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Drug Resistance Mechanisms to Gamma-secretase Inhibitors

in Human Colon Cancer Cells

by

Cindy R. Timme

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Arts and Sciences
University of South Florida

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Date of Approval:
August 23, 2013

Keywords: Oxaliplatin, GSI, Mcl-1, Bcl-xL, Autophagy

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DEDICATION

To my parents, Joseph and Veena Timme, I thank you for your strong belief in family values, for providing a warm, loving home that is always welcoming, and for all your love and support. To my dog, Ranger, for being (wo)man's best friend.

ACKNOWLEDGMENTS

I would like to thank my mentor Dr. Timothy Yeatman for his support, guidance, and the freedom to explore my ideas. We hit many dead ends during my dissertation work but he always remained positive and taught me to keep looking and try again. I would also like to thank Mike Gruidl for all his scientific guidance and for being a sounding board to challenge and enrich my ideas.

I am also grateful for my committee members, Dr. David Shibata, Dr. Mark Alexandrow, and Dr. Douglas Cress. I appreciate all your time, input, and advice. I would like to thank Dr. Robert Coffey for taking the time to serve as my external chair for my dissertation defense. In addition, I thank the Cancer Biology Program for their fantastic educational program.

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ABSTRACT

Colorectal cancer is the third leading cause of cancer-related mortality. Much progress has been achieved in combating this disease with surgical resection and chemotherapy in combination with targeted drugs. However, most metastatic patients develop drug resistance so new modalities of treatment are needed.

Notch signaling plays a vital role in intestinal homeostasis, self-renewal, and cell fate decisions during post-development and is activated in colorectal adenocarcinomas. Under debate is its role in carcinomas and metastatic disease. In theory, blocking Notch activation using gamma-secretase inhibitors (GSIs) may show efficacy alone or in combination with chemotherapy in the treatment of colon cancer.

In Chapter Three, we tested the capacity for GSIs to synergize with oxaliplatin in colon cancer cell lines and evaluated the underlying molecular mechanisms. GSI alone had no effect on colon cancer cell lines. Surprisingly, we show that GSIs blocked oxaliplatin-induced apoptosis through increased protein levels of the anti-apoptotic Bcl-2 proteins Mcl-1 and/or Bcl-xL. Restoration of apoptosis was achieved by blocking Mcl-1 and/or Bcl-xL with obatoclax (an anti-apoptotic Bcl-2 agonist) or siRNA. An unexpected result was the induction of cell death with the combination of GSI and obatoclax.

In Chapter Four, we examined the mechanism of GSI + obatoclax-mediated cell death. We found that apoptosis played a minimal role. Rather, we identified blockage

of cytoprotective autophagy played a causative role. Interestingly, we also saw autophagy induction in GSI-treated cells, which could explain the insensitivity of colon cancer cells to GSI. When autophagy was blocked in GSI-treated cells, cells became sensitive to GSI and cell death was elicited. The mechanism by which induction of autophagy occurs in GSI- treated cells is an area for further research.

Overall, our work questions the validity of the use of GSIs in the treatment of colorectal cancers. We show that GSIs may block apoptosis and induce cytoprotective autophagy simultaneously, resulting in increased drug resistance and cellular survival. Whether these two cellular survival processes occurs in patients needs to be examined before GSIs can be utilized in a clinical setting. If so, these two hurdles must be overcome.

CHAPTER ONE: INTRODUCTION

Cancer

Cancer comprises of a diverse and complex range of diseases caused by both intrinsic (such as germline or somatic mutations, epigenetics, and hormones) and extrinsic (such as cigarette smoke, ionizing radiation, and chemical carcinogens) factors. The lifetime risk of developing cancer is 1 in 2 for both men and women in the United States, accounting for an estimated 1.6 million new cases per year (Siegel et al. 2013). One in 4 deaths is due to cancer, making it the seconding leading cause of death following heart disease (Howlader et al. 2013). Cancer can originate at any site in the body, with the lungs, prostate, breast, and colon leading the pack. The road to cancer development is a multi-step and multi-lateral process involving multiple molecular pathways within the cell that transforms a normal cell into a malignant one. The classical “Hallmarks of Cancer” include self-sufficiency in growth signals, insensitivity to anti-growth signals, cell-death evasion, limitless replicative potential, angiogenesis, and tissue invasion and metastasis, with this list being recently updated to also include reprogramming of metabolic pathways, immune system evasion, chromosomal abnormalities and DNA instability, and inflammation (Hanahan et al. 2000; Hanahan et al. 2011). While substantial progress has been made in the prevention and treatment of cancer, the heterogeneity and plasticity of cancer cells has

impeded effective long-term strategies to manage or ‘cure’ many cancer types. Targeted molecular therapy, alone or in combination with conventional chemotherapy and/or other targeted therapies, has therefore been the current focus of medical research.

Colorectal Cancer

Colorectal cancer accounts for more than 50,000 deaths each year in the United States and remains the third leading cause of cancer-related mortality (Siegel et al. 2013). While surgical resection remains the gold standard of treatment for localized disease, chemotherapy combinations with oxaliplatin, irinotecan, 5-fluorouracil, the VEGF inhibitor bevacizumab, and EGFR inhibitors have led to significant improvements in survival at all stages (Saltz 2009; Garcia-Foncillas et al. 2010). However, most metastatic colorectal cancer patients eventually develop drug resistance to current therapies so new strategies are needed for treatment and improved survival of these patients.

Colon Architecture

Normal human colon has millions of self-renewing crypts, each containing around 2000 cells (Cheng et al. 1984; Potten et al. 1992). The crypt is maintained by a small population of stem cells located at or near the base of the crypt; they divide slowly and gives rise to transit-amplifying progenitor cells (Figure 1). These progenitor cells have a very limited capacity to self-renew; within three to four divisions they terminally differentiate into one of the mature epithelial lineages – absorptive

colonocytes/enterocytes, hormone-secreting enteroendocrine cells, and mucus-producing goblet cells (Potten et al. 1997). The differentiated cells migrate towards the surface of the colon where they die and are shed into the lumen. All the cells of the crypt, except the stem cell, are regenerated every 5-7 days (Barker et al. 2008).

