Regulatory role of sphingolipid metabolites, sphingosine-1-phosphate and C16:0 ceramide, in immunogenic cell death of colon cancer cells induced by Bak/Bax-activation.

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ABSTRACT

We have previously identified sphingolipid metabolism as a key intracellular process associated with immunogenic cell death (ICD) induced by mitoxantrone in colon cancer cells. Specifically, we have demonstrated that inhibition of the sphingosine kinases (SphKs) synergistically enhanced production of hallmarks of ICD including ectoCRT production. To better understand the mechanism associated with ICD enhanced by SphK1-inhibition, we focused on the ER stress-associated intracellular signaling pathways leading to ectoCRT production. It is known that ABT-263 and AZD-5991 (ABT/AZD) are inhibitors of Bcl-2/Bcl-X_L and MCL-1, respectively, leading to activation of Bak/Bax. Herein, we now provide evidence that treatment of DLD-1 colon cancer cells with ABT/AZD results in the production of ectoCRT indicative of ICD. Additionally, our data show that ABT/AZD-induced ectoCRT production is significantly enhanced by combination treatment with the SphK1 inhibitor, PF-543. Mechanistically, we demonstrate that combined treatment of ABT/AZD+PF-543 induces ectoCRT exposure in a caspase 8-dependent manner. Accordingly, we have identified a Bak/Bax activation-dependent pathway that leads to activation of a pro-survival SphK1/sphingosine-1-phosphate (S1P) signaling that attenuates ectoCRT production. Additionally, we have identified a regulatory role of ceramide synthase 6 (CerS6)-C16:0 Cer in transporting of dimeric CRT to the cell surface (ectoCRT). Together, these results indicate that sphingolipid metabolites, such as S1P and C16:0 Cer, have a key regulatory role for survival/death decisions of cancer cells in response to ICD-inducing chemotherapeutic agents such as mitoxantrone and ABT/AZD. Hence, targeting SphKs may be an innovative means to enhance the efficacy of ICD-inducing chemotherapeutic agents promoting anti-tumor innate/adaptive immune response since SphK inhibition blocks the anti-ICD effects of S1P while simultaneously accumulating sphingosine (Sph) leading to pro-ICD C16:0 Cer synthesis.

INTRODUCTION

Sphingosine-1-phosphate (S1P) is a pro-mitogenic/pro-survival sphingolipid with pleiotropic effects on cancer cell growth, survival, metastasis and chemotherapeutic resistance. More recently, it has been recognized that S1P has immunosuppressive roles in the tumor microenvironment (TME). Thus, targeting the enzymes responsible for S1P production, sphingosine kinase 1 and 2 (SphK1 and SphK2) has been extensively investigated pre-clinically as well as in human clinical trials. As a stand-alone therapy, SphK inhibition may have some therapeutic benefit, but as an adjuvant to standard chemo- or immuno-therapies, SphK inhibition has great potential. Indeed, a recent study has demonstrated that SphK1 inhibition enhances the effectiveness of immune checkpoint inhibitors through modulation of PGE₂ synthesis in the TME¹.

While modulation of immunosuppression in the TME suggests a potential therapeutic benefit for SphK inhibition as part of the armament against cancer, we recently discovered a previously unrecognized role for sphingolipids as regulators of cancer cell immunogenicity². To this end, we demonstrated that inhibition of SphK1 and/or SphK2 and therefore, S1P production, leading to ceramide (Cer) accumulation enhances the ability of certain chemotherapies (e.g., mitoxantrone) to induce a specific form of regulated cell death termed immunogenic cell death (ICD).

Historically, the dogmatic view of the mechanism-of-action of cancer chemotherapies has been that they induce apoptosis, a tolerogenic form of regulated cell death, and hence remain hidden from the innate immune system. However, recent studies have challenged this assertion by demonstrating that certain anti-neoplastic agents such as the anthracyclines (e.g., mitoxantrone), oxaliplatin, radiation and photo-dynamic therapy, can kill cancer cells in a manner that allows them to be recognized as “non-self” by the innate/adaptive immune system through induction of ICD. ICD holds great potential as a means of engaging the immune system to attack tumors. Nevertheless, the understanding of ICD is in its infancy relative to the apoptotic cell death.

Numerous investigations have shown that cancer cells exposed to ICD-inducing chemotherapeutic agents release adjuvants, termed danger-associated molecular patterns (DAMPs) in a coordinated fashion to induce activation of the innate/adaptive immune system³. In contrast, chemotherapeutic agents that are unable induce ICD fail to promote DAMP release. It is known that dendritic cells (DCs) and antigen presenting cells (APCs) express several pattern recognition receptors that act as sensors for the DAMPs exposed by dying cancer cells. These DCs/APCs initiate the immune response by presenting captured antigens and priming naïve T cells in lymphoid tissues to mount an attack on the cancer cells⁴.

The current understanding of ICD is based primarily on the mechanism by which mitoxantrone (MTX) induces ICD⁵. MTX-induced endoplasmic reticulum (ER) stress leads to activation of the protein kinase R-like endoplasmic reticulum kinase (PERK) which, in turn, mediates phosphorylation of eukaryotic initiation factor 2 α (eIF2 α), a process thought to be pathognomonic to ICD⁶. Through multiple intracellular processes, eIF2 α phosphorylation induces production of several DAMPs including exposure of the normally ER resident chaperone, calreticulin (CRT), at the PM surface (ectoCRT), the autophagy-mediated secretion of ATP to the extracellular milieu, and the extracellular release of high-mobility group box 1 (HMGB1). ER stress is transmitted to activation of caspase 8, cleavage of BAP31 and activation of Bak/Bax at the ER membrane. Upon Bak/Bax activation, CRT is translocated to the PM surface in a process requiring SNAREs. For an extensive review of the current understanding of ICD, see Galluzzi et al. 2020³.

