in pancreatic cell lines in 1992[13] (*in vitro*) and in xenograft models

in 1993[14]. Its pre-clinical success led to first in human trials reported in 1991[15].

The success of these led to phase II trials, which reported that out of 44 patients

receiving gemcitabine none saw a complete response, four saw a partial response

and median survival was listed as 5.6 months[16].   A two armed Phase III trial was

conducted from July 1992 – March 1994 comparing the clinical benefit of

gemcitabine to that of 5-fluorouracil, the drug of choice for treating pancreatic

adenocarcinoma prior to gemcitabine. It demonstrated that gemcitabine treatment

alone is more beneficial in the treatment of advanced pancreatic cancer than

treatment with 5-fluorouracil with 23.8% of those receiving gemcitabine treatment

experiencing clinical benefit compared to only 4.8% of those receiving 5-

fluorouracil[17]. These led to gemcitabine's 1996 FDA approval for first-line

treatment of advanced pancreatic cancer. Recently the standard of care

chemotherapy was improved from gemcitabine alone to the combination of

gemcitabine and nanoparticle albumin bound (nab)-paclitaxel, which improved the

median overall survival from 6.7 months to 8.5 months[18].

1.2.2   Metabolism and Mechanisms of Action of Gemcitabine

As illustrated in Figure 1-1, Gemcitabine enters the cell through several

nucleoside transporters, which include members of both the concentrative (sodium

dependent) and equilibrative (sodium independent) subclasses[19-21] excluding
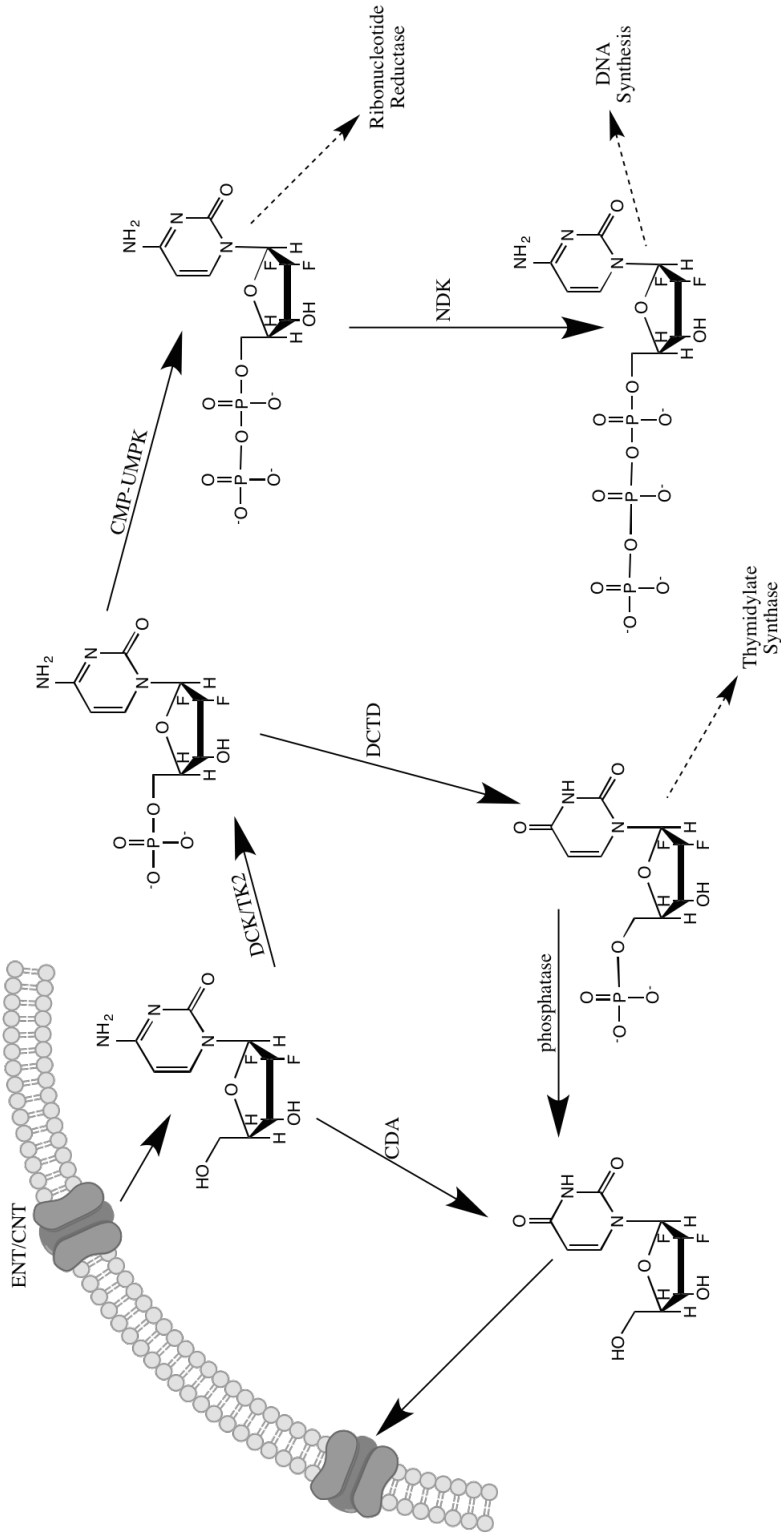
Figure 1-1: Gemcitabine Metabolsim. Upon entry into the cell through nucleotide transporters gemcitabine is phosphorylated by deoxycytidine kinase (DCK) and to a lesser degree by thymidine kinase 2 (TK2) to produce difluorodeoxycytidine monophosphate(dfdCMP). It is further phosphorylated to dfdCDP by cytidine monophosphate – uridine monphosphate kinase (CMP-UMPK), which can inhibit ribonulceotide reductase. dfdCDP is further phosphorylated by nucleotide diphosphate kinase (NDK) to produce dfdCTP which can be incorporated into the DNA during DNA synthesis. dfdCMP can be deaminated by deoxycytidine deaminase (DCDA) to produce difluorodeoxyuridine monophosphate (dfdUMP), an inhibitor or thymidylate synthase. The dephosphorylation of dfdUMP and the deamination of gemcitabine by cytidine deaminase (CDA) both yield difluorodeoxyuridine (dfdU), the major endpoint product of gemcitabine metabolism, which is excreted from the cell.

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the purine selective CNT2[19]. Upon entering the cell, gemcitabine is phosphorylated by either deoxycytidine kinase (dCK) (Km of 4.6μM as compared to 1.5μM for dCyd[22]) or thymidine kinase 2 (Km of 66μM for dfdC and 7μM for dCyd[23]) to produce di-fluorodeoxycytidine monophosphate (dfdCMP)[12], which can be further phosphorylated by UMP-CMP kinase to form the diphosphate, dfdCDP[24]. dfdCDP may be phosphorylated further by nucleoside diphosphate kinase to form the triphosphate dfdCTP, which will prohibit DNA elongation once it has been incorporated into the DNA during replication. dfdCDP has also been shown to inhibit DNA synthesis by inhibiting ribonucleotide reductase which in turn reduces the intracellular concentrations of deoxyribonucleotide triphosphates[25]. It has also been proposed that dfdUMP, the product of deamination of dfdCMP by deoxycytidylate deaminase, may inhibit thymidylate synthetase preventing the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate[26].

Of these three mechanisms of action, the incorporation of dfdCTP into DNA is recognized as the most important as it is essential for apoptosis[27]. The mechanism by which this occurs is well documented[26-28]. During the S-phase of the cell cycle dfdCTP competes with dCTP for incorporation into the DNA by DNA polymerases α and ε. Once dfdCMP has been incorporated, one more nucleotide is added before strand elongation is halted. The addition of the extra nucleotide neutralizes the 3′→5′ exonuclease activity of DNA polymerase ε[28]. This dfdCMP/DNA complex often halts polymerization leading to S-G1 phase arrest and apoptotic cell death[27] although it has been reported that the pause in DNA

synthesis is not necessarily fatal and polymerization can resume and go to completion leading to an incorporation of dfdCMP in the DNA[29].

The known means of cellular elimination of gemcitabine from the cell occurs through two main mechanisms: excretion of the products of deamination of both gemcitabine and dfdCMP by cytidine deaminase and deoxycytidine deaminase, respectively[15, 30].

1.2.3   Gemcitabine Resistance

Resistance to gemcitabine continues to pose a threat to successful treatment. It can occur through multiple mechanisms and can exist at the time treatment begins or be the result of acquired changes in molecular signaling pathways that result in increased detoxification of the drug or provide a molecular escape from apoptosis through either increased growth signaling, or modification of apoptotic signaling.

1.2.3.1   Resistance via Gemcitabine Transporters

Aberrations in the transporters responsible for the import of gemcitabine into the cell have been associated with both inherent and acquired resistance to the drug. As mentioned above, gemcitabine is imported into the cell via nucleoside transporters of which there are two families, the concentrative (CNT;SLC28) and equilibrative(ENT;SLC29). There are three members of the former and four of the latter – a combined five of which have been shown to transport gemcitabine across

membranes: ENT1, ENT2, ENT3, CNT1, and CNT3[31]. ENT1, ENT2, CNT1, and CNT3 have each been implicated in gemcitabine resistance.

ENT1 associated resistance has been the most heavily reported among the transporters. Though it has a relatively high K_m (160 µM) for gemcitabine[20], it is the most highly expressed of these transporters and, thereby, responsible for the majority of gemcitabine influx[32]. A lack of ENT1 in pancreatic adenocarcinoma is associated with a reduced clinical sensitivity to gemcitabine and reduced survival[33]. Conversely, patients with high levels of ENT1 expression responded better to gemcitabine-based chemoradiation experiencing longer disease-free survival as well as longer overall survival[34]. ENT2 has also been associated with decreased sensitivity to gemcitabine due to failure to localize to the plasma membrane[35].

The pyrimidine transporting concentrative transporters, CNT1 and CNT3, each have high affinity for the drug, with a K_m of 24 µM and $K_{0.5}$ of 60 µM, respectively[19, 36] but are expressed at much lower levels than ENT1. Despite their low levels of expression, their impact on tumor sensitivity is well noted. The same study that found increases in ENT1 expression correlated with higher survivability found that patients with higher CNT3 expression experienced longer overall survival. They also noted that having high expression of both ENT1 and CNT3 resulted in longer patient survival than high expression of just one of the two transporters thus providing a prognostic indicator for pancreatic adenocarcinoma[34]. Increased expression of CNT1 has also been associated with increased cellular susceptibility to gemcitabine. When pancreatic cancer cells that

already expressed high levels of ENT1 were transfected with CNT1, cDNA

nucleoside uptake increased along with sensitivity to gemcitabine[32].

Numerous genetic polymorphisms of these three nucleotide transporters

have been reported. However, only three impact the import of gemcitabine, and all

three of these occur in CNT1. A truncation of the gene occurs in 3% of the African-

American population and leads to a non-functional product. A 565 G>A single

nucleotide polymorphism (SNP) occurs in 28% of Caucasians, 19% of African-

Americans, and 35% of Asians and leads to a reduction in gemcitabine affinity. The

third polymorphism, a 1561 G>A SNP, does not yield any functional change *in vitro*,

however, clinically this SNP leads to an increased rate of myelotoxicity in non-small

cell lung carcinoma patients and occurs in roughly half the Caucasian population

and in 10% and 11% of the African-American and Asian populations,

respectively[26].

1.2.3.2   Resistance through alterations in gemcitabine metabolism

The modes of resistance related to gemcitabine metabolism are numerous.

Gemcitabine requires phosphorylation to the mono-, di-, and triphosphate forms to

achieve its cytotoxic effect. Therefore, it comes as no surprise that enzymatic

prevention and reversal of these phosphorylated states are associated with

resistance.

1.2.3.2.1 Regulation of phosphorylation: dCK, TK2, & 5′-NT

The most commonly reported modes of gemcitabine resistance involve

aberrations in its metabolism, among them is a deficiency in deoxycytidine

kinase[37]. In A2780 human ovarian carcinoma cells, a persistent increase in

concentration of continued gemcitabine exposure over several months produced a

gemcitabine resistant cell line. This cell line was maintained in 6μM gemcitabine

and its resistance was attributed to a complete down-regulation of dCK where the

resistant cell line no longer expressed the enzyme[38]. A similar effect was seen in

the human pancreatic carcinoma cell line PK9 which through increasing exposure

was able to maintain growth in 3.8μM gemcitabine and had an IC_{50} 2.8x10^5 greater

than its sensitive counterpart. It was discovered that the resistant cell line (RPK9)

had only 2% of the dCK concentration of the native PK9 cells[39]. dCK can also play

a role in intrinsic resistance. Van der Wilt et al reported a positive correlation with

dCk activity and sensitivity to gemcitabine in several cell lines of different origins,

and cells transfected with dCK cDNA became more sensitive to the drug compared

with the parent line[40].

Decreased expression of dCK has also been associated with a poorer clinical

prognosis. In a study of 32 patients, dCK was immunolabeled in tumor samples and

it was found that those with higher dCK expression were more responsive to

gemcitabine and experienced longer overall survival. However, it was also noted

that some tumors expressing high dCK were still not sensitive to gemcitabine

therapy. They also reported a decrease in dCK with increasing patient age and

suspected this was due to an increase in methylation of the dCK gene[41].

Several genetic polymorphisms have been identified throughout the dCK

locus as well as in the promoter region. Two SNPs in the promoter region, -360 C>G

and -201 C>T, have been associated with an increased event-free survival rate and

improved clinical response to cytarabine, another nucleoside analog that requires

phosphorylation by dCK, in acute myeloid leukemia patients[42]. Several SNPs in

the dCK coding region have been reported to decrease enzymatic activity *in vitro*.

However, there has been no report of any clinical significance these may have[26].

Additionally, a SNP in the 3′ UTR has been reported to lower dCK mRNA expression

and resulted in a decrease in cytarabine phosphorylation. This particular SNP

exhibits particularly high interethnic diversity. 77% of the African population carry

this SNP compared to 5% of the Caucasian population[43].

Upon entry into the cell, gemcitabine can receive its initial phosphorylation

by either dCK or thymidine kinase 2 (TK2), albeit to a much lesser degree as

gemcitabine is a comparatively poor substrate for TK2[23]. TK2 does, however,

readily phosphorylate deoxycytidine, generating dCMP. Additional phosphorylation

of dCMP produces dCTP a potent feedback inhibitor of dCK. Therefore, it can be

surmised that overexpression of TK2 would lead to a decrease in gemcitabine

toxicity due to dCTP associated feedback inhibition of dCK preventing the initial

phosphorylation of dfdC[44]. To date, however, there have been no clinical findings

correlating an overexpression of TK2 with gemcitabine resistance.

In addition to dCK and TK2, cases of intrinsic and acquired resistance to

gemcitabine due to an upregulation in 5′-nucleotidases (5′NT) have been reported.

5′-NT dephosphorylates nucleoside monphosphates such as dfdCMP to their

unphosphorylated state. Of the seven described 5′-NTs, two have been reported to

catabolize dfdCMP, cytosolic 5′ nucleotidase I (cN-IA) and cytosolic 5′(3′)-

deoxyribonucleotidase (cdN)[45]. Overexpression of cN-IA in HEK 293 cells yielded

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a 22-fold increase in the IC_{50} of gemcitabine[46]. And in a gemcitabine resistant variant of the human leukemia cell line, K562, that showed a 45-fold increase in the gemcitabine IC_{50}, the enzymatic activity of 5′-NT was found to have increased over 14-fold[47]. Additionally, it has been previously reported that dfdCMP is a poor substrate for the nucleotidase cN-II[48]. However, recent reports indicated that cN-II levels correlated with overall survival in NSCLC patients receiving gemcitabine therapy with patients expressing higher levels of cN-II having an increased average survival from 6 to 11 months[49]. This likely indicates that cN-II is involved in the management of dCMP reducing competition for gemcitabine incorporation into the DNA and limiting feedback inhibition of dCK.

1.2.3.2.2 Regulation through deactivation: CDA & dCDA

By catalytically removing the amino group, cytidine deaminase (CDA) and deoxycytidine deaminase (dCDA) also detoxify gemcitabine and dfdCMP, yielding dfdU and dfdUMP, respectively. dfdUMP is further metabolized to yield dfdU, the major end product of gemcitabine metabolism[30]. In one study, CCRF-CEM cells, a human T lymphoblastic cell line, over-expressing CDA were 2.4 fold less inhibited by gemcitabine, and inhibiting CDA with tetrahydrouridine restored sensitivity to another nucleoside analog, cytosine arabinoside[50]. Additionally, pancreatic cancer cells resistant to gemcitabine were found to have a 45-fold higher level of dCDA activity when compared to non-resistant cells[51]. A 2005 Phase I trial aimed at defining new treatment parameters for advanced pancreactic adenocarcinoma by combining a fixed dose rate of gemcitabine infusion with the infusion of peripheral blood progenitors 24hrs later discovered that CDA expression levels correlated with

circulating dfdU levels. They also found that greater expression of CDA

corresponded with a poor prognosis for the patient[52]. Additionally, several

coding single nucleotide polymorphisms have been discovered for both CDA and

dCDA, however, the most common one for each causes a decrease in enzymatic

function, a clinical concern for toxicity, but not resistance[53].

1.2.3.2.3 Regulation through competition: Ribonucleotide Reductase

Ribonucleotide reductase (RR) together with an electron donating

thioredoxin catalyzes the production of deoxyribonucleotide diphosphates (dNDPs)

from ribonucleotide diphosphates, an essential step for DNA synthesis. As

mentioned in 1.2.2, dFdCDP can directly inhibit RR thereby reducing dNDP

production. However, its overexpression can escape this inhibition and yield an

abundance of available dNDPs, which once phosphorylated further to the

triphosphate can compete with gemcitabine for incorporation into DNA during

synthesis.

RR is composed of two subunits most commonly the M1 and M2, however,

M1 can also form a functional RR with p53R2, a subunit transcribed in response to

DNA damage. Whether the M1 or M2 subunit is more important to gemcitabine

resistance is debated in the literature. What is clear, however, is that increased RR

activity produces resistance to gemcitabine. In two simultaneously generated

gemcitabine-resistant non-small-cell lung carcinoma (NSCLC) cell lines, RRM1 (the

non-catalytic subunit of RR) was the only mRNA over-expressed in both when

compared to their parental cell lines[54]. Knockdown of expression of the RRM1

subunit has resulted in increased sensitivity to gemcitabine in the lung

adenocarcinoma cell line H23, and in 20 patients with NSCLC RRM1 expression was inversely correlated with disease response to IndGC (a chemotherapeutic combination of gemcitabine and carboplatin)[55]. Experiments involving the induction of gemcitabine resistance in Colon-26a xenograft produced a 25-fold greater expression level of the RRM1 subunit of RR in the resistant tumor. However, this was accompanied by only a 2-fold increase in RRM2 expression, which correlated with the 2-fold increase in RR enzymatic activity[56].

Overexpression of the catalytically active M2 subunit has also been shown to cause gemcitabine resistance. A gemcitabine resistant oropharyngeal epidermoid carcinoma cell line, KB-gem, made resistant to gemcitabine via stepwise increases in exposure, had a 100-fold higher IC_{50} to gemcitabine than its parental cell line. These cells exhibited a 9-fold increase in M2 mRNA which corresponded to a 2-fold increase in protein expression[57]. In a clinical study involving 31 patients with non-resectable pancreatic carcinomas, the RRM2 mRNA levels of biopsies was examined. After normalization, 13 samples were classified as having higher levels of expression and 18 samples lower. The 18 patients with lower levels of RRM2 expression had a median survival time of 8.8 months compared to only 5.0 months for those 13 with higher expression levels. Patients with lower levels of RRM2 were more responsive to gemcitabine therapy than those with higher levels (50% showed at least a partial response compared to only 7%)[58]. Another study conducted using four pancreatic adenocarcinoma cell lines found that inhibition of RRM2 using RNAi technology increased sensitivity to gemcitabine along with decreasing invasiveness[59]. Additionally, a K562 variant was selected for gemcitabine

resistance by step-wise increases in drug concentration over a period of weeks. This resulted in a 45-fold increase in the concentration of drug that was needed to reach 50% cell death after 72 hours. Among other enzymatic changes, there was a 3.3-fold increase in the enzymatic activity of RR[47].

Additionally, pharmacological inhibition of cyclin-dependent kinases using flavopiridol (a nM inhibitor of cdks) leads to a decrease in E2F-1 mediated transcription resulting in a decrease in RRM2 mRNA. When combined with gemcitabine, flavopiridol enhanced apoptosis 10–15-fold in multiple adenocarcinoma cell lines[60]. Given that flavopiridol decreased RRM2 transcription, it is likely that the RRM2 promoter has E2F1 binding site. It has also been reported that the RRM2 promoter contains an upstream stimulatory factor binding motif inside which there is a non-canonical E-box to which another transcription factor, c-Myc, can bind[61].

It should also be noted that a functional RR can be comprised of RRM1 and a different active subunit, p53R2, which is induced in response to DNA damage[62]. This is of special interest given that an estimated 40-87% of pancreatic adenocarcinomas have a deleted or mutated p53 gene[63]. Thus emphasizing the importance of RRM2.

Several SNPs have been reported in the promoter and coding region of RRM1. The promoter allelotypes −524 T>C and −37 C>A have been reported to correlate with responsiveness to gemcitabine[64]. Interestingly, this is not due to a variation in intratumoral expression[65]. No polymorphisms affecting RRM2 expression or functionality have been reported.

Gemcitabine and other deoxycytidine analogs are considered inhibitors of RR because the enzyme falsely recognizes their diphosphate form as a substrate leading to temporary inactivation. Overexpression of RR outpaces these inhibitory effects and enables cellular resistance to these drugs. There are, however, several other RR inhibitors currently being investigated - the most promising of which is Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone) (Vion Pharmaceutical Inc., CT, USA). Triapine is over 1000-fold more potent at inhibiting RR than the original RR inhibitor, hydroxyurea[66]. It acts by first chelating available iron and then generating ROS that destabilize the tyrosyl free radical in the RRM2 and p53R2 subunits[67, 68]. Due to its pre-clinical success it is being evaluated in clinical trials in combination with cisplatin, doxorubicin, irinotecan, cytarabine, fludarabine, gemcitabine and others with and without radiotherapy. Unfortunately, combination therapy with gemcitabine has been met with mostly poor results[69-71].

1.2.3.3 Resistance through modification of growth or apoptotic signaling

Atypical intracellular signaling like those known to cancer often leads to the activation of transcription factors which result in an increase in signaling favoring growth and antagonizes apoptosis.

1.2.3.3.1 NF-κB

The constitutive activation of NF-κB is common to almost 70% of pancreatic cancers[72]. NF-κB is a transcription factor that is ubiquitously expressed. It exits as homo- and heterodimers comprised of any of the five members of the Rel/NF-κB family which include RelA(p65), RelB, c-Rel, NF-κB1(p50), and NF-κB2(p52). The

transcriptionally active p65/p50 dimer is the most abundant. Its function is critical to normal cell function as evidenced by the embryonic lethality of the double knockout of the p65 subunit in mice[73]. In quiescent cells the dimer is retained in the cytosol by inhibitory proteins such as IκBα, the most abundant member of the inhibitors of NF-κB (IκB) family. This inhibition is achieved by associating with the nuclear-localization domain of the dimer and removal of the inhibitor allows for activation of NF-κB. IκBα is removed via phosphorylation of two different serine residues (S32 & S36) by the catalytic subunits of the IκB kinase complex, a complex of three proteins, IKK-α, IKK-β, and IKKγ, which is formed in response to many different cellular stimuli. The phosphorylated IκBα releases the NF-κB dimer allowing it to be translocated to the nucleus, and IκBα is rapidly polyubiquitinated and degraded by the 26S proteosome.

NF-κB activation can affect many different pathways and cause a variety of genes to be transcribed. Specificity in this system is complex and not fully understood; however, several influential factors have been described. Each member of the Rel family contains a DNA binding element termed the Rel homology domain (RHD) and different combinations of dimers have differences in binding preferences[74]. Additionally, there are multiple sites for phosphorylation and other forms of post-translational modification on each member of the Rel family creating a multiplex of possible combinations providing signaling control and transcription specificity[75].

NF-κB can be activated through a variety of signaling mechanisms. Activation through the canonical pathway occurs as the result of extracellular

signaling through one of several receptors that include the toll-like receptors, interleukin-1 receptors, and the tumor necrosis factor receptors. Activation of NF-κB can also occur through alternative pathway by signaling through other members of the TNFR family such as TNFR2, B-cell activation factor receptor (BAFF-R), lymphotoxin β receptor (LTβR), and CD40 – all members of the TNFR family (Notably, TNFR1 does not activate the alternative pathway)[76].

In response to activation through the canonical pathways, the resultant intracellular signaling cascade leads to activation of the IKK complex. In normal physiology, NF-κB signaling allows the cell to survive genotoxic stress such as reactive oxygen species, withstand low-levels of lipopolysaccharide exposure, and adapt to an immune response and other environmental changes – it is an essential element to regulating the stress response. Without NF-κB, TNFα signaling leads to apoptosis due to TNFR's simultaneous activation of NF-κB and another transcription factor, AP-1[77]. AP-1 exists as a dimer composed of members of the Jun, Fos, Maf, and ATF families, and like NF-κB it controls gene regulation of many inflammation and apoptosis related genes with sometimes overlapping function as in the case of TNFR stimulation.

NF-κB can also be activated through an alternative pathway that culminates in the phosphorylation of NF-κB2 by IKK1, which leads to its degradation from its pro-form, p100, to its active form, p52. Activation of the alternative pathway occurs through one of several TNFR family receptors. The resultant signal transduction leads to the activation of NF-κB inducing kinase (NIK), which binds p100 and acts as

a scaffolding protein binding IKK1 as well. NIK also phosphorylates IKK1 inducing

its enzymatic activity, the phosphorylation of p100[78].