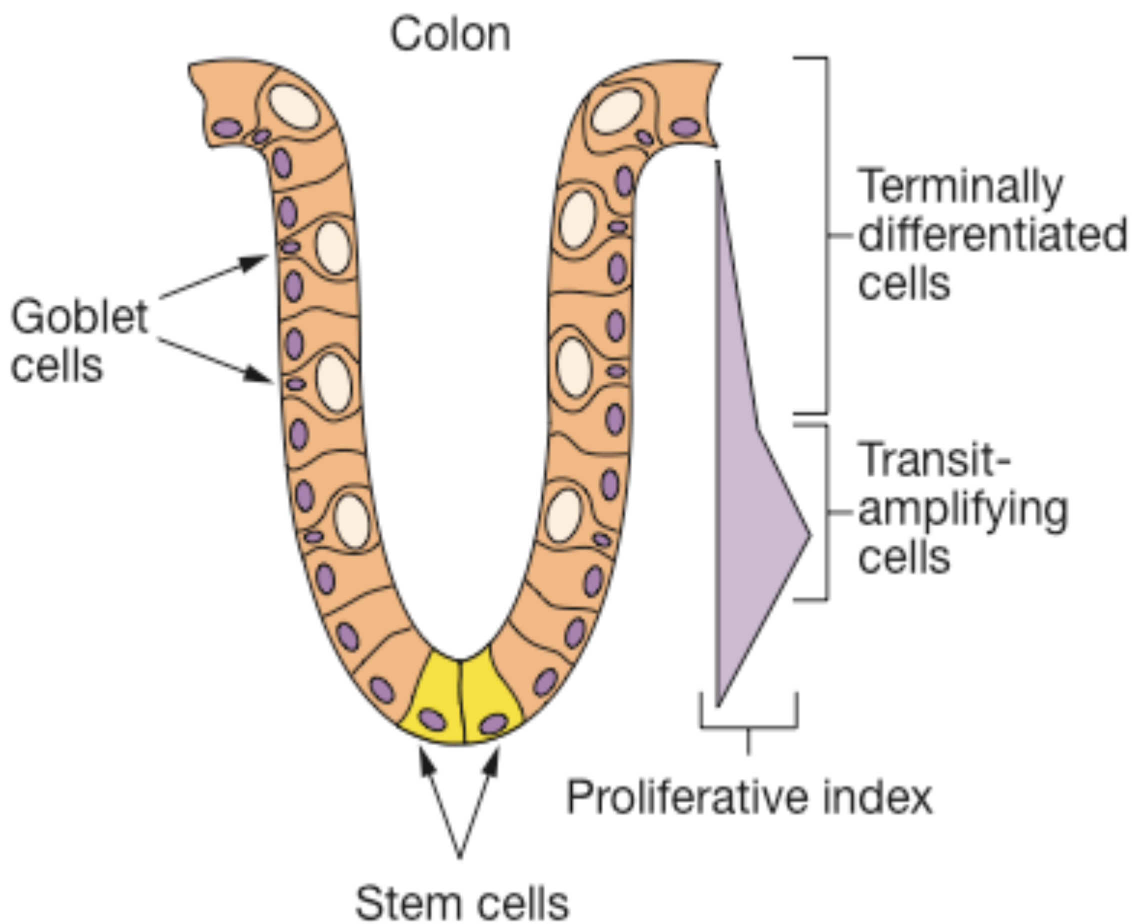


Figure 1: Architecture of the Colon Crypt
In the colon, stem cells are thought to reside at the base of the colon crypt and give rise to transit-aplifying progenitor cells. These transit-amplifying cells then terminally differentiate into either colonocytes, enteroendocrine cells, or goblet cells. Reprinted by permission from Nature Publishing Group (McDonald et al. 2006).

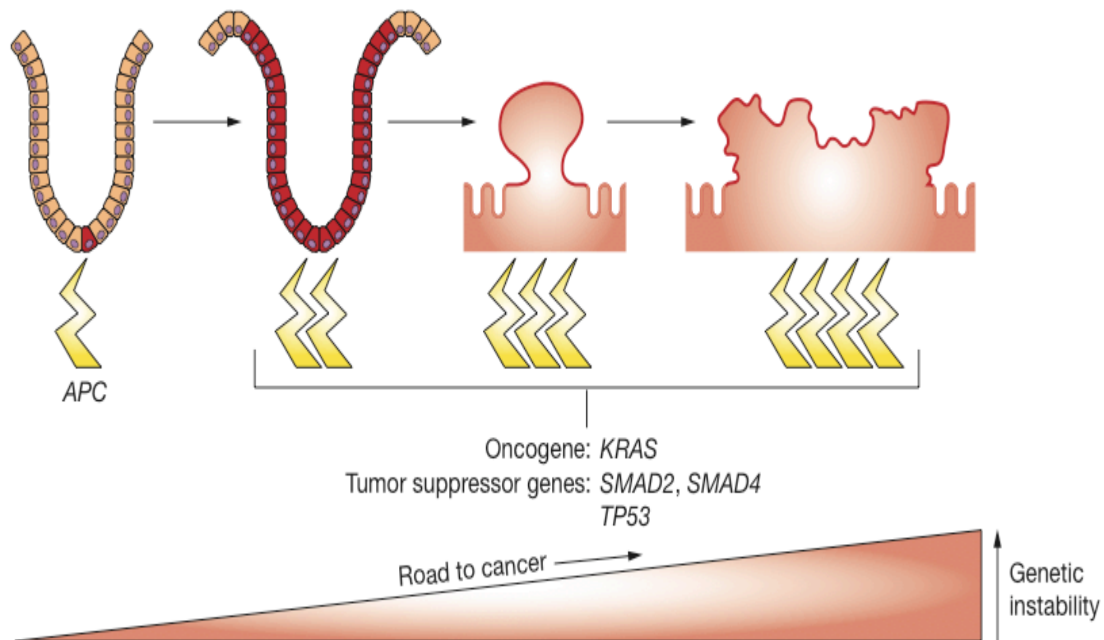


Figure 2: Sequence of Events to Colorectal (Adenoma:Carcinoma) Cancer
Mutations in the *APC* gene are considered to be an early event in colon carcinogenesis. Subsequent activation of oncogenes and repression of tumor suppressor genes, along with increasing genetic instability, leads to progression and development of adenocarcinoma. Reprinted by permission from Nature Publishing Group (McDonald et al. 2006).

Colorectal (Colon) Carcinogenesis

Dr. Bert Vogelstein introduced a multi-step model of colon carcinogenesis in 1988 (Figure 2). Mutations in the *APC* (adenomatous polyposis coli) gene are considered to be one of the earliest events in this process, occurring in up to 80% of sporadic colorectal carcinomas (Vogelstein et al. 1988). The APC tumor-suppressor protein downregulates the Wnt/β-catenin pathway, leading to increased cell proliferation and inhibition of cell death (He et al. 2004; Gregorieff et al. 2005). Further mutations in *K-RAS*, *SMAD*s, and *TP53* can occur during colorectal cancer progression. More recently, chromosomal instability (CIN) and microsatellite instability (MSI) have been described as distinct molecular mechanisms that give rise to colorectal cancer, each

displaying unique molecular features. Other pathways that drive carcinogenesis include Notch, TFG-β, EGFR, and PI3K signaling (Pritchard et al. 2011).

The Notch Pathway

The Notch pathway is an evolutionarily conserved pathway important in cell differentiation, proliferation, apoptosis, cell fate decisions, and stem cell renewal during development and cancer. The Notch receptor is a heterodimeric type 1 single-pass transmembrane protein that undergoes a series of proteolytic cleavage steps to form a mature receptor (Figure 3). There are four *Notch* family proteins (Notch1-4) that begin as a ~300 kDa precursor protein. The protein is shuttled to the *trans*-Golgi compartment where it is cleaved by furin-like convertases, generating N- and C-terminal fragments (NTF and CTF), which are subsequently joined by noncovalent bonds (Blaumueller et al. 1997; Logeat et al. 1998; Rand et al. 2000). The NTF also becomes heavily glycosylated to ensure proper folding and facilitate ligand binding (Vodovar et al. 2008). The mature receptor is then exported to the cell surface. Notch signaling begins with binding of the NTF to one of its five ligands (Jagged 1-2 and Delta-like 1, 3, and 4) located on adjacent cells, allowing for exposure of the second cleavage site on the extracellular portion of the CTF by TACE/ADAM metalloproteases (Brou et al. 2000; Mumm et al. 2000). This generates a membrane-anchored activated Notch form termed Notch extracellular truncation (NEXT). NEXT is then swiftly cleaved at a third site by gamma-secretase, an intramembrane aspartyl protease complex, releasing the Notch IntraCellular Domain (NICD) (Schroeter et al. 1998; De Strooper et al. 1999).