Our recent discovery of the role of sphingolipids, including Cer, in the process of ICD prompted us to reexamine the intracellular mechanism of ICD. In particular, we focused on the role(s) of Bak/Bax in ICD. Bak/Bax activation and Cer induced cell death have been associated in numerous studies^{7,8}. In addition to their better accepted role in intrinsic/extrinsic apoptosis associated with mitochondrial

localization, Bak/Bax are also localized to the ER⁹. Indeed, Bak/Bax activation at the ER has been associated with depletion of Ca²⁺ from ER stores¹⁰⁻¹⁴. Similarly, depletion of ER Ca²⁺ has been linked to ectoCRT exposure¹⁵. Thus, we examined whether direct activation of Bak/Bax could induce ICD and what role sphingolipids played in this process.

Herein, we report that at very low concentrations, inhibitors of pro-survival Bcl-2 family proteins (ABT-263 and AZD-5991 in combination), by themselves, do not induce ICD. However, these concentrations are sufficient to prime cells to undergo ICD when sphingolipid metabolism is blocked by inhibiting SphK1, which also does not induce ICD by itself. Thus, SphK1-generated S1P is a lipid metabolite that is critical for cancer cell survival under conditions that promote endoplasmic reticulum stress. We further elucidated the mechanism by which SphK1/S1P regulates the cellular response to ER stress and have determined that stabilization of cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein (c-FLIP) is a key pro-survival function of S1P. Moreover, we have also identified C16:0 Cer as a specific Cer species required for transport of DAMPs from their intracellular locales to the cell surface/extracellular environment, which is the hallmark of ICD.

MATERIALS AND METHODS

Compounds and Antibodies. ABT-263, AZD5991, Mitoxantrone, PF-543, Thapsigargin, KIRA6, GSK2606414, Necrostatin-1, Disulfiram and z-IETD-fmk were all purchased from Selleckchem (Houston, TX). Fumonisin B1 was from Cayman Chemicals (Ann Arbor, MI). All compounds were prepared in a DMSO vehicle. Anti-Calreticulin, anti-phospho eIF2 α Ser51, anti-full length Caspase 8, anti-Bap31, anti-Cleaved PARP, anti-GRP78/BiP, anti-Bcl-XL, anti-FLIP, anti-full length BID, and anti-total SphK1 antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-GAPDH, and anti-CerS6 antibodies were from Santa Cruz Biotechnologies (Dallas, TX). Anti-phospho-SphK1 Ser225 antibodies were from ECM Biosciences (Versailles, KY). Anti-CerS5 antibodies were from LSBio (Seattle, WA). Anti-Arginylated Calreticulin antibodies were from MilliporeSigma (Burlington, MA).

Cell Lines and Culture Conditions

Human colorectal DLD-1 (CCL-221) cells were obtained from ATCC, (Manassas, VA). HEK293 cells over-expressing SphK1 were described previously¹⁶. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

Crispr/Cas9 Knock-out of CerS5 and CerS6 in DLD-1 cells

CerS5 and CerS6 knock-out clones of DLD-1 cells were generated using the LentiCRISPR v2.0 system with guide RNAs (gRNAs; CerS5; 5'-GCTTGTCCTGATTCCTCCGA-3' and CerS6; 5'-GGCTCCCGCACAATGTCACC-3') purchased from GenScript (Piscataway, NJ USA). DLD-1 cells were transiently transfected using Lipofectamine 2000. After 48 h, cell were selected in puromycin (10 μ g/mL) for 24 h and maintained in 1 μ g/mL puromycin until single clones were isolated. Screening for CerS protein expression knock-out was conducted by western blot analysis using the appropriate CerS antibodies.

Detection of Cell Surface CRT

Analysis of cell surface exposure of CRT was described previously². Briefly, cells were seeded at about 3×10^5 cells/well in six well plates in complete DMEM for 24 hr, then transferred to DMEM containing 5% FBS and penicillin/streptomycin in the presence of treatments for 48 hours. Cells were collected by trypsinization, followed by three washes in PBS containing 2% FBS, stained with anti-calreticulin (D3E6) phycoerythrin (PE) conjugated antibody at 4°C for 1h, washed as above, and CRT was detected by Muse cell analyzer (SmartFlare detection settings).

Sphingolipid Analysis

DLD-1 cells were treated with ABT/AZD (0.5 μ M and 0.25 μ M respectively for low dose and 1.0 μ M and 0.5 μ M respectively for high dose) and/or PF-543 (5 μ M) for 24 h. Individual cell samples were collected by trypsinization, pelleted and washed with PBS and flash frozen. Sphingolipidomic analysis was conducted by the Lipidomic Shared Resource Facility (Medical University of South Carolina, Charleston SC). Sphingolipid levels are expressed as pmoles of sphingolipid per μ g of total protein.

Whole Cell Lysate Preparation

Total cell lysate was obtained by incubating treated and untreated DLD-1 cells in 1X RIPA (Cell Signaling Tech, Beverly MA), with phosphatase inhibitor cocktail and protease inhibitor tablets (Roche), for 30 min at 4°C and followed by removal of cell debris by centrifugation at 20,000 xg at 4°C. Protein concentrations were determined by BCA Assay (Pierce, Waltham MA).

Statistical Analysis

Where indicated, statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM. Results are reported as average values among replicates.

RESULTS

ABT-263 induces and AZD-5991 enhances cell surface exposure of calreticulin (ectoCRT). ER stress and ER Ca^{2+} depletion have been implicated in the production of cell surface CRT (ectoCRT) and activation of the pro-apoptotic Bcl-2 family members, Bak and Bax, have been identified as downstream factors required for ectoCRT exposure⁵. Bak and Bax have been localized to the ER membranes in addition to the mitochondrial membrane⁹. When activated by BH3-only proteins, Bak and Bax oligomerize to form channels that induce mitochondrial outer membrane permeability (MOMP)¹⁷. We reasoned that activation of ER resident Bak and Bax may, similarly, induce ER membrane permeabilization and ER Ca^{2+} depletion, without causing ER stress, and that their activation may be sufficient to induce ectoCRT exposure. We, therefore, assessed the ability of agents that activate these pro-apoptotic proteins to cause CRT cell surface exposure in DLD-1 cells. Bak and Bax are held inactive by Bcl-XL and Bcl-2, two of the anti-apoptotic Bcl-2 family proteins. Navitoclax (ABT-263; ABT) a BH3-mimetic inhibits the interactions between Bcl-2/XL with Bak and Bax thereby activating the latter¹⁸. Oligomerization and activation of Bak can also be prevented by its interaction with Mcl-1, the third anti-apoptotic Bcl-2 family member. AZD-5991 (AZD), another BH3-mimetic, has been shown to selectively bind Mcl-1 thereby resulting in Bak activation¹⁹.