As a means of controlling the activated dimer, NF-κB induces transcription of

IκBα, so in typical physiology following removal of a stressor NF-κB potentiates its

own deactivation[79], a negative feedback loop. However, in conditions of chronic

stress, such as chronic inflammation, the activating effect of the stressor overcomes

the feedback inhibition[75].

Deregulation of this system, as in oncogenesis, can lead to aberrant activation

of NF-κB, which has been shown to occur through a variety of means including

constitutive K-ras activity as well as positive feedback loops through both IL-1α and

TNFα signaling. Activation as a result of constitutively active K-ras, which occurs in

nearly all pancreatic cancers, signals constitutive NF-κB activation[80]. Paracrine

and autocrine inflammatory signaling through the IL-1 receptor can do the same. IL-

1α signaling through the IL-1 receptor activates NF-κB as which can then induce the

transcription of IL-1α, this can, and indeed does at times, result in a positive

feedback loop[81]. Additionally, the IL-1α promoter has a binding site for AP-1

which has been shown to induce its transcription in response to signaling through

the epidermal growth factor receptor (EGFR)[82] and K-ras creating another

positive feedback loop[83]. Autocrine production of IL-1β catalyzed by signaling as

a result of interaction between the L1 cell adhesion molecule (L1CAM, a

transmembrane glycoprotein) with integrins containing RGD binding domains on

adjacent cells has also been shown to induce constitute NF-κB production[84, 85].

Stimulation of the TNFR1 by TNF-α has been shown to cause constitutive activation

as well due to the NF-κB induction of TNF-α transcription, which then serves as an autocrine growth factor[86, 87]. In addition to these mechanisms, NF-κB has suggested to inhibit the transcription of CNT1[88], one of the nucleoside transporters that move gemcitabine across the cell membrane.

Gemcitabine resistance due to constitutive activation of NF-κB has been well noted. Additionally, gemcitabine (as well as other chemotherapeutics) has been found to induce NF-κB activation[89]. However, efforts at sensitizing resistant cells through inhibition of NF-κB by numerous methods have been quite successful, though this has not yet translated well in the clinic. In one study, gemcitabine resistant pancreatic cells were transfected with a mutated form of IκBα (IκBα super repressor), which irreversibly binds NF-κB. Following transfection, cells were treated with gemcitabine, which elicited a greater than two fold increase in the number of cells that underwent apoptosis when compared to a mock transfection[90].   Another study used siRNA to suppress NF-κB expression by 74% in MiaPaCa-2 cells and 54% in Panc-1 cells. When combined with gemcitabine apoptosis increased by 7.3% and 10.2%, respectively, when compared to a mock transfected control treated with gemcitabine alone after a 72-hour treatment[91].

Sulfasalazine, an anti-inflammatory drug already in use in the clinic for treating inflammatory bowl diseases, has been shown to inhibit the IKKs[92]. Administration of this drug was able to reduce intratumoral NF-κB expression in Capan-1 tumors *in vivo*. Co-treatment with gemcitabine in the same model produced a decrease in intratumoral vasculature and a decrease in Ki67 (an indicator of proliferation) staining[93]. Another study had similar findings in the

MiaPaCa-2 and Panc-1 cell lines following co-administration of sulfasalazine with gemcitabine[94].

Additionally, numerous derivates of natural products have also been found to suppress NF-κB activation and thereby potentiate gemcitabine activity. These include curcumin, (diferuloylmethane) isolated from *Curcuma longa,*[95, 96]; Dihydroartemisinin isolated from *Artemisia annua*[7]; Escin, a mixture of triterpene saponins isolated from *Aesculus wilsonii* Rehd[97]; Guggulsterone isolated from *Commiphora mukul*[98]; and Icariin, isolated from *Epimedi herba*[99]. While these have all been shown to inhibit NF-κB and sensitize cancer cells to gemcitabine, they are not strict NF-κB inhibitors, and have been shown to have effects on multiple pathways.

1.2.3.3.2 The mitogen-activated protein kinases ERK1/2

Like the NF-κB pathway, the mitogen-activated protein kinase (MAPK) pathway is an important messaging system, relaying signals from the cell membrane to the nucleus. Also like NF-κB, its deregulation has been implicated in gemcitabine resistance, although not nearly to the same extent. There are three well-characterized MAPK subfamilies, the extracellular signal-regulated kinase (ERK) family, the c-Jun N-terminal kinase (JNK) family, and the p38 kinase family. Signaling through the MAPK family often begins in response to extracellular stimuli such growth factors, hormones, neurotransmitters, inflammatory cytokines, environmental stressors and pathogen associated molecular patterns (PAMPs) like lipopolysaccharide.

A hallmark feature of the MAP kinase families is the multi-tier cascade of kinases, which allows for signal amplification. The first element of this cascade is made up of a very large and diverse group of enzymes called MAPK kinase kinases (MAP3K), these phosphorylate the second tier, MAPK kinases (MAP2K), which are sub-dived into MEKs and MKKs, these are responsible for the phosphorylation of MAPK. Activated MAPK can impact the cell in a variety of ways via both cytosolic and nuclear signaling. Phosphorylation of their target proteins can produce cellular changes as diverse as the stimuli that initiated the signal cascade. These include cell cycle control, growth and differentiation, as well as inflammatory response and apoptosis[100].

This signaling cascade is controlled by several elements. Scaffolding proteins allow for the assembly of a discrete set of MAPK family members which allows for rapid signal transmission while providing signal insulation which prevents promiscuous signaling between family members[101]. Additionally, MAPKs are proline directed kinases meaning that they will only phosphorylate a serine or threonine if it is immediately succeeded by a proline[102]. This reduces the number of phosphorylation sites they can impact. Control over this powerful system is also gained by the fact that a docking site on the MAPK must match its target protein decreasing the chance of an errant phosphorylation, adding selectivity to signal transduction[103].

Of particular interest to this research are the extracellular signal related kinases (ERK1 and ERK2), which are two distinctive enzymes, however, given their similarity in structure and seemingly overlapping functions they are often written of

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jointly. The ERK1/2 MAP kinases phosphorylate hundreds of proteins. Their action

is precipitated by a cascade of signals that begins with signaling through a receptor

tyrosine kinase (such as EGF or PDGF) which, once auto-phosphorylated, recruits an

adaptor protein that can then recruit a guanine nucleotide exchange factor, or GEF.

This GEF, in particular Son of Sevenless (Sos), engages one of the four Ras proteins

such as K-ras and exchanges its GDP for GTP.   GTP-bound Ras can then interact with

several downstream targets including one of the three Raf isoforms recruiting it to

the plasma membrane where it associates with multiple other proteins and

undergoes multiple post-translational modifications activating its enzymatic

ability[104]. Any one of the three Raf proteins is then able to phosphorylate MEK 1

and MEK2, which are responsible for the phosphorylation of ERK. The activation of

ERK1/2 requires dual-phosphorylation of the Thr-Glu-Tyr sequence in the

activation loop, which raises the activity level 1000-fold, and dephosphorylation of

either of these residues substantially decreases activity[105]. Following activation

ERK1/2 bind to target proteins via one or both of their docking sites: the D-site and

the F-site. Corresponding substrates possess either an FXFP sequence which

exclusively binds the F-site of ERK1/2 or a δ-domain (D-box) to which either

ERK1/2 or JNK may bind[106].

The list of ERK1/2 target proteins is lengthy. Included in this list are

numerous transcription factors including Elk-1 (which regulates c-fos

transcription)[107], c-Myc[108], HSF-1(inhibiting it)[109], ATF-2[110],

C/EBPα[111] as well as c-jun[112]. There are also many kinase targets of ERK1/2

including p90rsk[113], which phosphorylates, among other targets, IκBα causing

the release of NF-κB, freeing it from inhibition[114]. ERK1/2 can also

phosphorylate myosin light chain kinase leading to an increase in cell motility[115].

The kinase activity of ERK1/2 also has a large impact on the cell cycle. The MAPK is

required for phosphorylation of cyclin dependent kinase 2 and its nuclear

translocation[116]. Additionally, continued ERK1/2 expression is also necessary for

successful progression through the G1 phase of the cell cycle because of its

downstream impact on AP-1 activity, which is necessary for the inhibition of

transcription of 175 anti-proliferative genes[117].

Given the power of ERK1/2 to induce proliferation, cellular regulation of its

activation is of great importance.   ERK1/2 is involved in multiple self-potentiating

feedback loops. It can phosphorylate both MEK1/2 and Raf inactivating them,

preventing further ERK1/2 activation. It can also phosphorylate Sos disallowing its

recruitment to the plasma membrane. Erk1/2 also activate transcription factors

necessary for the production of MAP kinase phosphatases (MKPs) which

dephosphorylate ERK1/2[118]. Scaffolding proteins also regulate the activation of

ERK1/2. One such example is the kinase suppressor of Ras (KSR), which at

optimum levels of expression serves as a scaffolding protein between Raf, Raf's

inhibitor, 14-3-3, and ERK1/2 promoting activation of ERK1/2. However, when it is

expressed at levels greater than those needed for this mechanism, it inhibits

ERK1/2 activation[119]. Ras GTPase-activating-like protein 1 (IQGAP1), a

scaffolding protein, facilitates the activation of ERK following signaling through the

EGF receptor by binding b-Raf, MEK and ERK1/2. In its absence this signaling cascade fails to happen[118]. The β-Arrestins can also act as scaffolds for ERK1/2 signaling. While bound to this scaffold the cytosolic activity of ERK1/2 increases, but its nuclear activity is lost[120, 121]. This points to another facet of ERK1/2 control. Anchoring it in a particular sub-cellular compartment contains its kinase abilities to that area. In response to binding to PEA-15, an astrocytic protein containing a Death Effector Domain, ERK1/2 is prevented from entering the nucleus[122]. Sef, a component of the fibroblast growth factor (FGF) signaling pathway binds MEK1/2 with ERK1/2 preventing the dissociation of the activated ERK1/2 blocking its nuclear translocation[123]. Thus, controlling the localization of ERK1/2 provides control over the downstream targets it activates.

Due to mutated K-ras, Erk1/2 is often over activated in pancreatic cancer. This increased activity can lead to transformation of the cell by activating the above-mentioned pro-proliferation transcription factors, suppressing anti-proliferative genes through AP-1, increasing motility through phosphorylation of myosin light chain kinase, repressing pro-apoptotic Bcl-2 family members, and by inducing both the expression and activation of anti-apoptotic members of Bcl-2[124].

Additionally, ERK1/2 can contribute to gemcitabine resistance, not only by perpetuating the above-mentioned pro-proliferation phenotype, but also by increasing pyrimidine synthesis through direct activation of carbamoyl phosphate synthetase (CPS II) thereby increasing the pool of available pyrimidines for incorporation in the DNA[125]. Furthermore, inhibition of ERK1/2 has been shown to synergize with gemcitabine *in vitro* and reverse acquired resistance in Panc-1

cells[126]. In addition, a MEK inhibitor, U0126, has been shown to sensitize

gemcitabine resistant pancreatic cancer cells to gemcitabine[127].

Pharmacologically targeting the Ras-ERK pathway has been difficult. Two

farnesyl transferase inhibitors, lonafarnib and tipifarnib, were developed with the

aim of preventing the membrane localization of Ras[123]. These drugs failed in the

clinic because membrane localization of Ras is not necessarily dependent on

farnesylation. Geranylgeranylation of K-ras is sufficient for membrane localization.

Several RNA silencing technologies have been evaluated including ISIS-2503 which

initially showed promise in Phase I and II clinical trials[123], but has since been

abandoned. The Raf inhibitor NVP- AAL881 has shown promising pre-clinical

results reducing tumor growth and metastasis in an orthotopic pancreatic cancer

mouse model[128].

Inhibitors of MEK1/2 have been the main goal in controlling the activation of

ERK1/2. MEK1/2 has appeared as the most drugable target in this pathway due to

its unique physical features as revealed by crystal structure. Dozens of MEK

inhibitors are currently in development. One, Trametinib, has received FDA

approval for the treatment of melanoma patients with B-Raf mutations. In a phase

III trial Trametinib provided for longer progression free survival than the

chemotherapy group (either dacarbazine or paclitaxel) (4.8 months vs. 1.5

months)[129]. It acts by interfering with the phosphorylation of S217 by Raf[130].

The phosphorylation of this residue is one of the phosphorylations necessary for

activation of MEK1/2. An inactive MEK1/2 fails to activate ERK1/2.

1.2.3.3.3 The Bcl-2 Family

The Bcl-2 family of proteins controls the process of mitochondrial outer membrane permeabilization (MOMP), thereby controlling apoptosis. Apoptosis is one of the mechanisms leading to cell death. It is a controlled process characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, and membrane blebbing followed by the cell breaking apart into vesicles which can be phagocytized by surrounding cells. There are 25 known members of the Bcl-2 family, which are categorized into three groups based on their structure, specifically the combination of Bcl-2 homology (BH) domains (of which there are four), and their function: either pro- or anti-apoptotic. The three categories include multi-BH domain pro-apoptotic members, multi-BH domain anti-apoptotic members, and BH3-only pro-apoptotic members.

The modes leading to apoptosis can be categorized into one of two pathways, the intrinsic and extrinsic pathways. The extrinsic pathway is reliant on an external stimulus activating a death receptor (such as TNF-R1 or Fas). Activation recruits a complex of proteins ultimately resulting in the formation of the death-inducing signaling complex (DISC), which cleaves pro-caspases 8 and 10. The procession from here is determinant on cell type. Caspase 8 can directly cleave pro-caspase 3, an executioner caspase, which causes chromosomal condensation and DNA fragmentation. In other cell types caspase 8 can cleave the Bcl-2 family member Bid, which possesses only a BH3 domain. tBid can then translocate to the mitochondria where it can interact with other Bcl-2 family members activating the intrinsic pathway[131].

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The intrinsic pathway of apoptosis is induced for several reasons including, most notably, developmental cues and DNA damage. Irrespective the impetus, the result is a shift of the balance of Bcl-2 family of proteins from anti-apoptotic to pro-apoptotic. In the case of DNA damage, the damage induces the expression of p53, which in turn causes the expression of the protein PUMA (p53 upregulated modulator of apoptosis). PUMA is a BH3 only Bcl-2 family member. It interacts with anti-apoptotic members such as Bcl-2 or Bcl-xL freeing the pro-apoptotic Bax and Bak allowing them to form dimers and then pores in the outer membrane of the mitochondria permeabilizing it to the cytosol. This results in the release of the pro-apoptotic proteins cytochrome-c, SMAC/DIABLO, and HtrA2/Omi from their sequestration inside the mitochondria. Cytochrome-c binds with apoptosis protease activating factor-1 (Apaf-1) and pro-caspase 9 forming the apoptosome. This activates caspase 9 which can activate the effector caspases 3 and 7.

There are a number of BH3 only Bcl-2 family members capable of competing with Bax/Bak for binding with Bcl-2, Bcl-xL, and other anti-apoptotic Bcl-2 members. The activation of these BH3 family members is the initiator of intrinsic apoptosis. The network of factors controlling the inhibition of Bax/Bak dimer formation is complex, and though various pathways can activate BH3 only proteins, the response is conserved – freedom of Bax and Bak from the suppression of anti-apoptotic members allows them to form dimers and subsequently the MOMP.

The deregulation of Bcl-2 family members can have drastic implications in the cell and aberrant expression of various members has been repeatedly associated with cancer. In fact, the family name itself, B cell lymphoma-2, alludes to the origins

of its discovery, an involvement in cancer[132]. Unlike other oncogenes that promote cell growth, overexpression of Bcl-2 leads to an inhibition of cell death, which is crucial for tumor development. Higher levels of Bcl-2 expression and a higher ratio of Bcl-2:Bax have been correlated with poorer prognosis in multiple cancers[133]. Overexpression of Bcl-xL has also been associated with tumorgenesis and resistance[134]. Additionally, Mcl-1 overexpression has been indicated as a mediator of chemotherapy resistance in multiple cancers including ovarian carcinoma[135] and leukemia[136]. In addition to overexpression of anti-apoptotic Bcl-2 family members, inactivation of the pro-apoptotic members can supply a tumor cell with resistance to apoptosis. Mutations in p53, which abrogate its tumor suppressive ability, prevent it from inducing the transcription of the BH3 only family members PUMA and NOXA. Additionally, inactivating frameshift mutations in the gene encoding Bax have been observed in colorectal cancer[137].

Aberrant expressions of members of the Bcl-2 family are also implicated in resistance to gemcitabine. Bold et al reported that toxicity of gemcitabine was directly correlated with Bcl-2 expression in four pancreatic carcinoma cell lines. Cell lines expressing higher levels of Bcl-2 required significantly higher concentrations of gemcitabine to achieve 50% cell death[138]. And targeting Bcl-2 using siRNA in the YAPC pancreatic adenocarcinoma cell line enhanced the cytotoxicity of gemcitabine[139]. Likewise, human non-small cell lung carcinoma cells overexpressing the anti-apoptotic member Bcl-xL were found to be significantly resistant to gemcitabine[140]. Overexpression of Bcl-xL in the pancreatic adenocarcinoma cell line Colo-357 also produced a marked increase in

resistance to gemcitabine; however, overexpression of Bax in the same cell line sensitized them to gemcitabine[141].

Pharmacological correction of the imbalance of Bcl-2 members has been mainly aimed at producing BH3 mimetics that bind to the BH3 domain of anti-apoptotic Bcl-2 family members with the goal of freeing Bax and Bak to permeablize the outer membrane of the mitochondria. Most of these small molecule inhibitors are derivates of gossypol, a phenol isolated from the seeds of the cotton plant. Of these, only two, ABT-263 (navitoclax) and its derivative ABT-199, are currently being evaluated in the clinic. ABT-263 is orally bioavailable and inhibits Bcl-2, Bcl-XL and Bcl-w[142]. This drug has shown limited success as a single agent therapeutic[143], and thrombocytopenia, likely caused by inhibition of Bcl-xL in the platelets, has reduced the maximum tolerated dose[144]. However, clinical trials have continued in combination with other chemotherapeutic agents in multiple carcinomas. It has also been evaluated for safety in combination with gemcitabine and was found to be well tolerated[145]. Due to the thrombocytopenia caused by Bcl-xL inhibition, a Bcl-2 specific derivative of ABT-263 was designed by AbbVie designated ABT-199. ABT-199 has had better clinical success than its predecessor with 67% of CLL and 53% non-Hodgkins lymphoma patients showing at least partial responses to the drug with the dose limiting side-effect of tumor lysis syndrome – indicating profound apoptosis[144]. ABT-199 remains in clinical trials.

1.2.3.3.4 c-Myc

The *MYC* oncogene has been called the master regulator of pro-survival genes due to its far-reaching impact on gene transcription. A partial study of the

human genome found that Myc is involved in the transcription of at least 11% of all human genes[146]. These target genes affect all areas of cellular development including structural integrity: *LMNA* (lamin A/C), *ACTG1* (γ-actin); cellular defense:

MUC1 (mucin-1), *TXN* (thioredoxin); cell-signaling: *ICAM1* (CD54), *ITGB1* (integrin-β1); signal transduction: *APC*, *PTEN*; metabolism: *SLC2A4* (glucose transporter type 4), *FASN* (fatty acid synthase), *DCK* (deoxycytidine kinase), *TK1* (Thymidine Kinase[147]); both pro- and anti-apoptotic genes: *BAX*, *BCL2*, *BCL2L12*, *CASP8*, and *CASP9*; and a host of transcription factors: *JUN*, *E2F1*, *ELK1*, *HIF1A*, and *JUNB*[146].

1.2.3.3.4.1 - The life cycle of c-Myc

In the non-transformed cell, transcription of *MYC* is tightly regulated (Reviewed [148]), occurring in response to growth-promoting extra cellular signal transduction through many pathways which include the Wnt/APC/β-catenin/Tcf [149], Ras/MEK/Erk1/2[150-152], JAK/STAT[153], TGF-β[154], and PI3-K[155]. There are two promoters upstream of MYC, P1 and P2, which are separated by 150bp. Nearly 75% of all transcription initiates at P2[156]. There are binding sites for over 40 factors upstream of P2 that are, in part, responsible for transcriptional control. These include Nf-κB and E2F[156]. Additionally, transcription remains primed throughout much of the cell cycle with RNA polymerase II paused in a nucleosome free region proximal to the promoter[157]. Downstream response elements of the aforementioned signal transduction pathways mobilize RNA polII producing MYC mRNA. The stability of this mRNA is also tightly controlled with as few as one copy existing at any time in non-transformed cells[158]. Limiting the

half-life of the mRNA and c-Myc protein each to less than 30 minutes exerts additional control over c-Myc's pro-survival potential[156].

The c-Myc protein consists of an N-terminal transactivation domain comprised of two conserved myc box domains, MBI and MBII. The middle segment contains a PEST domain flanked on both sides by two additional myc boxes, MBIII and MBIV as well as a nuclear localization sequence. The C-terminus contains the basic helix-loop-helix leucine zipper DNA binding domain[159].

c-Myc is stabilized by phosphorylation of Ser62. Numerous kinases have been implicated in this phosphorylation including MAPK, JNK, CDK1 and CK2[160]. Phosphorylation at Ser62 is a pre-requisite for the sequential phosphorylation of Thr58 by GSK3. This second phosphorylation ultimately leads to c-Myc degradation by making the protein a target for the phosphatase PP2A, which removes the phosphorylation at Ser62[161]. The sole phosphorylation of c-Myc at Thr58 makes it a target for Fbw7, a component of the Skp-Cullin-F-box (SCF) ubiquitin ligase, which leads to ubiquitinylation and proteosomal degradation[162].

The initial ubiquitinylation of Myc does not necessarily result in immediate polyubiquitination. The ubiqutin ligase Skp2 can bind to c-Myc via one of two binding sites – one in the transactivation domain (MBII) as well one in the DNA-binding region. The initial ubiquitinylation stimulates c-Myc's transcriptional activity making Skp2 a transcriptional co-activator while at the same time marking it for eventual degradation[163].

A calpain cleavage site at lysine 298 gives additional control over c-Myc's transcriptional inhibitory activity. Cleavage removes the nuclear localization

sequence resulting in the removal of Myc from the nucleus. The cleavage product, termed Myc-nick, has been shown to increase the acetylation of α-tubulin by interaction with the protein and recruitment of the acetyltransferases GCN5 and TIP60. Binding of the adaptor protein TRRAP to c-Myc's MBII domain makes recruitment of these enzymes possible. The same study demonstrated the ability of Myc-nick to induce differentiation[164].

Acetyltransferases can also modify c-Myc. Binding of p300 to c-Myc has been shown to increase its stability. Acetylation of c-Myc by p300 was shown to both stabilize it[165] and, more recently, destabilize it, resulting in increased turnover[166]. Due to the number of lysine residues available for acetylation with c-Myc, it is likely that a mixture of the degree to which the protein is acetylated and its current binding partners determines the impact acetylation has on stability.

1.2.3.3.4.2 – Transcriptional activity of c-Myc

The transcriptional activity of c-Myc is dependent on the formation of heterodimers with MAX. Both c-Myc and MAX are basic helix-loop-helix zipper (bHLHZ) proteins.   Heterodimerization allows binding to E-box sequences: 5'–CACGTG[167] and thereby recruitment of transcriptional complexes which can result in the transcription of any of thousands of genes. The transcriptional impact of Myc depends on the location of the E-box relative to the promoter; with increasing distance c-Myc has less impact. While c-Myc is dependent on MAX for binding to E-boxes, MAX independent c-Myc activity has been detected in other promoter binding complexes interacting with NF-Y and RNA polymerase III[168, 169]. Competition for binding with MAX by the MXD family of proteins (as well as

MAX homodimers) allow for additional control of c-Myc. MXD1-4 (as well MNT) are also bHLHZ proteins. Binding of MXD-MAX dimers to E-boxes suppresses transcription by recruitment of the mSIN3 repressor complex[170]. By all accounts the MYC and MXD families seem to have opposing functions[159].

1.2.3.3.4.3 – Aberrant Regulation of c-Myc

MYC was first discovered in the 1970's as a sequence in several oncogenic viruses that caused cell transformation but was not associated with viral structural genes. Its association with cancer was cemented when it was discovered that elements of the avian leukosis virus inserted in front of the *MYC* locus resulting in its deregulation[159]. Combined these indicated that insertion of additional copies of *MYC* into the genome via viral transduction as well as disruption of the control of *MYC* expression by insertion of viral promoters or enhancers near the *MYC* promoter resulted in neoplasms identifying *MYC* as an oncogene. Hundreds of studies since then have added overwhelming evidences that deregulation of *MYC* is a hallmark of cancer.

Deregulation of *MYC* can occur through multiple modes. In addition to viral insertions, chromosomal translocations (as in Burkitt's Lymphoma) and gene amplifications[171] have also been implicated in uncoupling *MYC* from normal transcriptional control resulting in its overexpression. Overexpression can also be achieved through modulation of the signaling pathways that lead to *MYC* transcription such as those described earlier.

Overexpression of MYC leads to cell transformation by controlling a variety of pathways that lead to neoplasia. c-Myc drives the cell cycle. It induces several

cyclins as well as cyclin dependent kinases (CDKs) while acting as a repressor for the inhibitors of CDKs such as p21 and p15[172]. c-Myc promotes an increase in cell mass by causing an increase in protein synthesis[173], rRNA synthesis[174], and causes the production of a surplus of dNTPs by promoting the expression of the M2 subunit of ribonucleotide reductase[61]. c-Myc has also been reported to promote chromosomal aberrations such as amplifications[175], induction of telomeric aggregates which leads to non-reciprocal translocations[176] and induction of the DNA damage response[177]. Additionally, c-Myc has been shown to promote angiogenesis by promoting the expression of the miR-17-92 microRNA cluster, which suppresses the translation of the antiangiogenic thrombospondin-1[178].

