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Sphingosine Kinase 1 Inhibitor, A Novel Inducer of Autophagy

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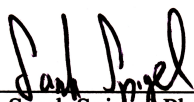
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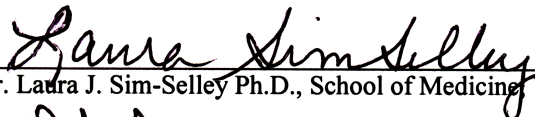
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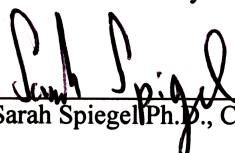
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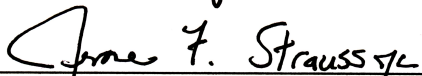
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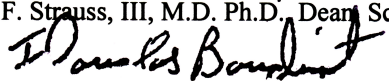
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July 9, 2009

SPHINGOSINE KINASE 1 INHIBITOR, A NOVEL INDUCER OF AUTOPHAGY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Virginia Commonwealth University
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Acknowledgement

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List of Abbreviations

3MA 3 methyladenine

4E-BP1 4E binding protein 1

Akt protein kinase B

AMPKinase 5' adenosine monophosphate-activated protein kinase

ATF6 activating transcription factor 6

Atg autophagy-related gene

BAD Bcl-2-associated death promoter

Bcl-2 B-Cell Lymphoma-2

BH3 Bcl-2 homology domain 3

BNIP3 BCL2/adenovirus E1B 19kDa interacting protein 3

BSA Bovine Serum Albumin

CERT Ceramide Transfer Protein

CHOP C/EBP homologous protein

DAPK death associated protein kinase

DMS N,N dimethylsphingosine

EGF epidermal growth factor

eIF2α eukaryotic initiation factor 2α

ER endoplasmic reticulum

ERK Extracellular Signal-Regulated Kinase

FAPP2 four-phosphate-adaptor protein

FBS Fetal Bovine Serum

FoxO3 Forkhead box O3

GFP Green Fluorescent Protein

Grp78/BiP Glucose Response Protein 78/Binding Immunoglobulin Protein

HDAC histone deacetylase

Hsc70 Heat shock cognate 70

IREα1 inositol-requiring kinase 1

JNK cJun N-terminal kinase

LAMP lysosome associated membrane protein

LC3 microtubule-associated protein light chain 3

MHC major histocompatibility complex

mTOR mammalian target of rapamycin

mTORC mammalian target of rapamycin complex

NF-κB nuclear factor kappa B

PARP Poly(ADP-ribose) Polymerase

PDGF platelet-derived growth factor

PDK phosphoinositide-dependent kinase

PERK protein kinase r-like ER kinase

PI3K Phosphoinositide 3-kinases

PI3P Phosphatidylinositol 3- phosphate

PIP3 Phosphatidylinositol (3,4,5)-trisphosphate

PKC Protein Kinase C

PRAS40 Proline-rich Akt substrate of 40-kDa

Raptor regulatory associated protein of mTOR

Rheb Ras homolog enriched in brain

Rictor rapamycin-insensitive companion of mTOR

ROS reactive oxygen species

Rsk1 p90 ribosomal S6 kinase

S1P sphingosine 1 phosphate

SDS-PAGE sodium dodecyl sulfide–polyacrylamide gel electrophoresis

SERCA Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase

sin1 stress-activated protein kinase-interacting protein

siRNA small interfering ribonucleic acid

SK1-I Sphingosine Kinase 1 Inhibitor

SKD1 Suppressor of K+ Transport Growth Defect1

SNARE soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor

SphK sphingosine kinase

SphK1 sphingosine kinase 1

SphK2 sphingosine kinase 2

TNF tumor necrosis factor

TRAF-2 tumor necrosis factor receptor-associated factor 2

ULK1 Unc-51-like kinase 1

UPR unfolded protein response

UVRAG UV radiation resistance associated gene

VEGF vascular endothelial growth factor

Vps34 vacuolar protein sorting 34

Vti1 vesicle transport through interaction with t-SNAREs

Wt wild type p53

XBP-1 X-box binding protein 1

Abstract

SPHINGOSINE KINASE 1 INHIBITOR, A NOVEL INDUCER OF AUTOPHAGY

By Daniel Meza B.A.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Sarah Spiegel, Ph.D.,
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Autophagy is the process of "cell self-eating" which has been implicated both in cell survival and cell death. Sphingosine kinase 1 (SphK1) regulates the intracellular balance between ceramide and sphingosine, bioactive lipids associated with cell death, and sphingosine-1-phosphate (S1P), whose actions are associated with survival and proliferation. Previous studies have implicated upregulation of SphK1 in the induction of autophagy. In this study, SK1-I, a SphK1 specific competitive inhibitor, induced autophagy in a concentration and time dependent manner in HCT116 colorectal carcinoma cells. This autophagic response was observed to be more intense in wild type p53

expressing HCT116 cells than in p53 null cells and ultimately led to non-apoptotic death in wild type and apoptotic death in p53 null cells. In agreement, cell death in wild type cells was not accompanied by cleavage of polyADP ribose polymerase, a hallmark of apoptosis.

Knockdown of Beclin 1 demonstrated that it and its binding partners do not have a significant role in the induction of autophagy in response to SK1-I treatment. Similarly, mTORC1 signaling was not observed. In contrast, SK1-I markedly decreased Akt phosphorylation. However, this might not be the sole factor important for SK1-I induced autophagy, as pharmacological inhibition of Akt only led to a comparatively weak autophagic response. Indeed, phosphorylation of the endoplasmic reticulum (ER) stress marker eIF2 α, was greatly reduced, suggesting that an ER mediated mechanism also contributes to SK1-I induced autophagy. Thus, SK1-I induced autophagy was likely triggered by ER stress signaling and led to non-apoptotic cell death in the more highly autophagic wild type 53 expressing cells. These results suggest that an isotype specific SphK1 inhibitor might be a useful adjunct for the treatment of cancer or other diseases in which enhancement of cytotoxicity or autophagy is desirable.

Introduction

Autophagy

Autophagy is catabolism of a cell's own contents through lysosomal degradation. Four broad categories of autophagy have been described: microautophagy, crinophagy, chaperone mediated autophagy and macroautophagy[1, 2]. These forms differ in their identification and trafficking of substrates to the lysosome. In microautophagy, the lysosomal membrane directly engulfs its substrate[3]. In crinophagy secretory vesicles fuse with the lysosome[4]. In chaperone mediated autophagy, cytosolic chaperones (Hsc70 complex) deliver proteins directly to the lysosome based on a pentapeptide consensus motif within the substrate[5]. Macroautophagy, the form of autophagy investigated in this study (henceforth referred to as autophagy), involves the formation of a distinctive double membraned vesicle, the autophagosome, which first envelops a portion of intracellular contents including cytosol and organelles[6], matures, then fuses with the lysosome.

Roles of Macroautophagy

Mammalian macroautophagy most commonly occurs in response to nutrient starvation and acts to support cell survival. Amino acid starvation (differing based upon the tissue and amino acid)[7] and growth factor withdrawal [8] have been shown to induce an

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autophagic survival response. At the organismal level, nutrient starvation-induced autophagy is proposed to be hormonally controlled, as, for example, in the liver, where suppression of autophagy by insulin and induction of autophagy by glucagon is observed[9].

It is also well known that basal autophagy plays an important housekeeping role as the primary mechanism for degradation of long-lived proteins[7]. Accordingly, it has been proposed that induction of autophagy could be a treatment for neurological disorders such as Huntington's, Alzheimer's, and Parkinson's diseases by degrading abnormal protein accumulations[10, 11]. Autophagy has also been theorized to support immune major histocompatibility complex 1 and 2 formation through the engulfment and degradation of intracellular pathogens[12].

Observations of autophagy induction by nutrient starvation, and the housekeeping role of autophagy in degrading long-lived proteins have led to the characterization of autophagy as a process of nonspecific mass degradation. Recently, however, it has been noted that mammalian autophagy plays a role in regulating intracellular homeostasis, including that of reactive oxygen species (ROS)[13-16] and attenuating the effects of pathogenic intracellular bacteria and viruses[17]. Proteomic analysis of autophagic cells indicates that degradation of cell contents via autophagy appears to be regulated at the protein complex/organelle level in a manner specific to the conditions triggering the autophagic response[18].

In keeping with this emerging homeostatic, organelle-focused understanding of autophagy, changes in endoplasmic reticulum homeostasis, related to both calcium release and protein misfolding, strongly influence autophagic response[19]. Furthermore, several

autophagy-related proteins appear specifically localized to the ER[18, 20, 21] . These characteristics of autophagy induction and localization will be considered in the following sections and may lend further insight into the mechanisms regulating autophagy.

In opposition to the homeostatic role proposed for autophagy, numerous recent studies have used inhibition of autophagy by the phosphatidylinositol 3-kinase (PI3Kinase) inhibitor 3-methyladenine (3MA) and downregulation of expression of autophagy related proteins to demonstrate that inhibition of autophagy prevents cell death under conditions which normally elicit non-apoptotic death[22] . These observations have been used to assert that autophagy is capable of inducing cell death in a non-apoptotic fashion.

The dichotomous role of autophagy in cell survival has become an important issue in understanding tumorigenesis[23] . Although autophagy appears to suppress tumor development in animals, its pro-survival role during nutrient scarcity may be essential for the survival of quickly proliferating tumor cells. Evidence that autophagy related genes are frequently deleted in cancer, and that cancer cells seem deficient in autophagy, suggest that autophagy is anti-tumorigenic. Several oncogenes, such as Bcl-2 and Akt, have been shown to play a central role in attenuating autophagy, while tumor suppressor genes, such as DAPK and Beclin 1, are pro-autophagic. In addition, chemotherapeutic agents, such as rapamycin have been shown to function via an autophagic killing mechanism[24] . Even so, the possibility exists that the autophagic response in these conditions is engaged to promote survival rather than death.

Autophagosome Formation and Maturation

The structural, enzymatic, and mechanistic elements of autophagy induction are beginning to be understood. These elements have typically been characterized as contributing to one of two phases of the autophagic process: autophagic vesicle formation or autophagosome maturation.

Autophagosome Maturation

While Atg5, LC3, and perhaps Beclin 1 complexes are now considered essential in regulating initial autophagic vesicle formation[25-27], enclosed vesicles undergo maturational changes leading to lysosomal fusion under the regulation of another, possibly distinct, set of factors. During maturation, autophagosomes acquire lysosomal membrane proteins, experience an increase in cholesterol content in their outer membrane and become more acidic[28-31]. It has been proposed that in mammals this maturation process occurs through autophagosome fusion with early and late endosomal vesicles[31]. It has been observed that hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) protein, known to be involved in receptor regulation by endocytosis[32], localizes to autophagosomes and its deletion inhibits autophagolysosme formation and multivesicular endosome formation[33]. Similarly, in mouse, a dominant negative form of suppressor of potassium transport growth defect 1 (SKD1, an ATPase) inhibits fusion of autophagosomes with endosomes causing a deficit in bulk protein degradation[34]. Fusion of autophagosomes with lysosomes in yeast is inhibited by knockdown of Rab7 small GTPase [35, 36] and Vti1p SNARE protein[37]. Lysosome Associated Membrane Protein 1 and 2 (LAMP1 and 2), which constitute the

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majority of lysosomal membrane proteins, are also thought to be involved in autophagosome fusion. However, its knockout increases autophagosome and cholesterol accumulation without attenuating long-lived protein degradation[29]. Throughout this process, microtubules are thought to play a role in the production of autophagosomes. It has been noted that microtubules carry autophagosomes toward the microtubule-organizing center[38]. Furthermore, traffic of autophagosomes requires cytoplasmic, microtubule-associated, histone deacetylase 6[39].

In mammalian cells, autophagic vesicle formation has commonly been associated with the activity and localization of three autophagy related proteins (Atg): Atg5, LC3 (Atg8), and Beclin 1(Atg6). The precise roles of these proteins in forming the autophagosome, however, are not yet well understood.

Atg5

Atg5 is a member of the Atg family first identified in yeast. Atg5 has been shown to be necessary for survival in mice[40] and has recently been implicated in both autophagy and apoptosis (after calpain cleavage)[41]. Atg5 protein capable of conjugation to another Atg protein, Atg12, is necessary for elongation of the isolation membrane (initiation of autophagosome formation) in mammals[25] (Fig. 1).