We treated DLD-1 cells with either ABT or AZD and determined ectoCRT exposure by flow cytometry (Figure 1A). ABT dose dependently increased ectoCRT exposure. ectoCRT exposure was significantly increased by 3 μM ABT relative to 1 μM ABT ($p=0.0003$) and to 3 μM AZD ($p<0.0001$). In contrast to ABT, AZD did not induce ectoCRT even at 3 μM , a concentration greater than 100x its IC_{50} value¹⁹. Similarly, as shown in Figure 1B, combining low doses of ABT (0.5 μM) with AZD (0.5 μM) significantly enhanced ABT-induced ectoCRT relative to 1 μM ABT alone ($p=0.0028$). These data show that ABT induces ectoCRT and that disrupting the Mcl-1:Bak interaction enhances ectoCRT displayed at the surface of DLD-1 cells.

Sphingosine kinase inhibition enhances the production of ectoCRT by non-effective doses of ABT/AZD. We have previously demonstrated that inhibition of sphingosine kinase 1 and/or 2 (SphK1/2) enhances ectoCRT production by the known ICD-inducing agent mitoxantrone MTX through accumulation of ceramide (Cer)². Bak, as well as Bax have been associated with induction of Cer synthesis through multiple studies^{7,8,20}. Cer has also been shown to form channels that are functionally regulated by the pro- and anti-apoptotic Bcl-2 family proteins^{21,22}. Moreover, as mentioned above Bak and Bax have been shown to form pores, albeit in the mitochondrial outer membrane¹⁷. Given that the ER is the site of Cer synthesis, we reasoned that activation of Bak and Bax might be enhanced by inhibition of the SphKs. In this scenario, SphK inhibition would block the conversion of Sph to S1P and lead to enhanced utilization of Sph by the Cer synthases to form Cer species and lead to the formation of channels/pores in the ER stabilized by Bak/Bax inducing ER Ca²⁺ leakage and induction of ICD.

As shown in Figure 2A, we identified concentrations of ABT and AZD that, by themselves, had no effect on production of ectoCRT, nor did they induce ectoCRT in combination. As we observed previously, the SphK inhibitor PF-543 (Pfizer) alone has no effect on production of ectoCRT. At these concentrations, however, the addition of PF-543 significantly ($p < 0.05$) and dose-dependently induced ectoCRT exposure as measured by flow-cytometry².

The mechanism of action of MTX-induced ectoCRT exposure has been shown to require the phosphorylation/inactivation of eukaryotic Initiating Factor 2 alpha (eIF2 α), activity of Caspase 8 (Casp8) and cleavage of the Casp8 substrate Bap31 (28kDa) yielding a 20kDa fragment^{5,6}. To determine whether ABT/AZD induced ectoCRT exposure occurred by the same mechanism as MTX-induce ectoCRT exposure, we next compared the effects of ABT/AZD $\pm$ PF-543 to that of MTX $\pm$ PF-543. As indicated in Figure 2B, ABT/AZD (0.5 μ M/0.25 μ M respectively; higher than Figure 2A) induced minimal phosphorylation of eIF2 α as compared to MTX (1 μ M or 4 μ M) alone. PF-543 did increase

phosphorylation of eIF2 α in response to ABT/AZD treatment, however, the effect was not as great as that observed in combination with 1 μ M MTX.

We next examined activation of Casp8 and cleavage of the substrate Bap31. PF-543 alone did not induce activation of Casp8. ABT/AZD and MTX (1 μ M) alone induced minimal activation of Casp8 as judged by the partial cleavage of Bap31. 4 μ M MTX alone enhanced the activation of Casp8 and increased Bap31 cleavage. The addition of PF-543 (5 μ M) significantly enhanced the activation of Casp8 by both ABT/AZD and MTX (1 μ M), resulted in at least 50% conversion of Bap31 to the cleaved 20kDa product and increased generation of the cleaved form of PARP. GRP78/BiP and Bcl-XL were employed as a loading controls and were not affected by Casp8 activation. Interestingly, ABT/AZD+PF-543 induced full activation of Casp8, yet produced the lowest levels of phosphorylated eIF2 α suggesting that eIF2 α phosphorylation is not directly correlated to the extent of ICD induction, at least for ABT/AZD. This is consistent with the idea that acting directly on Bax and Bak can circumvent the requirement for induction of ER stress attributed to other ICD-inducing agents, such as MTX.

We recently demonstrated that ectoCRT is present at the lipid raft fraction of the plasma membrane as a disulfide-linked dimer, in response to MTX $\pm$ PF-543². To ensure that ABT/AZD also induced disulfide-linked dimerization of ectoCRT, we assayed for the presence of dimeric CRT in whole cell lysates of DLD-1 cells, treated as above, under non-reducing conditions. As shown in Figure 2C, consistent with the activation of Casp8/Bap31 cleavage ABT/AZD induced and PF-543 enhanced CRT dimerization.

Our previous studies indicated that CRT was substantially present in the cytosolic fraction of DLD-1 cells, as a monomer, under untreated conditions. Upon MTX+PF-543 treatment monomeric, cytosolic CRT was significantly reduced with concomitant increases in the presence of exclusively dimeric CRT in the Triton X-100 insoluble (lipid raft) fraction. This indicated the possibility that

cytosolic CRT was the source for cell surface exposed ectoCRT. Cytosolic CRT arises from retrotranslocation of luminal CRT to the cytosol where it is post-translationally modified by the addition of an arginyl-group at the mature (signal sequence removed) NH₂-terminus^{23,24}. To further examine the nature of the dimeric form of ectoCRT, we examined whether dimeric CRT was NH₂-terminally arginylated using antibodies specific to Arg-CRT. As shown in Figure 2D, a fraction of the dimeric CRT is NH₂-terminally arginylated indicating that, at least some of the cell surface exposed CRT originates from the cytosolic fraction.