It is also believed that over-expressed c-Myc could bind to E-boxes indiscriminately, leading to the expression of genes that it does not normally serve as a transcription factor for in a non-transformed cell. Notably, this could lead to the aberrant expression of multiple metabolic genes by inducing NRF-1 target genes such as cytochrome c, Tfam, mtTFB1 and mtTFB2[179] as well as glycolytic and angiogenic genes traditionally activated by HIF-1α by binding to the HIF-1α response element, which it has been reported to do on the alpha-fetoprotein promoter[180].

1.2.3.3.4.4 – c-Myc and Apoptosis

While MYC is an oncogene capable of driving transcription of a massive number of pro-growth genes, the cell has a built-in safety mechanism to defend against this oncogenic potential in that overexpression of Myc can lead to apoptosis.

In fact, it is believed that barring a comorbid event, c-Myc overexpression leads to cell death[181]. This dichotomy reveals a role for Myc in both growth and apoptosis.

The exact mechanisms by which c-Myc overexpression leads to cell death are unknown. However, some of the elements controlling it have been uncovered. c-Myc can drive apoptosis through p53 dependent and independent modes. Overexpression of c-Myc can lead to p53-mediated apoptosis by increasing the expression of ARF, which diminishes the function of Mdm2, a negative regulator of p53[182]. Since there is no known binding site for c-Myc on the *CDKN2A* (also known as *Ink4a*) promoter (the gene from which ARF is produced), it has been suggested that increased ARF expression occurs secondary to increased production of E2F1 a known transcriptional target of c-Myc[181]. There is an E2F1 binding site upstream of *CDKN2A*[183]. Increased p53 expression can lead to one of two cellular fates – either the cell will enter G1 arrest to repair possible damage (i.e. DNA damage) or it will undergo apoptosis. A critical sensor involved in directing this fate is p21. p21 is a cyclin dependent kinase inhibitor a feature necessary in arresting the cell cycle. c-Myc represses the p21 promoter[184] diminishing the impact of p21 on cell cycle arrest. In the absence of the ability to pause the cell cycle a feedback mechanism dictates p53-mediated apoptosis.

Overexpression of c-Myc can lead to apoptosis in p53 independent manners as well. There is evidence of Myc-dependent suppression of the anti-apoptotic BCL-2 family members Bcl-2 proper and Bcl-xL[185]. Overexpression of c-Myc also induces expression of pro-apoptotic Bax[186]. Furthermore, c-Myc acts as a transcriptional repressor of c-FLIP increasing sensitivity to TRAIL[187].

Myc has also been implicated in gemcitabine resistance. A gemcitabine-resistant bladder cancer cell line was created by step-wise increases in gemcitabine dosage resulting in a cell line that could still proliferate in greater than 10µM gemcitabine. When exposed to gemcitabine these resistant KU19-19 cells showed a 30% increase in c-Myc. Inhibition of c-Myc using the inhibitor KSI-3716 (which interferes with Myc/Max/DNA complex formation) following gemcitabine treatment produced greater cell killing than either drug alone, however, concomitant treatment offered no additional benefits[188]. Additionally, the human colon carcinoma COLO 320DM overexpresses c-Myc and is resistant to gemcitabine. However, co treatment with a triplex forming oligonulceotide (TFO) targeting the MYC promoter (Myc2T) produces a greater inhibition of tumor growth in vivo than either drug alone, delaying growth to a predetermined size ($0.85cm^2$) by 20 days[189]. TFOs are sequence specific oligonucleotides that bind duplex DNA in the major groove of the target sequence forming a triple helix. By blocking polymerases, this leads to an inhibition of transcription and replication. Due to the structural impact of triplex formation, TFOs also cause DNA damage[190]. In the aforementioned colon carcinoma model the combination of Myc2T with gemcitabine proved especially effective due, in part, to the increased incorporation of gemcitabine into the DNA.

1.2.3.3.4.5 – Pharmacological Inhibition of c-Myc

Since its discovery c-Myc has been a prized target for pharmacological inhibition in cancer therapy. Given that the majority of its functionality arises when bound to MAX, interfering with this dimerization has been researched extensively.

However, the binding interface is quite large, a feature that is difficult to completely disrupt with a small molecule. Additionally, crystal structures of c-Myc failed to reveal binding pockets suitable for small molecule inhibition[191]. Some have prevailed in blocking dimerization by instead using small molecules to induce conformational changes in c-Myc preventing recognition by MAX[192]. The most promising of which is Mycro3, which inhibits the dimerization of Myc/MAX presumably by disrupting the binding surface of c-Myc[193]. Its *in vitro* success has led to remarkable pre-clinical results that demonstrated a profound shrinkage of tumor volume along with an increase in apoptosis, and a decrease in the cellular proliferation marker Ki67 in multiple mouse models of pancreatic ductal adenocarcinoma[194].

It has also been proposed that stabilizing MAX/MAX homodimers would result in decreased c-Myc transcriptional effects. Myc/MAX dimers are much more stable than MAX/MAX dimers[195]. Therefore, stabilizing MAX/MAX dimers would, presumably, limit the availability of MAX to bind with Myc limiting Myc's transcription capabilities. The small molecule NSC13728 has been shown to stabilize MAX/MAX dimers and reduced Myc-mediated transcription *in vitro*[196]. However, there have been no additional reports regarding this drug.

1.2.3.4 Summary of Gemcitabine Resistance

Resistance to the chemotherapeutic gemcitabine remains a complex problem. Multiple pathways can contribute to resistance. Rarely do single mutations alone perpetuate through tumorgenesis. Rather, tumors, especially aggressive ones often

contain multiple mutations leading to the belief that multiple mutations are needed for tumor growth. For instance, many follicular lymphomas are indolent in early stages – increasing in severity over their clinical course. This increase is highly correlated with a *BCL-2* transgene. There is a small subset of these that are particularly aggressive. This exacerbated phenotype is due to an additional aberration – a *MYC* translocation[197]. In fact, it seems that an alteration in apoptotic signaling is necessary for Myc induced transformation, because Myc also increases the transcription of pro-apoptotic proteins[172]. This so-called two-hit hypothesis implies that for tumor development to occur, the safety features the cell has in place to prevent such an occurrence must also be compromised. Multiple successive mutations lead to an invasive carcinoma.

The same concept often applies to gemcitabine resistance. Numerous studies have reported that in cases of such resistance more than one factor was likely to blame - whether it is aberrantly expressed proteins involved in metabolism, or multiple modifications of growth and apoptotic signaling. This leads to the conclusion that to best combat gemcitabine resistance multiple modes of resistance must be hindered at once.

1.3 Sphingolipids

1.3.1 Introduction to sphingolipids

Sphingolipids are a class of lipids utilized by the cell for both structural and signaling functions. They de novo synthesis of sphingolipids (Figure 1-1) begins in

the endoplasmic reticulum with the condensation of serine and palmitoyl-CoA by serine-palmitoyl transferase yielding 3-ketosphinganine. The reduction of this by 3-ketosphinganine reductase produces sphinganine. The acylation of the free amino group on sphinganine by one of the ceramide synthases, integral membrane proteins of the ER, produces dihydroceramides of varying acyl chain lengths according to the specific CerS from which it was produced (CerS1: C18, CerS2: C22-24, CerS3: C26 and higher, CerS4: C18-22, CerS5: C16, and CerS6: C16)[198]. The insertion of a *trans*-double bond at the 4,5 position of the sphingoid backbone by dihydroceramide desaturase produces ceramide. The majority of ceramides are then transported to the Golgi apparatus by ceramide transport protein (CERT) or vesicular trafficking.

Though referred to singularly, ceramide is a family of bioactive lipids considered to be the hub of sphingolipid metabolism. From this point hundreds of structurally different ceramide species can be produced[199]. β-linkage of the sugars glucose and galactose by glucosylceramide synthase and galactosylceramide synthase, respectively, produces the cerebrosides glucosylceramide and galactosylceramide, which form the basis of the glycosphingolipids of which there are numerous sub-categories. These are transported from the Golgi to the cell membrane by vesicular transport where they are involved in numerous interactions with cell surface proteins and receptors (both *cis* and *trans*) affecting contact inhibition of cell growth and motility/adhesion as well as signal transduction[200].
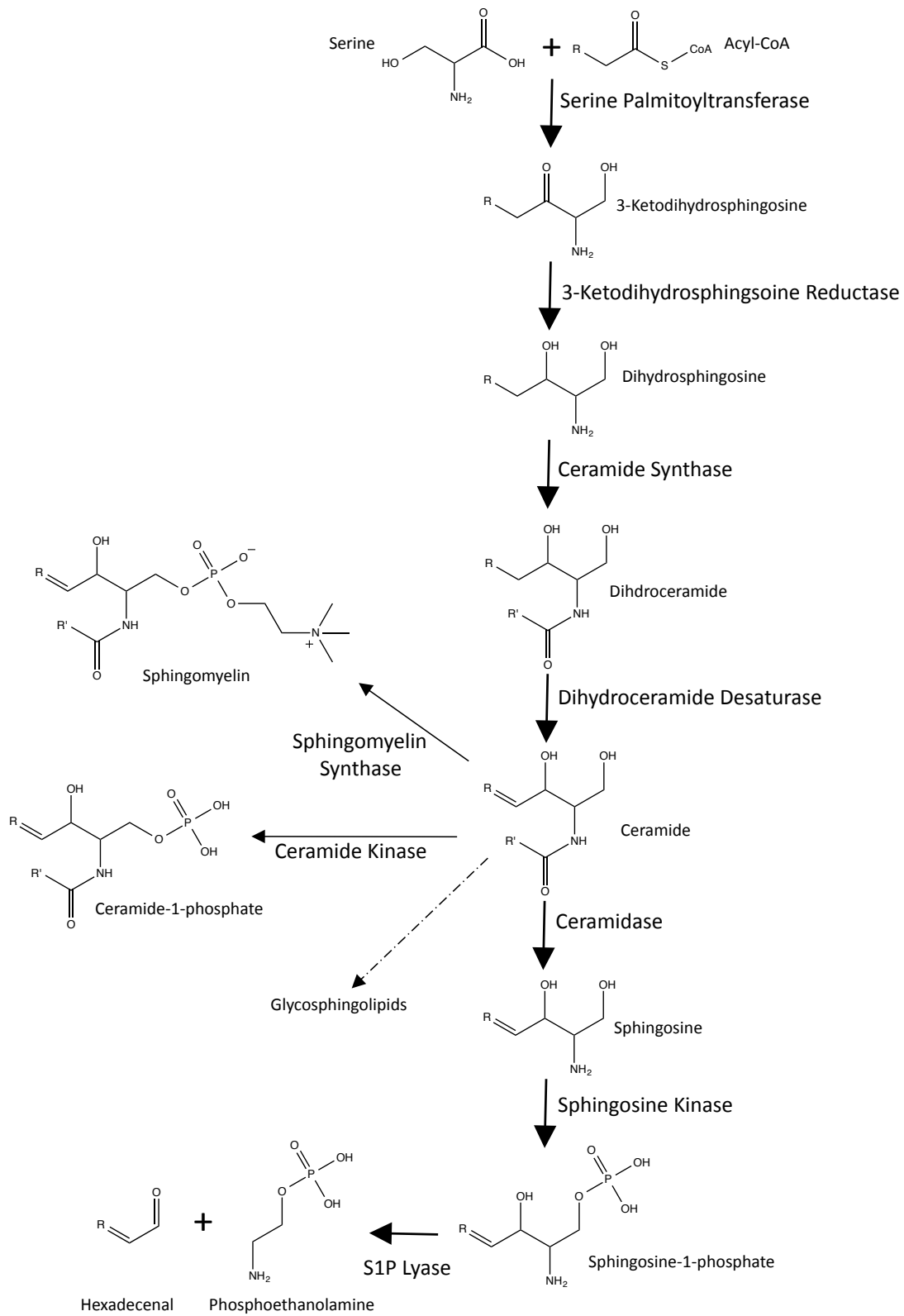
Figure 1-1. *De novo* synthesis of sphingolipids

Ceramide species with acyl chain lengths greater than 12 carbons long can also be phosphorylated by ceramide kinase to form ceramide-1-phosphate (C1P). C1P can impact membrane structure and fluidity as well as acting as an activator of cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$). Upon activation by inflammatory cytokines, cytosolic phospholipase A_2 translocates to the Golgi, which is rich in one of its substrate, phosphatidylcholine. C1P directly interacts with cPLA$_2\alpha$ diminishing its dissociation constant thereby increasing the amount of arachidonic acid (AA) produced by the cleavage of phosphatidylcholine[201]. AA is a precursor for eicosanoids, another group of lipids responsible for many of the affects of inflammation.

The addition of a phosphocholine group by one of the sphingomyelin synthases to ceramide produces sphingomyelin, the major lipid contributor to the structure of membranes and the most abundant sphingolipid. The functions of sphingomyelin go far beyond its structural support, though. Sphingomyelin along with glycosphingolipids and cholesterol are involved in the formation of membrane micro-domains known as lipid rafts. Lipid rafts are dynamic coordinating points for certain proteins. Though the same cast of sphingolipids exists in all intracellular membranes, they gain specificity for the recruitment of particular proteins by changes in relative concentration of different sphingolipids as well as variations in acyl-chain length and hydrophilic head-group (e.g. glucose, galactose, phosphoethanolamine, phosphocholine, etc.)[202]. Sphingomyelin can be hydrolyzed back to ceramide by four or five sphingomyelinases (SMases). It is debatable whether neutral SMase 1 truly serves as a sphingomyelinase *in vivo*[203].

Mice lacking the gene for nSMase1 (*Smpd2*) express normal tissue levels of sphingomyelin. However, SMase activity is decreased in the same compartment[204].

Ceramide can also be hydrolyzed removing the acyl chain by ceramidases (CDases) to form sphingosine, a bioactive sphingolipid previously shown to inhibit protein kinase C[205] as well as steroidogenic factor-1[206], and sphingosine has been shown to induce senescence and apoptosis. Sphingosine can be either acylated by CerS to reform ceramide or phosphorylated to form sphingosine-1-phosphate (S1P) by one of two sphingosine kinases. S1P is a well-studied sphingolipid, which can have intra- and extra-cellular targets and has been implicated in cell proliferation and growth. The hydrolysis of S1P by sphingosine-1-phosphate lyase, unlike the previous steps, is irreversible and the only exit from sphingolipid metabolism.

Several of the enzymes involved in sphingolipid metabolism are represented by multiple isoenzymes that localize to different sub-cellular compartments and have variable activity based on pH, allowing for greater control over the sphingolipid network. There are five different ceramidases. Acid ceramidase (ASAH1) is found in the lysosome, neutral ceramidase (ASAH2) localizes to the plasma membrane, and the three alkaline ceramidases (ACER1-3) localize to the Golgi and endoplasmic reticulum. Likewise, there are multiple sphingomyelinases: one acid (aSMase) which localizes to the lysosome and three neutral (nSMase1-3,). As mentioned above, nSMase1 is believed to not actually function as an sphingomyelinase. nSMase2 localizes to the Golgi, but upon palmitoylation

translocates to the plasma membrane, and nSMase3 localizes to the Golgi and ER. Directed sphingolipid trafficking through CERT and vesicular transport along with the controlled expression of sphingolipid metabolizing enzymes provides targeted manipulation of the sphingolipid microenvironment producing precision signal control.

Additional complexity is provided by the rapid interconversion between different sphingolipids mediated by a localized spatial proximity of the sphingome members (that is, sphingolipid metabolizing enzymes). This is furthered by the relative concentrations of sphingolipids. As mentioned earlier, sphingomyelin comprises the majority of cellular sphingolipids. In fact, its concentration is orders of magnitude greater than that of ceramide. The concentration of ceramide is greater than ten times that of sphingosine and sphingosine concentrations several fold greater than S1P[207]. Thus, one can imagine sphingomyelin as a storehouse for sphingolipids such that minor changes in the concentration of sphingomyelin can have drastic down stream effects. A small increase in the hydrolysis of sphingomyelin would create a substantial increase in a localized pool of ceramide. A similar increase in the hydrolysis of ceramide or phosphorylation of sphingosine creates a considerable impact on the concentration of the product sphingolipid, sphingosine and S1P, respectively. This allows for rapid signal amplification, which is important in downstream applications.

The effects of sphingolipids and their modification by the sphingome are not constrained to within the cell. S1P acts as a ligand for a family of g-protein coupled receptors known simply as the sphingosine-1-phosphate receptors. Sphingosine

and S1P both are amphipathic and, thus, soluble in either the membrane or cytosol and are secretable. Several enzymes are also secreted. These include acid SMase, which hydrolyzes sphingomyelin containing lipoproteins; Sphingosine kinase 1 (SphK1) is secreted from endothelial cells[208]; and CDases, which are also released by endothelial cells[209].

Collectively it can be appreciated that sphingolipids comprise a vast family of structural and signaling lipids full of complexities and capable of dynamic profile shifts.

1.3.2   Bioactive sphingolipids

Ceramide, sphingosine, and S1P are all bioactive sphingolipids. Ceramide can by hydrolyzed to form sphingosine by CDases. Sphingosine can be phosphorylated by sphingosine kinases yielding S1P. The reverse reactions also take place with S1P being dephosphorylated by S1P phosphatase and sphingosine acylated by ceramide synthase to yield sphingosine and ceramide, respectively.

1.3.2.1 Ceramides

The term ceramide applies to hundreds of molecules with different possible acyl tail lengths and different head groups produced and degraded by at least 28 different enzymes[207]. Speaking strictly in terms of ceramides without head groups, they are grouped into categories based on the length of their acyl tail. Though seemingly minor differences, these changes combined with differential

localization and metabolism generate such varying phenotypical responses that attempting to lump all ceramides together and define them as having one particular action isn't prudent. However, with that understanding, in general terms, increasing concentrations of ceramides are associated with at the very least senescence and most apoptosis.

Nomenclature for ceramides is as follows: nC_XCer where n describes the sphingoid base (e.g. 18:1 indicates a palmitoyl base with one double bond) no prefix implies a saturated palmitoyl sphingoid base, C_X indicates the length of the acyl chain added by ceramide synthase (e.g. C_{16}- indicates a 16 acyl chain). Additional modifications to either the acyl chain are noted before the Cer, or dhCer as the case may be, (e.g $18:1C_{24}2'OHCer$ would indicate a palmitoyl sphingoid base with one degree of saturation amide linked to a 24 carbon fatty acid with a hydroxylation at the 2' position).

$C_{18}Cer$ generated by CerS1 induces apoptotic cell death via activation of caspase 9 and 3 in head and neck squamous cell carcinoma (HNSCC) in response to gemcitabine/doxorubicin treatment[210]. In response to cisplatin CerS1 translocates from the ER to the Golgi, thus enriching the localized pool of $C_{18}Cer$ leading to the eventual activation of p38MAPK, though the linking mechanism is unresolved[211]. Ceramide has also been linked to TRAIL induced apoptosis. $C_{16}Cer$ produced by CerS6 is induced in response to the ligand binding to either of the TRAIL receptors (DR4 and DR5). This pool of $C_{16}Cer$ was shown to play a part in the release of caspase 3 from the plasma membrane in response to TRAIL[212]. However, $C_{16}Cer$ localized to the ER generated by CerS6 has been implicated in cell

survival by protecting the cell from ER mediated stress. Senkal et al showed that siRNA knockdown of CerS6 in HNSCC cells induced ER stress resulting in apoptosis and noted that $C_{16}Cer$ is up-regulated in HNSCC tumors when compared to surrounding tissue[213]. Thus, $C_{16}Cer$ can elicit opposing effects depending on its subcellular localization. A reduction of CerS2, which produces very-long-chain (VLC) ceramides (C22-24) in the ER and ER associated membranes (such as the mitochondrial associated membrane), has been associated with growth attributed to an attenuation in translation due to inhibition of the unfolded protein response (UPR)[214]. CerS2 has also been shown to form heterocomplexes with CerS5 and CerS6. In the same study, overexpression of CerS2 provided partial protection from ionizing radiation (IR) attributable to the increased VLC ceramides[215].

Additionally, when CerS2 was knocked down in the poorly invasive MCF7 cells, the cells became invasive whereas overexpression of CerS2 in MDA-MB-231 an aggressively invasive cell line greatly diminished its invasiveness.

Ceramides can also form channels in membranes. This is particularly relevant to permeabilization of the mitochondria. Ceramide channels are formed spontaneously in response to an increase in localized ceramide production. They are stable, rigid structures large enough to allow the escape of the proteins of inter-membrane space[216]. The size of the channel depends on the species that formed it. $C_{16}Cers$ form larger channels than the VLC $C_{24}Cers$. Mitochondrial localized increases in C16Cer can lead to an increase in MOMP and responsive increases in C24Cer can disrupt this[216]. In the case of overexpression of CerS2 mentioned above, the increased production of VLC ceramides likely prevented apoptosis in

response to IR by prohibiting the formation of ceramide channels[215]. Thus, generally speaking, very long chain ceramides seem to have a protective effect.

1.3.2.2 Sphingosine

Sphingosine is produced by either the hydrolysis of ceramide by one of the ceramidases or the dephosphorylation of S1P by S1P phosphatase. Sphingosine is an 18-chain carbon amino alcohol with a double bond between the 4th and 5th carbons. Compared to ceramide, the direct interactions of sphingosine are less well known. Exogenous addition of sphingosine inhibits protein kinase C (PKC) and steroidogenic factor 1 (SF1) while activating caspase 3 leading to the cleavage of PKC-δ to produce sphingosine dependent kinase (SDK). It also induces cell death with features of both apoptosis and autophagy. In the mid-1980s sphingosine was first shown to directly bind to PKC and inhibit its activation by competing with diacylglycerol at its binding site[205]. Inhibition of PKC by sphingosine inhibits cell growth and differentiation in promyelocytic leukemic cells[217]. SF1 is a cAMP sensitive transcription factor involved in promoting the transcription of enzymes involved in androgen biosynthesis. Sphingosine was shown to directly bind SF1 in vitro. In vivo, sphingosine diminished the association of a co-activator, SRC-1, and cAMP was shown to promote the dissociation of sphingosine[206]. Cleavage of PKC-δ by capase 3 (which can happen in response to exogenous sphingosine) releases its kinase domain, termed sphingosine dependent kinase because of its reliance on sphingosine for its kinase activity. SDK phosphorylates 14-3-3 preventing its dimerization and inhibition of BAX and BAD[218]. Relase of BAD and BAX inhibition

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promotes apoptosis. Sphinsoine has also been associated with the caspase 3 dependent cleavage of the tumor-suppressor protein prostate apoptosis response-4 (Par-4), which leads to apoptosis[219]. The mechanism by which up-regulation of sphingosine leads to activation of caspase 3 is curently unknown and whether activation of SDK precedes or follows caspase activation is unclear as other groups have published SDK activation leads to caspase activation due to the aforementioned 14-3-3 phosphorylation and resultant MOMP[220].

1.3.2.3 Sphingosine-1-phosphate

Like its metabolic precursors ceramide and sphingosine, sphingosine-1-phosphate (S1P) is a bioactive lipid. S1P has both intra- and extracellular binding targets, and S1P signaling drives proliferation and inhibits apoptosis. There are two isozymes that catalyze the phosphorylation of sphingosine: sphingosine kinase 1 and sphingosine kinase 2 (SphK1 and SphK2). S1P is degraded by either of two phosphatases, S1P phosphatase 1 and 2, and S1P lyase, which cleaves S1P yielding phosphoethanolamine and a fatty acid.

1.3.2.3.1 Sphingosine kinase 1 derived S1P

SphK1 is activated in response to signaling by numerous growth factors and cytokines such as EGF, PDGF, VEGF, and TNFα[221] and by crosslinking of the high affinity receptor for IgE (FcεRI). Receptor stimulation of their respective receptors results in ERK1/2 mediated phosphorylation of Ser225 of SphK1 increasing its activity and allowing for its translocation to the plasma membrane[222]. Calcium

and integrin-binding protein 1 binds activated SphK1 and facilitates this translocation[223]. When phosphorylated SphK1 arrives at the plasma membrane where it binds phosphatidylserine[224]. The S1P produced there can be exported from the cell through ATP binding cassette (ABC) transporters where it can act as an autocrine or paracrine signaling lipid. S1P binds five different G-protein coupled receptors named S1P-Receptor 1-5 ($S1P_{1-5}$).

$S1P_1$ couples exclusively to Gαi. The binding of S1P has been shown to elicit a number of effects. Most famously, signaling through $S1P_1$ is required for lymphocyte egress from lymph nodes. This has proved important in combating the autoimmune disease multiple sclerosis as down-regulation of SIP_1 by FTY720 sequesters lymphocytes in the lymphoid organs[225]. Cross-linking of FcϵRI in response to binding of IgE activates SphK1 leading to autocrine signaling of S1P through both $S1P_1$ and $S1P_2$. Signaling through $S1P_1$ in response to FcϵRI cross-linking leads to cytoskeleton rearrangement and migration toward the impetus[226].

$S1P_2$ couples to multiple Gα subunits: i, q, and 12/13[227]. $S1P_2$ has frequently been associated with vascularization. $S1P_2$ null mice exhibit disturbances in the stria vascularis, which forms the vasculature of the inner ear[228]. $S1P_2$ was linked to pathological angiogenesis of the eye following ischemic injury by inhibiting the production of eNOS and promoting the expression of cyclooxygenase-2, thus driving the inflammatory process[229]. $S1P_2$ has been linked to inflammatory processes in other ways as well. Signaling through $S1P_2$ leads to mast cell degranulation, which leads to anaphylaxis and resultant

pulmonary edema in mice. Pretreatment of mice with an $S1P_2$ antagonist, JTE-013,

drastically attenuated anaphylaxis in response to an anaphylaxis producing stimuli

due to an inhibition of mast cell degranulation[230]. Conversely, $S1P_2$ also seems

important for recovery from anaphylaxis. SphK1 null mice and $S1P_2$ null mice have

delayed clearance of histamine following anaphylaxis. They also had severe

hypotension during that time indicating that SphK1 derived S1P signaling through

$S1P_2$ impacts blood pressure control as well as recovery from anaphylaxis[231].