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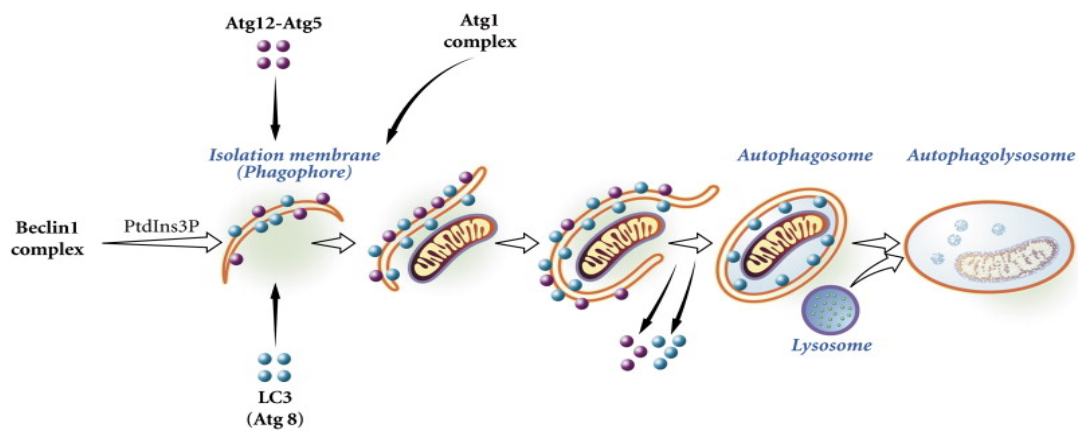
Figure 1: Atg5 and LC3II contribute to early formation of the autophagosome.[42] Beclin1 in complex with type 3 phosphotidyl inositol 3 phosphate kinase produces phosphatidyl inositol 3 phosphate (PtdINs3P), considered necessary for autophagosome formation and implicated in the localization of the isolation membrane. Atg12-Atg15 (autophagy protein) are shown to be necessary for autophagosome formation and act as E3 type ligases for lipidation of LC3I to LC3II. LC3II is necessary for elongation and completion of the autophagic vesicle. Atg5-Atg12 leaves the isolation membrane when it closes to form the autophagosome. LC3II is completely shed and degraded from the autophagosome after fusion with the lysosome.

In this ATP dependent process, Atg12 ubiquitin-like protein is covalently conjugated to Atg5 via the E1-ligase activity of Atg7 and the E2 like activity of Atg10[43-47]. The Atg5-Atg12 complex has been shown to localize to autophagosome precursors (the isolation membrane) and to dissociate from the autophagosome upon closure of the autophagic vesicle (isolation membrane)[48]. Atg5 is in complex with Atg16L and Atg12, yet knockdown of each of these proteins inhibits autophagosome formation at different stages[49]. Nonetheless, this complex has been shown to play necessary role in the next step of this process, LC3 lipidation.

LC3

LC3 is a soluble, microtubule associated protein[50] that was observed to associate with the inner and outer membrane of the elongating autophagosome[26]. For completion of the autophagic vesicle, cytosolic LC3I (Atg4 cleaved LC3) must be covalently conjugated to phosphatidyl-ethanolamine to form LC3II, a ubiquitin-like modification requiring Atg7 and Atg3[51]. In yeast and mouse, the Atg5-Atg12 conjugate functions as an E3-like enzyme for this lipidation reaction[48, 52]. In addition, the Atg5-Atg12 dimer binds Atg16L, homo-oligomerizing to form 800 kilodalton complexes in mammals[53, 54]. The intracellular site of LC3 lipidation appears to be determined by the localization of the Atg16L complex. Ectopic LC3 lipidation can be induced by changes in Atg16L localization[49]. In humans, Atg16L1, a single common coding variant of this protein, predisposes individuals to development of Crohn's disease (an inflammatory illness of the digestive tract) and shows

impairment in the capture of Salmonella within autophagosomes[55]. This LC3 lipidation system is also implicated in lipid droplet formation[56].

LC3II, although targeted to the Atg5-Atg12 associated autophagosomal precursors, is retained in the outer leaflet until autophagosome maturation[36] and is thought to be degraded on the inner leaflet only with lysosomal fusion[57]. As such, it has proven to be an important marker for pre-lysosomal autophagosomes.

Beclin (Atg6)

Beclin1 is a well characterized tumor suppressor gene[27, 58] homoallelically deleted in many human tumors. In breast cancer, Beclin1 haploinsufficiency causes defective autophagy, but restoration of Beclin1 induces autophagy and inhibits tumorigenicity[27]. Beclin 1's autophagic role is thought to be as binding partner of Vps34 type 3 PI3Kinase[59]. The association of Beclin 1 with Vps34 has been shown to induce the production of lipid scaffolding molecule phosphatidylinositol 3-phosphate (PI3P) which is considered necessary for autophagosome nucleation[59]. In yeast, Beclin1 orthologue-produced PI3P has been suggested to recruit proteins required for autophagosome formation based on association with FYVE or PX motifs[60, 61]. In rat cerebral cortex, Beclin 1 appears to localize to membrane rafts and dissociates from them during traumatic brain injury induced autophagic events[62]. In HCT116 colon cancer cells, Beclin 1 localizes to mitochondria and nucleus[63] other eukaryotic cells, Beclin 1 has been observed to localize to the trans-Golgi and ER that serve as foci of PI3P formation[59]. Serine threonine kinase p150 may mediate this membrane association of the Beclin 1 PI3kinase complex[64].

A number of Beclin1 binding proteins regulate the induction of autophagy through their influence on the association of Beclin1 with Vps34. This association is positively regulated by UV radiation resistance-associated gene tumor suppressor (UVRAG) and the activating molecule in Beclin 1-regulated autophagy protein 1 (Ambra1)[65, 66] resulting in increased induction of autophagy. In HCT116 cells, a heterozygous mutation inhibits UVRAG activity and restoration of UVRAG increases autophagy and attenuates cell proliferation[65].

Binding of Bcl-2 to Beclin1's Bcl-2 homology domain (BH3 domain) is a second and critical mechanism by which Beclin1's induction of autophagy is regulated. Binding of Bcl-2 to Beclin1 impedes the association of Beclin1 with Vps34. Furthermore, Bcl-2-binding-defective Beclin 1 mutants induce autophagy leading to caspase-independent autophagic cell death[67]. Specific Bcl-2 localization to the ER blocks autophagosome accumulation in response to Xestospongin B (an inositol 1,4,5-triphosphate receptor (IP3R) inhibitor), vitamin D analogue EB1089 (inducer of ER calcium release), ATP depletion, and starvation[20, 59, 68], whereas localization to other intracellular compartments does not. As such, Bcl-2 has emerged as the principal negative regulator of Beclin1 activity.

Bcl -2 binding to Beclin1 is itself mediated by two mechanisms. The first is competitive inhibition of Bcl-2 Beclin 1 binding by other BH3 proteins. This was demonstrated using Bad and BH3 mimetic compound ABT737. While BH3 binding of Bcl-2 is capable of inducing autophagy of mitochondria, it is not capable of inducing autophagy in the ER (notwithstanding its ability to disrupt ER Beclin1-Bcl-2 complexes)[20]

. The second mechanism by which Bcl-2 binding appears to be regulated is through phosphorylation of Bcl-2. In response to tamoxifen and starvation, Pattingre et al. observed increased accumulation of autophagic vesicles. This was explained by induction of Bcl-2 phosphorylation which caused its dissociation from Beclin 1. Ceramide activation of cJun N-terminal kinase (JNK) was required to phosphorylate Bcl-2 and to stimulate autophagy[69].

Beclin1's presence in the ER also allows it to interact with the IP3R (an ER membrane calcium channel) which, when in complex with Beclin1, appears to inhibit the induction of autophagy. This effect is increased by overexpression and binding of Bcl-2[21]. Furthermore, autophagy induced by pharmacological inhibition of receptor activity or IP3R downregulation due to nutrient starvation, is both Calcium and mTORC1 independent[21].

Interestingly, one case of Beclin 1 independent autophagy has been observed. Scarlatti et al. reported that Resveratrol, a phytoalexin with anti-cancer properties produced by plants in response to bacterial infection, causes an increase in autophagy in MCF7 cells. Knock down of Beclin 1 and Vps 34, and inhibition of Vps34 using specific inhibitor 3 methyladenine (3MA), had no effect on autophagic vesicle formation or degradation of long-lived proteins in Resveratrol treated cells[70].

Signaling Mediators of Autophagy

Potential upstream mediators of autophagosome formation integrate signals encoding the health of the cell and its environment. Two integrative signaling factors have

been shown to significantly correlate with autophagic induction. In many cases, these factors appear closely intertwined, suggesting that their interactions act as the cellular circuitry for deciding whether or not to mount the autophagic response. The first is mammalian target of rapamycin (mTOR).

mTOR

mTOR has been characterized as forming two complexes: mTORC1 and mTORC2[71, 72]. mTORC1 is composed of raptor (scaffolding protein), PRAS40 and mLST8[73]. Activity of the complex is directly inhibited by treatment with rapamycin which binds raptor and disrupts its interaction with mTOR[74].

mTORC1 is thought to integrate a number of nutrient and growth factor pathways including those containing Akt[75], ERK[76], AMP kinases[77] and Rsk1[78]. Sancak[79] theorized that in response to signaling events upstream, Rag small GTPase complex binds to Raptor which stimulates mTOR by changing localization of mTORC1 to perinuclear endomembrane structures where mTORC1 can be activated by Rheb small GTPase. The specific mechanism of activation is unknown. The direct substrates of mTORC1 are p70S6Kinase, 4E-BP1[80,81], and Pras 40[82]. P70S6 kinase, in turn, is the major protein kinase responsible for mTOR ser-2448 phosphorylation[83].

Direct mTORC1 inhibition by rapamycin has been shown to induce autophagy. mTORC1 inhibition is necessary for nutrient starvation induced autophagy[84-86]. Elevated mTORC1 activity results in cells with impaired ability to initiate autophagy in response to nutrient starvation[85]. Reduced mTORC1 activity has been observed in autophagic cells as

a result of ER stress associated with calcium activated AMPK signaling[68], and JNK upregulation[87], elevated SphK1 expression[88], elevated ceramide levels[69], and Safingol a SphK1 and PKC inhibitor[89]. Nonetheless, changes in mTORC1 activity have not been observed in autophagy induced by PKCθ in response to calcium[90], or IP3R in response to reduction in IP3R expression[91].

mTORC2 is composed of mTOR, rictor, mSin1, mLST8 and PRR5[92-95]. In response to growth factors but not nutrients, mTORC2 phosphorylates Akt on serine 473 [95] (this may be a separate population of Akt than that upstream of mTORC1). In addition, turn motif phosphorylation involved in folding of Akt and conventional PKC is dependent on mTORC2 function[96]. Overall, mTORC2 activity has been primarily associated with actin cytoskeletal rearrangement involving PKCα and GTPases Rho and Rac which signal through unknown effectors[97, 98]. Recently mTORC2 has proven to have direct relevance to autophagy as well. mTORC2 inhibition induces FoxO3 transcription of BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3)[99], a BH3-only protein[100] whose upregulation is necessary and sufficient for induction of autophagy.

Endoplasmic Reticulum

The endoplasmic reticulum also appears to have a central role in regulating autophagy (Fig 2). In addition to localization of Beclin and its binding partners to the ER, changes in ER homeostasis mediated by factors regulating the unfolded protein response and ER calcium balance, also induce autophagy. The ER is a central regulatory apparatus for protein folding and trafficking, intracellular calcium homeostasis, and synthesis of

unsaturated fatty acids, sterols and phospholipids. Disruption of any of these functions results in ER stress[101]. Increasingly, the sensors of ER homeostasis have been implicated in the induction of autophagy. Homeostatic imbalances of the ER are caused in different ways by a variety of pharmacological agents: thapsigargin disrupts ER calcium homeostasis, tunicamycin inhibits protein n-glycosylation, Brefeldin A blocks protein transport to the Golgi, polyglutamine proteins form protein aggregates [102] and long chain lipid molecules, such as palmitate[103], induce decreases in luminal calcium[104]. In response to these insults, three membrane proteins act as parallel sensors triggering transcriptional effects: protein kinase r-like ER kinase (PERK), which phosphorylates eukaryotic initiation factor 2α (eIF2α) and induces C/EBP homologous protein (CHOP) transcription[105], inositol-requiring kinase 1 (IREα1), which binds tumor necrosis factor receptor-associated factor 2 (TRAF-2) inducing splicing of XBP-1 transcription factor and stimulating JNK activity[101], and activating transcription factor 6 (ATF6), which translocates to the nucleus and induces transcription of ER-specific member of heat shock protein 70 family Grp78/BiP (Glucose Response Protein 78/Binding Immunoglobulin Protein)[106]. Each appears to have differential sensitivity to stress stimuli, PERK being activated most quickly in response to calcium changes, PERK and IREα1 responding equally quickly to protein misfolding, and ATF6 responding slowly to protein misfolding[107]. It has been theorized that ER chaperone GRP78/BiP mediates this effect through its binding inhibition of PERK, IREα1 and ATF6. BiP is also required for stress-induced autophagy[108].
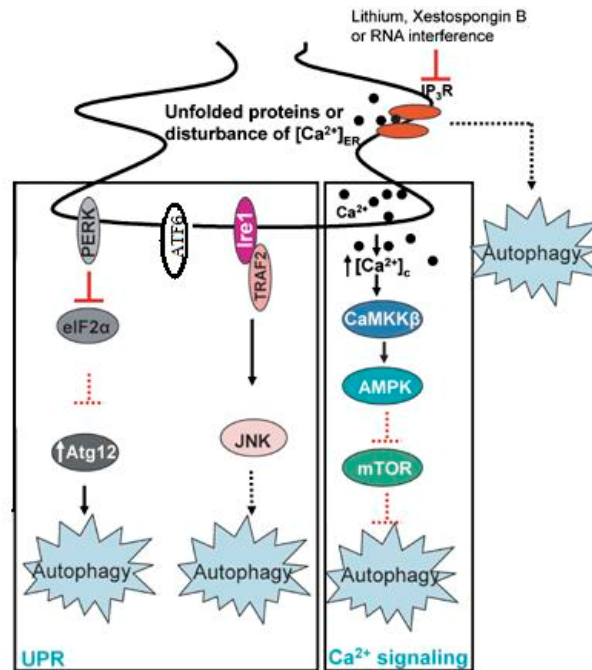
Figure 2: ER stress pathways implicated in autophagy[109] There are three ER stress responsive proteins whose activity appears to be affected by ER calcium levels, accumulation of misfolded proteins, inhibition of transport from the ER etc., disruptions of ER equilibrium which are known to result in changes in transcription that define ER stress. They are: protein kinase r-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring kinase 1 (IRE1). While ATF6 knockdown has not been shown to affect autophagy in response to ER disequilibrium, knockdown of PERK and IRE1, along with eIF2alpha and JNK downstream targets, has been shown to inhibit autophagy and are therefore thought to be necessary for autophagy in response to different forms of ER stress. Independent of these three proteins are autophagic responses to cytosolic calcium increases which can be induced by calcium release from the ER and are calcium/calmodulin dependent kinase kinase-beta (CaMKKBeta), AMP kinase, and mTOR dependent. Direct inhibition of ER-bound inositol (1,4,5) triphosphate receptor also appears to induce autophagy.