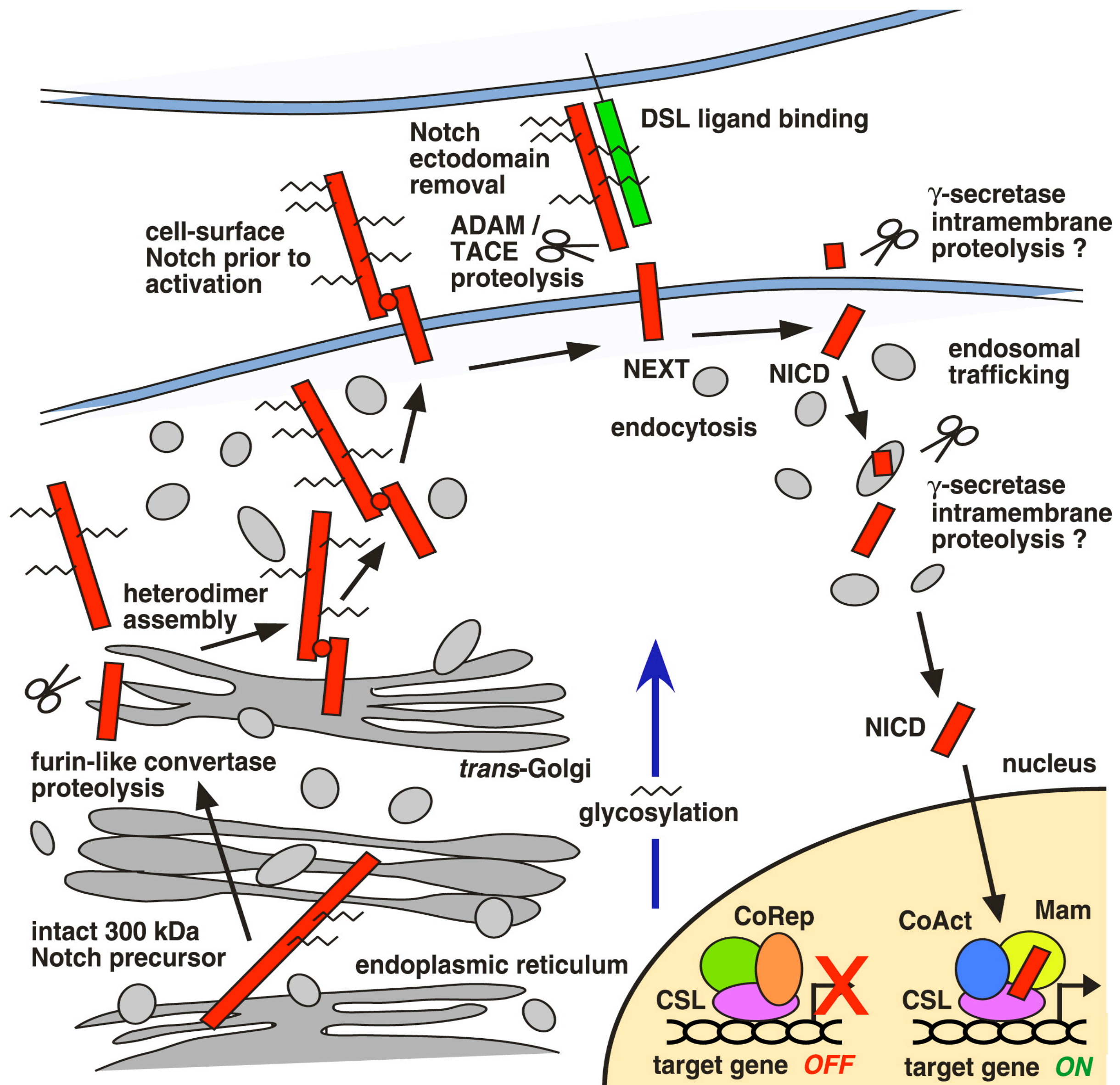


Figure 3: Overview of Notch Receptor Synthesis and Signaling
The precursor Notch protein is cleaved by furin-like convertases in the *trans*-Golgi compartment to produce an N-terminal extracellular fragment and a C-terminal transmembrane fragment. These two fragments are joined through noncovalent linkage to form the mature heterodimeric receptor and shuttled to the cell surface. Notch signaling is initiated through binding of its receptor to its ligand located on an adjacent cell, allowing for exposure of the second cleavage site by TACE/ADAM metalloprotease followed by intramembranous cleavage at a third site by gamma-secretase. Cleavage by gamma-secretase releases the Notch IntraCellular Domain (NICD). NICD translocates to the nucleus, binds to the transcription factor CSL (CBF-1), displaces The intracellular activated form of Notch, NICD, translocates to the nucleus and binds to various corepressors, and recruits MAML-1 and p300/CBP to initiate transcription of Notch target genes. Reprinted by permission from Elsevier (Fortini 2009).

The transcriptional factor CSL (CBF-1/suppressor of hairless/Lag-1), displacing various co-repressors and recruiting MAML-1 (mastermind-like-1) and p300/CBP to induce transcription (Tamura et al. 1995; Fryer et al. 2002; Wallberg et al. 2002). Notch target genes include the HES (hairy/enhancer of split) and HEY family members, c-myc, p21, and cyclin D1, to name a few (Jarriault et al. 1995; Borggrefe et al. 2009).

Gamma-Secretase Complex

Gamma-secretase is a multiprotein transmembrane complex consisting of at least 4 core components: presenilin (PSEN), Pen-2, nicastrin (NCT), and Aph-1 (Figure 4). The complex catalyzes the intramembranous cleavage of type I transmembrane proteins such as APP (amyloid precursor protein, implicated in the development of Alzheimer’s Disease), Notch, E-cadherin, ErbB4, CD44, and c-Met (for review, see (Haapasalo et al. 2011)). Presenilin1, an aspartyl type protease, is the catalytic domain of gamma-secretase and contains nine transmembrane domains (Wolfe et al. 1999; Laudon et al. 2005). Nicastrin is another type I integral membrane protein (Yu et al. 2000). Aph-1 has 7 transmembrane domains while Pen-2 has two (Francis et al. 2002; Goutte et al. 2002). All four proteins are necessary and sufficient for gamma-secretase activity (Kimberly et al. 2003; Takasugi et al. 2003). Nicastrin is essential for stability of the complex; *NCT*-null mouse fibroblasts retain on 50% gamma-secretase activity compared to wildtype but the trimeric complex is highly unstable (Zhao et al. 2010). Aph-1 is also important to regulate stability (LaVoie et al. 2003). Pen-2 also increases stability of the complex and may contribute to the proteolytic activity of the complex (Bammens et al. 2011). More than 50 additional proteins are thought to associate with

gamma-secretase, regulating its activity, subcellular localization, and substrate specificity (Chen et al. 2002; McCarthy et al. 2009)