These combined data indicate a role for $S1P_2$ in both severity of anaphylaxis and

recovery from it.

Like $S1P_2$, $S1P_3$ couples to $G\alpha i$, $G\alpha q$, and $G\alpha 12/13$. Activation of $S1P_3$ in

response to the glucocorticoid dexamethasone in human fibroblasts results in

activation of the PI3K/Akt pro-survival pathway as well inhibiting apoptosis

through an increased expression the anti-apoptotic Bcl-2 member Bcl-xL[232]. Like

the other S1P receptors, $S1P_3$ has also been implicated in the inflammatory process.

S1P is released form the cell in response to signaling through the protease-activated

receptor 1. This leads to amplification of pro-inflammatory signaling through

$S1P_3$[233]. S1P signaling through $S1P_3$ has also been shown to impact

vasoconstriction by increasing intracellular Ca^{2+} concentration and signaling

through Rho kinase in human coronary artery smooth muscle cells[234].

$S1P_4$ couples to $G\alpha i$ and $G\alpha 12/13$ in response to S1P. Less is known about

$S1P_4$, but unlike the other S1P receptors, it has been shown to have

immunosuppressive effects. Signaling through $S1P_4$ was shown to inhibit the

proliferation of T cells as well as their secretion of effector cytokines while

stimulating the release of IL-10, a suppressive cytokine[235].

S1P$_5$, like S1P$_4$, couples to both Gαi and Gα12/13 in response to S1P. S1P

signaling through S1P$_5$ was shown block the migration of oligodendrocyte precursor

cells indicating that S1P is important in cellular commnication events involved in

brain development[236]. Signaling through S1P$_5$ also inhibits the migration of

esophageal cancer cells[237]. However, signaling through S1P$_5$ has a positive effect

in natural killer (NK) cells. Its expression is necessary for NK cell exit from the bone

marrow[238]. S1P signaling through S1P$_5$ has also been shown to trigger ER stress

in the PC3 prostate cancer cell line leading to autophagic cell death[239].

SphK1 derived S1P also has distinct intracellular functions. TNFα binding to

TNF receptor 1 recruits TNF-associated factor 2 (TRAF2) to the intracellular domain

of the receptor. TRAF2 can then bind SphK1. The S1P produced by SphK1 acts as a

cofactor for TRAF2 activating its E3 ubiquitin ligase function. TRAF2 mediates the K-

63 polyubiquitination of receptor interacting protein 1(RIP1). The

polyubiquitination of RIP1 serves as a scaffold for TGFβ-activated kinase 1 and the

IκB kinase (IKK) complex allowing for the activation of NF-κB[240]. Interestingly,

TNFα has also been shown to induce the degredation of SphK1 in a cathepsin B

dependent manner[241].

Given that SphK1 derived S1P can drive cell growth and proliferation, it

comes as no surprise that its deregulation has been observed in numerous cancers.

Its ability to transform fibroblasts, which can then form tumors in immunodeficient

mice, earned it the title of oncogene[242]. SphK1 overexpression has been observed in lung cancer, colon cancer, and glioblastoma[243] as well as breast cancer[244].

1.3.2.3.2 Sphingosine kinase 2 derived S1P

Like SphK1, SphK2 catalyzes the phosphorylation of sphingosine. SphK2 can also phosphorylate two additional sphingoid bases: dihydrosphingosine and phytosphingosine[245]. *SPHK1-/-* and *SPHK2 -/-* mice develop normally indicating that the isomzymes are able to compensate for one another to some degree. However, knocking out both genes simultaneously is embryonic lethal[246]. There are five reported splice variants of SphK2. It has been reported to localize to several subcellular compartments including the cytosol [247], the endoplasmic reticulum[248], and the mitochondria[249]. However, the primary localization seems to be the nucleus due to a nuclear localization sequence[250]. This may be cell type dependent, though.

SphK2 derived S1P has been reported to both inhibit and promote cell growth. SphK2 was first reported to suppress growth and promote apoptosis. This was discovered by overexpression of the enzyme and attributed to a putative BH3 domain absent in SphK1 which was able to bind with Bcl-xL preventing its suppression of pro-apoptotic BH3-only Bcl-2 family members such as Bax and Bak[251]. Mutation of a single leucine residue in the BH3 domain of SphK2 drastically decreased apoptosis. Knockdown of SphK2 has also resulted in increased apoptosis in glioblastoma cells[252], colorectal carcinoma cells, and MCF7 breast cancer cells[253]. This seemingly dichotomous effect can likely be attributed to the overexpression of SphK2. When it was overexpressed in the initial report

declaring it pro-apoptotic the normal subcellular distribution of SphK2 was not

retained allowing for non-physiological functions such as cleavage of its BH3 only

domain, which would then promote apoptosis[253].

Like SphK1, SphK2 is activated by phosphorylation from Erk1/2[254]. In the

mitochondria, SphK2 derived S1P binds specifically to prohibitin 2, which regulates

the assembly of complex IV (cytochrome-*c* oxidase, COX) of the electron transport

chain[249]. S1P derived from SphK2 has also been associated with the formation of

amyloid plaques in Alzheimer's disease. The major component of these plaques,

amyloid-β, is a cleavage product of the amyloid-β precursor protein (APP). Cleavage

of APP is carried out by the β-secretase β-site APP cleaving enzyme 1 (BACE1).

SphK2 derived S1P has been shown to bind the transmembrane domain of BACE1,

which allostericly modifies the catalytic domain increasing its activity[255].   Thus,

SphK2 overexpression could contribute to Alzheimer's disease.

SphK2 was also shown to play a role in the ischemic preconditioning

response in mouse brains. Inhibition of SphK2 with the specific SphK2 inhibitor

ABC294640 mitigated the protective effects of isoflurane preconditioning in mouse

stroke model where C57BL/J mice received a transient middle cerebral artery

occlusion[256]. It was later shown that preconditioning upregulates SphK2 and

induces autophagy[257]. Thus, inhibition of SphK2 hampers the preconditioning

response.

Additionally, it has been reported that caspase 1 mediated cleavage of SphK2

in cells undergoing apoptosis allows for the translocation of a still catalytically

active fragment of SphK2 to the plasma membrane where it binds

phosphatidylserine and mediates the phosphorylation of sphingosine[258]. S1P can be exported from the cell and serve as a chemoattractant for monocytes and macrophages by interacting with S1P receptors[259].

Importantly, SphK2 has been shown to bind histone H3 and impact its acetylation in the MCF7 breast cancer cell line. siRNA knockdown of SphK2 was reported to decrease the acetylation of H3-K9, H4-K5, and H2B-K12. Addition of S1P to isolated nuclei of siSPHK2 cells resulted in restoration of acetylation of these three residues. This same study showed that S1P and dihydro-S1P were able to inhibit histone deacetylases 1 and 2 and that siRNA knockdown of SphK2 enhanced HDAC activity. Additionally, the authors showed that activation of SphK2 by phorbol 12-myristate 13-acetate (PMA, a potent activator of protein kinase C, upstream of ERK1/2 mediated SphK2 phosphorylation) enhanced the co-localization of SphK2 with HDAC1. PMA is a known inducer of the transcription of the cyclin dependent kinase inhibitor p21 and the transcription factor c-fos. When combined with siRNA knockdown of SphK2 PMA failed to induce the transcription of p21 and c-fos. This correlated with a reduction in acetylation of H3 associated with the promoters of p21 and c-fos[260]. This agreed with this lab's earlier findings showing knockdown of SphK2 prevented doxycyclin-induced p21 expression[253]. These results are supported by Igarashi et al who reported that overexpression of SphK2 and SphK1 with a fused nuclear localization sequence (NLS), but not SphK1, cause an inhibition of DNA synthesis[250]. SphK2 derived S1P has, thus, been demonstrated to impact gene transcription through regulation of HDAC activity.

Given that a definitive role for SphK2 in proliferation is still murky it comes as no surprise that its role in carcinogenesis is also unclear - with some research groups finding SphK2 to be a tumor suppressor and others a mediator of tumor growth. Treatment of *SPHK2* -/- mice with the carcinogen azoxymethane and the inflammation inducing toxin dextran sodium sulfate was reported to produce more numerous, larger, and more aggressive tumors than wild-type mice receiving the same treatment. This was reported to coincide with an increase in STAT3, NF-κB and IL-6 expression in hematopoietic cells of the knockout mice, which drove the colitis and associated cancer. SphK2 has also been described as oncogenic in acute lymphoblastic leukemia (ALL). Knockdown of *SPHK2* and pharmacological inhibition using the specific inhibitor ABC294640 both attenuated development of leukemia in an ALL mouse models. This was attributed to a reduction in the expression of *MYC* (and thereby c-Myc target genes) by way of reduced acetylation levels of H3, linking SphK2 to the expression this important oncogene. This same study also showed the importance of SphK2 in the development of ALL. When BCR/ABL-transduced B-cell progenitor cells were implanted in 29 sublethally irradiated mice 22 developed ALL with a median survival of 42 days. When the same BCR/ABL translocation was introduced to *SPHK2* (-/-) cells which were then implanted in 29 sublethally irradiated mice, only 16 developed ALL and these experienced a median survival of 58 days. Cells recovered from the *SPHK2*(-/-) mice had reduced c-Myc expression compared to their wild-type counterparts[261]. The expression of SphK2 was linked to actin rearrangement in MCF-7 cells with expression promoting a structural rearrangement of actin into membrane

ruffles/lamellipodia indicative of a more migratory phenotype[262]. SPHK2 expression was also shown to negatively correlate with disease free survival and overall survival in NSCLC patients[263]. Additionally, inhibition of SphK2 has delayed tumor growth in mouse xenograft models of pancreatic, kidney, liver, and colon cancers[264-266].

1.3.2.3.3 Sphingosine Kinase & S1P inhibitors

Because of the importance of S1P in growth signaling, a great deal of research has been done with the aim of inhibiting S1P signaling. Numerous therapeutic strategies have been developed that target either one or both sphingosine kinases as well as those that inhibit S1P rector signaling.

N,N-dimethylsphingosine (DMS) is a naturally occurring N-methyl derivative of sphingosine that inhibits both SphK1 and SphK2 with Ki of ~16μM and ~14μM, respectively[267]. It inhibits SK activity in human platelets preventing platelet aggregation in response to exogenous S1P[268]. Its inhibition of SK activity has been shown to induce apopotosis in leukemic and colonic carcinoma cell lines[269, 270]. Two additional sphingosine analogs, L-*threo*-dihydrosphingosine and N,N,N-trimethyl-sphingosine have also been described as sphingosine kinase inhibitors, however, they also inhibit protein kinase C which may be responsible for the observed phenotype[199]. SKI-II also inhibits both isoforms with Ki of 16μM (SphK1) an 8μM (SphK2)[267], but it has also been shown to inhibit dihydroceramide desaturase[271]. Treatment of prostate cancer and breast cancer cells with SKI-II induced apoptosis *in vitro*[272, 273] and *in vivo*[274].

The specific SphK1 inhibitor PF-543, which is over 100-fold more selective

for SphK1 than SphK2, has a Ki of 3.6 nM (SphK1). Though it did not affect the

growth of 1483 HNSCC cells, it was able to inhibit the formation of S1P in human

whole blood by >90% compared to control[275]. SK1-I is a specific competitive

inhibitor of the ATP-binding site of SphK1 with a Ki of 10µM that has been shown to

induce apoptosis in the U937 and Jurkat leukemic cell lines. Treatment of these cells

with SK1-I decreased ERK1/2 and Akt phosphorylation. Additionally, treatment of

AML xenograft bearing mice with SK1-I reduced tumor growth[276]. Decreased

Akt activation and increased apoptosis were also seen in glioblastoma cell lines

following treatment with SK1-I. The inhibitor also slowed glioblastoma xenograft

growth[277]. Importantly, in a murine model of breast cancer, treatment with SK1-I

reduced metastasis and decreased overall tumor burden. The observed phenotype

was attributed to a decrease in circulating S1P, which plays a role in angiogenesis

signaling[278].

FTY720 (fingolimod, Gilenya®) is a S1P receptor functional antagonist.

Upon entry into the cell it is phosphorylated by SphK2. phospho-FTY720 can inhibit

SphK1 or exit the cell via ABC transporters to bind S1P receptors (except $S1P_2$).

Binding results in the internalization of the receptor and prolonged receptor down-

regulation muting extracellular S1P signaling[279]. Prevention of S1P receptor

signaling, mainly that of $S1P_1$ has had a dramatic clinical impact in preventing the

release of lymphocytes from lymphoid tissue thereby attenuating the autoimmune

response responsible for multiple sclerosis[279, 280]. An analog of FTY2720, (R)-

FTY720 methyl ester, was recently reported to have specific inhibitory activity

against SphK2. Inhibition of SphK2 with this drug prevented actin rearrangement in MCF-7 cells in response to S1P thereby inhibiting motility[262].

ABC294640 is a specific inhibitor of SphK2. It has a Ki of 9.8µM for SphK2[281] while inhibiting SphK1 by only 10% at 100 µM[267]. Inhibition of SphK2 by ABC294640 was first reported to reduce VEGF induced vascular leakage in a rat model of diabetic retinopathy[282]. SphK2 and S1P are overexpressed in ulcerative colitis, an inflammatory disease of the colon. ABC294640 has been shown to decrease S1P in the colons of a dextran sulfate sodium (DSS) induced chronic colitis mouse model in part due to attenuation of TNFα induced expression of VCAM and ICAM thereby decreasing lymphocyte infiltration diminishing the inflammatory response[283]. When the colon carcinogen azoxymethane was added to this model, ABC294640 diminished tumor incidence and multiplicity[266]. ABC294640 was also effective at diminishing the severity of trinitrobenzene sulfonic acid induced Crohn's disease, another form of inflammatory bowel disease (IBS), in mice and rats. Treatment with ABC294640 reduced the colonic levels of TNFα and IL-1β[284]. Notably, SphK2 expression was induced in the bowels of mice and rats in response to DSS and TNBS induced IBS.

TNFα activation of SphK2 has also been associated with arthritis. In two rat models of arthritis, a collagen-induced and an adjuvant-induced, ABC294640 was able to diminish the severity of outcome. ABC294640 attenuated both bone and cartilage degredation in the adjuvant-induced model and provided additive protection when combined with a sub-optimal dose of methotrexate, the gold standard of rheumatoid arthritis drugs[285]. When injected into joints,

monosodium iodoacetate produces a reliable, reproducible model of osteoarthritis. Treatment with ABC294640 was able to reduce both the histological severity and pain associated with osteoarthritis in rats receiving injections of monosodium iodoacetate in the knee[286]. In the hTNFα mouse model of inflammatory arthritis, though, the use of ABC294640 worsened the arthritic phenotype, and genetic ablation of SPHK2 had a minimal, insignificant effect on the development of arthritis[287].

ABC294640 has also demonstrated profound anticancer effects. This was first shown by proliferation and migration inhibition in A498 cells and tumor growth inhibition in a JC xenograft in BALB/c mice[281]. ABC294640 has been shown to promote autophagic cell death in A-498 kidney carcinoma cells, PC-3 prostate cancer cells, and MDA-MB-231 breast adenocarcinoma cells as well as in xenografts of A-498 in SCID mice as evidenced by increases in Beclin-1 and the cleavage of the microtubule-associated proteins 1A/1B light chain 3A (LC3). This same study demonstrated a decrease of MAPK and Akt pathway activation in response to ABC[288].

The use of ABC294640 in treating cancers of hematopoietic origin has recently shown great promise. Treatment of primary effusion lymphoma (PEL) cells positive for Kaposi's sarcoma-associated herpesvirus (KSHV) (BCBL-1 cell line), induced dose dependent apoptosis that corresponded with decreases in phosphorylation of ERK1/2, Akt, and NF-κB (p65). When these cells were injected in NOD/SCID mice, ABC294640 treatment delayed disease progression as indicated

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by spleen size, weight gain, and ascites volume. ABC294640 treatment vastly attenuated the pathogenesis of KSHV^{+} PEL[289].

SphK2 is also overexpressed in multiple myeloma (MM). Treatment of both immortalized MM cell lines as well as freshly isolated CD138^{+} myeloma cells from myeloma patients resulted in an inhibition of proliferation. ABC294640 increased caspase 3 activation and caspase 9 cleavage in OPM1 MM cells indicative of apoptotic cell death. Treatment of MM cell lines with ABC294640 also increased mRNA and protein expression of the pro-apoptotic Bcl-2 family member Noxa, which is regulated by p53. The same study also showed ABC294640 promotes proteosomal degradation of myeloid cell leukemia 1 (Mcl-1), a Bcl-2 family protein overexpressed in the majority of MM patients. Thus, ABC294640 shifts the balance of these apoptotic mediators in the direction of apoptosis. ABC294640 also enhanced the proteosomal degradation of c-Myc. Additionally, the authors demonstrated that ABC294640 induced apoptosis of MM cells even in the presence of bone marrow stromal cells, which support the growth of MM cells. These findings prompted *in vivo* studies using a luciferase expressing MM.1S cell line xenograft where ABC294640 suppressed tumor growth[290].

ABC294640 has also been evaluated in preclinical testing against acute lymphoblastic leukemia (ALL). ABC294640 inhibited proliferation and induced cell death in ALL cell lines as well as ALL patient samples, but, importantly, not bone marrow mononuclear cells. The manner of cell death was caspase-independent, however not completely autophagic. Therefore the manner of cell death was inconclusive. Next, a gene expression profile of the impact of ABC294640 inhibition

of SphK2 on gene expression was compiled which revealed an inhibition of c-Myc and c-Myc-regulated genes. *MYC* downregulation in response to ABC294640 treatment was confirmed by qRT-PCR. This same study showed that inhibition of SphK2 by ABC294640 prevented S1P mediated inhibition of HDAC activity resulting in a reduction in the association of *MYC* with histone 3 acetylated on lysine 9 and thereby a reduction in *MYC* expression. Xenografts of ALL in NOD/SCID mice treated with ABC294640 had significantly less disease than vehicle control following 21 days of treatment[261].

Combination of ABC294640 with other anti-cancer drugs has shown promising results. Combination with the tyrosine kinase inhibitor sorafenib resulted in synergistic cell killing in A-498 kidney carcinoma and BxPC-3 pancreatic adenocarcinoma cells by means of apoptosis, and the combination attenuated the growth of xenografts of each cell line, separately, in SCID mice to a greater degree than either drug alone[264]. This same combination proved effective in two hepatocellular carcinoma xenografts. The mode of cell death was shown to be autophagic. However, as with the A-498 and BxPC-3 cells, ABC294640 decreased the levels of p-Erk1/2 provoking the idea that ABC294640 mediated cell death could come as a result of diminished MAPK signaling[265]. Combination of ABC294640 with the microtubule stabilizer paclitaxel was shown to greatly increase caspase-9 signaling and apoptosis when compared to either drug alone[291]. ABC294640 has also been combined with doxorubicin, vincristine, imatinib, and bortezomib in ALL. Only additive effects were observed with the DNA intercalating agent doxorubicin and the mitosis inihibitor vincristine. However, synergistic cell death was produced

with the proteasome inhibitor bortezomib and the multi-tyrosine kinase inhibitor (ie Bcr-Abl inhibitor) imatinib when either was combined with ABC[261].

ABC294640 has also been shown to bind to the antagonist ligand-binding domain of the estrogen receptor thereby inhibiting the growth of the estrogen receptor positive breast cancer cell line MCF-7 both *in vitro* and *in vivo*[292]. ABC294640 was also effective at inducing apoptotic cell death in triple-negative MCF-7 cells (MCF7-TN-R) by diminishing pro-survival signaling through the NF-κB pathway. *In vitro* levels of apoptosis were further increased by addition of either etoposide or doxorubicin after pretreatment with ABC294640. Additionally, Ki67 staining was decreased in MCF7-TN-R orthotopic tumors treated with ABC294640 when compared to control[293]. However, ABC294640 is ineffective against the luminal, endocrine-resistant MDA-MB-361 breast cancer cell line. This same study also showed ABC294640 inhibition of Bcl-2 in MCF-7 tumors[294]. ABC294640 was also shown to inhibit proliferation in androgen resistant PC-3 and LNCaP prostate cancer cell lines[295].

Ischemia repurfusion injury (IR) occurs following reoxygenation of anoxic tissues. As a result of IR, cells produce inflammatory cytokines and express adhesion molecules to attract lymphocytes. They also increase the production of reactive oxygen species, which can lead to mitochondrial depolarization and apoptotic cell death. Treatment of cultured primary mouse hepatocytes with ABC294640 prior to an anoxia/reoxygenation challenge greatly reduced cell death. The livers of mice pre-treated with ABC294640 an hour prior to a 1-hour ischemic challenge to 70% of the liver suffered drastically less necrosis, apoptosis, and

mitochondrial depolarization after 6 hours of reperfusion. There was also a decrease in the upregulation of the inducible form of nitric oxide synthase (iNOS) as well as a decrease in NF-κB activation and a decrease in the production of TNFα[296]. IR also plays a role in primary graft non-function, a complication of liver transplantation. In orthotopic liver transplantations performed in Lewis rats, rats treated with ABC294640 had a decrease in circulating S1P when compared to the vehicle control, but more than the sham. The expression of TLR4, phospho-NF-κB (p65), and ICMA-1 was diminished in the transplanted livers of mice receiving ABC294640. The mRNA expression of the cytokines TNFα, IL-1β and CXCL-10 was also diminished in the ABC294640 treated mouse livers. Additionally, there was a decrease in necrosis as well as monocyte/macrophage infiltration in the transplanted livers of mice receiving ABC294640. Overall, transplant recipients receiving ABC294640 had improved liver function compared to the vehicle control[297]. Together these data demonstrate a protective role for ABC294640 in IR injury.

The use of ABC294640 in the treatment of atherosclerosis has also been examined. After 16 weeks of use in LDL-R(-/-) mice on a Western diet ABC294640 caused a 30% decrease in plasma S1P levels, however, this did not result in attenuation of atherosclerotic plaque formation[298]. ABC294640 has also been tested in a mouse model of lupus nephritis. Again, its use corresponded with a decrease in circulating S1P as well as dihydro-S1P levels, but this did not result in any statistically relevant decreases in pathology[299].

1.4 Cyclin Dependent Kinase Controlled Cell Cycle Progression

Briefly, the cell cycle is divided into phases: two gap phases (G1 and G2) separated by a synthesis phase (S). Following G2 the cell undergoes mitosis and then returns to interphase. This process is largely governed by cyclins and cyclin-dependent kinases (cdks) due to their upstream control of transcription of genes necessary for cell cycle progression. While the expression of many cdks remains fairly constant throughout the cell cycle, expression of cyclins is tightly controlled being elevated and inhibited in a specific, progressive manner. In the non-transformed cell, induction of cell division occurs through extracellular signaling by growth factors binding to growth factor receptors, which can induce activation of Ras. Ras signaling can ultimately lead to induction cyclin D expression, which then binds to cdk4/6. Upon further activation by cdk activating kinase (CAK), cdk4/6 phosphorylates the retinoblastoma protein, Rb[300]. Phosphorylation of Rb causes it to release its binding partners, E2F(1-3) transcription factors. E2F along with its binding partner DP-1 promotes the transcription of proteins necessary for progression into S-Phase including cyclin E[301].

Cyclin E binds to cdk2, which also has numerous targets including, again, Rb serving as a positive feedback mechanism. cyclin D-cdk4/6 also sequesters Cip/Kip proteins, which inhibit cdk2. Thus cyclin D-cdk4/6 promotes cyclin E-cdk2 mediated progression both transcriptionally and post-translationally[300]. In addition to Rb, cyclin E-cdk2 also phosphorylates p27[Kip1] an inhibitor of cdk2 and cdk4, which leads to their degredation[301]. Cyclin E-cdk2 also activates nuclear

protein of the ATM locus (NPAT) and nucleophosmin, which stimulate transcription of histones and duplication of the centrosome, respectively, both of which are required for S-phase entry[302]. Additionally, cylcin-E/cdk2 stimulates assembly of the DNA replication complexes at origins of replication. E2F also binds the transcriptional repressor of cyclin A, removing it[303].

As cyclin A levels rise they bind cdk2 and later in the cell cycle cdk1. This complex phosphorylates a number of targets including E2F and DP-1, which deactivates the transcription factors[304]. Neutralization of E2F activity allows for progression into S-phase. Cyclin-A/cdk2 and cyclin-a/cdk1 phosphorylate components of the DNA replication complex driving DNA replication. It also inhibits non-assembled DNA replication complex components that aren't binding DNA, preventing their future binding ensuring the DNA is only replicated once[305]. The onset of S-phase triggers transcription of cyclin B, which begins to accumulate in the cytoplasm. The end of replication marks the end of S-phase and the beginning of the second Gap.

Phosphorylation of the phosphatase CDC25 by cyclin A/cdk2 leads to dissociation of CDC25 from its inhibitor 14-3-3. CDC25 is responsible for removal of inhibitory phosphorylations on cyclin-B associated cdk1[306]. Cyclin-B/cdk1 can phosphorylate this same target resulting in a positive feedback and activation of cyclin-B/cdk1 leads to its translocation to the nucleus. Translocation of active cyclin-B/cdk1 corresponds with the degradation of cyclin-A complexes. Active cylin-B/cdk1 has at least 75 intracellular targets. Its activity promotes and inhibits the activity of numerous transcription factors. It regulates mitotic spindle

formation and is involved in telomere maintenance and DNA repair[307]. The targets of cyclin-B/cdk1 drive progression through mitosis. Cyclin-B/cdk1 drives mitosis all the way to anaphase. Then cyclin-B/cdk1 targets several phosphorylation sites in the anaphase-promoting complex/cyclosome (APC/C), which promotes binding to CDC20 leading to its activation. APC/C[CDC20] is the major ubiquitin ligase complex during mitosis. With cyclin-B as a target, activation of APC/C[CDC20] by cyclin-b/cdk1 leads to its own degradation during late anaphase[302].