Both PERK and IREα1 have been found necessary for induction of autophagy. In one case, PERK and eIF2α were required for autophagic response induced by aggregation of polyglutamine repeat proteins, but IREalph1 and ATF6 were not[102]. Alternatively IREα1, TRAF2 and JNK were required for autophagy in response to thapsigargin and tunicamycin, while PERK and ATF6 were not[110].

It has been proposed that increased cytosolic calcium due to ER calcium release is responsible for autophagy signaling by the ER[68]. Calcium release from the ER can be induced by a number of different agents, including vitamin D analogues, thapsigargin inhibition of ER calcium transporter (SERCA)[111], ATP induced opening of IP3R channels[68], and accumulation of unfolded proteins in the ER lumen[112].

One explanation of ER calcium release induced autophagy attributes regulation of this response to calcium/calmodulin-dependent kinase kinase-beta activation of AMPK that leads to inhibition of mTORC1[68].

Another possibility is PKCθ phosphorylation which is required for the ER-stress induced autophagic response but unnecessary for amino acid starvation induced autophagy. PKCθ phosphorylation induces autophagy in a calcium dependent manner independent of mTORC1. PKCθ phosphorylation is not dependent upon ER sensors PERK, IRE1 or ATF6, but its autophagic effect is inhibited by knockdown of IRE1 and eIF2α[90].

Increases in cytosolic calcium also activate the death associated protein kinases (DAPK)[113] and calpain proteases[114], both of which induce autophagosome formation.

Sphingosine-1- Phosphate (S1P)

S1P is a bioactive lipid signaling molecule containing a sphingoid long chain base attached to a phosphate group. S1P has been shown to have a remarkable array of roles within the cell. Once considered only the end metabolite of all sphingolipids, it is now known to regulate cell growth[115, 116], suppress apoptosis[117], contribute to cytoskeletal rearrangements and cell motility[118-120], invasion, angiogenesis and vascular maturation[121, 122], and trafficking of lymphocytes[123]. It has also been proposed to play an important role in the regulation of intracellular calcium[124], which may contribute to its varied influences. By contrast, its precursors, ceramide (N-acylsphingosine) and sphingosine are associated with cell-growth arrest, stress responses, and apoptosis. Thus a regulatory balance has been theorized between S1P and ceramide[125].

Many of the effects of S1P are mediated by five G-protein coupled receptors ($S1P_{1-5}$) for which S1P and dihydroS1P are the high affinity ligands[125]. The varying effects of S1P have been ascribed to the differential distribution of these receptors on each cell and their differential coupling to G proteins. $S1P_1$ couples to G_i[126], $S1P_{3-5}$ to G_i or $G_{12/13}$ and $S1P_2$ may couple to G_s, G_q or $G_{12/13}$[127]. This engages signaling cascades which involve small GTPases[128], adenylate cyclase[127], inhibition of phospholipase C [129] or inhibition of Akt and ERK1/2 phosphorylation[130, 131]. In addition, cells are capable of secreting S1P, which is thought to act in an autocrine/paracrine fashion on these receptors[132]. Little, however, is known about S1P's intracellular targets.

S1P is not biosynthesized de novo and can only be formed from sphingosine produced during turnover of complex sphingolipids by phosphorylation catalyzed by two

sphingosine kinase isoenzymes, SphK1 and SphK2 (Fig. 3). De novo ceramide synthesis begins with the rate limiting condensation of L-serine with palmitoyl-CoA at the cytosolic leaflet of the ER[133]. The product, 3-keto-sphinganine, is reduced and N-acylated to dihydroceramide. A trans 4,5 double bond is inserted into dihydroceramide by desaturase to form ceramide[134]. Inhibition of this desaturase produces non-Beclin dependent autophagy[135]. Ceramide production can be induced by hypoxia, various pharmacological agents and tumor necrosis factor α[136, 137]. Ceramides are shuttled to the Golgi by CERT, a cytoplasmic protein with a phosphatidylinositol-4-phosphate binding domain[138]. Ceramides are then converted into sphingomyelins by sphingomyelin synthase on the lumenal side of the Golgi, or to glucosylceramides (whose accumulation is associated with lysosomal storage disorders) on the cytosolic surface of the Golgi[139].
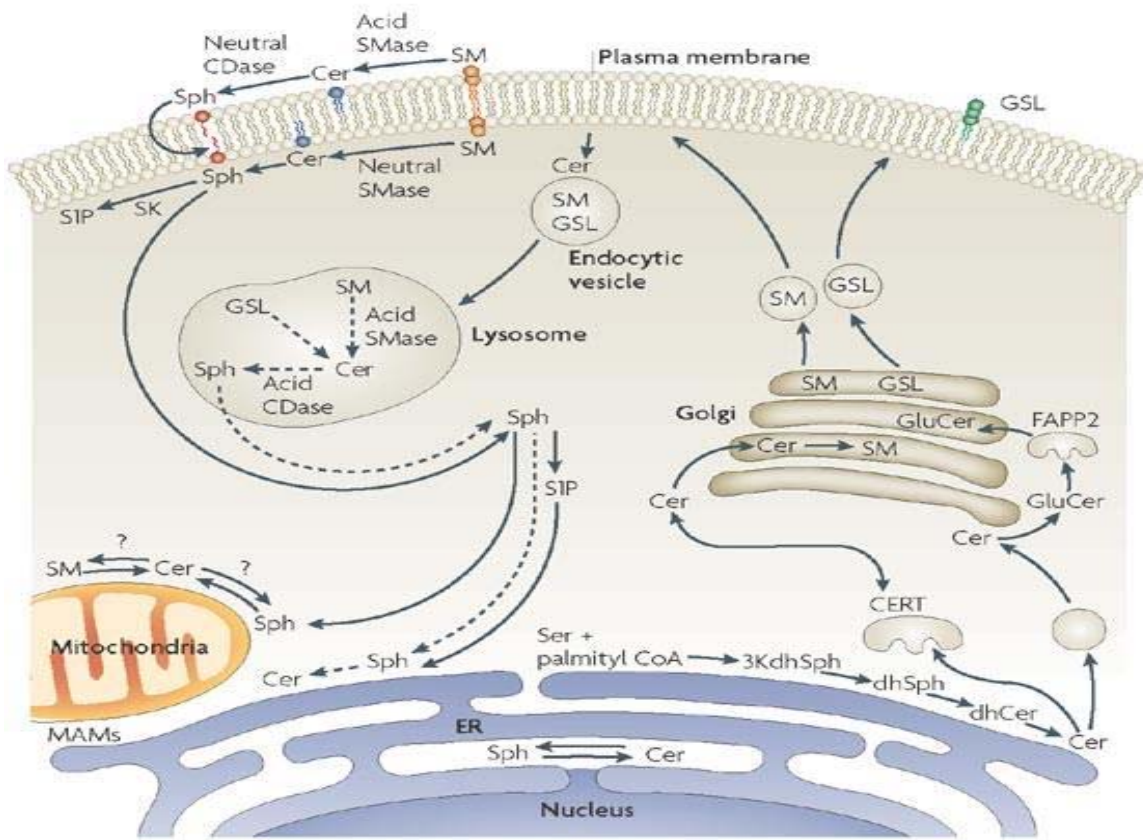
Figure 3: Sphingolipid Synthesis [140] Sphingosine 1 Phosphate (S1P) is the product of sphingosine (Sph) phosphorylation by sphingosine kinases (SK) which occurs at the plasma membrane and may also occur at other intracellular locations. Sph is the result of removal of a fatty acid chain from ceramide (cer). Ceramide is synthesized via sphingomylin (SM) recycling by sphingomylinases (SMase) at the inner and outer leaflets of plasma membrane, lysosome, and perhaps other sphingomylin containing membranes (such as of the mitochondria) and via a denovo synthesis pathway involving the ER (which begins with condensation of L-serine, Ser, with palmitoyl-CoA, palmityl CoA, to produce 3-keto-sphinganine, 3KdhSph, which is reduced to dihydrosphingosine ,dhSph, and N-acylated to dihydroceramide, dhCer, which is desaturated to ceramide). Ceramide from denovo synthesis is transported to the Golgi via CERT and between golgi compartments via FAPP2. Golgi ceramide is modified to form SM and glucosylceramides (GluCer) used to form other glycosphingolipids (GSL).

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GlcCer is transported between Golgi compartments by FAPP2[141]. Sphingomyelin, which is localized to cell membranes throughout the cell, is an important structural lipid and serves as a reservoir for ceramide for the synthesis of sphingosine and S1P. This function is performed by neutral, acid, and alkaline sphingomyelinases[142] and is rapidly increased by chemotherapeutic agents, ultraviolet irradiation and other cell stresses[143, 144]. To produce sphingosine, ceramidase removes the fatty acid chain from ceramide[142]. The ATP dependent synthesis of S1P follows, accomplished by SphK1 and SphK2. Ultimately, S1P is dephosphorylated back to sphingosine by S1P phosphatase isotype 1 or 2 which reside in the ER[145, 146]. S1P can also be degraded by an integral ER membrane protein, S1P lyase, forming hexadecenal and phosphoethanolamine which is reused for the biosynthesis of phosphatidylethanolamine[147].

SphK2

The most recently identified isotype of sphingosine kinase is SphK2. Although we will be considering the role of a SphK1 specific inhibitor, SphK2 is of interest as it appears to play both an opposing and complementary role to SphK1 in survival[148]. SphK2 is larger and has a different amino terminus and central region than SphK1[149]. Furthermore, it possesses a BH3 like Bcl-2 family binding domain implicated in inducing apoptosis[150]. SphK2b splice variant expression and nuclear translocation is increased in response to serum deprivation and causes induction of apoptosis[151]. SphK2 is less substrate specific than SphK1, phosphorylating substrates other than sphingosine including FTY-720, converting this pharmacological agent into a S1P receptor agonist[152-154]. Lastly, SphK2

overexpression and downregulation results in inhibition of cell growth and induction of apoptosis[155, 156].

SphK1

In contrast to SphK2, SphK1 has been implicated in the pro-survival, proliferative effects of S1P that were previously described. Owing to studies of basal SphK1 activity and attenuation of growth response in SphK1 mutants unable to localize to the plasma membrane, it seems likely that SphK1 effects are mediated by its membrane localization[157, 158]. Phosphorylation on Ser225 by ERK2 is essential for this membrane affinity[159]. This is consistent with the observation that sphingosine, SphK1's substrate, resides in the plasma membrane. SphK1 is stimulated by a wide variety of plasma membrane receptor ligands, particularly growth factors, including platelet-derived growth factor (PDGF)[160], vascular endothelial growth factor (VEGF)[161], epidermal growth factor (EGF)[162], and hepatocyte growth factor[163], as well as cytokines, tumor necrosis factor α[164], hormones[165, 166], GPCR ligands, such as lysophosphtidic acid [167] and S1P[168], as well as other ligands[169]. ERK1/2 phosphorylation by TNFα also appears to require SphK1 activity[170]. In addition, SphK1 directly interacts with TRAF2 (for TNF induced NF-κB activation protecting against apoptosis)[171], Calcium/calmodulin (which is important for SphK1 agonist induced membrane translocation)[172], platelet endothelial cell adhesion molecule 1[173], delta catenin[174], aminoacylase[175], SphK1 interacting protein[176], and RPK118[177]. Specific knockdown of SphK1 by small interfering RNA triggers apoptosis in multiple tumor cell lines[178-182]. Effector caspase and cytochrome c release was induced as well as significantly

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increased levels of ceramide and sphingosine in cells with suppressed SphK1 expression[179]. Thus SphK1 is implicated as a regulatory molecule in the signaling of a number of different pro-survival signaling cascades and its knockdown often leads to apoptotic cell death.