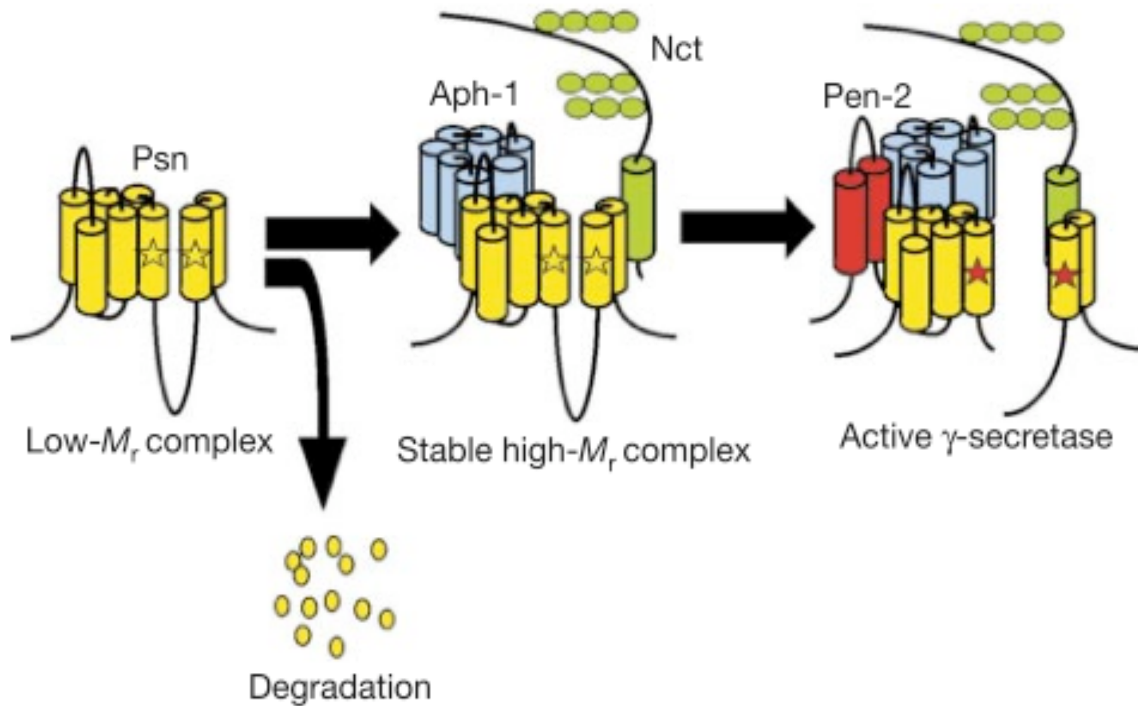


Figure 4: Schematic of Gamma-secretase Assembly and Activation
The gamma-secretase complex consists of at least four core proteins: Presenilin (PSEN), Pen-2, Nicastrin (NCT), and Aph-1. Presenilin is the catalytic domain of the complex but is highly unstable and rapidly degraded. Binding of NCT and Aph-1 to Presenilin help stabilize the complex. Finally, binding of Pen-2, while also potentially increasing stability, contributes to the proteolytic activity of the mature complex. Reprinted by permission from Nature Publishing Group (Takasugi et al. 2003).

Notch Signaling: Oncogenic and Tumor-Suppressive

Aberrant activation of Notch signaling has been implicated in a variety of solid tumors, including breast (Reedijk et al. 2005; Stylianou et al. 2006), melanoma (Bedogni et al. 2008), ovarian (Wang et al. 2010), and prostate (Santagata et al. 2004; Wang et al. 2010). It was first linked to T-cell acute lymphoblastic leukemia (T-ALL), where

>50% of tumors harbored activating mutations within *Notch1* (Ellisen et al. 1991; Weng et al. 2004). In contrast, reduced Notch signaling has been implicated in HPV-positive cervical cancer (Talora et al. 2002), neuroendocrine tumors (Kunnimalaiyaan et al. 2007), small cell lung cancer (Sriuranpong et al. 2001), hepatocellular carcinoma (Qi et al. 2003), and skin squamous cell carcinoma (Lefort et al. 2007). Indeed, the use of gamma-secretase inhibitors in clinical trials for Alzheimer's Disease (the amyloid precursor protein – APP –implicated in this disease is a gamma-secretase target) showed an increase risk of skin cancer development (McKee); decreased gamma-secretase activity is also associated with increased skin cancer occurrence in mice (Li et al. 2007). Therefore, the biological outcome/function of Notch signaling as oncogenic or tumor-suppressive is highly cell context specific (reviewed in (Radtke et al. 2003; Leong et al. 2006)).

Notch Signaling in Normal Colon

The Notch pathway is one signaling pathway that plays a major role in homeostasis of the colon crypt compartment (Radtke et al. 2005). One function is to maintain the intestinal stem/progenitor cells; these are the cells that express Notch receptors and downstream target genes (Schroder et al. 2002; Sander et al. 2004). Transgenic mice expressing NICD under the control of an intestine-specific Villin promoter exhibited significantly increased proliferation in crypt cells, impaired differentiation into goblet and enteroendocrine cells, and upregulated Hes1 (Fre et al. 2005). Deletion of CSL by cre-mediated recombination in the intestinal epithelium of mice revealed a massive differentiation of the proliferative transit-amplifying cells into

goblet cells; similar phenotypic results were obtained by blocking Notch signaling with a gamma-secretase inhibitor in mice with normal colons and in adenomas arising in *Apc*^{Min} mice (van Es et al. 2005). These findings are consistent with observations in *HES1*-null mice and rats treated with gamma-secretase inhibitors showing goblet cells metaplasia (Jensen et al. 2000; Milano et al. 2004).

Aberrant Activation of Notch Signaling in Colon Cancer

The Notch pathway has been implicated in colon cancer tumorogenesis. Colorectal cancer tumors in humans show higher expression of *NOTCH1*, *JAGGED* ligands, and *HES1* compared to normal by *in situ* hybridization and qPCR (Reedijk et al. 2008; Qiao et al. 2009). Transgenic expression of NICD in *ApcMin* mice, which form multiple adenomas (but do not progress into carcinoma), show increased number of adenomas, suggesting that Notch signaling may play a role in initiation but not progression of intestinal tumors (Fre et al. 2009). This group also showed elevated levels of Notch-1 and Hes-1 in adenomas compared to carcinomas in a small cohort of human patients. These finding are consistent with other studies in humans showing high Notch signaling in adenomas and early-stage colon carcinomas but low in later-stage, advanced and metastatic colon carcinomas (Reedijk et al. 2008; Rodilla et al. 2009). The molecular mechanisms underlying this distinction remain unclear.

Apoptosis

Apoptosis is an evolutionarily conserved multi-step form of type I programmed cell death that culminates in the self-destruction of a cell. This process is important in

normal development, aging, and tissue homeostasis by removing damaged or redundant cells. For example, cells in the bone marrow and intestine undergo apoptosis to make way for new cells every day. The deregulation of apoptosis is associated with disease: increased apoptosis in neurodegenerative disorder and inhibition of apoptosis in cancer, for example (Thompson 1995).