Progression through the cell cycle is tightly controlled. Control is gained by controlling not only the production of cyclins, but also the degradation of both cyclins and cyclin/cdk complexes and nuclear export of cyclin/cdk complexes. Phosphorylation of cyclins in their conserved destruction box domain makes them targets for polyubiquitination by the SPF complex, which leads to their degradation. This happens to cyclin-E/cdk2 in early to mid S-phase[308]. Cyclin A is also targeted for ubiquitination beginning in late-G2 and concluding before the end of metaphase[309]. Numerous binding partners can also interfere with the activity of cyclin/cdk complexes. As mentioned earlier, p27, a member of the cyclin-dependent kinase inhibitor (CKI) family, is a potent inhibitor of cdk2. While there are other important CKI family members, of particular importance to this project is p21[WAF1/CIP1] (hereto referred to simply as p21).

p21 can bind to and inhibit (among other targets) cyclin complexes with either -cdk2, -cdk4, and cdk6 effectively inhibiting cell cycle progression in the G1/S phases[310]. p21 can be induced by p53 in response to DNA damage, preventing cell division. Its transcription can also be induced by a number of other factors

including SP1/Sp3 (in response to TGFβ signaling, increases in Ca2+ concentration, and HDAC inhibitors such as butyrate and trichostatin A), STAT1/STAT3 (in response to EGF, INF-γ, or IL6 signaling) and E2F1/E2F3 [311, 312].

1.5 Summary of literature review

Cancer cell resistance to the mainline chemotherapeutic drug gemcitabine is one of the major contributors to the dismal life expectancy of those diagnosed with pancreatic cancer. Resistance is possible through a variety of means most prominently by alterations in gemcitabine metabolism followed by deregulated growth signaling and aberrant apoptotic signaling. Any one of the mentioned modes of resistance is capable of derailing gemcitabine treatment. Finding a pathway that cripples multiple modes of resistance is of upmost interest to those attempting to correct for gemcitabine resistance. The interaction of sphingolipids with these pathways is well documented and represents a promising target for therapeutic sensitization to gemcitabine. By altering the sphingolipid profile it may be possible to disrupt multiple pathways of resistance at once restoring cancer cell sensitivity to gemcitabine.

S1P can drive numerous mechanisms of gemcitabine resistance. S1P has repeatedly been shown to promote the activation of proliferation signaling through NF-κB activation through both SphK1[313-315] and SphK2[293]. ERK1/2 phosphorylates both SphK1 and SphK2 leading to their activation. Their product, S1P, has also been shown to promote ERK1/2 signaling through S1P receptors,

likely S1P$_3$, multiple times[316-318]. S1P has been shown to upregulate Bcl-2

expression while sphingosine has been shown to promote apoptosis through

downregulation of Bcl-2 and Bcl-xL[319]. Additionally, S1P has been linked to

subversion of gemcitabine incorporation by promoting the expression of E2F target

genes such as c-Myc and RRM2. Exogenous addition of sphingosine induces

dephosphorylation of Rb thereby preventing the expression of E2F target genes *MYC*

and *RRM2*[61, 320, 321].

The specific SphK2 inhibitor ABC294640 has previously been shown to

reduce phosphorylation of ERK1/2[264, 265], phosphorylation of NF-κB (p65)[293],

and increase cellular sphingosine levels all of which contribute to its well-

documented antitumor activity. Therefore, it may complement gemcitabine therapy

by attenuating multiple modes of resistance providing the rationale for targeting

S1P production as a method of sensitizing gemcitabine resistant cells to gemcitabine.

Chapter Two

Combined drug effects of gemcitabine and sphingosine kinase inhibitors and

development of a mouse model of pancreatic cancer

ABSTRACT

Pancreatic cancer is the fourth leading cause of cancer related death in the United States. The current five-year survival rate is a dismal 6% due largely to late diagnosis and chemoresistance. Recently the combination of gemcitabine and paclitaxel was designated the new standard of care for the treatment of pancreatic cancer. However, chemoresistance still persists. Deregulation of the MAPK/ERK and NF-κB pathways as well as aberrant regulation of the balance of expression of Bcl-2 family members and the balance of levels of bioactive sphingolipids mediate resistance to both gemcitabine and paclitaxel. The sphingosine kinase 2 inhibitor ABC294640 suppress ERK1/2 and NF-κB activation as well as alter the balance of Bcl-2 family of proteins such that apoptosis is promoted. This led to the hypothesis that ABC294640 would produce synergistic cell death when combined with gemcitabine through alterations in either ERK1/2 or NF-κB activation or altering the balance of the Bcl-2 family of proteins. Here we show that combination of either the specific sphingosine kinase 1 inhibitor PF-543 or ABC294640 with either gemcitabine or paclitaxel results in synergistic killing of BxPC-3, MiaPaCa-2, and Panc-1 cells. We also show that the impact of sphingosine kinase inhibitors with and without gemcitabine on the above modes of resistance is inconsistent amongst these three cell lines and, therefore, not likely the source of the observed synergy. We also show that BxPC-3, the most sensitive of the three cell lines to gemcitabine, has a higher concentration of dihdroceramide validating previous reports that show such a feature may sensitize cells to chemotherapy.

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INTRODUCTION

Pancreatic cancer remains one of the most difficult cancers to treat. With a five-year mortality rate of nearly 95%, new treatment options are a necessity. Late detection of cancer and chemoresistance to the mainline chemotherapeutic, gemcitabine, are the major contributors to the high mortality rate.   Recently the combination of gemcitabine and nanoparticle albumin bound (nab)-paclitaxel became the standard of care for treating pancreatic cancer[18]. Gemcitabine is a di-fluorinated analog of deoxycytidine, which upon intracellular phosphorylation can promote apoptosis by both disrupting nucleotide production through the inhibition of ribonucleotide reductase as well as by incorporating into DNA during replication leading to strand breaks[28, 29]. Paclitaxel stabilizes the cellular cytoskeleton by binding β-tubulin thereby preventing dynamic microtubule rearrangement leading to an induction of apoptosis[322].

Resistance to gemcitabine is well documented. It occurs mainly through cellular alterations in its metabolism, a feature that can be either innate or acquired[37]. Aberrations in growth and apoptotic signaling have also been shown to contribute to tumor resistance. These include alterations in the NF-κB pathway[7, 90, 95, 323], the MAPK/ERK pathway[126, 127] and the balance of Bcl-2 family members[138-140, 324, 325]. The ratio of the bioactive sphingolipids ceramide and sphingosine-1-phosphate are also predictive of a cell's responsiveness to gemcitabine[326]. Resistance to paclitaxel can also occur through alterations in the NF-κB pathway[327-329] and disruptions in the balance of Bcl-2 family members[330, 331].

Sphingolipids are pleiotropic signaling lipids involved in multiple cellular processes. The *de novo* synthesis of sphingolipids begins with the palmitoylation of serine producing 3-ketodihydrosphingosine, which is then reduced to dihydrosphingosine. Acylation of dihydrosphingosine by ceramide synthase yields dihydroceramide (dhCer). A 4,5-trans-double bond is inserted into the sphingoid backbone of dhCer by dihydroceramide desaturase producing ceramide. Ceramide is the hub of sphingolipid synthesis from which hundreds of different sphingolipids can be produced. For the purposes of this research, ceramide metabolism can proceed by cleavage of ceramide into sphingosine by ceramidase. Sphingosine can then be phosphorylated by one of two sphingosine kinases (SphK1 and SphK2) yielding sphingosine-1-phosphate (S1P). This pathway can run in the opposite direction as well, however, S1P can be degraded by S1P-lyase removing it from the pathway. The latter three species (ceramide, sphingosine, and S1P) are considered to be bioactive sphingolipids due to their comparatively higher level of involvement in signaling events although signaling roles for other sphingolipids species are also known (Reviewed [207]).

Previously, our lab has demonstrated that genetic ablation of SphK2 has profound anticancer effects[332]. We have also developed a specific inhibitor of SphK2, ABC294640, which has been shown to induce cell death in a number of tumor cell lines representing many types of cancer including kidney adenocarcinoma[267], hepatocellular carcinoma[265], B-cell lymphoma[289], multiple myeloma[290], primary effusion leukemia[261], prostate cancer[295] and breast cancer[273]. By the inhibition of S1P formation ABC294640 has been shown

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to diminish activation of ERK1/2 and NF-κB[288, 293]. Additionally, it is known to

decrease concentrations of anti-apoptotic Bcl-2 family members[290, 294]. Here we

report differing lipidomics profiles of three pancreatic cell lines (BxPC-3, MiaPaCa-2,

and Panc-1) and how sphingosine kinase inhibitors in combination with

gemcitabine alter several growth and apoptotic signaling pathways. Importantly,

we show that the specific SphK1 inhibitor PF-543 and ABC294640 synergize with

both gemcitabine and paclitaxel *in vitro* to produce increased cell killing.

MATERIALS AND METHODS

Reagents and cell lines. ABC294640 was supplied by Apogee Biotechnology

Corporation. Gemcitabine HCl, and 2,2,2-tribromoethanol were purchased from

Sigma. Paclitaxel and PF-543 were purchased from Calbiochem. Sulpharhodamine

B was purchased from MP Biomedicals. BxPC-3, MiaPaCa-2 and Panc-1 were

obtained from ATCC. BxPC-3 were maintained in RPMI 1640 medium supplemented

with 10% fetal bovine serum. MiaPaCa-2 were maintained in DMEM supplemented

with 10% fetal bovine serum and 5% horse serum. Panc-1 cells were maintained in

DMEM supplemented with 10% fetal bovine serum. All cell culture medium also

contained 25μg/mL gentamicin.

Cytotoxicity assays. Cells were plated in 96-well plates at a density of 3000/well in

200 μL of complete media. Drugs were added the following day. Following 96-

hours of treatment the media was removed and 100 μL of 10% trichloroacetic acid

(TCA) was added to each well. The plates were held at 4°C overnight. The following

day the TCA was removed, and the plates were rinsed three times in water. When the plates were dry 100 μL of sulpharhodamine B was added to each well. This was allowed to sit at room temperature for 30 minutes. Following this the plates were rinsed three times in 1% acetic acid. After the plates were dry 100 μL of 10 mM TRIS was added to each well to solubilize the retained SRB. These were left on an orbital shaker for 30 minutes after which time the optical density of each well was determined using a SpectraMax M5 Spectrophotometer (Molecular Devices) reading at 562 nm.

Immunoblotting. Cells were lysed using RIPA buffer (50 mM Tris, 150mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% NP-40, pH=7.4) with an added protease and phosphatase inhibitor cocktail (Halt, Thermo Scientific) per the manufacturers recommendations. The lysates were vortexed four times over 15 minutes and then centrifuged at 23,000g for 25 minutes. The supernatant was transferred to another tube. The protein concentration of the lysates was equalized using the Pierce BCA protein assay kit (Thermo Scientific). Following equalization Laemmli sample buffer was added to each sample all of which were then heated to 95°C for 8 minutes. 30 μg of protein per sample was loaded into each well of a 10-well 10% Mini-PROTEAN TGX pre-cast gel (Bio-Rad). Electrophoresis was performed for 50 minutes at 150 V in Tris-glycine-SDS running buffer (Bio-Rad). The gels were then equilibrated in Tris-glycine transfer buffer (Bio-Rad) for 20 minutes before being transferred to Immun-Blot PVDF membrane (Bio-Rad) using a Trans Blot semi-dry transfer cell (Bio-Rad) run at 15 V for 28 minutes. Upon the completion of transfer

membranes rinsed in dH20, Tris buffered saline (TBS), and TBS + 1% Tween20 (TBST) sequentially for five minutes each. The membranes were then blocked in 5% BSA (in TBST). Antibodies were diluted in 5% BSA/TBST according to the manufacturers recommendations and the blots were exposed to the antibody dilutions overnight at 4°C. The following day the blots were rinsed 3x for 5 minutes each in TBST. Secondary antibody was diluted 1:30,000 in 5% BSA/TBST and added to the membranes for 1 hour at room temperature. The membranes were then washed 3x for 5 minutes each in TBST. Each membrane was then incubated for 5 minutes in Pierce enhanced chemiluminescence western blotting substrate (Thermo Scientific) before being imaged in a dark room using HyBlot® CL autoradiography film (Denville Scientific).

Lipidomics analysis. After reaching 90% confluence cells were harvested and washed in PBS three times. A small aliquot was stored for protein equalization of the samples. Following the removal of PBS the samples were transported on ice to the Lipidomics Core facility at MUSC where the sphingolipid content was measured via mass spectrometry as described previously[333].

Statistics and graphing. IC$_{50}$ calculations, all graphs, as well as graph statistics were made using Prism (Graph Pad). Combination index calculations were made using Calcusyn (Biosoft).

RESULTS

Sphingosine kinase inhibitors synergize with gemcitabine and paclitaxel

For three pancreatic cell lines (BxPC-3, MiaPaCa-2, and Panc-1) we established 96-hour IC_{50} concentrations for gemcitabine and paclitaxel as well as for two sphingosine kinase inhibitors specific for each isozyme: PF-543 for SphK1 and ABC294640 for SphK2 (Figure 2-1). Among these four drugs gemcitabine had the greatest variance in IC_{50} with BxPC-3 being the most sensitive at 7.4 nM, Panc-1 moderately sensitive at 111 nM, and MiaPaCa-2 being the most resistant with an IC_{50} greater than 400 nM. MiaPaCa-2 was also the most resistant to paclitaxel with an IC_{50} of nearly 1.8 nM compared to 1.08 nM and 1.03 nM for BxPC-3 and Panc-1, respectively.

The SphK2 specific inhibitor ABC294640 has a K_i of roughly 10 μM for the inhibition of SphK2[281]. All three cell lines were nearly equally sensitive to ABC294640 with a range of 23-35 μM. PF-543 has a reported K_i of 3.6 nM for the specific inhibition of SphK1. At higher concentrations it inhibits SphK2 as well (K_i 356 nM)[275]. A concentration of 1 μM of PF-543 has previously been shown to not alter the growth of several cancer cell lines even after nine days of continuous exposure[275]. Here we shown that a concentration between 17-22 μM effectively kill 50% of cells after 96-hours in these three cell lines.

The efficacy of combinations of these drugs was then evaluated in all three of these cell lines (Figure 2-2). Combining gemcitabine with either ABC294640 or PF-543 at or above each drug IC_{50} produced synergistic cell killing with higher drug concentrations yielding greater synergy. Recently the combination of gemcitabine

Gemcitabine Sensitivity

	BxPC-3	MiaPaCa-2	Panc-1
IC50 (nM)	7.4	404	111

Paclitaxel Sensitivity

	BxPC-3	MiaPaCa-2	Panc-1
IC50 (nM)	1.1	1.8	1.0

ABC294640 Sensitivity

	BxPC-3	MiaPaCa-2	Panc-1
IC50 (µM)	28.5	35.2	23.2

PF-543 Sensitivity

	BxPC-3	MiaPaCa-2	Panc-1
IC50 (µM)	17.8	18.9	22.1
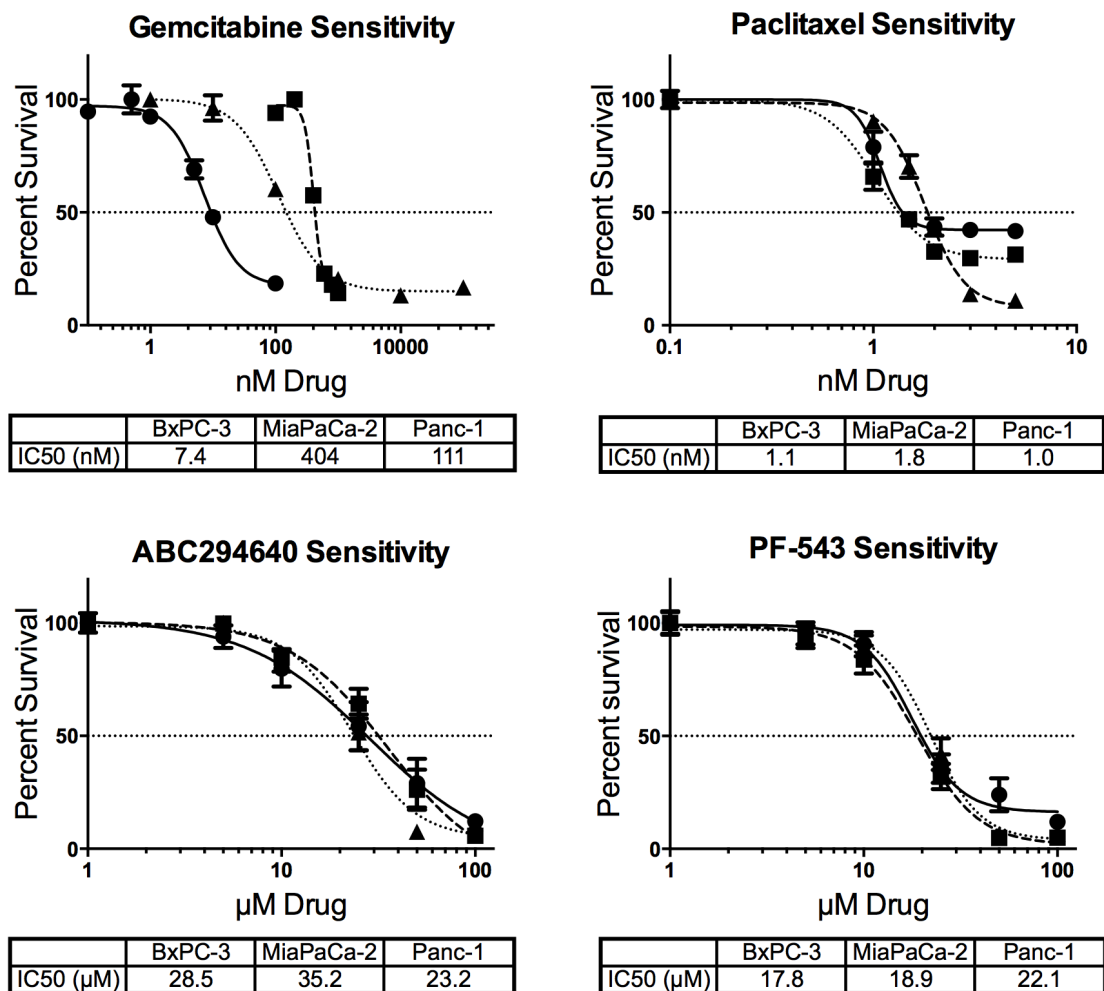
Figure 2-1: Cytotoxicity of gemcitabine, paclitaxel, ABC294640, and PF-543. IC$_{50}$ graphs for Gem, PTX, ABC294640, and PF-543 in three pancreatic cancer cell lines: BxPC-3 (●), MiaPaCa-2 (■) and Panc-1 (▲). Each graph represents at least three independent experiments.

and nab-paclitaxel became the standard of care for treating pancreatic cancer. Our results confirm the effectiveness of this combination against pancreatic cancer cells showing that when combined at their respective IC_{50} concentrations these drugs produce a synergistic response in BxPC-3 and MiaPaCa-2. Additive benefits were seen in Panc-1 when the cells were treated with IC_{50} concentrations of gemcitabine and paclitaxel. Synergy was observed when the concentrations were slightly increased, though. The combination of paclitaxel with ABC294640 was synergistic at or above the IC_{50} in both BxPC-3 and MiaPaCa-2. However, the combination of these drugs was at best only additive in the same range for Panc-1. The combination of paclitaxel with PF-543 produced synergistic cell killing at or above each drug IC_{50}. And the combination of the two sphingosine kinase inhibitors produced very strong synergism at and above each drug IC_{50} in all three cell lines.

Changes in activation of NF-κB and ERK1/2 in response to gemcitabine and SK inhibitors

ABC294640 has previously been reported to diminish NF-κB activation in MCF-7TN-R cells[293]. So, we sought to examine the effect of ABC294640 in three pancreatic cancer cell lines. Protein samples were isolated from each cell line after having been exposed to ABC294640, PF-543, Gemcitabine, Gemcitabine + ABC294640, Gemcitabine + PF-543, or DMSO control for 24 hours. All cells were treated at their respective 96-hour IC_{50}. Western blots were performed using these samples. The blots were probed for p-NF-κB (p65), NF-κB (p65), p-ERK1/2, and ERK1/2 (Figure 2-3a).
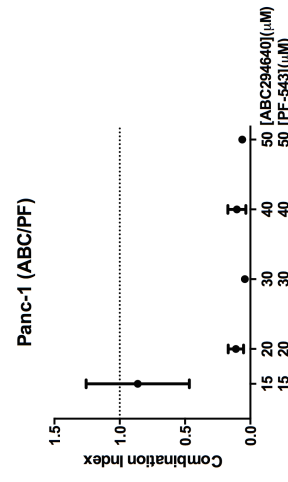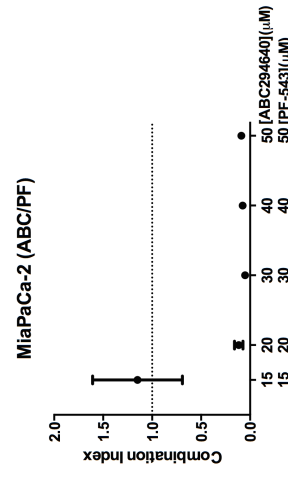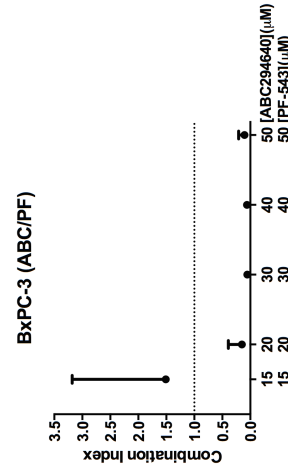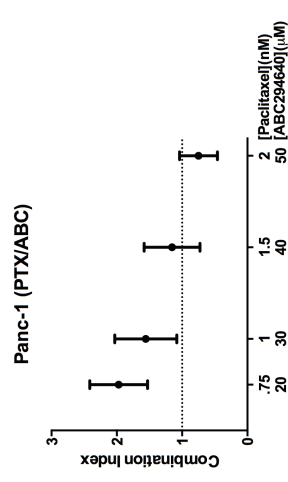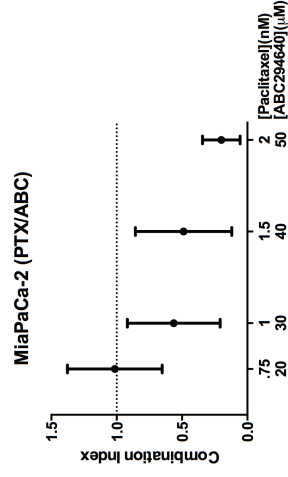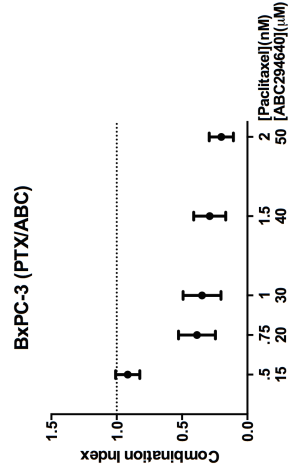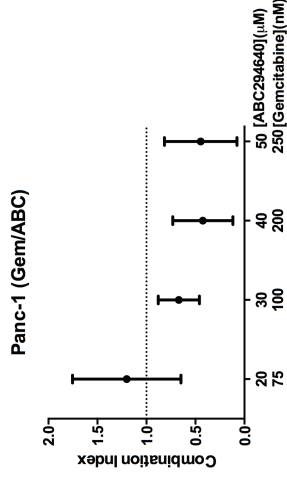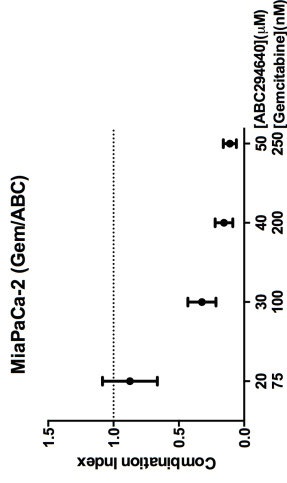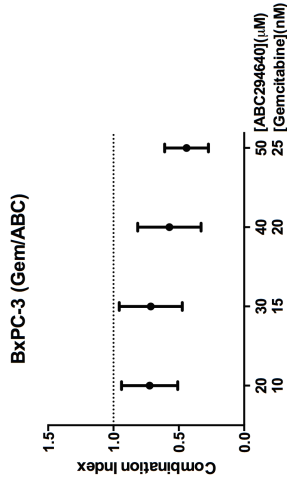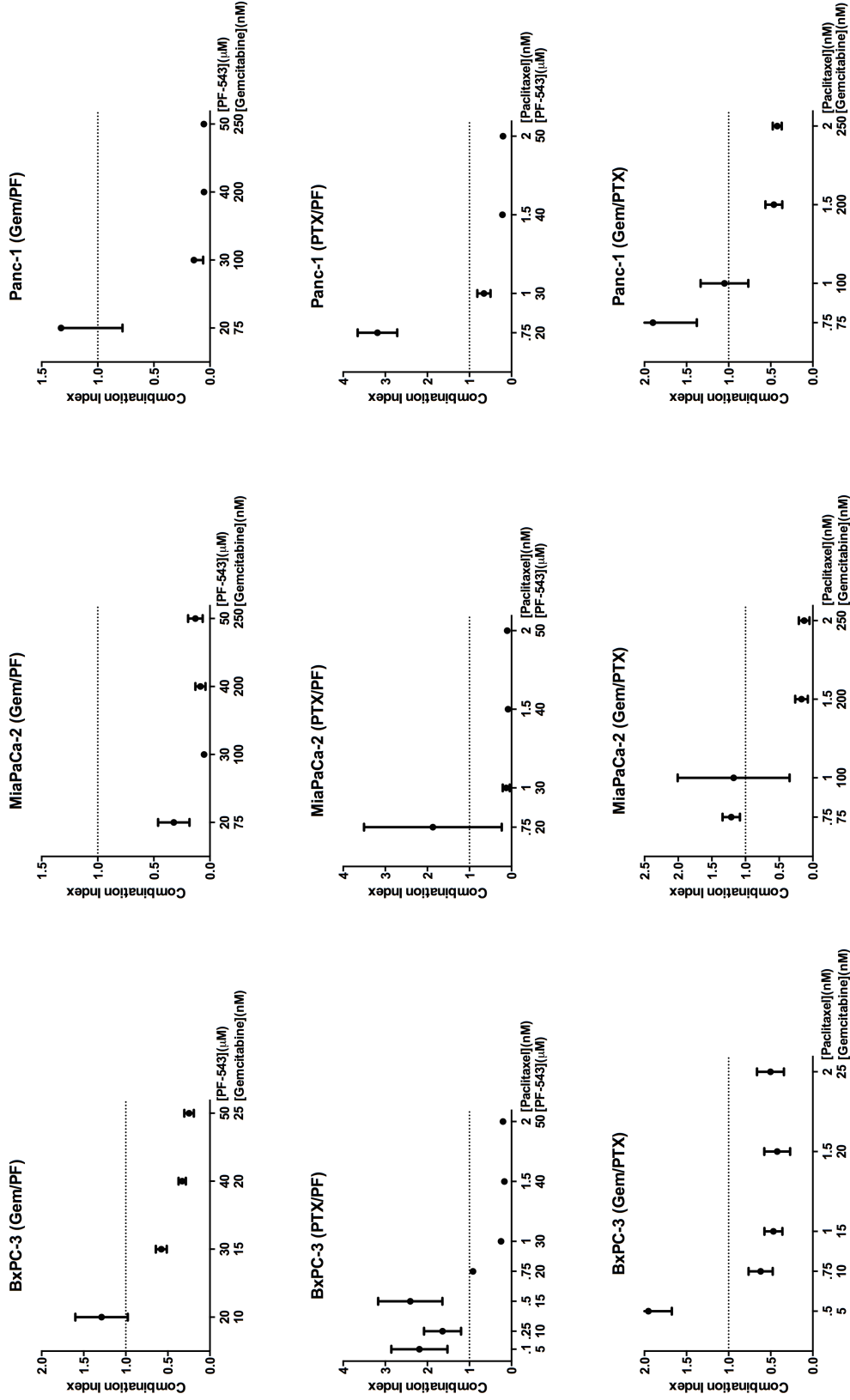
Figure 2-2: Combination of gemcitabine (Gem), paclitaxel (PTX), ABC294640 (ABC) and PF-543 (PF). Combination Index graphs for all six possible two-drug combinations of gem, ptx, ABC, and PF in three pancreatic cancer cell lines. Each graph represents at least three independent experiments.