Sphingolipid metabolism and autophagy

Elements of the S1P metabolic pathway also have a role in inducing autophagy. Increases in ceramide associated with tamoxifen treatment result in accumulation of Beclin1, inhibition of Akt phosphorylation and autophagy. This effect is inhibited by pharmacological inhibition of long chain ceramide production by fumonisin B1 and replicated by short cell-permeant ceramide[183]. In addition, mTORC1 activity is inhibited, however in contrast to nutrient starvation, Bcl-2 overexpression does not inhibit autophagy[183].

Interestingly, SphK1 overexpression is also capable of inducing autophagy. In this case, while mTORC1 activity is inhibited, there is little change in beclin1 expression and no decrease in Akt phosphorylation. Nor is the amount of endogenous ceramide shown to change. These effects are inhibited by SphK1 RNA interference and the pan SphK inhibitor N,N-dimethylsphingosine (DMS) which in turn increases cell death with PARP cleavage. In cells undergoing nutrient starvation, upregulation of SphK1 was also observed[88]. It therefore appears that increases in ceramide result in autophagy associated with cell death while SphK1 activation and increased S1P formation may serve to protect cells.

Pharmacological inhibitors of SphK1 have also been shown to induce autophagy. Although not a SphK1 specific inhibitor, Safingol, a synthetic stereoisomer of sphinganine, has been shown to inhibit PKC and SphK. Safingol treatment of the HCT116 colon cancer cell line induces autophagy followed by cell death with concomitant decreases in Akt phosphorylation, ERK phosphorylation and mTORC1 activity. Safingol did not however induce increases in ceramide, S1P or dihydroceramide and autophagy induced by PKC depletion with siRNA was not of comparable intensity to that induced by Safingol [89]. Thus, the activity of S1P appears central to cell survival and its biosynthetic pathways overlap cell structures sensitive to autophagic induction.

SK1-I

SK1-I is a water soluble sphingosine analogue shown to competitively inhibit S1P production by SphK1 without inhibiting the catalytic activities of SphK2, PKC or a number of other protein kinases (Fig 4). SK1-I decreases growth and survival in human leukemia U937 and Jurkat cells and caspase inhibitors and overexpression of Bcl-2 protect against apoptosis induced by SK1-I[184].
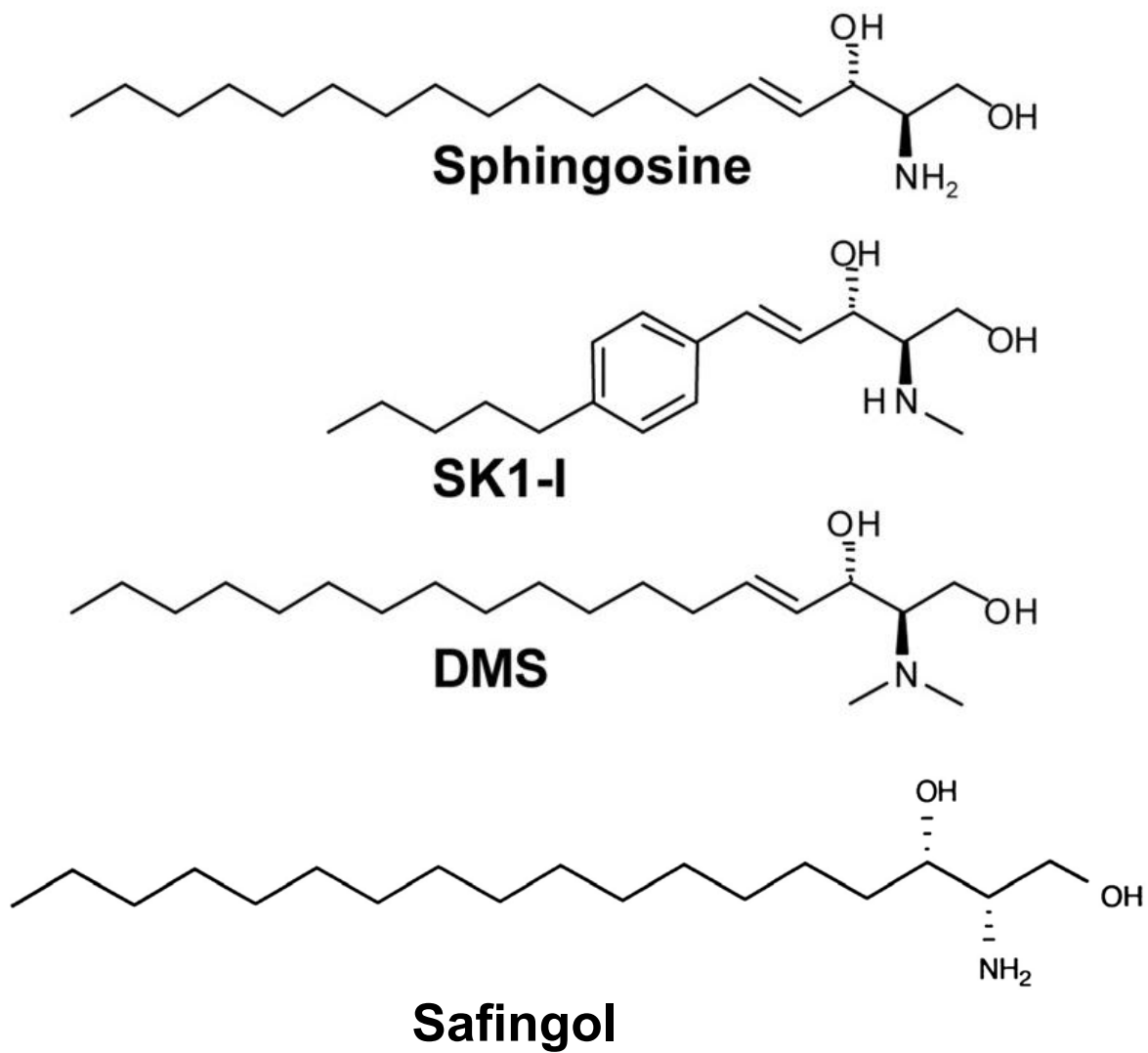
Fig4: Structure of SK1-I [184]

Statement of Intent

Autophagy is the process of "cell self-eating" thought to provide essential nutrients to the cell under conditions of nutrient restriction. Recent observations, however, suggest that significantly elevated rates of autophagy induce apoptosis-independent cell death. Sphingosine Kinase 1 (SphK1) catalyzes the synthesis of sphingosine 1 phosphate, a bioactive lipid signaling molecule which has been shown to suppress apoptosis and stimulate cell growth, proliferation and motility. Moreover, it has been shown that SphK1 induced autophagy protects cells from apoptosis during nutrient starvation[88]. This study seeks to determine whether Sphingosine Kinase 1 Inhibitor (SK1-I), a specific inhibitor of SphK1, regulates autophagy, to identify the mechanism of its action, and to evaluate the impact of SK1-I induced autophagy on HCT116 colon cancer cell viability.

Materials and Methods

Materials

SK1-I, (2R,3S,4E)-*N*-methyl-5-(4'-pentylphenyl)-2-aminopent-4-ene-1,3-diol (BML-258), was synthesized as the HCl salt by BIOMOL International (Plymouth Meeting, PA). Propidium iodide for membrane integrity analysis and bisbenzimide Hoechst 33342 for nuclear staining were obtained from Sigma Aldrich (St. Louis, MO). LY94002 was obtained from Biomol (Plymouth Meeting, PA).

Cells

Wild type and p53 null HCT116 human colorectal carcinoma cells were obtained from American Type Culture Collection (Manassas, Va). Cells were cultured and maintained in logarithmic growth phase in Dulbecco's Modified Eagle Medium 11995 containing 4,500 mg/L D-glucose, L-glutamine, and phenol red (DMEM, Invitrogen, Carlsbad CA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad CA). During SK1-I treatment, cells were cultured in DMEM supplemented with 0.1% fatty acid free BSA (Sigma Aldrich, St. Louis, MO).

Cell Transfection

Cells were plated on poly-L-lysine covered glass coverslips in a 6 well plate at 1 million cells per well. After 24 hours, cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad CA). Cells were transfected with pEGFP-C1-rLC3 plasmid (kindly given by T. Yoshimori, National Institute of Genetics, Mishima, Japan). 24 hours after transfection, cells were cultured in DMEM containing 0.1% BSA and treated with SK1-I as indicated in figure legends.

RNA interference

Cells were plated on poly-L-lysine covered glass coverslips in a 6 well plate at 300,000 cells per well. After 24 hours, cells were transfected using Oligofectamine according to the manufacturer's protocol (Invitrogen, Carlsbad CA) with either 100 nM Smartpool siRNA for Atg5 (Thermo Scientific Dharmacon, Lafayette, CO) or 100nM siRNA for Beclin 1 (Qiagen, Valencia, CA). Cells were transfected with pEGFP-C1-rLC3 24 hours after siRNA transfection using Lipofectamine 2000. 24 hours later, cells were then cultured in DMEM containing 0.1% BSA and treated with SK1-I as indicated in figure legends.

Confocal Microscopy

Cells grown on glass cover slips were fixed using 3.7% paraformaldehyde (Sigma Aldrich, St. Louis, MO) in phosphate buffered saline. Cover slips were mounted using 10 nM N-propyl gallate (Sigma Aldrich, St. Louis, MO). Images were acquired with a Zeiss LSM 510 confocal microscope with a 63x objective lens. Excitation (ex.) and emission

(em.) filter settings were: GFP ex. 488 nm, em. long pass 505. The number of GFP-LC3 positive cells with more than 5 GFP-LC3 dots was determined. Quantification was performed by Dr. Sandrine Lépine.

Western blot analysis

Adherent and detached cells were collected and resuspended in cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate, 1 mM sodium orthovanadate, 1%Triton X-100, Sigma protease inhibitor cocktail). Lysates were sonicated and centrifuged. After protein quantification (Bio-Rad, Hercules, CA), equal amounts of protein were separated by 10% sodium dodecyl sulfide–polyacrylamide gel electrophoresis (SDS-PAGE) and then transblotted to nitrocellulose. Blots were incubated with primary antibodies overnight in Tris-buffered saline (TBS) containing 3%BSA.

The following were used as primary antibodies at 1:1000 dilution (except in the case of Phospho-Akt, 1:500): Phospho-Akt(Ser473) (rabbit polyclonal), Akt (rabbit polyclonal), Phospho-mTOR(Ser2448)(rabbit polyclonal), mTOR (rabbit polyclonal), Phospho-Erk1/2 (Thr202/Tyr204) (rabbit polyclonal), Erk2(rabbit polyclonal), Phospho-eIF2α (Ser 51) (rabbit polyclonal), Clathrin Heavy Chain (rabbit polyclonal) (Cell Signaling, Beverly, MA), Beclin (mouse polyclonal) (BD Pharmingen, San Diego, CA), and anti-PARP (rabbit polyclonal) (BIOMOL International), APG5L (rabbit polyclonal) (Abgent, San Diego, California), LC3 (rabbit polyclonal) (Novus Biologicals, Littleton, CO).

After washing, cells were incubated for one hour at room temperature with peroxidase conjugated anti–rabbit or anti-mouse IgG (1:10 000; Jackson Immunoresearch Laboratories, West Grove, PA). Immunocomplexes were visualized using Super Signal West substrate (Thermo Fisher Scientific, Rockford, Illinois) with Kodak (Rochester, NY) or Phenix Research Products (Candler, NC) X-ray film.

Cell Death Assay

Cells were plated at four hundred thousand cells per well in a 6 well plate. Cells were given 24 hours to adhere. Cells were then cultured in DMEM containing 0.1% BSA in the absence or presence of 10 μM SK1-I for 24 hours. Apoptotic and necrotic cells were quantified by fluorescence microscopy after labeling with Hoescht 33342 (10 μg/ml) and propidium iodide (50μg/ml). Cells were visualized with a Nikon eclipse TE300 fluorescence microscope. Cells exhibiting blue condensed or fragmented nuclei or red condensed nuclei were considered apoptotic. Red nuclei without signs of condensation or fragmentation were considered necrotic.

Statistical Analysis

For each experiment, data from samples were calculated and expressed as the mean plus or minus standard deviation (SD). The significance of differences between experimental conditions was determined using the Student *t* test for unpaired observations.

Results

SK1-I Induces autophagy in a time and dose dependent manner

Previous studies have shown that upregulation of SphK1 expression associated with increases in S1P result in increased autophagic vesicle formation[88]. As such, it was of interest to investigate the effect on autophagy of the isotype specific sphingosine kinase 1 inhibitor, SK1-I[184].

In this study we used HCT116 wild type p53 and p53 null cells as our model system. First we verified that p53 null cells did not express p53. This was demonstrated by western blot (Fig 5A) of p53.