The morphological and biochemical features that characterize apoptosis include cell shrinkage, membrane blebbing, chromosome condensation, nuclear fragmentation, mitochondrial membrane disruption, activation of caspases, and exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane (Kerr et al. 1972; Martin et al. 1995; Saraste et al. 2000). The resultant apoptotic cell is removed by phagocytosis, providing the engulfing cell with recyclable cellular material while avoiding inflammation.

Apoptosis can be triggered by two pathways: the extrinsic pathway or the intrinsic pathway. The extrinsic pathway of apoptosis is activated upon binding of a specific ligand to death receptors. The intrinsic pathway is activated by cellular stress and involves the mitochondria. Apoptosis is tightly regulated and leads to the execution of step-wise caspase activation. Caspases, cysteinyl aspartate-specific proteases, are synthesized as inactive precursors known as procaspases and are activated upon cleavage. They can be separated into two functional groups: initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspases-3, -6, -7) (Cohen 1997). Active caspases can then cleave other caspases to amplify the signal or cleave other proteins, resulting in the morphological and biochemical features of apoptosis (Enari et al. 1998; Coleman et al. 2001; Taylor et al. 2008).

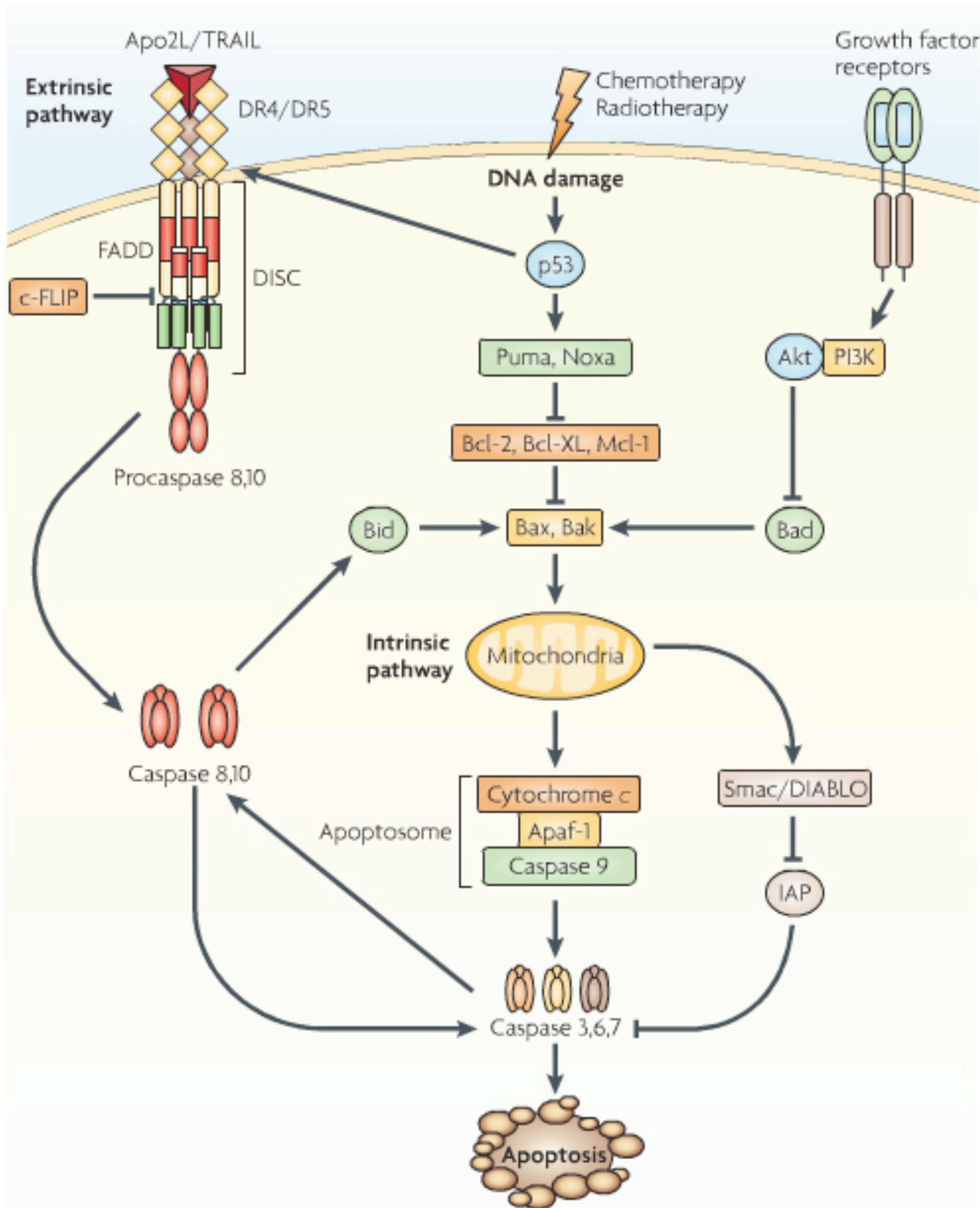


Figure 5: The Intrinsic and Extrinsic Pathways of Apoptosis
The extrinsic apoptotic pathway is activated upon binding of a specific ligand to death receptors and subsequent recruitment of cytoplasmic adaptor proteins and initiator procaspase-8 and/or -10. The initiator procaspases are cleaved to produce mature, active caspase-8 and/or -10 that, in turn, cleave and activate effector caspase-3, -6, and/or -7. The intrinsic pathway is initiated by extra- or intracellular stress, leading to activation of Bax and/or Bak at the mitochondria and subsequent cytochrome c release. Cytochrome c forms a complex with Apaf-1 and caspase-9 and this complex cleaves and activates effector caspase-3, -6, and/or -7. Reprinted by permission from Nature Publishing Group (Ashkenazi 2008).

The Extrinsic Apoptotic Pathway

The extrinsic, or receptor-mediated, pathway involves activation of the tumor necrosis factor (TNF) family member receptors by their extracellular ligands (Figure 5). These receptors, often referred to as death receptors, all contain cysteine-rich extracellular domains responsible for ligand binding and a cytoplasmic death domain (DD). Members include TRAIL, FasL, TNFR1-2, and DN3-5 (Locksley et al. 2001). Ligand binding initiates receptor trimerization and activation, leading to the recruitment of cytoplasmic adaptor proteins such as FADD (Fas-associated death domain) or TRADD (TNF-associated death domain) to the DD of the receptor (Ashkenazi et al. 1998). FADD also contains a death effector domain (DED) that recruits DED-containing initiator caspase-8 and/or caspase-10. The resultant complex formed is termed death-inducing signaling complex (DISC) and is responsible for proteolytic processing and activation of the initiator caspases leading to further cleaving and activation of the effector caspases (Ashkenazi 2002). The apoptotic signal can be further amplified by cleavage of BID by caspase-8 into a truncated form (tBID). This tBID protein translocates into the mitochondria and interacts with Bak and Bax to activate the intrinsic apoptotic pathway (Yin 2000).

The Intrinsic Apoptotic Pathway

The intrinsic, or mitochondrial, pathway is activated by extracellular and intercellular stresses, including growth factor withdrawal, oxidative stress, hypoxia, radiation, and chemotherapy (Figure 5).