No drug or combination of drugs seemed to elicit a change in the activation of NF-κB in BxPC-3 cells, although, there did seem to be a decrease in total NF-κB in response to treatment with ABC294640. Treatment with ABC294640 produced an increase in NF-κB phosphorylation in MiaPaCa-2 cells, which, while still elevated, was attenuated by the addition of gemcitabine.   Gemcitabine alone produced a decrease in NF-κB phosphorylation in Panc-1, however, addition of either sphingosine kinase inhibitor abrogated this effect.

ABC294640 has also previously been shown to decrease the phosphorylation of ERK1/2[288]. Interestingly there was an increase in ERK1/2 activation when exposed to ABC294640 in both MiaPaCa-2 and Panc-1. This feature was reproduced when gemcitabine was added to MiaPac-2 cells. However, this combination did not produce an increase in ERK1/2 phosphorylation in Panc-1. Treatment with PF-543 produced a slight increase in ERK1/2 activation in BxPC-3 while gemcitabine produced a slight decrease. The combination of gemcitabine and PF-543 produced a decrease in activation of ERK1/2 in both BxPC-3 as well as MiaPaCa-2. Gemcitabine alone produced no change in either MiaPaCa-2 or Panc-1. In contrast to its effects on BxPC-3, PF-543 caused a decrease in ERK1/2 phosphorylation in MiaPaCa-2.

Changes in apoptotic and autophagic markers in response to gemcitabine and SphK inhibitors

Given that ABC294640 has been previously reported to diminish the expression of anti-apoptotic Bcl-2 family members and promote the synthesis of
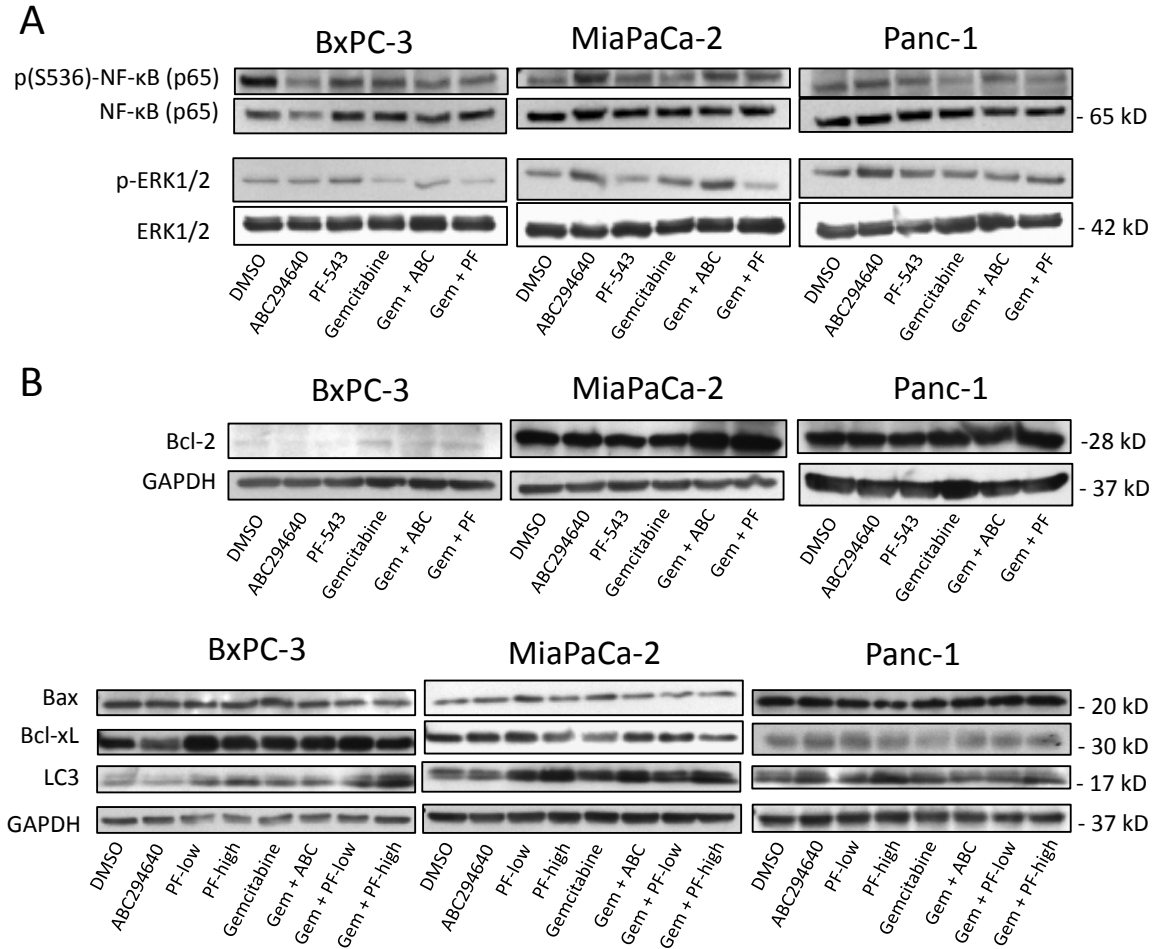
Figure 2-3: Cell signaling changes due to sphingosine kinase inhibitors alone and in combination with gemcitabine. (A) The effects of SK inhibitors with and without gemcitabine on the phosphorylation of NF-κB and ERK1/2. (B) The effects of sphingosine kinase inhibitors with and without gemcitabine on the expression of three Bcl-2 family members Bax and Bcl-xL as well as their effects on cleavage of LC3.Cells were treated for 24 hours with a dose that corresponded to their 96-hour IC50. PF-high is the same treatment as PF-543. PF-low indicates 20 nM PF-543. All blots are representative of at least two separate experiments.

pro-apoptotic members we next examined the impact of sphingosine kinase

inhibitors on three Bcl-2 family members. Again, cells from each cell line were

treated with gemcitabine, ABC294640, PF-543, and combinations of gemcitabine

with each sphingosine kinase inhibitor. Preliminary findings prompted the addition

of two treatment groups: a low concentration of PF-543 that would only target

SphK1 and this low concentration combined with gemcitabine.   It has previously

been reported that 20 nM of PF-543 was sufficient to inhibit >95% of SphK1

enzymatic activity[275]. Therefore, we sought to determine if the inhibition of

SphK1 alone was sufficient to produce the observed effects of PF-543 at

concentrations required for cell death. Immunoblots were used to observe changes

in the concentrations of three Bcl-2 family members as well as changes in the

cleavage of LC3, a marker of autophagy, which ABC294640 has previously been

reported to induce (Figure 2-3b).

Previous studies have shown that treating MDA-MB-468 xenografts with

ABC29440 causes a downregulation in intratumoral Bcl-2[294]. Thus, we first

examined the impact of SphK inhibitors with and without gemcitabine on the

protein expression of Bcl-2 in these three pancreatic cancer cell lines. The most

notable difference was that BxPC-3 expressed drastically little Bcl-2 when compared

to the other two cell lines. However, there was no observable drug effect from any

drug or drug combination on the expression of Bcl-2. This led to the examination of

the impact of these drug and drug combinations on another anti-apoptotic Bcl-2

family member, Bcl-xL. Gemcitabine and the high concentration of PF-543 produced

a decrease in Bcl-xL in MiaPaCa-2. Many drugs and drug combinations produced

changes in Bcl-xL in BxPC-3, none of which were consistent with those seen in

MiaPaCa-2. In BxPC-3 ABC294640 caused a decrease in Bcl-xL while both individual

concentrations of PF-543, as well as gemcitabine and the combination of

gemcitabine and ABC294640 produced increases in Bcl-xL. The combination of

gemcitabine with PF-543, however, produced no changes in Bcl-xL when compared

to the control. There was a slight decrease in the concentration of Bcl-xL in Panc-1

cells in response to gemcitabine, but no other treatment produced such an effect.

Only MiaPaCa-2 cells showed a change in total concentration of pro-apoptotic

Bcl-2 family member Bax in response to any treatment. There was a slight increase

in total Bax in response to both gemcitabine and the lower concentration of PF-543.

Interestingly, though, this effect was not seen when these two drugs were combined.

Accumulation of the degradation components of microtubule-associated

protein 1A/1B light chain 3A (LC3) is indicative of autolysosomal activity

demonstrating fusion of autophagosomes with lysosomes, a hallmark of autophagy.

Both low and high concentrations of PF-543 produced an increase in LC3 in all three

cell lines both alone and in combination with gemcitabine. The high concentration

produced a greater increase in all three cases and the addition of gemcitabine only

produced an additional increase in LC3 cleavage in BxPC-3. ABC294640 has

previously been reported to induce autophagy in A-498 kidney carcinoma cells[288].

We found that when combined with gemcitabine, which itself caused slight

increases in LC3 in all three cell lines, ABC294640 increased LC3 accumulation in

MiaPaCa-2 cells.
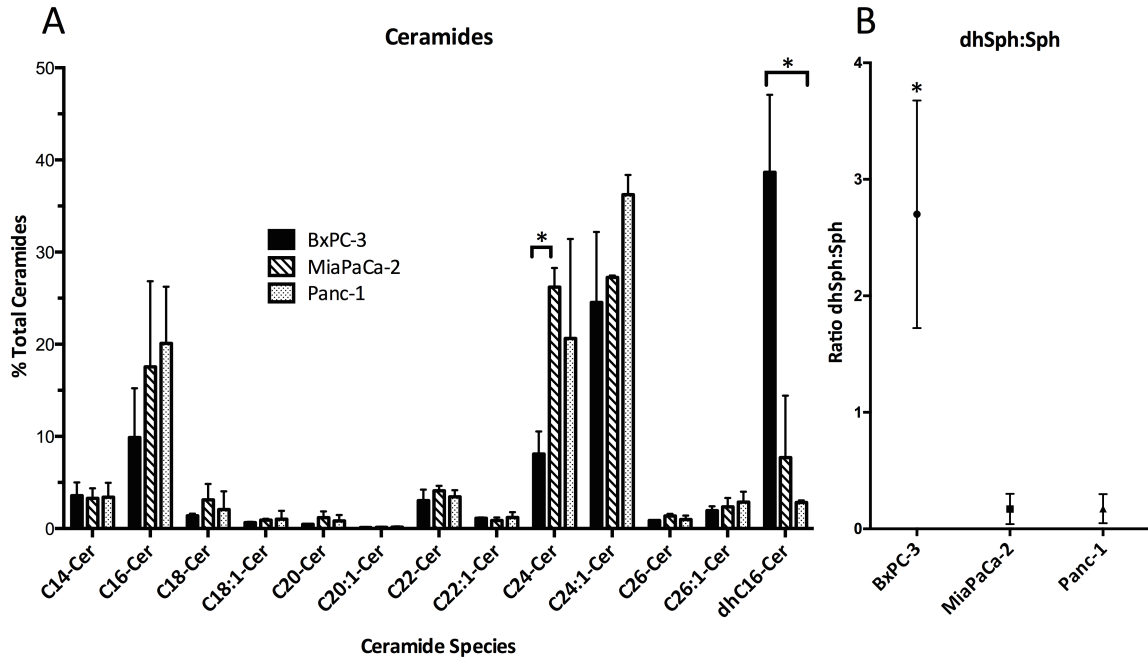
Figure 2-4: Pancreatic cancer cell line sphingolipid profiles. (A)Ceramide profiles of three different human pancreatic cancer cell lines. (B) Ratio of dihydrosphingosine to sphingosine for the same three cell lines. Each sample was normalized to its protein concentration. Averages and standard deviation represent two experiments. (*p < 0.05)

Lipidomics analysis of three pancreatic cancer cell lines

The balance of sphingolipids is well known to impact a cell's fate and its sensitivity to apoptotic stimuli. Therefore, we assessed basal sphingolipid levels for three pancreatic cancer cell lines. The total mass of 17 different sphingolipid species per sample was measured by a modified LC/MS method for the BxPC-3, MiaPaCa-2, and Panc-1 cell lines (Figure 2-4). MiaPaCa-2 and Panc-1 had similar profiles but BxPC-3 differed in its relative concentration of several different species as a percent of total sphingolipids. Most noticeably, the concentration of dhC16-Cer varied greatly among the three (Figure 2-4a). While making up over 40% of the total percent of sphingolipids of BxPC-3, dhC16-Cer accounted for roughly 8% and 3% of total sphingolipids for MiaPaCa-2 and Panc-1, respectively. To compensate for this huge amount of dhC16-Cer BxPC-3 had drastically less C24-Cer and sphingosine. However, dihydrosphingosine accounted for over three times the percent of total sphingolipids in BxPC-3 as it did in the other two cell lines (Figure 2-4b).

Discussion

Pancreatic cancer is a notoriously difficult cancer to treat. Recently the combination of gemcitabine and nab-paclitaxel became FDA approved as the standard of care for treating this disease. Here, we demonstrated that these two drugs synergize in pancreatic cancer cells at and above their IC_{50} concentrations. Each of these drugs also potently synergized with sphingosine kinase inhibitors

producing increased cell killing. The mechanism for this observed synergy between gemcitabine and sphingosine kinase inhibitors was then further investigated.

Previous work by our collaborators and us has demonstrated that ABC294640 can cause a decrease in NF-κB phosphorylation[293]. A decrease in the phosphorylation of ERK1/2 in response to ABC294640 has also been noted[288]. Each of these observations were made when cells were exposed to higher concentrations than were used in these experiments. Previous work has also demonstrated that ABC294640 can induce autophagy. Here we show that higher concentrations may be necessary to achieve this. Additionally, all previous reports on the usage of ABC294640 were made in cell lines of different origin than pancreas provoking the idea that these effects could be cell type specific.

Little work has been conducted to determine the signaling impact of PF-543. Here we show that like inhibition of SphK2, targeted inhibition of SphK1 may have cell type specific effects. Consistently, though, concentrations sufficient for the specific inhibition of SphK1 induced LC3 cleavage in all three cell lines. However, this was insufficient to yield autophagic cell death as indicated by the orders of magnitude higher IC_{50} concentration. Given that autophagy can also be a survival mechanism, this data indicates that while SphK1 inhibition is a noxious stimuli the cell may use autophagy as a means of eliminating excess sphingosine thereby escaping death signaling. Our findings on the effect of PF-543 on LC3 are in contrast to others who have reported that SK1 inhibition alone was insufficient to result in autophagy; observing specifically that 24-hour treatment of HGC 27 cells with 1 μM

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PF-543 was unable to stimulate autophagosome production as measured by cleavage of LC3[271].

The intracellular effects of dihydroceramide (dhCer) are still unclear. Genetic ablation of dihydroceramide desaturase, which results in increased levels of dhCer, has been reported to promote resistance to apoptotic stimuli[334]. dhCer has been shown to inhibit the formation of ceramide channels[335]. However, increased dhCer has also been associated with autophagy[336]. Of the sphingolipid compositions measured in our studies BxPc-3 had by far the highest percentage of dhCer. It also had increased levels of dihydrosphingosine indicating a possible deficiency in dihydroceramide desaturase. BxPC-3 was also the most sensitive cell line to gemcitabine. It is likely that the high concentration of dhCer predisposed BxPC-3 to gemcitabine induced cell death. Whether gemcitabine sensitivity and intracellular dhCer concentrations are linked is worthy of further investigation.

From these studies it is clear that combining sphingosine kinase inhibitors with both gemcitabine and paclitaxel produces synergy at and above their respective IC_{50} concentrations, and it is unlikely that this is due to changes in the activation of ERK1/2 or NF-κB. Neither of the sphingosine kinase inhibitors seemed to impact the balance of Bcl-2 family of proteins, however, further evaluation is needed. These results indicate that the observed synergy between sphingosine kinase inhibitors and gemcitabine are not likely mediated by a decrease in ERK1/2 or NF-κB activation or by alteration of the Bcl-2 family of proteins. Uncovering the mechanism behind this synergy requires further investigation.

Chapter Three

Sphingosine kinase 2 inhibition using ABC294640 synergizes with gemcitabine via

p21-mediated suppression of E2F target genes *MYC* and *RRM2* in pancreatic cancer

Abstract

Pancreatic cancer remains extremely difficult to treat, with the average lifespan following diagnosis being only 3-6 months resulting in a death to incidence ratio of 0.94. A major reason for this high mortality rate is resistance to the main chemotherapeutic agent used to treat this disease, gemcitabine. Alterations in nucleoside and gemcitabine metabolism, specifically over-expression of ribonucleotide reductase, have been implicated as the main mechanism of resistance to this drug. Previously we showed that inhibition of sphingosine kinase 2 with the specific inhibitor ABC294640 is synergistically cytotoxic with gemcitabine toward three pancreatic cell lines. Here we hypothesize that synergy between ABC294640 and gemcitabine occurs in response to ABC294640 mediated inhibition of modes of resistance related gemcitabine metabolism. Treatment with ABC294640 resulted in decreased expression of both *RRM2* and *MYC* in all three cell lines. Additionally, expression of c-Myc protein and phosphorylation of Rb at S780 both decreased in a dose-dependent manner in response to ABC29460, while acetylation of H3-K9 and p21 levels increased. Pretreatment with either the protein phosphatase 1 inhibitor okadaic acid or the ceramide synthase inhibitor fumonisin B1 failed to prevent the ABC294640 drug effect on Rb phosphorylation. These data indicate a role for sphingosine kinase 2 in E2F and c-Myc mediated transcription through alteration of histone acetylation and thereby p21 expression. These effects of ABC294640 suggest that it may be an effective agent for pancreatic cancer, particularly in combination with gemcitabine.

Introduction

For the last ten years the incidence rate of pancreatic cancer has been rising in the US while the five year survival rate remains near 6%[337]. The two main reasons for this alarming statistic are the lack of early warning signs of the disease which leads to a delay in diagnosis, often after the cancer has already grown beyond the borders of the pancreas, and resistance to the mainline chemotherapeutic drug, gemcitabine, a di-fluorinated nucleoside analog of deoxycytidine. After intracellular phosphorylation, gemcitabine induces cell death predominantly by incorporating into the DNA, leading to strand termination and subsequent apoptosis[29]. Chemoresistance to gemcitabine, whether inherent or acquired, occur most often through modification of gemcitabine metabolism (reviewed in [37]), most notably through overexpression of the catalytic subunit (RRM2) of ribonucleotide reductase.

Ribonucleotide reductase (RR) catalyzes the reduction of ribonucleotides yielding deoxyribonucleotides, and is the rate-limiting step to DNA synthesis. Its enzymatic activity is controlled mainly through transcriptional regulation[338]. A gemcitabine resistant cell line generated by incremental increases in exposure to gemcitabine was found to have a 9-fold increase in RRM2 expression and 2-fold greater protein of the same subunit[57]. Additionally, expression levels of RRM2 in tumors were shown to be predictive of treatment responsiveness to gemcitabine[58]. The inhibition of RRM2 by RNAi technology[59] and an upstream inhibitor of RRM2 transcription, flavopiridol[60], have been shown to restore sensitivity to gemcitabine.

The transcription factor c-Myc has a plethora of target genes that play roles in proliferation, the stimulation of mitochondrial biogenesis, and glucose metabolism. Its overexpression is common to many cancers[339] including pancreatic cancer[340] and contributes to gemcitabine resistance. Inhibition of this proto-oncogene sensitizes cells to gemcitabine[188, 189].

Sphingolipids are pleiotropic signaling lipids involved in many signaling systems. The *de novo* synthesis of sphingolipids begins with the palmitoylation of serine yielding 3-ketodihydrosphingosine, which is reduced to dihydrosphingosine (dhSph), which is acylated to produce dihydroceramide (dhCer). dhCer is converted to ceramide (Cer) by dihydroceramide desaturase. Cer can be further metabolized to generate additional types of sphingolipids. Of most importance to this research, however, is the ceramidase-mediated cleavage of ceramide to yield sphingosine. Sphingosine can be phosphorylated by one of two sphingosine kinases to form sphingosine-1-phosphate (S1P). Cleavage of S1P by S1P-lyase removes it from this system. The last three of these members propagate antagonizing signaling events such that excess sphingosine and/or ceramide induce apoptosis while S1P is considered pro-proliferation (Reviewed[207]).

Sphingolipids have previously been shown to affect upstream modulators of both RRM2 and c-Myc transcription. Both MYC[320, 341] and RRM2[342, 343] have E2F binding sites on their promoter regions subjecting them to partial control by the retinoblastoma family of proteins. Specifically, hypophosphorylated Rb binds E2Fs preventing the transcription of its target genes[344]. Two classes of proteins determine the phosphorylation status of Rb. Cyclin dependent kinases are

responsible for phosphorylation[345] while protein phosphatases dephosphorylate Rb[346]. The exogenous addition of sphingosine induces dephosphorylation of Rb, and addition of the ceramidase inhibitor fumonisin B1 potentiates these effects[321]. Addition of C_2-ceramide also leads to the dephosphorylation of Rb as a result of an increase in p21 expression and a corresponding decrease in the expression of MYC[320].

In our lab we have previously shown that the sphingosine kinase 2 selective inhibitor ABC294640 has extensive anti-tumor activity[265, 281], that these effects mimic SphK2 ablation, and that loss of SphK2 impacts tumor survival more profoundly than loss of SphK1[332]. Alongside, our collaborators have shown a role for ABC294640 in the decrease of intracellular c-Myc in both acute lymphocytic leukemia[261] and multiple myeloma[290]. Here, we show that inhibiting sphingosine kinase 2 increases gemcitabine sensitivity in three pancreatic cell lines (BxPC-3, MiaPaCa-2, and Panc-1) associated with increasing acetylation of lysine 9 on histone 3 leading to an increase in p21. This prevents the phosphorylation of Rb leading to the sequestration of E2F1 thereby decreasing the transcription of both MYC and RRM2 followed by a concomitant decrease in protein expression of c-Myc and to a lesser extent RRM2.

Materials and Methods

Cell Lines. All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2. BxPC-3 cells were grown in RPMI 1640 medium containing 10% fetal

bovine serum and 25 μg/mL gentamicin. MiaPaCa-2 cells were grown in DMEM

containing 10% fetal bovine serum, 5% horse serum, and 25 μg/mL gentamicin.

Panc-1 cells were grown in DMEM containing 10% fetal bovine serum and 25 μg/mL

gentamicin.

Reagents. Apogee Biotechnology Corporation supplied ABC294640. Gemcitabine

HCl was purchased from Sigma. Antibodies were purchased from Cell Signaling: c-

Myc (9402s), p21 (2947P), pRb (S780) (9307P), Rb (9309P), GAPDH (2118s), Santa

Cruz: Ubiquitin (sc-8017) and Abcam: RRM2 (ab57653). wtMyc plasmid was

graciously provided by Dr. Besim Ogretman, Medical University of South Carolina.