In order to visualize autophagosome accumulation, we observed changes in localization and lipidation of LC3, a protein whose attachment to the isolation membrane (unenclosed autophagosomal precursor) is considered necessary for autophagosome formation[51]. Shortly after translation, LC3's carboxy terminal region is cleaved creating LC3I. Upon induction of autophagy, LC3I is lipidated with phosphatidylethanolamine forming LC3II. LC3II specifically localizes to the developing autophagosome membrane [51,185]. GFP-LC3 also undergoes this process thereby labeling autophagosomal structures with GFP in a manner consistent with direct immunohistochemical staining[185]. Thus, in order to determine whether SK1-I induces autophagosome formation, we transfected HCT116 colon cancer cells with a plasmid expressing GFP conjugated LC3 and autophagy was quantified using confocal microscopy. In vehicle treated cells in which no autophagy

was induced, GFP staining appeared as a diffuse light green fluorescence present in the cytosol. In cells with autophagy, GFP fluorescence appeared as concentrated green puncta. One limitation of this method is that GFP-LC3 is prone to form aggregates which resemble GFP-LC3II puncta[186]. Furthermore, cells are expected to engage in some basal level of autophagy. Therefore, we quantified the number of cells with more than GFP-LC3 puncta in a double blind manner. We observed that SK1-I induced an elevated level of autophagic vesicle formation in a time and dose dependent manner (Fig. 5). Because p53 has been shown to be involved in autophagy[187], we examined whether the effect of SK1-I on autophagy was influenced by the presence of p53 expression. Both wild-type and p53 null HCT116 cells had significant levels of autophagy within 3 hours of exposure to 10μM SK1-I (Fig 5b). 35 percent of cells expressing wild type p53 and 32 percent of p53 null cells were autophagic. Wild type cells continued to become increasingly autophagic until approximately 24 hours at which time 67 percent were autophagic and rates leveled off. p53 null cells became increasingly autophagic until 12 hours at which time approximately 40 percent of cells exhibited maximal autophagy (Fig. 5b). Similarly, significant increases in response to SK1-I concentration were observed in both wild type and p53 null cells when cultured in 10μM SK1-I for 6 hours as compared to cells cultured at 3 or 5μM SK1-I (Fig 5c). At 10μM SK1-I, 41 percent of wt cells and 23 percent of p53 null cells were autophagic. Thus there was a significantly higher percentage of punctate staining in wild type cells than in p53 null cells in response to SK1-I treatment (Fig. 5).

SK1-I induction of autophagy was confirmed using western blot (Fig 6). As a result of its lipidation, LC3II has greater mobility than LC3I in SDS-PAGE[185]. Increases

in LC3 lipidation and thus accumulation of autophagosomes can therefore be evaluated based on the differential induction of LC3I and LC3II in response to treatment. LC3 lipidation increased as a function of SK1-I time and dose as seen by an increase in LC3II intensity relative to LC3I (Fig. 6). Interestingly, in SK1-I treated cells, earliest increases in lipidation were observed at 15 minutes (Fig. 6a). The lowest concentration at which SK1-I elicited a response appeared to be 10μM (Fig. 6b). As such, 10μM SK1-I was used for all other experiments.

SK1-I does not trigger classical Beclin 1 or mTORC1 signaling

Increases in Beclin1 protein have commonly been associated with autophagy induction[27, 42, 183]. We therefore examined changes in Beclin1 protein expression during the three hour period in which autophagy was seen to be initiated. Western blot of whole cell lysates showed that Beclin 1 protein levels were not upregulated in HCT116 wt or p53-/- cells after exposure to SK1-I (Fig. 7 A, B). Previous studies have also shown that in cases in which Beclin1 protein levels do not change, interactions with other protein factors mediate autophagic induction in a Beclin1 dependent fashion (UVRAG[65], Ambra[66], Bcl-2[67]). To eliminate this possibility, Beclin1 protein expression was downregulated using short interfering RNA for Beclin1. The efficiency of Beclin 1 downregulation was confirmed by western blotting for Beclin1 protein (Fig 8). Examination of LC3 lipidation by western blot revealed that downregulation of Beclin 1 did not attenuate induction of autophagy with SK1-I (Fig. 8). Beclin1 and its interactions with other regulatory proteins

(i.e. UVRAG, Ambra, or Bcl-2) was therefore considered unlikely to mediate autophagy induction by SK1-I.

Next, the role of rapamycin sensitive mTOR complex (mTORC1) was considered. mTORC1 integrates numerous growth factor and nutrient signaling pathways and regulates the transcriptional response to these pathways[188]. Most commonly, mTORC1 is considered to be downstream of Akt and ERK [75, 76, 189] although the exact mechanism of its activation is unclear. In mammalian cells, inhibition of mTORC1 is sufficient to induce autophagy and is required for autophagy due to nutrient starvation[84-86].

Changes in mTORC1 activity were evaluated by blotting for Phospho-mTOR(Ser2448). During the first three hours of SK1-I treatment (when there is a significant increase in autophagy), mTOR phosphorylation was not seen to be reduced relative to control (Fig. 9). We therefore conclude that there is no significant change in mTORC1 activity in response to SK1-I treatment.

Non-canonical mTORC2 signaling is observed

The downstream signaling of the rapamycin insensitive mTOR complex (mTORC2) was then examined. mTORC2 downregulation was shown to induce FoxO3 nuclear translocation in transfected myofibers, which correlated with expression of autophagy inducer BNIP protein. A resulting increase in autophagosome formation was observed independent of mTORC1 inhibition[99].

mTORC2 has been identified as the primary complex to phosphorylate Akt on Ser473[95]. Phosphorylation of Akt ser473 was evaluated by western blot as a downstream

target of mTORC2 activity. It was observed that phosphorylation of Akt at Serine 473 was consistently lower in SK1-I treated cells than in vehicle treated cells (Fig. 10a). Furthermore, induction of LC3 lipidation was observed at the same time point as this decrease in Akt phosphorylation (Fig. 10b).

Akt is not involved in autophagy

In order to investigate the effects of potential Akt inhibition on autophagy induction, cells were treated with LY294002 a specific inhibitor of type1 PI3kinase [190] to inhibit Akt phosphorylation. Akt (Ser 473) and downstream target mTOR(Ser2448) phosphorylation were examined. LY294002 resulted in more intense reductions in Akt and mTOR phosphorylation than did SK1-I (Fig. 11). Even so, autophagic response to SK1-I treatment was observed to be much stronger than that induced by inhibition of Akt phosphorylation and mTORC1 inactivation (Fig. 11). An additive effect by LY294002 and SK1-I treatment on LC3 lipidation was observed. Taken together, this data suggests that inhibition of phosphorylation of Akt may not be a major contributor to SK1-I induced autophagy.

SK1-I induced ER stress

ER stress and the unfolded protein response (UPR) have been implicated in autophagy[109]. Furthermore, the UPR does not require Beclin1 for activation[191]. During ER stress, protein kinase r-like ER kinase (PERK) is activated in response to decreases in calcium or protein misfolding. In turn it phosphorylates transcription factor eIF2alpha[107,]

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[192]. eIF2alpha phosphorylation is also necessary for Atg 12 mediated LC3 conversion during autophagy due to aggregation of polyglutamine repeat proteins in the ER[102]. EIF2alpha phosphorylation was examined by Western blot. eIF2alpha showed significantly elevated levels of phosphorylation in comparison with untreated cells beginning within 15 minutes of treatment with SK1-I (Fig. 12), similar to the time course observed in LC3 lipidation (Fig 2a). In addition, eIF2alpha phosphorylation was seen to increase in response to 6 hours treatment with 10μM SK1-I rather than 5μM SK1-I, just as was observed for LC3II appearance (Fig. 6b). These results indicate that SK1-I induces ER stress that could be responsible for SK1-I induced autophagy.

Atg5 is necessary for SK1-I autophagic induction

To determine whether other elements of the canonical autophagy induction pathway were necessary for accumulation of LC3 associated vesicles in SK1-I treated cells, small interfering RNA for Atg5 was used. Atg5 has been shown to localize to the isolation membrane and be necessary for autophagosome formation[25]. Small interfering RNA for Atg5 inhibited induction of autophagy as measured by LC3 lipidation (Fig. 8). This confirmed that LC3 lipidation was dependent upon Atg5, a known participant in autophagic vesicle nucleation.

SK1-I induces non-apoptotic cell death in wild type HCT116 cells and apoptosis in p53 null cells

Cell survival in response to SK1-I was then examined. Propidium Iodide staining in combination with Hoechst 33342 has been shown an effective method of visualizing membrane permeablization, and nuclear condensation, respectively[193]. Nuclear condensation is considered a cytological marker of apoptosis[193]. Membrane permeablization without nuclear condensation is considered to indicate non-apoptotic cell death[193]. By propidium iodide staining, 94 percent of wild type cells cultured in 10μM SK1-I for 24 hours were observed to be membrane permeablized without nuclear condensation (Fig. 13). P53 null HCT116 cells treated with SK1-I were observed to have a higher percentage of apoptosis (35 percent) than control (3 percent) or wild type cells (3 percent), with insignificant amounts of membrane permeablization (Fig. 13). While p53 null cells underwent apoptosis, wild type cells appeared highly susceptible to a non-apoptotic form of cell death.

To confirm the observation of non-apoptotic cell death in wild type HCT116 cells, Poly-(ADP-ribose) polymerase (PARP) cleavage was evaluated by western blot. PARP is a nuclear protein implicated in DNA repair. PARP is one of the earliest proteins targeted for specific caspase cleavage and the best characterized caspase substrate in measuring apoptotic cell death[194]. No PARP cleavage was detected in wild type HCT116 cells up to 24 hours (Fig. 14) indicating that the permeablization observed in these cells is not a result of apoptotic cell death. ERK phosphorylation was also observed as an indicator of cell survival. ERK phosphorylation was reduced in both wild type and p53 null cells (Fig. 15), suggesting that this does not contribute to the distinct apoptotic responses of p53 null

HCT116. Altogether these results demonstrate that SK1-I induced a non-apoptotic cell death in HCT116 wt cells while inducing apoptosis in HCT116 p53 null cells.
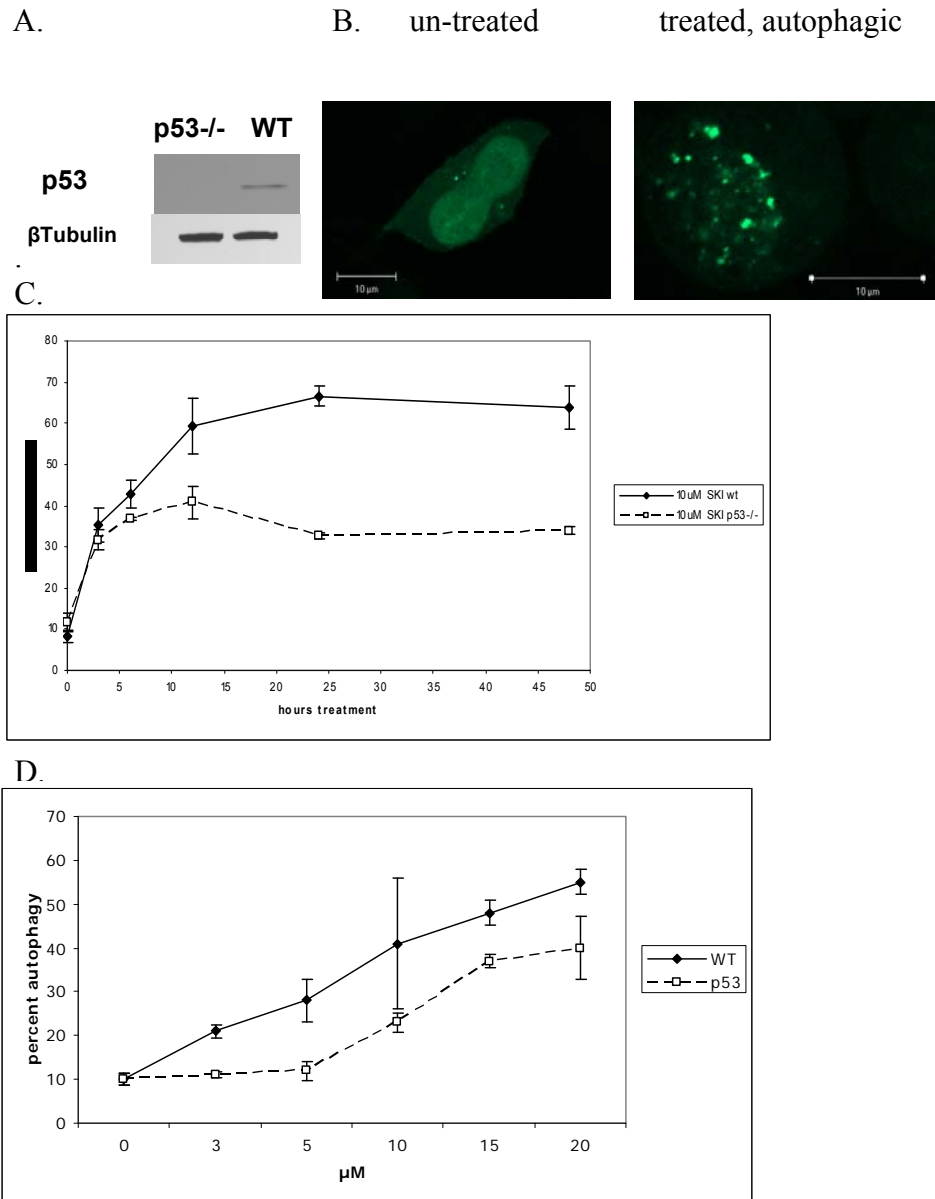
Figure 5. SK1-I Induces autophagy with different intensity in human wild type p53 expressing HCT116 colon carcinoma cells, and in their p53-null counterparts.
A. Equal amounts of lysate proteins from naive wild type and p53 null HCT116 cells were resolved by SDS-PAGE and analyzed by western blotting with antibodies against p53. Blots were stripped and reprobed for βTubulin to demonstrate equal loading and transfer. B-D. LC3-GFP transfected wild type and p53null HCT116 cells cultured in DMEM supplemented with 0.1%BSA were treated with 10μM SK1-I in for the indicated time (B,C) or with the indicated concentrations of SK1-I for 6 hours (D). Cells were examined by confocal fluorescence microscopy. (B) Representative images are shown. Scale bars, 10 μm. (B) Percentage of cells showing GFP-LC3 fluorescence in puncta was quantified. Data are means ± SD.