ABT/AZD alone are minimally effective at induction of ectoCRT exposure. One possible explanation for this is that ABT/AZD do partially activate Bak/Bax to induce minimal pore formation and Ca²⁺ leakage from the ER, but the activity of ER Ca²⁺ pumps (i.e., SERCA) are sufficient to maintain a ER:cytosol Ca²⁺ gradient preventing dimerization of cytosolic CRT and transport to the cell surface. To test this possibility, we next employed the SERCA inhibitor thapsigargin to prevent Ca²⁺ reentry into the ER in the presence of ABT/AZD at low doses. As shown in Figure 2E, ABT, AZD and their combination, again, had minimal effect on ectoCRT production. PF-543 significantly enhanced the effects of the ABT/AZD combination as observed previously. The SERCA inhibitor, thapsigargin, by itself had minimal effect. However, in combination with low dose ABT/AZD inhibition of the cells ability to maintain ER Ca²⁺ homeostasis significantly enhanced the effects of ABT/AZD. This implies that ABT/AZD acts at the ER membrane to induce Ca²⁺ leakage from the ER to the cytosol triggering ectoCRT production. Consistent with this, thapsigargin enhanced the ABT/AZD-induced dimerization of Arg-CRT (Figure 2F).

SphK inhibitors synergize with ABT/AZD to induce c-FLIP_{L/S} depletion upstream of Caspase 8 activation. Casp8 activation is regulated by proteolytic processing through autocatalytic- or cross-processing and by the presence of a ubiquitously expressed endogenous inhibitor, cellular FLICE inhibitory protein (c-FLIP)²⁵. How Casp8 is activated intracellularly during ICD is not clear.

Thus we examined whether levels of c-FLIP were affected by ABT/AZD±PF-543. As shown in Figure 3A, low dose ABT/AZD had minimal effect on levels c-FLIP and PF-543 alone did not affect c-FLIP stability. In combination, however, ABT/AZD+PF-543 almost completely abolished c-FLIP levels in DLD-1 cells leading to Casp8 activation cleavage of the Casp8 substrates Bap31 and BID. Consistent with its role as an inhibitor of Casp8, and therefore upstream of Casp8, inhibition of Casp8 did not affect c-FLIP levels although it did completely block cleavage Bap31 and BID.

Inhibition of Casp8 attenuated formation of CRT dimers, including the Arg-CRT dimer (Figure 3B), induced by ABT/AZD alone and ABT/AZD+PF-543. Interestingly, previous studies have suggested that Caspase 8 activation occurs upstream of Bak/Bax activation and that Bak/Bax exert their effects at the mitochondria⁵. Figures 3A and 3B indicate that Bak/Bax activation may be upstream of c-FLIP depletion and Caspase 8 activation. If this were the case, then Caspase 8 inhibition should attenuate ectoCRT exposure induced by ABT/AZD alone and in combination with PF-543. Indeed, as shown in Figure 3C, inhibition of Caspase 8 dose dependently reduced ectoCRT exposure induced by ABT/AZD alone and in combination with PF-543. These results are consistent with the hypothesis that Bak/Bax induced activation of Caspase 8 occurs at the ER and is enhanced by alteration of sphingolipid levels through inhibition of SphK1 activity.

To better understand whether inhibition of S1P formation or accumulation of Cer might be responsible for the effects of ABT/AZD+PF-543 on c-FLIP stability, we employed the Cer synthase inhibitor, fumonisin B1 (FB1). As shown in Figure 3D, in the presence of FB1, ABT/AZD+PF-543 induced c-FLIP depletion and Bap31 cleavage indicating that synthesis of Cer is not required for depletion of c-FLIP induced by inhibition of SphK1. We further examined the effects of FB1 on dimerization of CRT and observed that FB1 enhanced the formation of dimeric CRT by ABT/AZD+PF-543 (Figure 3E).

The enhancement of CRT dimer formation implies that cell surface exposure of ectoCRT would also be increased by FB1 treatment. However as shown in Figure 3F, FB1 significantly attenuated the cell surface exposure of ectoCRT. This also correlated with a decrease of dimeric CRT in the Triton X-100 insoluble plasma membrane lipid raft fraction in ABT/AZD+PF-543 conditions co-treated with FB1 (data not shown). One possible explanation for this observation would be that translocation of dimeric CRT from the intracellular space to the plasma membrane is altered by inhibition of Cer synthesis. The increase of dimeric CRT under conditions of FB1 treatment could indicate that in addition to cell surface exposure of ectoCRT, a portion of the dimeric CRT is being shed into the culture medium, either directly or inside of exocytic vesicles. Inhibition of transport to the cell surface would block the shedding process resulting in more dimeric CRT retained intracellularly.

Together, these data indicate that inhibition of SphK1 enhances the depletion of c-FLIP leading to Casp8 activation. This suggests that SphK1 has a pro-survival/anti-ICD role to overcome ER stress and prevent ectoCRT production. Furthermore, because cell surface exposure of ectoCRT was attenuated by inhibition of Cer synthesis, this implies that Cer is an important factor required for cell surface transport of ectoCRT. Thus, sphingolipids appear to have biphasic effects as both anti- (S1P) and pro- (Cer) ICD sphingolipids, consistent with their better understood roles in apoptotic cell death.

Inhibition of Inositol Requiring Enzyme 1 alpha (IRE1α) blocks activating phosphorylation of SphK1 and enhances ectoCRT production by ABT/AZD. To better elucidate the role of SphK1/S1P as an inhibitor of the events leading to ICD, we next examined the regulation of SphK1 activity. SphK1 has a constitutive basal activity that is enhanced by phosphorylation at Ser225. To date, Erk1/2 is the only kinase known to phosphorylate SphK1 in response to growth factor stimulation (e.g., TNFα and phorbol esters)²⁶. Thus, we considered the role of Erk1/2 as an activator of SphK1. Preliminary studies indicated that inhibition of Erk1/2 had no effect on the phosphorylation of SphK1 and Ser225 in HEK293 cells over-expressing SphK1 (data not shown).