The following primers were purchased from Qiagen: MYC (PPH00100B), RRM2

(PPH14649A), SPHK1 (PPH02491A), and SPHK2 (PPH21192A)

GAPDH(PPH00150F).

Cytotoxicity assays. 96-well plates were seeded with 3000 cells/well. Cells were

treated with either gemcitabine, ABC294640, or a combination of the two the

following day. After 96 hours of treatment, media was removed from the cells,

which were then washed in PBS and fixed in 10% trichloroacetic acid overnight at

4°C. The TCA was then removed and the cells were washed in H_2O three times

before adding sulpharhodamine B (4 g/L in 1% acetic acid) for 30 minutes. The

cells were then de-stained with 1% acetic acid and left to dry. 100 μL of 10 mM

TRIS was then added to each well of a 96 well plate and the absorbance for each well

at 560 nm was measured using a SpectraMax M5 spectrophotometer (Molecular Devices).

Protein Isolation and Immunoblots.   After 24 hours of treatment, cells were washed three times and harvested from either 100 or 150 mm plates. Lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% NP-40, pH=7.4 plus protease and phosphatase inhibitors) was added to each plate while sitting on ice. The cells were lysed and the lysate transferred to 1.5 mL microcentrifuge tubes, which were then vortexed for 10 seconds four times over 15 minutes. The lysates were then centrifuged at 23,000 x g for 25 minutes at 4°C. The supernatant was transferred to another microcentrifuge tube. A BCA kit (Pierce) was used to determine protein concentration. Protein concentrations were then equalized using lysis buffer. Following equalization, 30 μg of protein sample were added per well in 10-well 10% Mini-PROTEAN® TGX™ pre-cast gels (Bio-Rad) and electrophoresis was performed at 150 V for 50 min. The protein was then transferred to polyvinyl difluoride membranes using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked for 1 hour in 5% BSA and then incubated with primary antibody for 1-2 hours according to the manufacturer's recommended dilution (1:1000). After washing, the membrane was incubated with a horseradish peroxidase conjugated secondary antibody for 1 hour (1:30,000). After washing, 1 mL of an enhanced chemiluminescent substrate (Thermo Scientific) was applied to the membrane and imaging was carried out in a dark room with HyBlot® CL autoradiography film (Denville Scientific).

Quantitative PCR. Following treatment, cells were collected and total RNA was collected using the RNeasy kit (Qiagen). RNA content was measured using a NanoDrop 1000 (Thermo Scientific). 1 μg of RNA was used to synthesize cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was aliquoted and combined with primers, nuclease free water and SsoFast EvaGreen Supermix and qRT-PCR was carried out on a MyiQ Real-Time PCR system (Bio-Rad). The cycling parameters consisted of 1 enzyme activating cycle of 95°C for 5 minutes followed by 45 cycles of denaturation (95°C for 45 sec) and annealing/extension (60°C for 1 min). The cycle threshold was determined for each sample and the values were normalized to GAPDH.

Statistics and graphing. IC_{50} calculations, all graphs, as well as graph statistics were made using Prism (Graph Pad). Combination index calculations were made using Calcusyn (Biosoft).

Results

The SphK2 inhibitor ABC294640 enhances gemcitabine cytotoxicity.

Previously, our lab has described the anticancer effects of multiple sphingosine kinase inhibitors[267], and ABC294640, an SphK2 selective inhibitor, was found to have broad anti-cancer activity[281]. Its combination with the multiple kinase inhibitor sorafenib yielded synergistic cytotoxicity toward A498

cells, as well as the pancreatic adenocarcinoma cell line, BxPC-3[264]. With its proven anti-tumor potential, we combined ABC294640 with the nucleoside analog gemcitabine to evaluate their usefulness together. In order to quantify the benefit of combining the SphK2 inhibitor with gemcitabine, we established 96-hour IC_{50} concentrations for each drug in BxPC-3, MiaPaCa-2, and Panc-1 cells and arrived at 7.4 nM, 404 nM, and 111 nM for gemcitabine and 28.5 μM, 35.2 μM, and 23.2 μM for ABC294640, respectively (Figure 3-1a). Combination of ABC294640 with gemcitabine resulted in synergistic cell killing (Combination Index < 1.0) in all three cell lines (Figure 3-1b). Interesting is the impact of combining ABC294640 with gemcitabine in the cell line most resistant to gemcitabine, MiaPaCa-2, in that addition of 30 μM ABC294640 with 100 nM gemcitabine resulted in a CI value of nearly 0.3, indicative of strong synergism.

Inhibition of SphK2 leads to transcriptional suppression of c-Myc.

Overexpression of c-Myc in the pancreas was shown to produce ductal adenocarcinoma in transgenic mice[347], and cells over-expressing c-Myc have been reported to be resistant to gemcitabine[188]. c-Myc is a transcription factor downstream of p38 kinase with well-documented oncogenic potential (reviewed in [339]). Its overexpression induces the transcription of genes that promote proliferation, stimulate mitochondrial biogenesis, and regulate glucose metabolism. Recently, it was shown that inhibition of SphK2 using ABC294640 results in decreased c-Myc expression[261]. Here, we show that inhibition of sphingosine kinase 2 by the inhibitor ABC294640 causes a concentration-dependent decrease in

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A

Gemcitabine Sensitivity

	BxPC-3	MiaPaCa-2	Panc-1
IC50 (nM)	7.4	404	111

ABC294640 Sensitivity

	BxPC-3	MiaPaCa-2	Panc-1
IC50 (µM)	28.5	35.2	23.2

B

BxPC-3 (Gem/ABC)

20	30	40	50 [ABC294640](µM)
10	15	20	25 [Gemcitabine](nM)

MiaPaCa-2 (Gem/ABC)

20	30	40	50 [ABC294640](µM)
75	100	200	250 [Gemcitabine](nM)

Panc-1 (Gem/ABC)

20	30	40	50 [ABC294640](µM)
75	100	200	250 [Gemcitabine](nM)
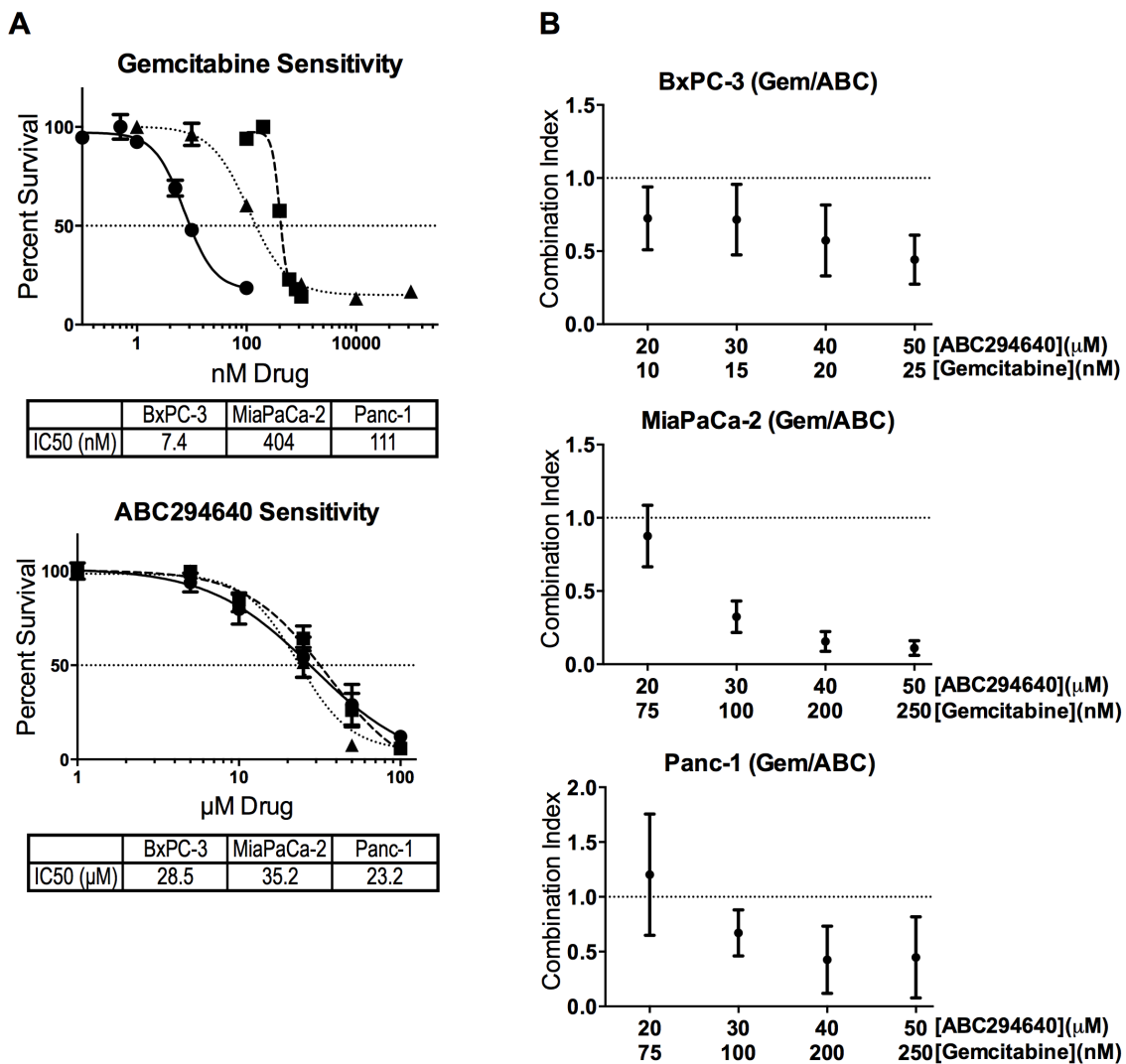
Figure 3-1. Cytotoxicity of ABC294640 and gemcitabine alone and in combination. Panel A. BxPC3 (●), MiaPaCa-2 (■) or Panc-1 (▲)pancreatic cell lines were exposed to gemcitabine and/or ABC294640 at the indicated concentrations for 96-hours . Cell survival was then quantified using the SRB assay. **Panel B**. Combination index values of the combination of ABC294640 and gemcitabine in three pancreatic cell lines were calculated using Calcusyn. All experiments were done in triplicate, and values represent mean ± SEM. (This figure is a condensation of Figures 2-1 and 2-2)

MYC mRNA, as well as c-Myc in three pancreatic cell lines in 24 hours. BxPC-3 cells were most responsive, experiencing a near 50% reduction in mRNA as well as protein expression when treated with 30 μM ABC294640 (Figures 3-2a & 3-2b). MiaPaCa-2 and Panc-1 had more muted decreases in mRNA levels, but similar decreases in protein expression when treated with 30 μM ABC294640. When comparing the native protein expression levels of c-Myc among the three cell lines, BxPC-3 was observed to express drastically less c-Myc than the other two cell lines (Figure 3-2e).

Given the varying results in *MYC* mRNA levels between the three cell lines, proteosomal degradation was addressed as a potential cause of c-Myc suppression. Each cell line was treated with its respective IC$_{50}$ dose of ABC294640 with and without the proteosome inhibitor MG-132. The presence of proteosome inhibitor did not abrogate the effect of ABC294640 in BxPC-3, suggesting a proteosome independent pathway. However, addition of MG-132 increased the accumulation of c-Myc in both MiaPaCa-2 and Panc-1 cells following ABC294640 treatment (Figure 3-3).

ABC294640 inhibits expression of RRM2 mRNA.

Overexpression of the catalytic unit of ribonucleotide reductase (RRM2) has often been cited as one of the major sources of gemcitabine resistance[57-59]. Ribonucleotide reductase catalyzes the reduction of ribonucleotides to deoxyribonucleotides. It's overexpression results in an increased pool of dNTPs that outcompete gemcitabine for incorporation into elongating DNA during

100
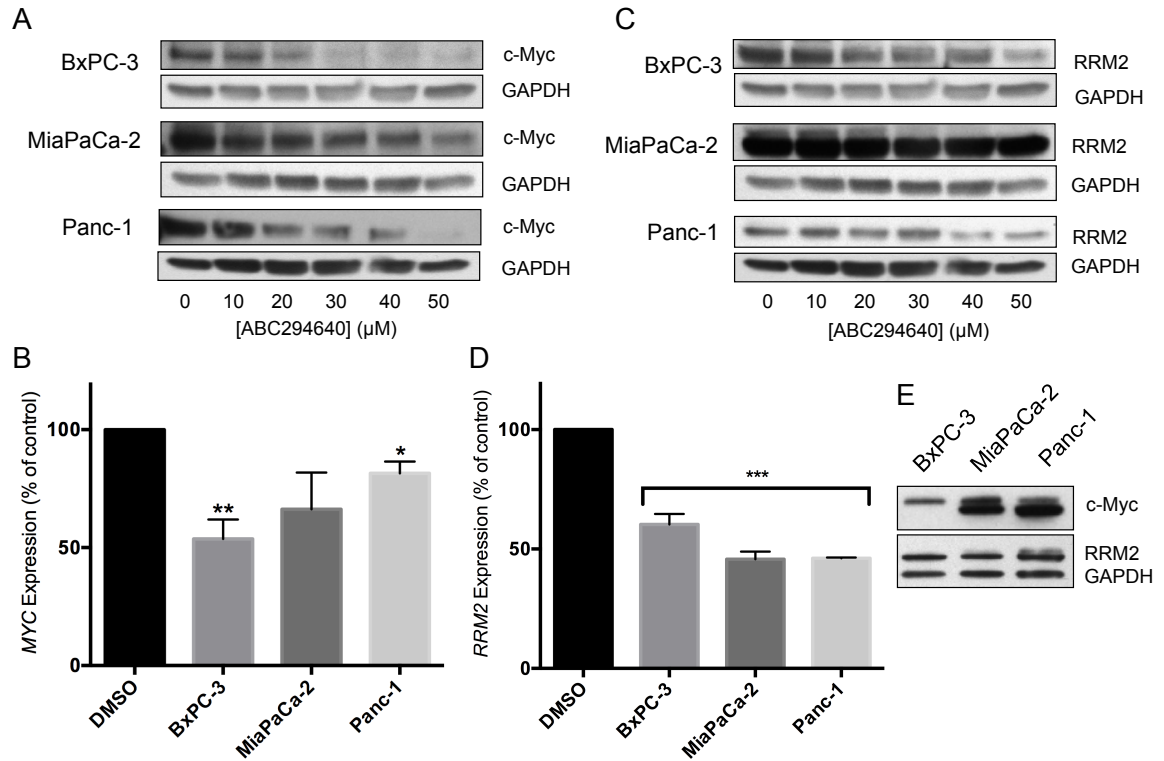
Figure 3-2. Effects of ABC294640 on expression of c-Myc and RRM2. Panels A and C. BxPC-3, MiaPaCa-2 and Panc-1 cells were treated with the indicated concentration of ABC294640 for 24 hr. Cells were then harvested and analyzed for protein expression levels of c-Myc and GAPDH (Panel A) or RRM2 and GAPDH (Panel C). Western blots were performed on three independent replicates. **Panels B and D**. BxPC-3, MiaPaCa-2 and Panc-1 cells were treated with 30 µM ABC294640 for 24 hr. Cells were then harvested and analyzed for mRNA expression levels of *MYC* (Panel B) and *RRM2* (Panel D) (p < 0.05*,0.005**, or 0.0001***). qRT-PCR was performed on two independent replicates.
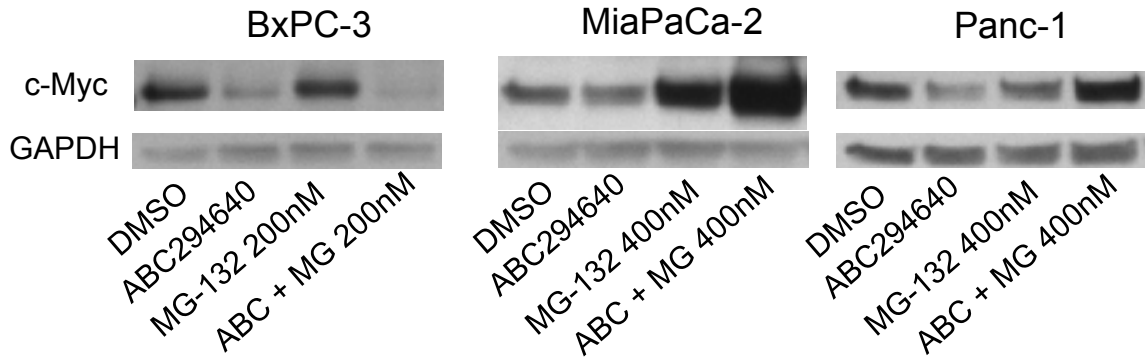
Figure 3-3. Effects of ABC294640 on proteasomal degradation of c-Myc. The indicated pancreatic cancer cells were incubated with DMSO (control) or MG-132 for 2 hr and then treated for 24 hr with 30 μM (BxPC-3) or 40 μM (MiaPaCa-2 and Panc-1) ABC294640. Cells were then harvested and analyzed for protein expression levels of c-Myc and GAPDH. Western blots were performed in two independent experiments.

replication. Like c-Myc, transcription of the M2 subunit of ribonucleotide reductase is partially dependent on the E2F transcription factor[341, 342]; there are two E2F binding sites in the *RRM2* promoter region[61]. Therefore, we examined the impact of ABC294640 on *RRM2* transcription and translation. Treatment of the BxPc-3, MiaPaCa-2 and Panc-1 cells with ABC294640 resulted in a near 50% reduction in *RRM2* mRNA in all three cell lines, which was matched by a decrease in protein expression of RRM2 (Figures 3-2c & 3-2d). Therefore, addition of ABC294640 leads to a dose-dependent decrease in two E2F responsive genes: *MYC* and *RRM2*.

ABC294640 decreases Rb phosphorylation in a dose dependent manner.

The Retinoblastoma protein, Rb, is a tumor suppressor protein responsible for sequestering the transcription factor E2F for the majority of the cell cycle. E2F can control the transcription of c-Myc[320, 348] and RRM2[341, 342]. Phosphorylation of multiple serine residues on Rb causes release of E2F and is correlated with the activation state of E2F[344]. Importantly, sphingosine, but not S1P has also been shown to activate Rb[321]. We found that a 24-hour treatment of BxPC-3, MiaPaCa-2, and Panc-1 cells with increasing concentrations of ABC294640 resulted in a dose-dependent decrease in the phosphorylation of Rb at S780, which would result in increased sequestration of E2F. (Figure 3-4a and b).
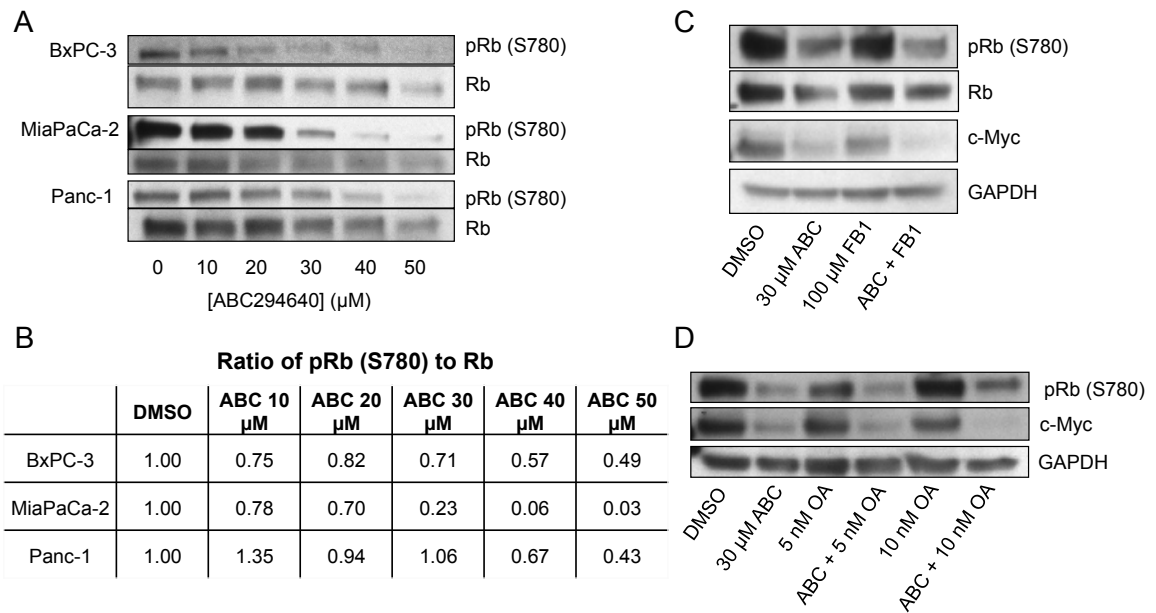
Figure 3-4. Effects of ABC294640 on phosphorylation of Rb. **Panel A**. BxPC-3, MiaPaCa-2 and Panc-1 cells were treated with the indicated concentration of ABC294640 for 24 hr. Cells were then harvested and analyzed for protein expression levels of total Rb and pRb (S780) by western blotting. **Panel B.** The ratio of pRb S780 to Rb in BxPC-3, MiaPaCa-2, and Panc-1 following treatment with the indicated concentration of ABC294640 for 24 hr. **Panel C**. BxPC-3 cells were treated with 100 µM fumonisin B1 and/or 30 µM ABC294640 for 24 hr. Cells were then harvested and analyzed for protein expression levels of total Rb, pRb (S780), c-Myc and GAPDH by western blotting. **Panel D.** BxPC-3 cells were treated with the indicated concentration of OA and/or 30 µM ABC294640 for 24 hr. Cells were then harvested and analyzed for protein expression levels of pRb (S780), c-Myc and GAPDH by western blotting.

ABC294640 mediated c-Myc suppression is not a result of the salvage pathway.

It has previously been shown that exogenous addition of short chain ceramides causes accumulation of long-chain ceramides formed via the salvage pathway leading to inhibition of c-Myc in A549 cells, a human lung adenocarcinoma cell line[349]. Ceramide has been shown to directly activate protein phosphatase 1 (PP-1) which dephosphorylates Rb both *in vitro*[350] and *in vivo*[351]. Combining these findings suggests that the addition of exogenous ceramide may result in direct activation of PP-1, which dephosphorylates Rb leading to the sequestration of E2F and decreased transcription of c-Myc.

Previous studies showed that exposing cells to sphingosine caused a reduction in Rb phosphorylation[321]. Given that ABC294640 is a SphK2 inhibitor, we sought to next determine whether the resultant increase in sphingosine is responsible for the observed decrease in pRb (S780), or whether the increasing pool of sphingosine is being salvaged to form ceramide which is activating protein phosphatases. We, therefore, co-treated BxPC-3 cells with fumonisin B1 (FB1), an inhibitor of ceramide synthases, to prevent the conversion of sphingosine to ceramide. Cells were pretreated for 2 hours with 100 µM FB1 and then treated with ABC294640 for 24 hours. Immunoblots were performed examining changes in the phosphorylation status of Rb as well as changes in c-Myc. FB1 did not prevent the dephosphorylation of Rb or the downstream suppression of c-Myc by ABC294640 suggesting that this response is mediated in a sphingosine-dependent manner, rather than a ceramide-dependent mechanism (Figure 3-4c).

Suppression of c-Myc is not due to inhibition of protein phosphatases.

To further investigate the mechanism for the decrease in Rb phosphorylation, we inhibited protein phosphatase 1 (PP-1) using the established phosphatase inhibitor Okadaic acid (OA)[351-353]. PP-1 is the enzyme responsible for the dephosphorylation of Rb, thereby allowing it to sequester E2F. Therefore, to investigate the involvement of PP-1 in the mechanism by which SphK2 inhibition leads to down-regulation of c-Myc, BxPC-3 cells were treated with 5 or 10 nM OA with or without 30 μM ABC294640. Okadaic acid caused a slight increase in Rb phosphorylation at S780 indicating an inhibition of protein phosphatase activity. However, OA did not substantially prevent the dephosphorylation of Rb, and OA did not prevent the ABC294640 effect on c-Myc (Figure 3-4d). These data suggest that the decrease in Rb phosphorylation was not due to an increase in protein phosphatase activity, but rather a decrease in cyclin dependent kinase function.

ABC294640 induces p21.