A.
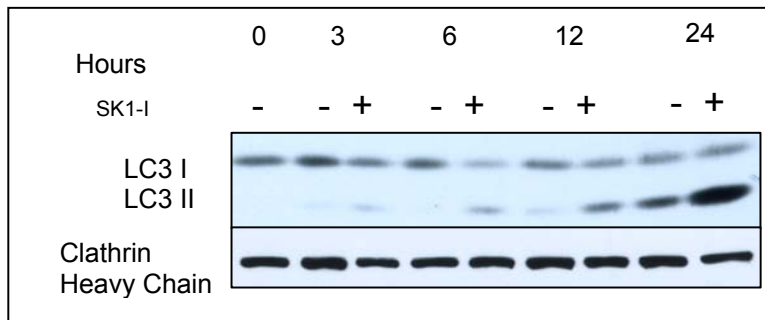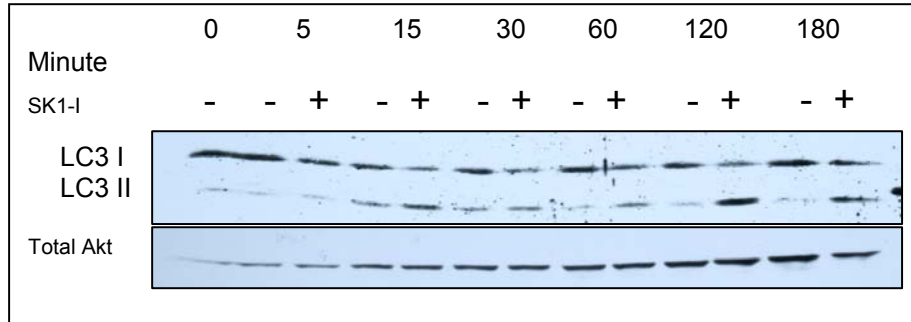
B.
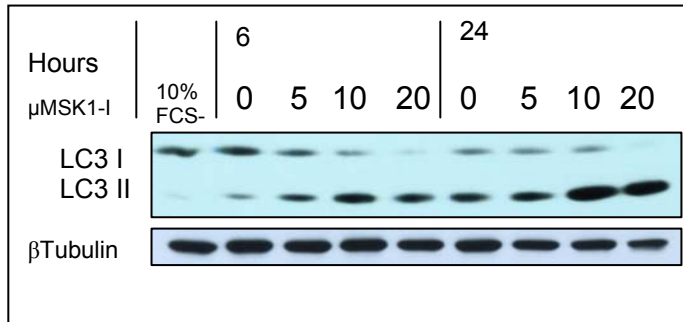
Figure 6: SK1-I induces LC3 lipidation in HCT116 cells.
A,B. Naive HCT116 cells cultured in DMEM supplemented with 0.1%BSA were treated without or with 10μM SK1-I for the indicated times (A) or with the indicated concentrations of SK1-I for 6 or 24 hours (B). HCT116 cells were also cultured with 2%FCS for 6 hours and 24 hours as a control. Equal amounts of lysate proteins were resolved by SDS-PAGE and analyzed by western blotting with antibodies against LC3. Blots were stripped and reprobed for total Akt, Clathrin Heavy Chain, or βTubulin to demonstrate equal loading.

A.   wt   **B. p53 null**

| | 0 | 5 | 15 | 30 | 60 | 120 | 180 | | 0 | 5 | 15 | 30 | 60 | 120 | 180min |
Beclin1
Tubulin
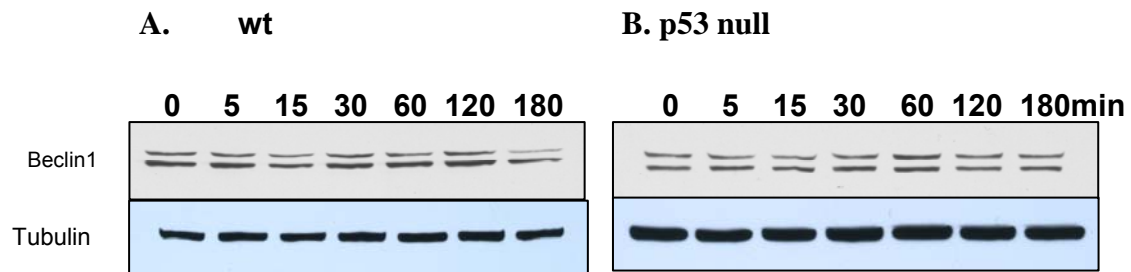
Figure 7: SK1-I induces autophagy without increasing Beclin1.
Wild type (A) and p53 null (B) HCT116 cells cultured in DMEM supplemented with
0.1%BSA were treated without or with 10μM SK1-I for the indicated times. Equal
amounts of lysate proteins were resolved by SDS-PAGE and analyzed by western blotting
with antibodies against Beclin1. Blots were stripped and reprobed for βTubulin to
demonstrate equal loading.
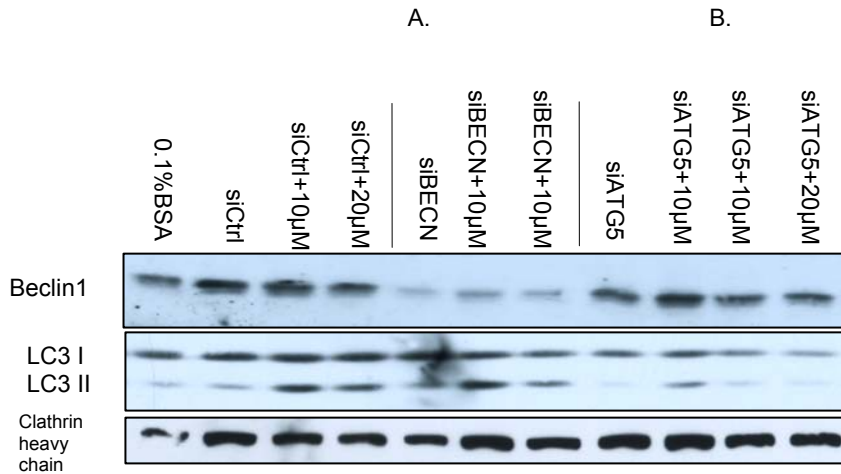
Figure 8: Atg5 but not Beclin1 is required for SK1-I-induced autophagosome formation.

HCT116 wt cells were transfected with siControl, siBeclin or siATG5 then transfected with LC3-GFP. Cells were cultured for 24 hours then treated with without or with the indicated concentrations of SK1-I in DMEM supplemented with 0.1%BSA for 6 hours. Equal amounts of lysate proteins were resolved by SDS-PAGE and analyzed by western blotting with antibodies against LC3. Blots were stripped and reprobed for Beclin1 to ensure protein expression knockdown. Blots were stripped and reprobed for Clathrin Heavy Chain to demonstrate equal loading.
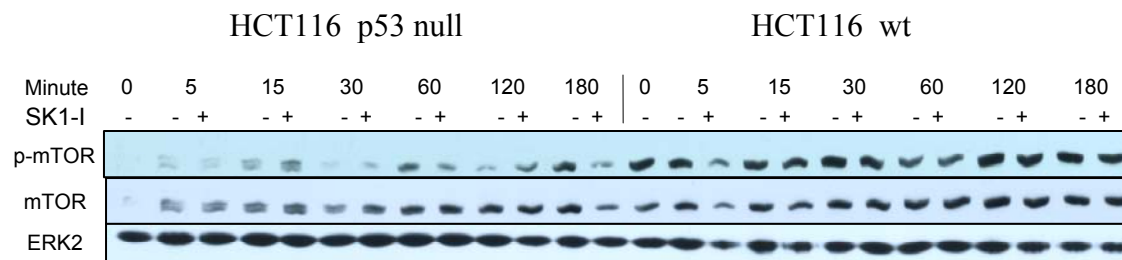
Figure 9: SK1-I does not inactivate mTOR complex 1.
Wild type and p53 null HCT116 cells cultured in DMEM supplemented with 0.1%BSA were treated without or with 10μM SK1-I for the indicated times. Equal amounts of lysate proteins were resolved by SDS-PAGE and analyzed by western blotting with antibodies against Phospho-mTOR(Ser2448). Blots were stripped and reprobed for mTOR for comparison with phosphorylation. Blots were stripped and reprobed for Erk2 to demonstrate equal loading.

A. HCT116 p53 null   HCT116 wt

B.   HCT116 wt
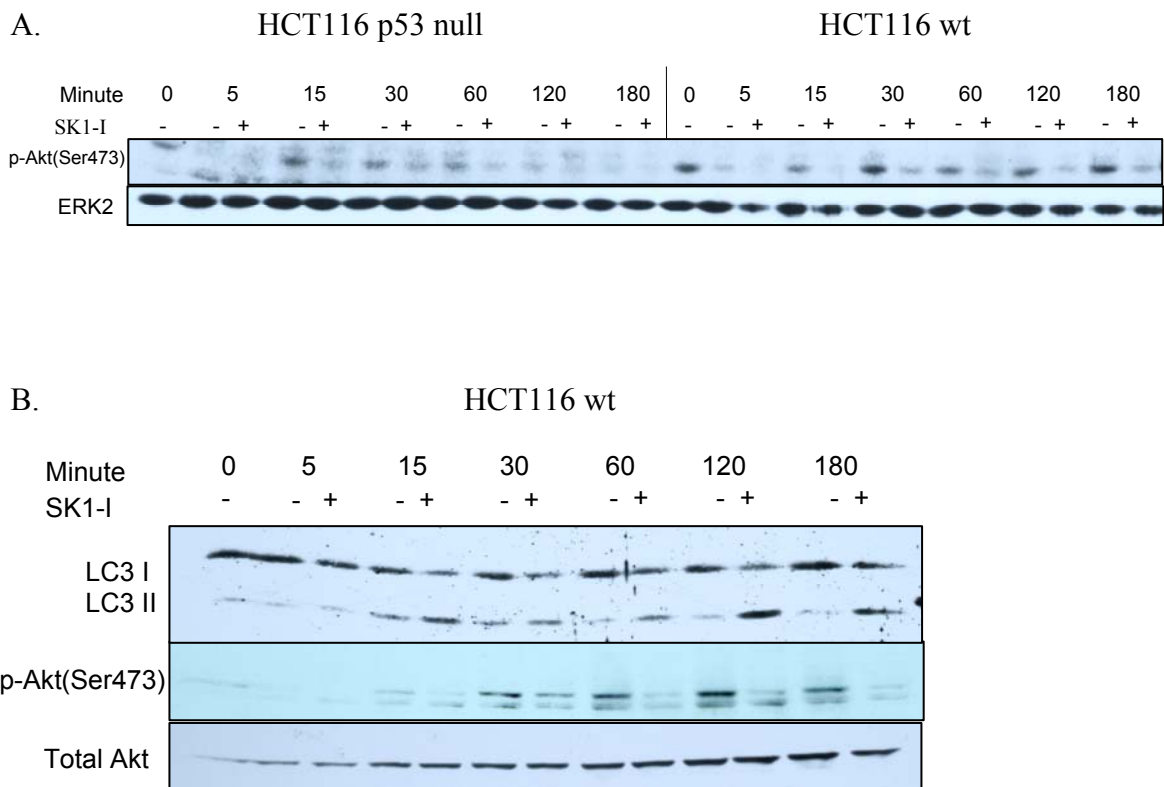
Figure 10: SK1-I treatment decreased phosphorylation of Akt concomitantly with LC3 lipidation.
Wild type and p53 null HCT116 cells were treated without (-) or with 10μM SK1-I (+) for the indicated times. Equal amounts of lysate proteins were resolved by SDS-PAGE and analyzed by western blotting with antibodies against Phospho-Akt (Ser473) (A and B). Blots were stripped and reprobed for Erk2 (A) or total Akt (B) to demonstrate equal loading.
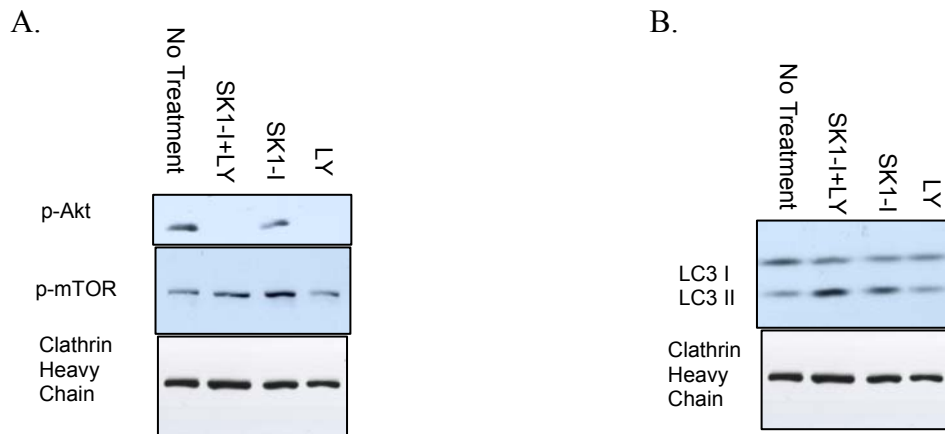
Figure 11: SK1-I induces autophagy independently of Akt and mTORC1
CT116 cells cultured in DMEM supplemented with 0.1%BSA were treated with 10μM
LY294002 or 10μM SK1-I or a combination of both for 30 minutes. Equal amounts of
lysate proteins were resolved by SDS-PAGE and analyzed by western blotting with
antibodies against Phospho-mTOR(Ser2448), LC3 and Phospho-Akt. Blot was stripped
and reprobed for Clathrin Heavy Chain to demonstrate equal loading.