The members of Bcl-2 family tightly regulate apoptosis through the intrinsic pathway. They can be divided into two classes: inhibitors of apoptosis and promoters of apoptosis. Anti-apoptotic Bcl-2 members include Bcl-2, Bcl-xL, Mcl-1, and Bcl-w and share structural similarity within all four of their Bcl-2 homology (BH) domains. Pro-apoptotic Bcl-2 members can be further subdivided into multi-domain proteins, like Bax, Bak, and Bok, and into BH-3 only proteins, like Puma, Noxa, Bim, Bad, and Bid (Petros et al. 2004). The anti-apoptotic members inhibit apoptosis by inhibiting Bax and Bak, thereby maintaining mitochondrial integrity. The BH-3 only proteins act as stress sensors; two models have been proposed to explain how they promote apoptosis. First, in the displacement model, the BH3-only proteins bind to anti-apoptotic Bcl-2 family members, releasing them from Bax and Bak. In the second model, the BH-3 only proteins directly activate the multi-domain, pro-apoptotic proteins Bax and Bak (Strasser 2005; Shamas-Din et al. 2011).

Once Bax and Bak are dissociated from anti-apoptotic Bcl-2 proteins, they undergo a conformational change, translocate to the mitochondria, and oligomerize to form a pore (Hsu et al. 1998; Lalier et al. 2007). This activation and subsequent pore formation is required for mitochondrial outer membrane permeabilization (MOMP) and results in the release of cytochrome c (and/or other apoptogenic proteins AIF, endonuclease G, HtrA2/Omi, and Smac/Diablo) into the cytoplasm. There, cytochrome c binds to Apaf-1, causing Apaf-1 oligomerization and recruitment of procaspase-9 (forming the apoptosome). Procaspase-9 clustering in the apoptosome results in its cleavage to an activated form and subsequent cleavage of effector caspases. Smac/Diablo and HtrA2/Omi bind to inhibitors of apoptosis (IAP) proteins, such as XIAP

and cIAP, preventing their inhibition of effector caspases; AIF and endonuclease G participate in DNA fragmentation. Initiation of the intrinsic apoptotic pathway is therefore a delicate balance between pro- and anti-apoptotic factors (Shore et al. 2005).

Autophagy

Autophagy is an evolutionary conserved cellular pathway responsible for the degradation and recycling of cellular material and organelles. Autophagy plays a role in stress responses (including nutrient deprivation, growth factor withdrawal, hypoxia, and chemotherapy) to promote survival, in cellular homeostasis to remove misfolded or aggregated proteins and damaged organelles, and during host-defense to degrade intracellular pathogens (Moreau et al. 2010). Three distinct forms of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy.

Autophagy plays a dual role in carcinogenesis. On one hand, it's believed to play a tumor suppressive role to limit tumor initiation. However, once neoplastic lesions have been established, autophagy instead facilitates tumor progression and may be critical for continued growth and sustained cellular metabolism. Autophagy is also induced in response to chemotherapy to limit drug efficacy. Aberrant control of autophagy is now considered to be another hallmark of cancer (Levine et al. 2008; Kimmelman 2011).

Upon autophagy induction, a small vesicular sac called the isolation membrane or phagophore elongates and subsequently encloses a portion of the cytoplasm. This results in the formation of a double-membrane structure, termed the autophagosome.

The autophagosome matures when the outer membrane fuses with a lysosome (to form the autolysosome), leading to the degradation of the enclosed materials together with the inner autophagosomal membrane by lysosomal hydrolases. Amino acids and other small molecules that are generated by autophagic degradation are delivered back to the cytoplasm for recycling or energy production (Figure 6)(Yang et al. 2010).

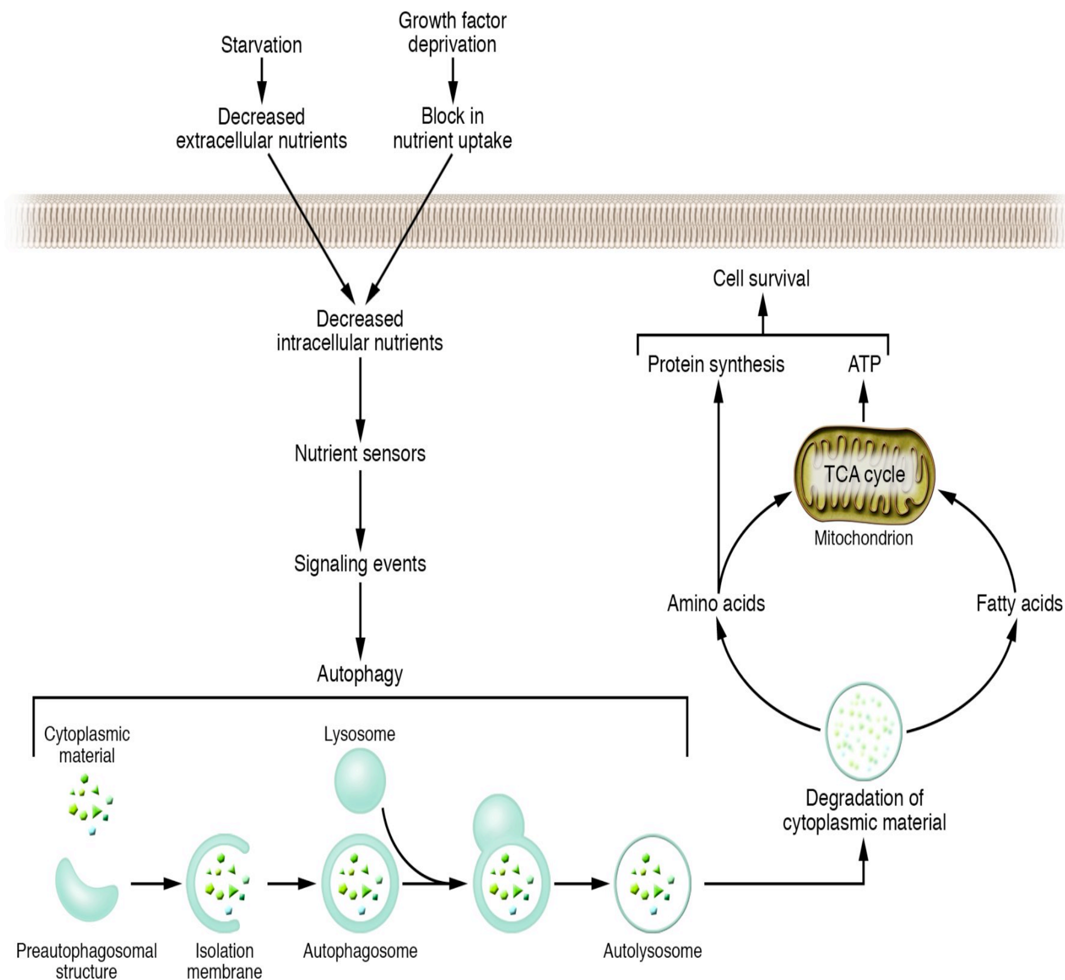


Figure 6: The Process of Autophagy
Upon activation of autophagy by starvation or growth factor deprivation, for example, cytoplasmic material becomes enclosed in a double-membrane structure termed the autophagosome. The lysosome fuses with the autophagosome and leads to the degradation of the enclosed cytoplasmic material. The resultant amino acids and other small molecules generated can be used for energy-production or recycling. Reprinted by permission from American Society of Clinical Investigation (Levine et al. 2005).