Rb activity is regulated by its phosphorylation state, which is maintained by a balance between cyclin-dependent kianses (cdks) and protein phosphatases 1 and 2. Inhibition of protein phosphatases using OA failed to prevent the hypophosphorylation of Rb in response to treatment with ABC294640, suggesting that inhibition of cdks was the likely mode by which the observed decrease in Rb phosphorylation occurred. We next then looked at the cdk inhibitor p21, which binds to the cyclinD/cdk4/6 complex inhibiting the kinase activity. Treatment of BxPC-3, MiaPaCa-2, and Panc-1 cells with increasing concentrations of ABC294640
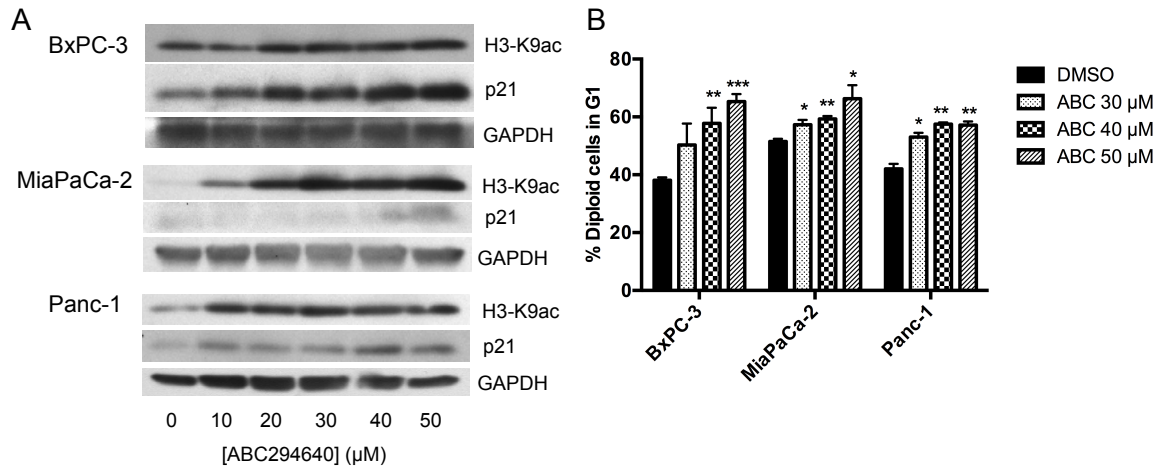
Figure 3-5. Effects of ABC294640 on H3K9ac, p21, and cell cycle. Panel A. BxPC-3, MiaPaCa-2 and Panc-1 cells were treated with the indicated concentration of ABC294640 for 24 hr. Cells were then harvested and analyzed for protein expression levels of H3-K9ac, p21 and GAPDH by western blotting. **Panel B.** BxPC-3, MiaPaCa-2 and Panc-1 cells were treated with the indicated concentration of ABC294640 for 24 hr. Cells were then harvested and analyzed by flow cytometry, and the percentage of cells in G1 is indicated ($p < 0.05$*,0.005**, or 0.0005***). All experiments were performed in triplicate.

(0-50 µM) resulted in a concentration-dependent increases in p21 expression as determined by immunoblotting (Figure 3-5a). Increasing p21 is, therefore, the probable mechanism for hypophosphorylation of Rb.

Because elevation of p21 is associated with G1 arrest, we examined the effect of increasing concentrations of ABC294640 on the cell cycle distributions of the three pancreatic cancer cell lines. We found that increasing concentrations of ABC294640 result in an increasing number of cells in the G1 phase for all three cell lines, indicating a G1 arrest (Figure 3-5b).

An increase in p21 is often the result of increased p53 activity, and all three of the cell lines under study have inactivating mutations in p53. We examined the impact of increasing concentrations of ABC294640 on total p53 as well as phospho-p53 (S15); however, there were no observable changes in either after ABC294640 treatment (data not shown).

ABC294640 induces acetylation of lysine 9 on histone 3

Recently, it was shown that SphK2 overexpression drives MYC overexpression, which inhibits histone deacetylases allowing for the association of H3-K9ac with *MYC* increasing its transcription[261]. Increasing lysine acetylation as a result of inhibition of histone deacetylases has also been shown to increase p21 expression[354]. Our data are consistent with both of these, i.e. inhibition of SphK2 by ABC294640 results in a concentration-dependent increase in acetylation of lysine 9 on histone 3 (Figure 3-5a), which corresponds with the increase in p21 expression and a decrease in c-Myc. Increasing concentrations of ABC294640

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resulted in increases in acetylation suggesting that inhibition of SphK2 results in inhibition of histone deacetylase activity.

Discussion

Treatment options for non-resectable pancreatic cancer are limited and increase the average patient lifespan by only 6-8 months. Acquired resistance to gemcitabine is a major contributor to these poor results indicating the need for a means to overcome this resistance. Here we show that the inhibition of SphK2 using the selective inhibitor ABC294640 synergizes with gemcitabine to produce increased pancreatic cancer cell killing *in vitro.* Interestingly, synergy was reached with much lower concentrations in the most resistant cell line, MiaPaCa-2, where synergy was noted at ¼ the gemcitabine IC_{50} concentration. Synergistic cell killing by combining ABC294640 with gemcitabine is in agreement with our previous findings and those of others that ABC294640 can be effectively combined with other cancer drugs. Herein, we support a model in which nuclear S1P derived from SphK2 contributes to chemotherapeutic resistance by potentiating the expression of pro-proliferative genes such as *MYC* and *RRM2*. Pharmacologic inhibition of SphK2 leads to a decrease in the expression of these genes sensitizing pancreatic cancer cells to gemcitabine.

The mechanism by which inhibition of SphK2 leads to c-Myc and RRM2 suppression likely involves inhibition of HDAC activity, which leads to a direct increase in p21 transcription and decrease in *MYC* transcription. The increase in

p21 results in a decrease in Rb phosphorylation due to the inhibition of cyclin/CDK complexes. Hypophosphorylated Rb sequesters E2F preventing its transcriptional activity resulting in a decrease in c-Myc and RRM2. The data presented here support a role for sphingosine in the suppression of E2F mediated transcription. Given the plethora of genes that E2F serves as a transcription factor for, attenuation of its activities by the prohibition of its release from Rb is an attractive target for chemotherapy. Additionally, the E2F transcription factors are transcriptional targets of c-Myc[181]. Therefore, Myc suppression can also indirectly lead to a decrease in the transcription of E2F target gene. c-Myc also acts as a transcriptional suppressor of p21[172], and so inhibition of c-Myc would lead to a release of p21 suppression. Additionally, there is an upstream stimulatory factor binding motif within the RRM2 promoter (within which there is a non-canonical E-box motif) to which c-Myc can bind and stimulate transcription[61]. In total, c-Myc can have a transcriptional impact on every member of this pathway (Figure 3-6) creating a redundancy in this system, ensuring cell death.

SphK2 and SphK2-derived S1P have been shown to regulate HDACs[260], and we postulate that inhibition of SphK2 by ABC294640 reduces the association of S1P with HDAC2 leading to a decrease in the association of *MYC* with H3K9ac and, therefore, decreased expression[261]. HDAC inhibitors are known to cause an induction of p21[354]. As demonstrated in Figure 5, SphK2 inhibition by ABC294640 inhibited HDAC activity. Therefore, ABC294640 inhibits *MYC* transcription on two fronts. Not only is the HDAC inhibition directly decreasing *MYC* transcription, but E2F mediated transcription is also effected. Additionally, a

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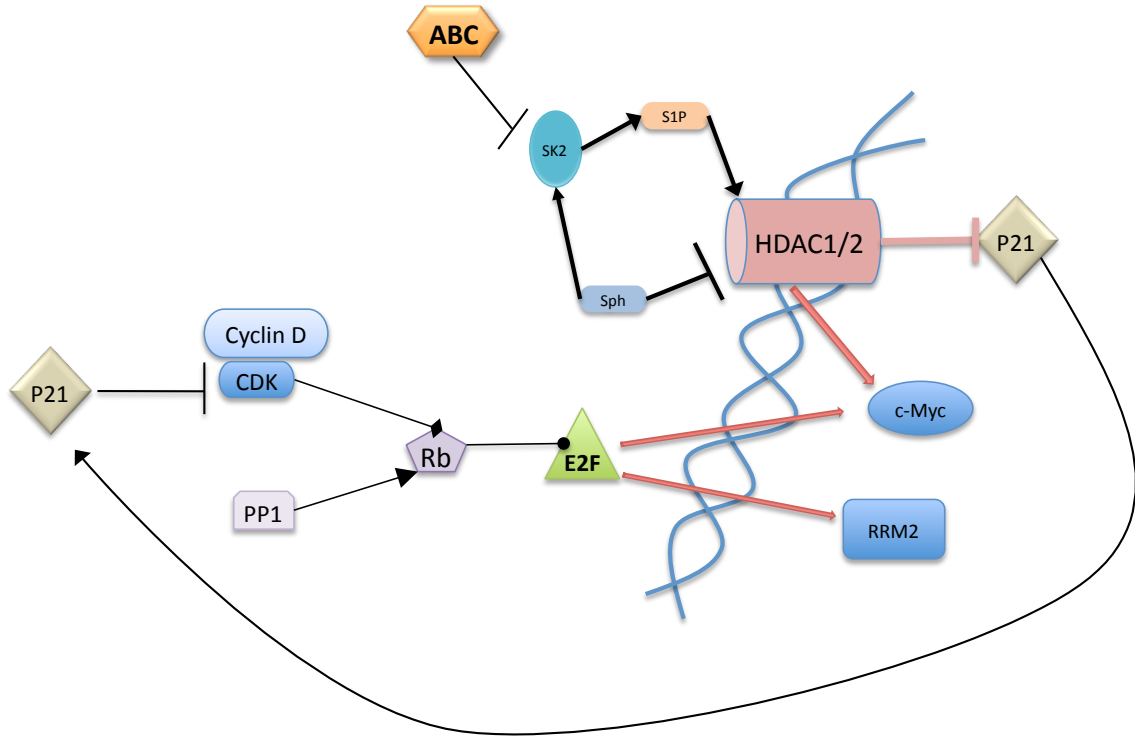
Figure 3-6. Postulated mechanism of action of ABC294640. ABC294640 acts by inhibiting SphK2 in the nucleus leading to HDAC inhibition because of loss of nuclear S1P and/or elevation of nuclear sphingosine levels. This results in an increase in p21, which binds CDK/Cyclin-D1 complexes preventing the phosphorylation of Rb. The resulting hypophosphorylated Rb binds E2F preventing its transcriptional activity. It should also be noted that c-Myc inhibits the transcription of p21 and enhances the transcription of CDKs, Cyclin-D1, and E2F1, 2 and3. The overall result is a decrease in proliferative signaling thereby resulting in inhibition of cancer growth.

third mechanism of ABC294640 mediated c-Myc inhibition, proteosomal

degradation, has been previously observed in some cell lines[290]. We report here

that the proteosome contributes to the decrease in c-Myc in some, but not all, cell

lines indicating that multiple potential mechanisms for ABC294640 mediated c-Myc

inhibition are at work in pancreatic cancer cells. This multi-directional mechanism

of c-Myc inhibition, the redundancy of cell cycle arrest provided by c-Myc inhibition,

as well as the inhibition of *RRM2* transcription make ABC294640 ideal for

combination therapy with gemcitabine.

Herein, we show that inhibition of SphK2 by ABC294640 results in an

increase in p21 that results in G1 arrest. This agrees with our earlier findings, which

showed that SphK2 inhibition via siRNA resulted in an increase in cells in the G1

phase in A498 cells[332]. The fate of p21 expression as a result of SphK2 inhibition

may be cell type specific though. Recently it was shown that inhibition of SphK2 by

siRNA led to decreased p21 in the MCF7 breast cancer cell line[260]. However,

exogenous treatment of Hs 27 human fibroblasts with C_2-ceramide, a substrate for

ceramidases for the production of sphingosine, resulted in increases in p21 and

growth arrest[320]. Inhibition of SphK2 with the specific inhibitor ABC294640 in

these three pancreatic cell lines results in a concentration-dependent increase in

p21. It is probable that the differences in these experimental data are due to

intrinsic differences in the cell types used.

Sphingolipid interaction with proteins has been documented numerous

times[355, 356]. Herein, we provide evidence that changing the nuclear

sphingolipid microenvironment by inhibition of SphK2 diminishes HDAC activity

leading to an increase in acetylation of lysine 9 on histone 3 and a dose-dependent increase in p21 expression and decrease in c-Myc expression. SphK2 has previously been shown to directly interact with HDAC[260]. Our data indicate a possible role for an activity altering sphingosine interaction. Additionally, these observed effects of increasing p21 and diminished c-Myc are indicative of HDAC inhibition[357, 358]. As well, HDAC inhibitors have previously been used to increase the efficacy of gemcitabine[359].

The combination of gemcitabine with ABC294640 creates an environment conducive to tumor cell death. Furthermore, ABC294640 attenuates multiple modes of gemcitabine resistance. Antoon et al. demonstrated the ability of ABC294640 to overcome NF-κB mediated chemoresistance in breast cancer[293]. NF-κB is overexpressed in 70% of pancreatic cancers[7] and a well-documented source of gemcitabine resistance[90]. Treatment of ovarian cancer cells with ABC294640 caused an increase in the expression of BAX[291] tilting the balance of the bcl-2 family of proteins, which are known to contribute to gemcitabine resistance[138], in the pro-apoptotic direction. And we show here that ABC294640 reduces the expression of c-Myc and RRM2. Our combined data yield a compelling argument for continued study of these synergistic drugs in combination.

As a whole, these data contribute to our understanding of the roles of SphK2 in tumor biology, increasing the evidence in support of a role for sphingolipids as regulators of gene transcription. We have also elucidated a mechanism of synergy with gemcitabine. Few drugs counteract multiple modes of drug resistance

rationalizing the future combined use of gemcitabine and ABC294640 for pancreatic

cancer chemotherapy.

Chapter Four

Conclusions and Future Directions

Conclusions

Pancreatic cancer is among the most difficult cancers to treat. This can be attributed to two major causes 1) the delayed onset of symptoms which often results in the disease not being discovered until the later stages and 2) chemoresistance to the mainline chemotherapeutic gemcitabine. This body of work explored circumventing the later. Resistance to gemcitabine most often occurs through aberrations in the enzymes involved in its metabolism – mainly through overexpression of the M2 subunit of ribonucleotide reductase. Gemcitabine resistance also occurs through alterations in growth and apoptotic signaling such that growth is constitutively signaled and apoptosis inhibited. These include aberrations in the NF-κB family and MAPK family, such that growth is promoted, and disregulation of the Bcl-2 family of proteins, which results in the balance of anti-apoptotic to pro-apoptotic family members shifting towards anti-apoptotic. Additionally, over expression of the proto-oncogene c-Myc has been shown to promote resistance to gemcitabine.

The balance of bioactive sphingolipids is also important in determining cell fate. An imbalance, as in an excess of S1P, promotes cell growth. S1P has been shown to promote growth through NF-κB and ERK1/2 signaling. It has also been shown to tip the balance of the Bcl-2 family of proteins towards opposition of apoptosis. Additionally, it has been well established that preventing the formation of S1P by inhibition of SphK2 induces cell death. Therefore we hypothesized that inhibition of SphK2 using the specific inhibitor ABC294640 would synergize with gemcitabine to produce increased cell killing. ABC294640 did, indeed, synergize

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with gemcitabine, however, the data presented in Figure 2-3 show that ABC294640

did not impact these systems equally in three different pancreatic cancer cell lines

suggesting that the mechanism behind the observed synergy lied elsewhere.

Over the past year, ABC294640 has twice been reported to inhibit the

expression of c-Myc through both transcriptional regulation and enhanced

proteosomal degradation[261, 290]. Given that c-Myc is known to promote

resistance to gemcitabine we examined the impact of ABC294640 in three

pancreatic cancer cell lines where we observed a concentration-dependent decrease

in c-Myc. Uncovering the connection between S1P and c-Myc led to the discovery

that ABC294640 caused a concentration-dependent increase in p21, and a

corresponding decrease in Rb phosphorylation at S780. Hypophosphorylated Rb

binds E2F preventing transcription of E2F target genes and, thereby, the

progression of the cell cycle, an effect we were able observe.

Overexpression of RRM2 has repeatedly been shown to confer resistance to

gemcitabine. Given the nature of RRM2's function, nucleotide production, it is up-

regulated in preparation for mitosis. This is facilitated, in part, by E2F. So, we

sought next to see if ABC294640 impacted RRM2 transcription, which it did.

Therefore, ABC294640 impacts the expression of a potent oncogene in c-Myc and

one of the most commonly reported mediators of gemcitabine resistance, RRM2.

The forced sequestration of E2F caused by ABC294640 allows for inhibition of both.

Our understanding of the role of sphingolipids in mediating resistance to

chemotherapeutics is growing. It is know well established that S1P has specific

nuclear targets, HDAC1 and HDAC2. Our findings conflict with those showing that

S1P promotes p21 expression. The data presented here demonstrate that the inhibition of S1P leads to an increase in acetylation H3K9, which promotes expression of p21. ABC, though not likely directly, is promoting lysine acetylation by either inhibiting HDAC activity or promoting histone acetyltransferases (HAT) activity. In agreement with my data, HDAC inhibitors have previously been shown to promote p21 expression.

The data presented here bolster the idea that nuclear S1P promotes proliferation and that inhibition of its production by ABC294640 sensitizes cells to the cytotoxic effects of gemcitabine due to an inhibition of E2F mediated transcription. These data also provide rationale supporting further pre-clinical evaluation of the combination of ABC294640 and gemcitabine.

Future Directions

There is still much to be done in solidifying the mechanism of synergy between ABC294640 and gemcitabine. Verifying that ABC294640 diminishes E2F mediated transcription using an E2F luciferase reporter construct would strengthen the claim that ABC294640 causes a decrease in E2F mediated transcription. We know that ABC294640 specifically inhibits SphK2 and that SphK2 associates with HDAC1 and HDAC2. However, specific competition studies showing that ABC294640 prevents binding of S1P to HDACs would provide a linking proof between ABC294640 and histone acetylation. We've also shown that ABC294640 causes a dose dependent increase in p21. However, its impact on cyclins, cdks and other cdk inhibitors such as p15, p16, and p27 is unknown. Determining the combinations of these affected by ABC294640 would help may unveil a specific role

for SphK2 derived S1P in cell cycle regulation.   It should also be determined whether suppression of p21 through genetic ablation would abolish sensitivity to ABC294640.

In addition to unraveling the role of S1P in cell cycle progression, determining the target genes of S1P mediated histone deacetylation could establish a therapeutic profile of those most likely to benefit from ABC294640. Genetic profiling is becoming an increasingly more popular tool in the clinic. As therapeutic decisions become more reliant on this methodology having an understanding of S1P target genes would allow for patient stratification, flagging patients with aberrations in S1P target genes as those who may benefit most from ABC294640.   It could also indicate potential future drug combination partners.

This work also shows that BxPC-3 has elevated dhCer and dhSph. This cell line was also the most sensitive to gemcitabine. Evaluating the expression and activity of dihydroceramide desaturase in BxPC-3 as compared to MiaPaCa-2 and Panc-1 may provide evidence supporting a role for dhCer and dhSph (and thereby dihydroceramide desaturase) in sensitivity to gemcitabine. Genetic ablation of dihydroceramide desaturase in MiaPaCa-2 and Panc-1 may also support this notion.

This project has elucidated mechanisms by which ABC294640 enhances susceptibility of pancreatic cancer cells to gemcitabine in vitro. An important next step will be to validate this mechanistic information in preclinical models. Current mouse models fit into two main categories: xenografts and genetic models. Xenografts include both subcutaneous and orthotopic xenografts. Numerous genetically modified mouse models exist which target tumorgenesis to the pancreas

by utilizing a pancreas specific promoter element upstream of a tumor promoting gene (such as mutated Kras). Each model possesses advantages and disadvantages. Subcutaneous xenografts are relatively inexpensive and allow for the use of human pancreatic cancer cells. However, the cells are not in the pancreas, and, therefore, may respond differently to treatment. Orthotopic xenografts allow for the growth of human cancer cells in pancreatic tissue, but are currently technically difficult to produce reliably and growth is difficult to track. Both xenograft types necessitate the use of immunocompromised mice; therefore, the inflammatory aspect of pancreatic cancer is unaccounted for when evaluating therapeutics. Genetically engineered mice are expensive, however, they allow for control over the genetic impetus for tumorgenesis and the mice are immunocompetent. Tumors can take several months to develop, though, and tracking growth is difficult[360].

Our lab has developed a luciferase expressing, orthotopic pancreatic cancer mouse model by implanting Panc-1 cells transfected with the pGL4.50[luc2] plasmid into SCID mice. When provided with substrate the Panc-1-luc cells emit luminescence 371 fold above background and the light emitted is directly correlated with the number of cells present (Figure 4-1a). When these cells were implanted into the tails of the pancreases of six SCID mice, tumors were readily formed. When luciferin was administered intraperitoneally to the mice tumor growth could be monitored using a bioluminescence imager and in this pilot study the growth rates were similar among all six mice (Figure 4-1b).

While a luciferase expressing orthotopic xenograft model is useful, it lacks the inflammatory element known to promote pancreatic tumor growth. To build on
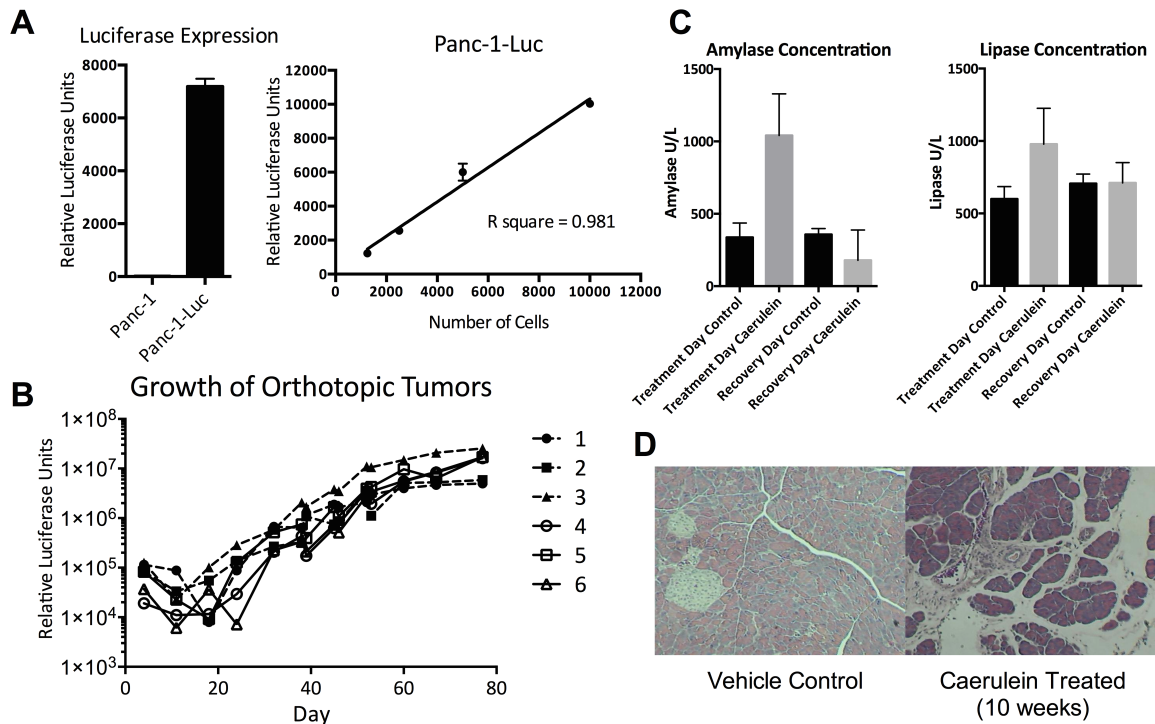
Figure 4-1: Development of a luciferase expressing orthotopic mouse model of pancreatic cancer and caerulein induced pancreatitis. (A) Luciferase activity of Panc-1 and Panc-1 clone expressing luciferase and the linearity of Panc-1-luc luciferase signal relative to cell number. (B) Tumor growth of orthotopic tumors as measured by bioluminescence imaging. (C) Average serum concentrations of amylase and lipase following treatment with either vehicle control or caerulein on both the treatment day and the day following treatment. (D) H&E stain of pancreatic tissue samples from C57BL/6 mice treated with either vehicle control or caerulein for 10 weeks.

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this model we have transfected Pan02 cells, a pancreatic cancer of C57BL6/J origin, with the same luciferase gene as the one introduced into the Panc-1-luc cells. Implanting these Pan02-luc cells into the pancreas of a C57BL6/J mouse would produce an orthotopic, syngeneic model. Such a model would provide a more representative tumor environment than the orthotopic xenograft due to the presence of a competent immune system.

Given both the anti-proliferative and anti-inflammatory benefits of ABC294640 and the highly inflammatory nature of pancreatic cancer, we have also begun work assessing the benefit of ABC294640 on pancreatitis. Pancreatitis is the result of both intra-pancreatic trypsinogen activation and aberrant NF-κB activation, which induces a local inflammatory response. These lead to apoptotic death of acinar cells and a larger systemic inflammatory response. Chronic pancreatitis is the result of sustained NF-κB activation[361]. Patients with chronic pancreatitis have a much higher risk of developing pancreatic cancer when compared to the general population[362]. The most common mode of inducing chronic pancreatitis in mice is through repeated injections of the cholecystokinin mimetic caerulein. Caerulein causes both aberrant activation of trypsinogen and NF-κB activation in acinar cells.

Using a bi-weekly, 10-week, scheduled administration of caerulein repeated episodes of acute pancreatitis were induced in C57BL/6 mice which led to a chronic pancreatitis phenotype as assessed by histological examination.   Disease progression was monitored through serum amylase and lipase levels. Blood samples were obtained an hour after the final dose of caerulein on the treatment

day and again 24 hours later. As expected serum amylase and lipase levels were both elevated on the day of treatment, indicative of pancreatitis. These then returned to near normal levels within 24 hours (Figure 4-1c). Following the 10-week treatment course, histological examination of the pancreases showed disruptions in the organization of acini, ductal formations, and islets of Langerhans as well as large areas of necrosis (Figure 4-1d). Future studies will utilize this model to examine the efficacy of ABC294640 in the treatment of pancreatitis. Also, given the high percentage of patients who experience symptoms of pancreatitis and pancreatic cancer as co-morbidities, we aim to further our luciferase expressing orthotopic, syngeneic model by adding the complexities of caerulein induced pancreatitis. We believe this complicated, highly inflammatory model will more accurately reflect human disease, and, therefore, be a useful in pre-clinical drug evaluation.

This body of work has provided evidence demonstrating that ABC294640 synergizes with gemcitabine via suppression of E2F mediated transcription through increased acetylation of H3K9. This leads to inhibition of the expression of the proto-oncogene *MYC* as well as that of gemcitabine resistance mediator *RRM2*. Moving forward, this evidence strongly supports further pre-clinical evaluation of the efficacy of the antineoplastic combination of ABC294640 and gemcitabine.

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