A.　　　　　　　HCT116 p53 null　　　　　　　　　　　HCT116 wt

| Minute | 0 | 5 | 15 | 30 | 60 | 120 | 180 | 0 | 5 | 15 | 30 | 60 | 120 | 180 |
| SK1-I | - | - + | - + | - + | - + | - + | - + | - | - + | - + | - + | - + | - + | - + |

p-eIF2 α

ERK2

B.　　　　　　　　　HCT116 wt

| | 6 hours | 24 hours |
| SK1-I | 0　5　10　20 | 0　5　10　20 |

LC3 I
LC3 II

p-eIF2α

βTubulin
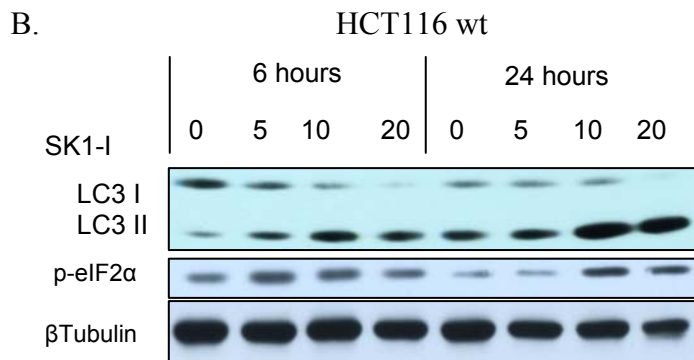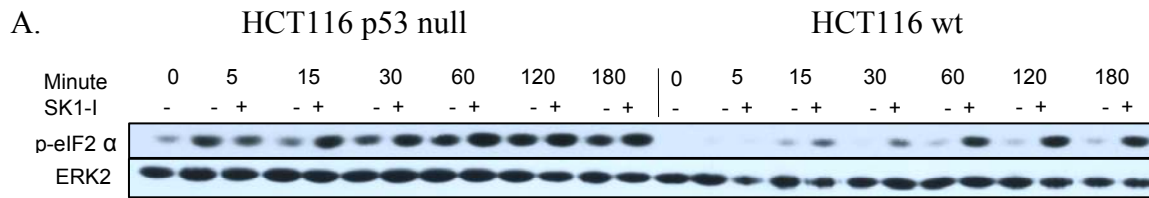
Figure 12: SK1-I treatment induces ER stress

Wild type and p53 null HCT116 cells cultured in DMEM containing 0.1% BSA were
treated without (-) or with 10μM SK1-I (+) for the indicated times (A) or with the indicated
concentration of SK1-I for 6 hours and 24 hours (B). Equal amounts of lysate proteins
were resolved by SDS-PAGE and analyzed by western blotting with antibodies against
Phospho-eIF2α (A) or Phospho-eIF2α and LC3 (B).   Blots were stripped and reprobed for
Erk2 (A) or βTubulin (B) to demonstrate equal loading.

A.   Apoptosis
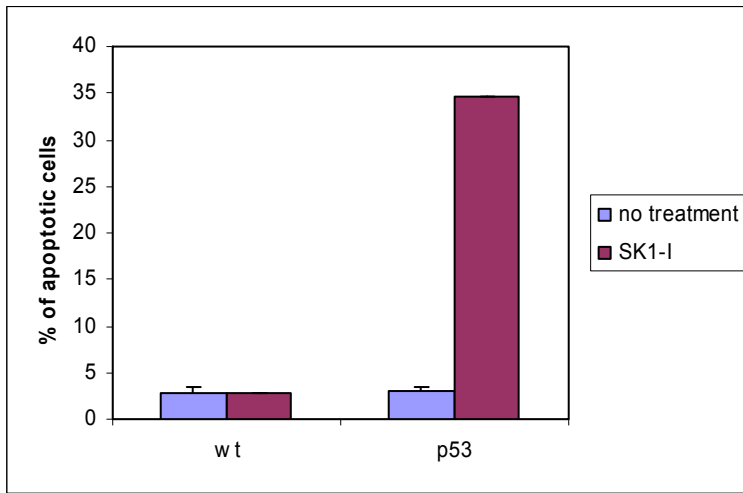
B.   Non-apoptotic Permeablization
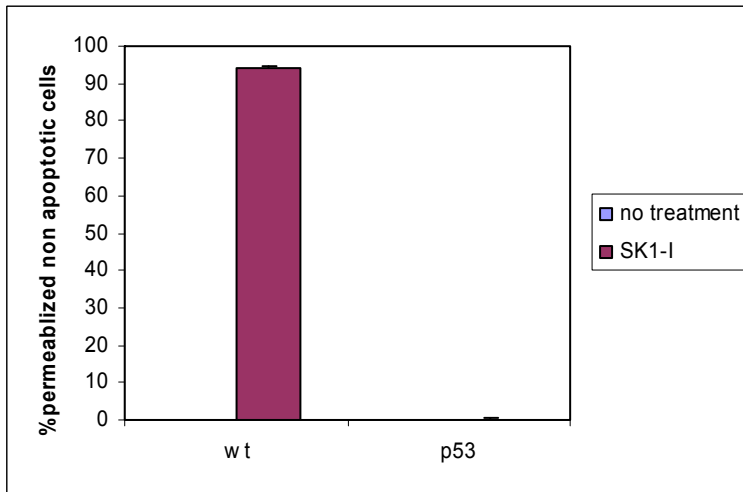
Figure 13

C. **untreated HCT116 wt (Hoechst Blue, PI red)**
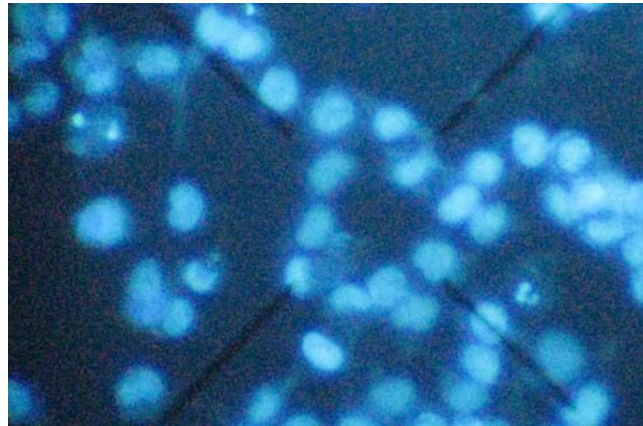
SK1-I HCT116 wt (Hoechst Blue, PI red)
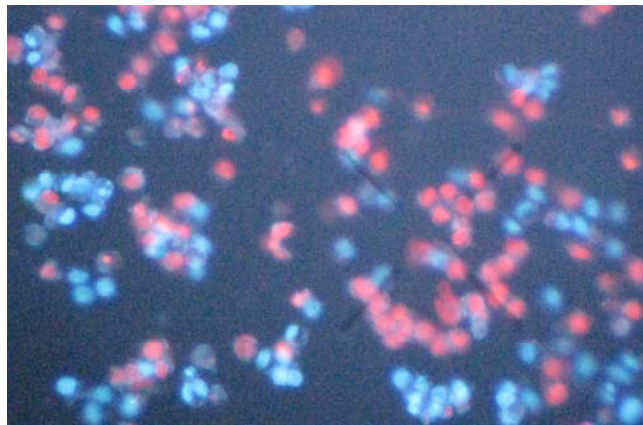
Figure 13: SK1-I induces non-autophagic death in wild type HCT116 cells and apoptosis in p53-/- HCT116 cells.
Wild type and p53 null HCT116 cells were treated with 10μM SK1-I for 24 hours. Cells were fixed and stained with propidium iodide and Hoechst and percentage of cells with permeablized membranes (A) or condensed nuclei (B), respectively, were counted. C. representative field of untreated wild type HCT116 cells (top) or 10μM SK1-I treated for 24 hours (bottom).
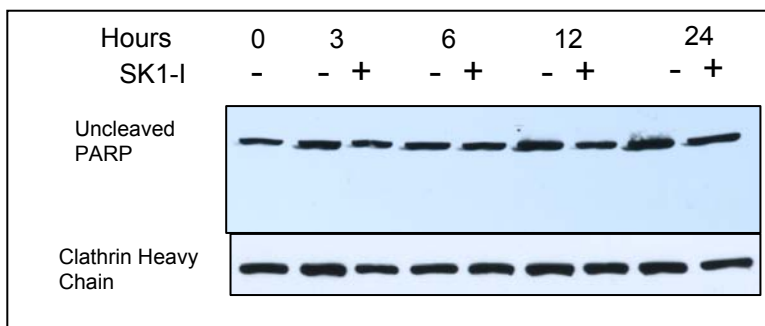
Figure 14: SK1-I did not induce PARP cleavage in wt cells.
HCT116 cells were treated without (-) or with 10μM SK1-I (+) for the indicated times.
Equal amounts of lysate proteins were resolved by SDS-PAGE and analyzed by western
blotting with antibodies against PARP. Blots were stripped and reprobed for Clathrin
Heavy Chain to demonstrate equal loading.
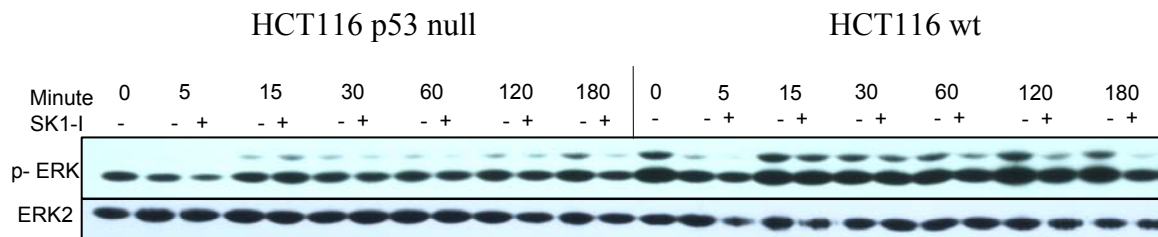
Figure 15: SK1-I decreases ERK1/2 phosphorylation
Wild type and p53 null HCT116 cells were treated without (-) or with 10μM SK1-I (+) for
the indicated times. Equal amounts of lysate proteins were resolved by SDS-PAGE and
analyzed by western blotting with antibodies against Phospho-Erk1/2. Blots were stripped
and reprobed for Erk2 to demonstrate equal loading.

Discussion

Autophagy Induction by SK1-I: an Unexpected Result

Autophagy has traditionally been associated with the adaptive responses of nutrient deprived cells. This nutrient response was primarily associated with increased Beclin 1 activity and decreased mTORC1 activity[84-86]. Recently, however, autophagy has been associated with maintaining cell homeostasis and in some cases contributing to cell death[22]. This study places the effects of the isotype SphK1 inhibitor SK1-I within this new paradigm and investigates its mechanism of action. In many respects, the findings of this study differ significantly from the previously reported observations of autophagy induced by SphK1 upregulation [88] and ceramide increase[183], suggesting a novel mechanism of autophagy induction.