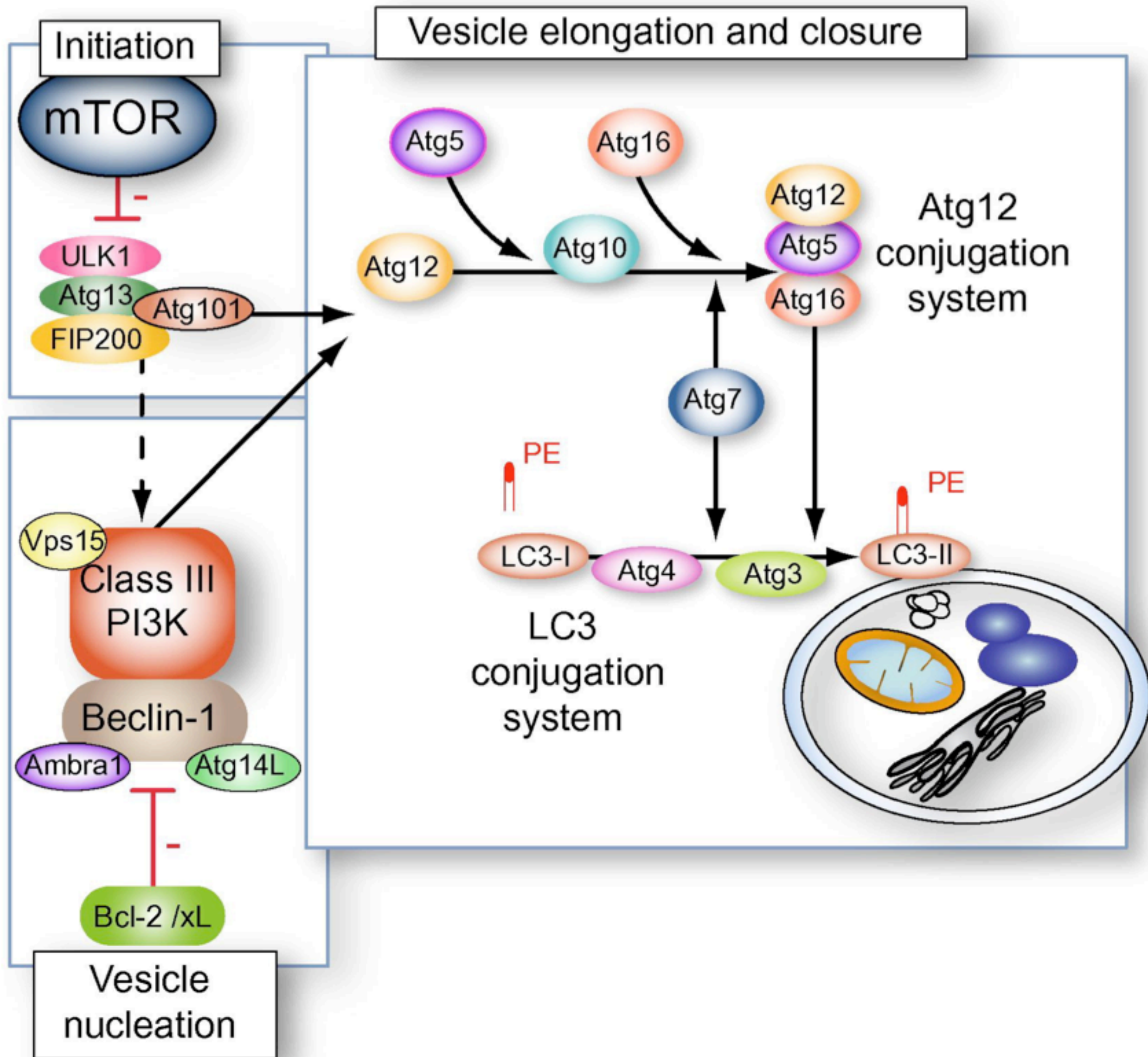


Figure 7: The Core Molecular Machinery of Autophagy
There are three basic steps leading to autophagosome formation: initiation, vesicle nucleation, and vesicle elongation and closure. Initiation begins when mTOR is inactivated, allowing for the formation of the ULK1 complex. Vesicle nucleation begins by the formation of the Beclin1-Vsp34 PI3K-Atg14L-Vsp15 complex. Vesicle elongation and closure follows via two conjugation systems: Atg12 and LC3. They lead to the conjugation of phosphatidylethanolamine (PE) to LC3-1, forming the autophagic vesicle-associated form (LC3-II). Reprinted by permission from Elsevier (Rautou et al. 2010).

Core Molecular Machinery of Autophagy

As mentioned earlier, autophagy occurs at basal, constitutive levels in many cells and is upregulated under physiological stress. The initiation of autophagy occurs mainly through downregulation of mTOR (target of rapamycin) signaling and the Vps34 (a class III phosphatidylinositol-3-kinase (PI3K)) – Atg6/Beclin-1 complex (Yang et al. 2010).

ATG genes

The basic molecular machinery of autophagy involves many ATG (autophagy associated proteins) genes. They were first identified in yeast and show strong evolutionary conservation from yeast to humans. The Atg proteins regulate the entire process of autophagy initiation, maturation, and degradation of substrates. They are divided into four core subgroups: 1) The Atg1/unk-51-like kinase (ULK1), 2) Vsp34/Beclin-1 (Atg6), 3) two ubiquitin-like proteins (Atg12 and Atg8/LC3) conjugation complexes, and 4) two transmembrane protein, Atg9 and VMP1 (vacuole membrane protein 1) (Figure 7).

Atg1 exists in a complex with Atg13 and Atg7. Atg13 is phosphorylated by mTOR signaling to modulate complex formation. Initiation begins when mTOR becomes inactivated; Atg13 becomes dephosphorylated and increases Atg1-Atg7-Atg13 complex formation, initiating autophagy. Vesicle nucleation begins by formation of the autophagy-specific Vsp34-Beclin-1-Atg14L-Vsp15 complex. Vsp15 (vacuolar sorting protein 15) (the yeast homologue of mammalian p150) is a protein kinase that activates Vsp34. Vsp34 is a lipid kinase that generates phosphatidylinositol (3)-phosphate (PtdIns(3)P) at the PAS (perivacuolar sites known as phagophore assembly

sites) to allow recruitment of other Atg proteins. Agt14L is thought to direct the complex to the phagophore and also initiate recruitment of other Atg proteins (Yang et al. 2010). The nucleation step can be blocked by treatment with the PI3K inhibitor 3-MA (3-methyladenine).

The two ubiquitin-like conjugation systems, Atg12-Atg5 and Atg8/LC3-phosphatidylethanolamine (PE), regulate the elongation and expansion of autophagosome formation. Atg7 and Atg10 (E1 and E2-like enzymes, respectively), conjugate Atg12 to Atg5. The Atg12-Atg5 complex non-covalently interacts with Atg16L. The second system involves cleavage of Atg8/LC3 (microtubule-associated protein light chain 3) at its C terminus by Atg4. This generates the cleavage product LC3-I, a cytosolic protein with a C-terminal glycine reside. PE is conjugated to the glycine residue in a reaction that requires Atg7 and Atg3 (an E2-like enzyme). This lipidated form of LC3 (LC3-II) is attached to both the outer and inner phagophore membrane. Removal of LC3-II from the outer membrane of the autophagosome facilitates its fusion to the lysosome (Yang et al. 2009; Yang et al. 2010). Upon completion, LC3-II is degraded within the autolysosome. Monitoring of LC3-II levels is one of the best markers to assess autophagic flux (Rubinsztein et al. 2009).