If Erk1/2 were not responsible for SphK1 activation, we next considered the possible role of kinases involved in ER stress and the unfolded protein response (UPR). Interestingly, SphK1 has recently been shown to form a complex with TRAF2 and IRE1 α in response to ER stress²⁷. IRE1 α is one of the three major arms of the UPR pathway and it possesses both protein kinase and RNase activities. To date, it is thought that IRE1 α autophosphorylation is the only activity of the IRE1 α kinase domain. We tested whether inhibition of IRE1 α kinase activity would affect basal phosphorylation of SphK1 in the HEK293 over-expression system. To our surprise, inhibition of IRE1 α kinase activity with KIRA6 completely abrogated basal SphK1 phosphorylation as low as 2.5 μ M while inhibition of Erk1/2, using PD98059 (10 μ M) had no effect of phosphorylation of SphK1 at Ser225 using anti-phospho Ser225 antibodies (Figure 4A). Inhibition of IRE1 α did not affect levels of total SphK1, implying that IRE1 α or a down-stream interacting kinase was responsible for the bulk phosphorylation of SphK1.

Bak and Bax have been shown to interact with and activate IRE1 α ⁹. This is consistent with the hypothesis that ABT/AZD could activate SphK1, via IRE1 α , to counter-balance the pro-ICD inducing effects of ABT/AZD and thus, inhibition of SphK1 would synergistically/additively enhance the production of ectoCRT by ABT/AZD. We therefore, examined the effects of IRE1 α inhibition on ectoCRT production by ABT/AZD $\pm$ PF-543. As shown in Figure 4B, inhibition of IRE1 α did not induce ectoCRT production alone or in combination with PF-543 demonstrating that insult to the ER (i.e., ER stress or ER Ca²⁺ release) is required for ectoCRT production. ABT/AZD alone, at the concentrations employed, had no effect on ectoCRT production. However, inhibition of IRE1 α (KIRA6) significantly enhanced ectoCRT production. The addition of KIRA6 to ABT/AZD+ PF-543 did not further enhance ectoCRT production consistent with the hypothesis that IRE1 α is up-stream of SphK1 activity and is possibly directly responsible for SphK1 activation.

Interestingly, inhibition of another UPR kinase (protein kinase regulated by RNA (PKR)-like ER kinase (PERK)), one of four kinases that phosphorylate eIF2 α at Ser51, also enhanced ectoCRT production by ABT/AZD. This is in direct opposition to the effect of PERK inhibition on MTX-induced ectoCRT production. As with IRE1 α , PERK inhibition did not further enhance the effects of ABT/AZD+PF-543 on ectoCRT production. These data are consistent with the role of ER stress in induction of ectoCRT/ICD, however, other form of regulated cell death including necroptosis and pyroptosis have been reported to be immunogenic. To preclude the possibility that either of these forms of cell death were induced by ABT/AZD, we employed the necroptosis inhibitor, necrostatin-1 and the pyroptosis inhibitor, disulfiram and examined their effects of ABT/AZD+PF-543 induced ectoCRT production. Inhibition of these regulated cell death pathways had no effect on production of ectoCRT by ABT/AZD+PF-543 consistent with the induction of *bona fide* ICD by targeting Bak/Bax and SphK1.

ABT/AZD induces Cer accumulation only at high concentrations. We previously observed an increase in multiple Cer species in response to combined treatment of MTX+PF-543 in DLD-1 cells². We believe that the observed increase occurs due to ER stress stimulation of CerS 1-6 activity in combination with the accumulation of Sph arising from inhibition of SphK1. Our results above suggest that low concentrations of ABT/AZD may not directly induce ER stress, per se, but may instead induce ER Ca²⁺ release. Other studies have demonstrated that IC₅₀ concentrations of ABT-263 can directly stimulate the production of C16:0 Cer through Bak-mediated activation of CerS activity⁸. Thus, we performed sphingolipidomic analysis of DLD-1 cells with ABT/AZD (subIC₅₀) alone and in combination with PF-543. As shown in Figure 5, PF-543 alone induced the expected decrease in S1P/dhS1P levels and concomitantly induced an increase in Sph/dhSph levels. Cer levels were unchanged by PF-543 alone consistent with our previous studies and with the fact that PF-543 is non-cytotoxic²⁸. Low doses of ABT/AZD alone had minor effects on C18:0 Cer levels, but did not affect other Cer species. In combination with PF-543, ABT/AZD (low) induced increases in C14:0 and C18:0 Cer, however, C16:0 Cer levels were not altered relative to vehicle treatment. As a control for the previously reported increase

in Cer synthesis induced by ABT-263, we also treated cells with ABT/AZD at high doses and observed that they do, indeed, induce increases in Cer species. The fact that low doses of ABT/AZD do not induce Cer synthesis in combination with PF-543 is consistent with our hypothesis that ABT/AZD, at these concentrations, do not induce ER stress but do induce ER Ca^{2+} release. This suggests that ER Ca^{2+} release is the trigger for ICD induction and that SphK1/S1P is cytoprotective under conditions of dysregulated Ca^{2+} homeostasis.

CerS 6 deletion impairs transport of ectoCRT to the cell surface. Fumonisin B1 treatment blocked cell surface translocation of ectoCRT (Figure 3F) indicating that Cer was required for ectoCRT transport. However, our sphingolipidomic analysis indicated that ABT/AZD+PF-543 did not significantly induce Cer accumulation. This suggests that basal levels of Cer species are sufficient for ectoCRT transport. Furthermore, this also indicates that the process that drives ectoCRT transport to the cell surface is a normal cellular function, not one that is directly caused by Cer accumulation. Nevertheless, we next wanted to determine which, if any, Cer species were involved in the process of ectoCRT transport to the cell surface.