SK1-I treatment increased autophagy significantly in cells expressing or lacking p53. This increase in autophagy was unusual in three aspects. Firstly, very rapid induction of autophagy was observed. By western blotting to examine LC3 lipidation, it was possible to observe LC3 conversion in response to SK1-I treatment within 15 minutes. This finding was difficult to compare with previous investigations of autophagy as they often only evaluated autophagy after longer periods of time. Nevertheless, Tasdemir et al. 2008[195] , showed that in HCT116 cells wild type for p53, lithium (IP3R antagonist),

tunicamycin, rapamycin or ABT737 (Bcl-2 inhibitor, BH3 mimetic small molecule) typically required more than 1 hour to demonstrate changes in LC3 lipidation[195]. In other experiments, ligand induced JNK activation [196] or IP3R antagonism [91] was observed to require 12 hours of treatment for autophagy induction. Other studies evaluating sphingolipid metabolites such as that investigating 100 μM C_2-Cer induced autophagy and nutrient starvation, only evaluate autophagy after 4 hours of treatment[69].

Secondly, autophagy was induced more profoundly in cells expressing wild type p53 than in cells which p53 was absent. Tasdemir et al [195] have proposed that cytoplasmic p53 is degraded during the autophagic process. In HCT116 cells, it was observed that cytosolic p53 inhibition, depletion or deletion causes a maximal level of autophagy that cannot be enhanced by chemical agents used to trigger nutrient deprivation or ER stress [195]. Furthermore, induction of autophagy by these agents can be inhibited by preventing the degradation of p53. This inductive effect is, however, cell cycle dependent and only causes autophagy in G1or S phase. In contrast, nuclear p53 is known to promote transcription of pro-autophagy factors such as the DAPK proteins[197]. It is difficult, therefore to implicate p53 directly in this differential induction of autophagy except perhaps as regulator of the cell cycle, allowing cells to spend more time in highly autophagic G1 or S phase, and as potential transcription factor. It was, however, noted (as seen in differential phosphorylation levels of eIF2α, mTOR, Akt and ERK proteins and unreported blotting of EGF receptor phosphorylation), that wild type p53 and p53 null cells displayed significant differences in the basal status of signaling pathways normally associated with autophagy. These differences, which may or may not be directly

associated with p53 status, may also account for some difference in autophagy intensity induced by SK1-I.

Lastly, inhibition of SphK1 using RNA interference or DMS in MCF7 breast cancer cells was previously reported to attenuate the autophagic response induced by nutrient starvation or SphK1 upregulation[88]. Our observation of a rapid increase in autophagy using SK1-I is quite distinct from this earlier report. However, observations by Coward et al (2009) that safingol, an unspecific SphK and PKC inhibitor, induces autophagy more strongly than can be accounted for by PKC inhibition alone, and without concomitant increases in sphingosine, S1P, or ceramide metabolites[89], lead one to wonder what effects SK1-I has on sphingolipid metabolite levels at early time points and whether its accumulation or modification may account for its effects (it could, for example, it be N-acylated to become a dihydroceramide analogue as does safingol [198] and induce autophagy through a similar mechanism as resveratrol which shares beclin1 independent characteristics.   In addition, the anti ceramide-tunnel forming characteristics of dihydroceramide would help to account for the lack of apoptosis in wild type cells[199]). Interestingly, SK1-I also induced rapid autophagy in glioblastoma cells (D. Kapitonov, unpublished) and SK1-I treatment induced a very rapid decrease in S1P (within 20 minutes). This was followed by an increase in sphingosine at 1 hour and a later increase in ceramide (D. Kapitonov et al, submitted to Cancer Research). Therefore, the potent effect on autophagy of SK1-I could be due to a lack of intracellular S1P or increases in sphingosine and ceramide. In order to investigate this possibility in HCT116 cells, mass-spectophotometric analysis of cells treated with SK1-I at early time points should be

performed examining SK1-I and sphingolipid metabolites. In turn, detected differences in quantity and structure of these metabolites should be introduced as alternative treatment conditions and compared with the SK1-I effect.

Absence of Beclin1 or mTORC1 effects offer mechanistic insights

Although Beclin 1 is considered to have a significant role in regulating autophagy, no such involvement was observed with SK1-I. Previously, genetic upregulation of Beclin levels have been shown to induce autophagy[27]. Futhermore, in response to ceramide, a robust increase in Beclin1 has been observed[183]. Even without changes in Beclin expression, starvation induced autophagy is inhibited by Bcl-2 binding to Beclin [67] and Bcl-2 binding is regulated by its ceramide dependent phosphorylation[69]. Ambra and UVRAG also regulate autophagy by mediating the interaction between Beclin 1 and Vps34[65, 66]. The observed down regulation of total Beclin1 expression in this study, without significant inhibition of autophagy, however, suggests that these Beclin 1 regulatory mechanisms do not have a significant role in the SK1-I induced autophagic effect. While not conclusive, these results also cast doubt on the possibility that ceramide may be the primary cause of SK1-I induced autophagy. This further emphasizes the need for investigation of SK1-I induced changes in sphingolipid metabolism during early SK1-I treatment. Furthermore, it may be of interest to compare the effects of Resveratrol, which induces a Beclin 1 independent, dihydroceramide mediated, form of autophagy[70,135], with those of SK1-I.

Inhibition of mTORC1 is also commonly observed with induction of autophagy, and yet was not observed in SK1-I treated cells. In mammalian cells, inhibition of mTORC1 is sufficient to induce autophagy and is required for autophagy due to nutrient starvation[84-86]. Elevated mTORC1 activity results in cells with impaired ability to initiate autophagy in response to nutrient starvation[85]. Reduced mTORC1 activity has been observed in autophagic cells as a result of ER stress associated with Ca activated AMPK signaling[68], elevated SphK1 expression[88], elevated ceramide levels[69], and treatment with Safingol[89]. Our results are consistent with the notion that autophagy is not being induced by a nutrient starvation pathway. We also observed that Akt activity and downstream signaling through mTORC1 does not account for autophagy induced by SK1-I. Complete downregulation of Akt phosphorylation and downstream decrease in mTOR phosphorylation resulted in little increase in LC3 lipidation while SK1-I treatment, with comparatively much less inhibition of Akt and mTOR phosphorylation , resulted in a much higher rate of LC3 lipidation. Furthermore, treatment with both the Akt inhibitor and SK1-I appeared to produce an additive effect on LC3 lipidation. Owing to Akt's importance in S1P receptor signaling, this may support the possibility of an intracellular mechanism of action.

Interestingly, changes in mTORC1 activity have not been observed in autophagy induced by PKCθ in response to calcium[90], or IP3R in response to reduction in IP3R expression[91]. Therefore, further investigation of these ER associated mechanisms of autophagy may provide some insight into the mechanism of SK1-I induced autophagy.

Translational effects, cytoskeletal changes, and a role for the ER

Ultimately, inhibition of autophagy by knockdown of Atg5 confirmed that SK1-I effects were dependent upon the conserved mechanism of initial autophagosome formation. Therefore, investigation of other, less conventional autophagic pathways seemed warranted. The observation that Akt serine 473 dephosphorylation and eIF2α phosphorylation occur concomitantly with the first observed increase in LC3 lipidation suggest a role for mTORC2 and ER stress in mediating autophagy induced by SK1-I.

mTORC2 is of particular interest in SK1-I induced autophagy because it is considered to play a role in regulation of the actin cytoskeleton [97] much as has SphK1[200]. In addition, growth factor independent mTORC2 activity has been shown to be necessary for carboxyl-terminal folding and stabilization of newly synthesized Akt and PKC[96]. mTORC2 downregulation has been shown to induce FoxO3 nuclear translocation in transfected myofibers, which correlates with expression of autophagy inducer BNIP protein. Autophagosome formation is observed in response to mTORC2 inhibition without mTORC1[99]. Changes in cytoskeletal organization, inhibition of protein synthesis and autophagic protein expression could all contribute to an autophagic effect. Therefore, further investigation of co-localization, constituitive activation and downregulation of mTORC2's binding constituents in relation to SphK1 may shed light on a new intracellular mechanism of SphK1 action.

In response to perturbations in protein folding/glycosylation, shuttling of proteins to the Golgi, ER calcium balance, and synthesis of fatty acids, sterols and phospholipids, ER stress is induced[19]. Each of these perturbations may also induce autophagy[109]. There is

some evidence that calcium release from the ER is the primary signal initiating the ER mediated autophagic response[109]. ER stress results in eIF2α phosphorylation[192]. Furthermore, eIF2α phosphorylation has been shown necessary for LC3 conversion by induction of Atg12 during ER stress caused by aggregation of polyglutamine repeat proteins[102]. Based on the finding that eIF2α is phosphorylated in response to SK1-I treatment, ER stress due to protein misfolding and modification, ER integrity, lipid synthesis and trafficking, and calcium homeostasis should be further investigated as mediators of SK1-I autophagy. Furthermore, treatment of HCT116 cells with non-specific SphK inhibitor N,N-dimethyl-D-erythro-sphingosine (DMS) a sphingosine analogue, has been shown to induce cytosolic calcium release by an IP3R independent mechanism[201]. As such, one first step in this process is to confirm that cytosolic calcium chelation cannot inhibit autophagy and that signaling through a known ER stress sensor is necessary for SK1-I induced autophagy as would be demonstrated by sensor knockdown[102, 110]. Furthermore, because the mitochondria are in such close apposition to the ER, and the ER plays a role in mitochondrial homeostasis, mitochondrial changes may also be considered.

Autophagic Death? Need for Further Investigation of the Mechanisms of Death

Lastly, SK1-I causes non-apoptotic and apoptotic death including reductions in Akt and ERK phosphorylation. In addition, death was induced differentially in wt and p53 null cells. This may be accounted for by the intensity of autophagy induction in each cell type and other observed differences between wt and p53 null cells. However, to determine the relationship between autophagy, apoptosis, and cell death, inhibition of autophagy using

siAtg5 and observations of the resulting cell viability and death signaling may be worthwhile.

In sum, SK1-I rapidly induces autophagy with non-canonical signaling involving decreased mTORC2 activity and ER stress. The result is cell death whose differential expression in wild type p53 and p53 null cell lines parallels their differences in autophagic intensity. The relationship of these observations to sphingolipid metabolism and putative changes in ER homeostasis or mTORC2 activity warrants further investigation as does the clinical utility of SK1-I in the treatment of cancer or other conditions in which cytotoxicity or autophagy may have therapeutic value.
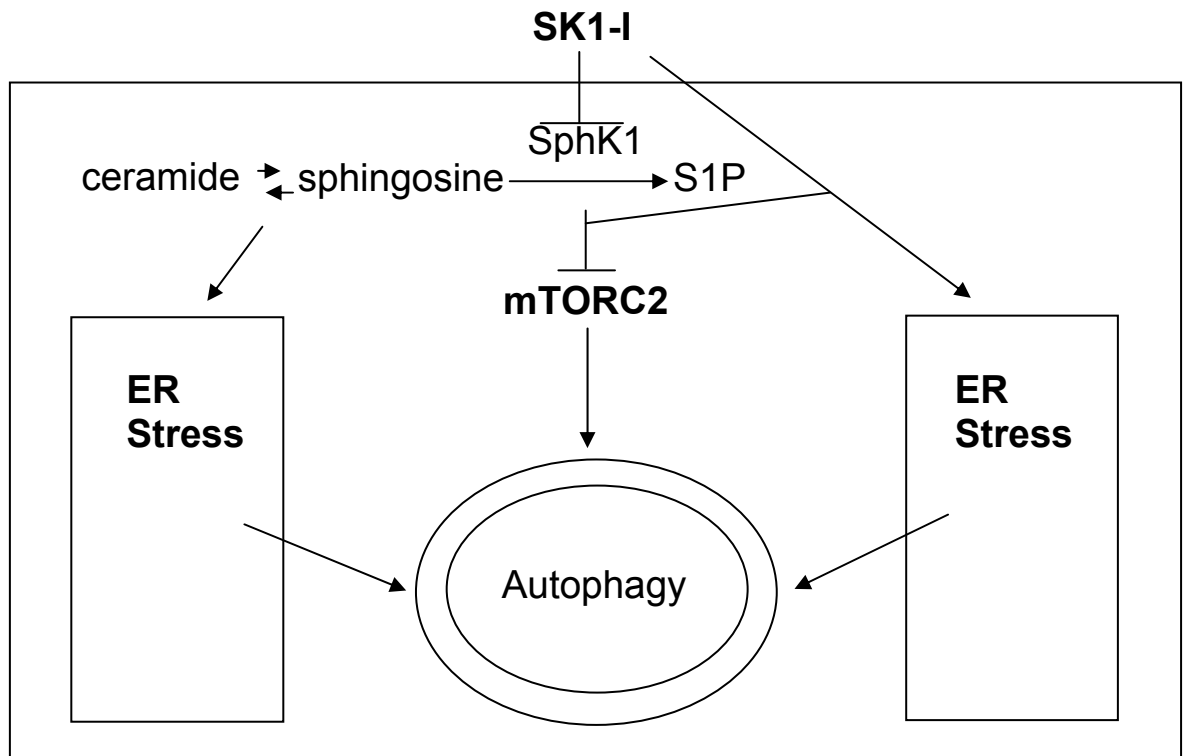
Figure 16: Schema of Autophagy induced by SK1-I

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VITA

Daniel Meza is an unorthodox student of science, having devoted himself previously to the education of children. It is his hope that the investigative abilities he has learned as a student of Biochemistry and Molecular Biology will enhance his ability to serve children by using these scientific tools and methodologies.