Given that ICD is a form of regulated cell death and that C16:0 Cer has long been associated with apoptosis and other forms of cell death, we focused on the effects of C16:0 Cer and the Cer synthases responsible for its production (CerS5 and CerS6). To this end, we generated CerS6 knock-out cell lines in DLD-1 cells using Crispr/Cas9. As shown in Figure 6A, individual clones were confirmed to have deletion of the CerS6 protein and one clone was selected for further evaluation. To confirm that CerS6 KO altered levels of C16:0 Cer production, we conducted sphingolipidomic analysis in wild-type DLD-1 and CerS6 KO clones. As shown in Figure 6B, we observed an ~80% reduction in levels of C16:0 and dhC16:0 Cer indicating that CerS6 is the Cer synthase primarily responsible for the production of C16:0 in DLD-1 cells.

We also generated and selected a CerS5 KO clone for comparison to the CerS6 KO clone (Figure 6C). We next examined the cell surface exposure of ectoCRT in wild-type DLD-1 and CerS5 and CerS6 knock-out clones. Interestingly, the CerS5 KO had higher levels of ectoCRT, particularly in response to ABT/AZD alone. Conversely, the CerS6 KO had significantly less ectoCRT exposure (Figure 6D). Since both CerS5 and CerS6 can produce C16:0 Cer, we considered the possibility that CerS6 may be up-regulated in CerS5 KO cells. As shown in Figure 6E, CerS6 protein levels are increased in the CerS5 KO cell line relative to wild-type DLD-1 cells. This suggests that C16:0 Cer, produced by CerS6, is required for transport of ectoCRT to the cell surface. This observation is in line with our data that inhibition of Cer synthesis blocked ectoCRT exposure without affecting dimerization of intracellular calreticulin.

To exclude the possibility that CerS6 KO might affect early events in the ICD process, we conducted a time course analysis of wild-type DLD-1 and CerS6 KO cells treated with vehicle (V), low dose ABT/AZD (L), low dose ABT/AZD+PF-543 (C) and high dose ABT/AZD (H). Comparing the wild-type DLD-1 and CerS6KO cell lines indicates that C16:0 Cer reduction does not affect the SphK1 inhibitor-induced depletion of c-FLIP, nor the activation of Casp8 as judged by the cleavage of its substrate Bap31 (Figure 6F). Dimerization of intracellular calreticulin also proceeded with the same kinetics in wild-type and CerS6KO cells. Together, these data indicate that C16:0 Cer is selectively involved in the process of transport of ectoCRT from its intracellular source to the plasma membrane.

DISCUSSION

We recently demonstrated that MTX, a well characterized inducer of ICD causes Cer accumulation through recycling of Sph to form Cer. Inhibition of the SphKs blocked conversion of Sph to S1P and further enhanced Cer formation and production of dimeric ectoCRT². Elsewhere, we have demonstrated that synthetic cannabinoids induce ICD through induction of *de novo* synthesis of Cer and that inhibition of the SphKs enhances these effects (manuscript submitted to Cannabis and Cannabinoids Research). Herein, we extend our recent observations that sphingolipid metabolism plays a key role in the induction of ICD and provide evidence for the mechanistic roles of both S1P and C16:0 Cer in the process of ICD.

Inhibition of SphK by itself is non-cytotoxic, however in response to minimal cellular insult (i.e., non-cytotoxic doses of ABT-263 and AZD-5991) SphK activity and the production of S1P becomes critical for cell survival. Our data demonstrate that S1P has pro-survival/anti-ICD function that blocks the earliest steps in the pathway leading to ICD. Inhibition of SphK leads to the depletion of c-FLIP an endogenous inhibitor of Casp8 and induces Casp8 activation in response to ABT/AZD. Importantly, Cer formation was not required for this event indicating that the stabilization of c-FLIP is attributed to S1P.

How S1P stabilizes c-FLIP is unknown at the present time. It is possible that S1P binds directly to c-FLIP. S1P, as a small hydrophobic molecule with a hydrophilic PO₄ head group, could bind to c-FLIP in such a way as to mimic a phosphorylated amino acid (e.g., phosphoSer, phosphoThr or phosphoTyr). Phosphorylation of amino acids commonly affects the stability of proteins leading to or preventing their proteolytic processing²⁹.

Another possibility would be that S1P affects the activity of ubiquitin ligases that regulate c-FLIP stability. To this end, c-FLIP has been recently shown to be linearly ubiquitinated (M1-ubiquitinated) and stabilized by the linear ubiquitin chain assembly complex (LUBAC)³⁰. The activity of LUBAC has been best studied in regulation of TNF α signaling in which heterotypic K63/M1 chains predominate³¹. In fact, studies suggest that K63 ubiquitin oligomers are the preferred substrate of LUBAC. Interestingly, S1P is a co-factor required for the E3 ubiquitin ligase activity of TRAF2, which preferentially produces K63 polyubiquitin chains³². Thus, K63 ubiquitination of c-FLIP, by TRAF2, may be a prerequisite for M1-ubiquitination. In support of this, TRAF2 has been shown to bind to c-FLIP, however, ubiquitination of c-FLIP by TRAF2 was not measured in this study³³.

TRAF2 and SphK1 have recently been identified as interacting partners of the UPR protein IRE1 α and S1P was shown to be critical for NF- κ B activation in response to ER stress²⁷. Consistent with this, we observed that inhibition of IRE1 α kinase domain activity completely abrogates SphK1 phosphorylation at Ser225, an event that increases SphK1 catalytic activity above basal levels²⁶. As mentioned above, ER localized Bak and Bax have been shown to bind to and induce activation of IRE1 α ⁹. Therefore, we propose that at low concentrations, ABT/AZD-induced activation of Bak/Bax leads to IRE1 α activation and phosphorylation of SphK1. SphK1 interacts with TRAF2, which in turn binds S1P, becomes active and mediates K63-polyubiquitination of c-FLIP that nucleates M1-ubiquitination by LUBAC to stabilize c-FLIP. Through this mechanism, minimal activation of Bak and Bax, by low dose ABT/AZD, makes cells dependent on SphK1/S1P-induced stabilization of c-FLIP for their survival. Inhibition of SphK1/S1P blocks heterotypic K63/M1 ubiquitination of c-FLIP leading to K48-polyubiquitination and proteasomal degradation and activation of Casp8 (Diagrammed in Figure 7).

Cer accumulation has been associated with induction of apoptosis for decades. Our recent studies suggested that Cer accumulation also has a role in ICD. In this study, we determined that Cer is

important for transport of CRT from inside the cell to the cell surface. Specifically, C16:0 Cer seems to be required for the cell surface exposure of ectoCRT, but is not required for intracellular generation of dimerized CRT. Interestingly, in contrast to our previous studies, we did not observe an overall increase in Cer levels after combined treatment with ABT/AZD and PF-543. We believe that this is due to the fact that MTX, and possibly synthetic cannabinoids, induce ER stress and/or ROS species generation in addition to ER Ca^{2+} depletion. ER stress/ROS may stimulate Cer synthesis either through the recycling pathway if SphK activity is inhibited or by stimulating the *de novo* synthesis pathway. We believe that ABT/AZD, on the other hand, induce ER Ca^{2+} depletion in the absence of ER stress/ROS.

Even in the absence of Cer accumulation, our data indicate that C16:0 Cer plays a role in ectoCRT transport to the cell surface. Blocking Cer synthesis (i.e., reducing Cer below basal levels) using FB1 and specifically deleting CerS6 both reduced cell surface exposure of ectoCRT. Thus, endogenous levels of C16:0 Cer are sufficient to allow the translocation of ectoCRT to the cell surface. This indicates that the process by which ectoCRT translocates might be a normal cellular process. Previous studies have demonstrated that ectoCRT is transported through anterograde transport pathways. Our data indicate that ectoCRT is NH_2 -terminally arginylated. The addition of Arg to CRT occurs at the mature NH_2 -terminus Glu-18 after ER signal sequence removal. NH_2 -terminal arginylation occurs in the cytosol, rather than the ER lumen, and is catalyzed by arginyltransferase 1 (ATE1)³⁴. Thus, cytosolic CRT is a leaderless-protein. In support of this, Arg-CRT has been localized to the cytosol in association with stress granules. If cytosolic Arg-CRT is the source of ectoCRT, then it is likely that cell surface transport occurs through a mechanism other than anterograde transport.

Leaderless protein secretion occurs normally in cells and is typified by secretion of mature $\text{IL1}\beta$ ³⁵. Recent studies have demonstrated that $\text{IL1}\beta$ is secreted through secretory autophagy (distinct from macroautophagy) and involves a specific set of SNARE proteins (Sec22b (R-SNARE), Syntaxin 3/4 and SNAP23/29 (Qbc-SNARES)). Interestingly, SNAP23 was previously identified as a protein

required for ectoCRT exposure⁵. Thus, it is possible that ectoCRT transports to the cell surface inside of secretory autophagosome vesicles. ectoCRT associated with the cell surface mediates engulfment by antigen presenting cells and induces ICD.

Taken together, our results begin to clarify the roles of S1P and Cer in the process of ICD. Furthermore, they suggest that inhibition of SphK/S1P formation may enhance the efficacy of ICD-inducing agents in vivo. Importantly, this study also demonstrates for the first time that targeting the anti-apoptotic Bcl-2 family proteins Bak and Bax induces ICD. It will be critical to evaluate how induction of ICD affects overall patient response to agents such as Navitoclax (ABT-263) as they proceed toward FDA approval as anti-cancer agents.

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FIGURE LEGENDS

Figure 1: AZD enhances ABT263-induced CRT in DLD-1 cells. (A). Surface exposed CRT (ectoCRT) mean fluorescence intensity (MFI) was determined in vehicle treated (DMSO) DLD-1 cells or cells treated with ABT or AZD for 48hrs, at the indicated doses. (*p=0.0003, **p<0.0001) **(B).** Surface exposed CRT was determined in untreated (DMSO) DLD-1 cells or cells treated with either ABT (1μM) or AZD (1μM) alone or in combination (0.5μM each) for 48hrs (*p=0.0028). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM.

Figure 2. SphK Inhibition enhances the production of an N-terminally arginylated dimeric form of ectoCRT by direct targeting of Bax/Bak with the Bcl-2 family BH3-mimetic inhibitors ABT-263 (Bcl-2 and Bcl-XL) and AZD-5991 (Mcl-1). **A.** In DLD-1 cells, ABT and AZD alone or in combination had no effect on the cell surface exposure of ectoCRT. PF-543 alone also had no effect on ectoCRT exposure as high as 20 μM. In combination with ABT/AZD, PF-543 dose dependently induce cell surface exposure of ectoCRT (*p<0.05). **B.** To determine whether ABT/AZD±PF-543 activated Caspase 8, we examined levels of Pro-Caspase 8 (Full length) and cleavage of the Casp8 substrate Bap31. ABT/AZD and MTX alone induce Casp8 activity and Bap31 cleavage. PF-543 enhanced these effects for both ABT/AZD and MTX (n=3). **C.** ABT/AZD and MTX induce the formation of dimeric CRT (non-reducing conditions –βME) and PF-543 enhances these effects (n=3). **D.** Monomeric CRT migrates as two distinct species (Monomer and Monomer+). The Monomer+ form is arginylated at the N-terminus as determined by antibodies specific for the N-term Arg-CRT form (Millipore ABS1671). Dimeric CRT is predominantly comprised of the N-term Arg-CRT form denoted by Dimer+ suggesting that homodimerization of CRT occurs from a cytosolic form of CRT (n=3). **E.** Activation of Bak/Bax induces pore formation allowing for ER luminal Ca²⁺ leakage to the cytosol. The activity of SERCA can attenuate cytosolic Ca²⁺ levels by replenishing the ER. Inhibition of SERCA with Thapsigargin (TH) enhances ABT/AZD induced ectoCRT exposure

(*= $p < 0.05$). **F.** Inhibition of SERCA activity enhances ABT/AZD induced dimerization of Arg-CRT (n=3). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM.

Figure 3. SphK inhibitors synergize with ABT/AZD to induce c-FLIP_{L/S} depletion upstream of Caspase 8 activation. DLD-1 cells were treated with ABT/AZD (AA; 0.5/0.25 μ M, respectively) $\pm$ PF-543 (5 μ M) for 16h. AAPF induced depletion of c-FLIP_{L/S} and induced activation of Caspase 8 as determined by cleavage of the Casp8 substrates Bap31 and BID. Inhibition of Casp8 (z-IETD-fmk; 10 μ M) blocked Bap31 and BID cleavage but c-FLIP_{L/S} depletion was independent of Casp8 activity suggesting that SphK generated S1P stabilizes c-FLIP under conditions of ER stress (n=3). **B.** ABT/AZD+PF-543 induced dimerization of Arg-CRT was also blocked by Casp8 inhibition (**D+**, **D** dimer and **M+** **M** monomeric forms of CRT are indicated). **C.** Casp8 inhibition dose-dependently reduced ABT/AZD alone and ABT/AZD+PF-543 induced ectoCRT exposure. **D.** Inhibition of Cer synthesis using fumonisin B1 (FB1; 70 μ M) had no effect on ABT/AZD+PF-543 induced depletion of c-FLIP and cleavage of the Casp8 substrate, Bap31. **E.** FB1 treatment enhanced dimerization of cell associated CRT in response to ABT/AZD+PF-543 treatment. **F.** FB1 significantly reduced cell surface exposure of ectoCRT induced by ABT/AZD+PF-543 treatment (*= $p < 0.0001$). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM.

Figure 4. Inhibition of IRE1 α blocks activating phosphorylation of SphK1 at Ser225 in a HEK293 over-expression system. **A.** HEK293 cell over-expressing FLAG-SphK1¹⁶ were treated with the IRE1 α kinase domain inhibitor KIRA6 at the indicated concentrations. Phosphorylation of SphK1 at Ser225 was determined using phospho-specific S225 antibodies (ECM Bio). IRE1 α inhibition completely blocked SphK1 phosphorylation as low as 2.5 μ M. Erk1/2 has been previously demonstrated to mediate SphK1 phosphorylation at Ser225²⁶, however, PD-98059 (20 μ M) had no effect on phosphorylation of SphK1. **B.** Inhibition of IRE1 α (KIRA) and PERK (GSK2606414), two

arms of the UPR, enhanced cell surface exposure of ectoCRT (*= $p < 0.05$). Pyroptosis (Disulfiram) and necroptosis (Necrostatin-1) inhibitors had no effect on exposure of ectoCRT indicating ICD is distinct from these forms of RCD. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM.

Figure 5. Sphingolipidomic analysis of DLD-1 cells treated with ABT/AZD alone or in combination with PF-543. Sphingolipidomic analysis was conducted on DLD-1 cell samples treated with ABT/AZD low (0.5 μ M/0.25 μ M) alone and in combination with PF-543 (5 μ M) or ABT/AZD high (1.0 μ M/0.5 μ M) for 24h, (n=3). Values are presented as pmoles of sphingolipid/ μ g of total cellular protein.

Figure 6. Knock-out of CerS6 attenuates cell surface exposure of ectoCRT. A. Knock-out clones of DLD-1 cells were generated with Crispr/Cas9 gRNAs targeted to CerS6. Knock-out was confirmed by western blot analysis using anti-CerS6 antibodies. Clone #2 was chosen for further analysis. **B.** Sphingolipidomic analysis of CerS6 knock-out clone #2. Sphingolipid levels are expressed as fold change relative to wild-type DLD-1 cells. **C.** Knock-out clones of DLD-1 cells were generated with Crispr/Cas9 gRNAs targeted to CerS5. Knock-out was confirmed by western blot analysis using anti-CerS5 antibodies. Clone #3 was chosen for further analysis. **D.** CerS6 knock-out reduced and CerS5 knock-out enhanced cell surface exposure of ectoCRT determined by flow-cytometry (n=3). Representative results of at least 3 individual experiments. **E.** Western blot analysis of CerS6 expression in CerS5 and CerS6 knock-out clones normalized to GAPDH. **F.** Time course analysis. Wild-type DLD-1 and CerS6 knock-out cells were treated for the indicated times with vehicle (**V**), ABT/AZD low (0.5 μ M/0.25 μ M; **L**), ABT/AZD low + PF-543 (5.0 μ M; **C**) and ABT/AZD high (1.0 μ M/0.5 μ M). Depletion of c-FLIP, cleavage of Bap31 and dimerization of CRT were determined by western blot analysis using appropriate antibodies.

Figure 7. Proposed Model for S1P-mediated pro-survival/anti-ICD signaling. According to our results, ER membrane localized Bak/Bax activation occurs upstream of Caspase 8 activation within the “canonical” ICD pathway. ABT/AZD at high concentrations can directly induce ER lumenal Ca^{2+} depletion and activate Caspase 8 leading to ectoCRT exposure and ICD. At lower, non-cytotoxic concentrations of ABT/AZD, ER membrane localized Bak/Bax induced Caspase 8 activation is counter-balanced by Bak/Bax induced activation of IRE1 α kinase activity. IRE1 α phosphorylates SphK1 at Ser225 to enhance its catalytic activity above basal levels. SphK1, localized to the cytosolic face of the ER membrane, interacts with TRAF2 and produces S1P locally to activate TRAF2 K63 ubiquitin ligase activity to polyubiquitinate c-FLIP. K63-Ub c-FLIP serves as the substrate for M1 linear ubiquitination, by LUBAC, which stabilizes c-FLIP and inhibits Caspase 8 activity. Thus, induction of ectoCRT dimerization, cell surface exposure and ICD are inhibited. Inhibition of SphK1 activity using SKIs such as PF-543 (among others) blocks S1P production and TRAF2 activation leading to c-FLIP degradation/depletion. Caspase 8 auto-activates leading to ectoCRT dimerization/exposure and ICD.

FIGURE 1

FIGURE 2

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FIGURE 5

FIGURE 6

FIGURE 7