Mammalian Atg9 and VMP1 are two transmembrane proteins required for the biogenesis of autophagosomes and recruitment of the Vsp34-Beclin1-Atg14L-Vsp15 complex to the phagophore, respectively. Atg9 shuttles between the *trans*-Golgi network and late endosomes and peripheral sites. This shuttling is cycling of Atg9 localization is thought to contribute to the delivery of membranes to the forming autophagosome (Yang et al. 2010).

CHAPTER TWO: MATERIALS AND METHODS

Cell Lines and Chemicals

The human colorectal cancer cell lines HCT-116, HCT-15, HT-29, and Colo205 were obtained from NCI; SW480 was obtained from ATCC. All cell lines were grown in RPMI 1640 supplemented with 10% FBS and L-glutamine and grown in a 37°C humidified camber 5% CO_2. Cells were maintained for no more than 30 passages after thawing and tested as mycoplasma-free. HCT-116 and SW480 cell lines were authenticated using short tandem repeat (STR) DNA typing according to ATCC's "Authentication of Human Cell Lines: Standardization of STR Profiling" (2012).

Oxaliplatin (Sigma) was suspended in 5% dextrose. The gamma-secretase inhibitors MRK-003 (a generous gift from Merck Inc), DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)-S-phenylglycine]-t-butyl ester; Santa Cruz), GSI-XII (Calbiochem), and GSI-XX (DBZ; Calbiochem) and the Bcl-2 inhibitor obatoclax (GX15-070; Selleck) were suspended in DMSO. 3-MA (Santa Cruz) was suspended in water and dissolved by heating at 100°C. Aliquots were stored at -20°C and reheated before use. Bafilomycin A1 and z-VAD-FMK were purchased from Santa Cruz. Propidium iodide and acridine orange were purchased from Sigma.

Apoptosis Assays via Flow Cytometry

Apoptosis was measured by flow cytometry by detecting activated Caspase-3. Cleaved Caspase-3 was measured using the Active Caspase3-FITC Apoptosis Assay Kit (BD# 550480) according to the manufacturer's protocol. Briefly, floating and adherent cells were collected, washed in PBS, and fixed in Cytofix/Cytoperm buffer for 20 minutes on ice. Cells were then washed in Perm/Wash buffer and incubated with Cleaved caspase3-FITC antibody for 30 minutes at room temperature. Data were acquired on a FACS Scan or Calibur and analyzed with FlowJo software.

Apoptosis was also measured by AnnexinV-FITC (or AnnexinV-APC) (BD# 556420 or 550475) and 7-AAD staining according to the manufacturer's protocol. Adherent and non-adherent cells were collected and incubated in 1X Annexin-V Binding Buffer (10mM Hepes pH 7.4, 150mM NaCl, 2.5nM $CaCl_2$) with AnnexinV-FITC (or AnnexinV-APC) and 7-AAD for 15 minutes at room temperature. Data were acquired on a FACS Scan or Calibur and analyzed with FlowJo software. Dot plots with the percentages of each quadrant were reported for each sample.

Apoptosis was also measured by Propidium iodide (PI) staining for cell viability. Cells were harvested by trypsinization and washed with PBS. Cells were suspended in 2 μg/ml PI in PBS and kept on ice. Data were acquired on a FACS Scan or Calibur and analyzed with FlowJo software.

Western Blotting

Cell pellets were lysed in standard RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS) containing

protease and phosphatase inhibitors – 1 μg/ml each of aprotin, leupeptin, and pepstatin and 1 mM each of sodium fluoride, sodium vanadate, phenylmethylsulfonyl fluoride, and sodium pyrophosphate - for 30 minutes on ice. Cell extracts were clarified by centrifugation at 15,000g for 10 minutes at 4°C. Protein concentration was determined via the Bradford Assay and lysates were mixed 1:1 in 2X Laemmeli Sample Buffer and heated at 95°C for 5 minutes. 30 μg of protein was resolved onto SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked in 1X TBST (Tris buffered saline with 0.05% Tween-20) supplemented with 5% milk and primary antibodies were incubated overnight at 4°C in 1% BSA or 5% milk dissolved in TBST. Membranes were washed and incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Jackson ImmunoReasearch Laboratories). The signals were visualized using SuperSignal West Pico Chemiluminescent detection reagent (Pierce/ThermoScientific).

Primary Antibodies

The primary antibodies used for western blot were: rabbit anti-Mcl-1 (#5453), rabbit anti-Bcl-xL (#2764), rabbit anti-PARP (#9542), rabbit anti-LC3A/B (#4599); rabbit anti-Atg5 (#8540), and rabbit anti-Beclin1 (#3495) from Cell Signaling; mouse anti-β-actin (#A5441) from Sigma; and mouse anti-p62 (#sc-28359) from Santa Cruz.

Cytochrome C Release

Adherent and non-adherent cells were harvested by scrapping and pelleted by centrifugation. For preparation of cytosolic extracts, the cell pellet was immediately

suspended in a cytochrome c permeabilization buffer (20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM Sucrose) containing 200 µg/ml Digitonin (Sigma# D141) supplemented with protease and phosphatase inhibitors (see above in Western blotting) and incubated on ice for 5 minutes. Lysates were clarified by centrifugation at 1000g for 5 minutes at 4°C to remove unlysed cells, cell debris and nuclei. The supernatant transferred to a new tube and centrifuged at 20,000xg for 20 minutes at 4°C to remove mitochondria and any remaining debris. The supernatant was transferred to a new tube and 20-30 µg of protein was subjected to western blot analysis using anti-cytochrome c (BD# 556433) antibody. An anti-COX IV (Cell Signaling# 4580) antibody was used as a control to measure mitochondrial contamination of cytosolic extracts.

Cell Survival and Colony Formation Assays

Short-term cell survival was measured by MTT assay. Cells were seeded into 96-well plates in quadruplicate and drug treated the next day for 48 hours. MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma# M2128) was added at a final concentration of 0.5 µg/uL per well and incubated for 3-4 hours in a humidified chamber at 37°C in the dark. The plate was centrifuged, supernatant aspirated, 100 µL / well of DMSO added, and the absorbance measured at 540 nm. Data were normalized relative to untreated controls, which were taken as 100% survival.

Colony / foci formation assays were used to assess long-term cell survival. Cells were seeded into 12-well plates, allowed to attach overnight, and drug treated for 24 hours. Attached cells were then trypsinized, counted, and 1000 cells were plated in

triplicate in 6-well plates and allowed to grow for 8 or 12 days. Cells were fixed with 4% paraformaldehyde for 15 minutes with 0.1% crystal violet.

Cell Cycle Analysis

Adherent and non-adherent cells were collected, fixed in ice-cold 70% ethanol, and kept at 4°C overnight. Cells were then stained with 20 μg/ml propidium iodine, 200 μg/ml RNase A, and 0.1% Triton X-100 in PBS for 30 minutes in the dark at room temperature. Data was acquired on a FACS Scan or Calibur and analyzed with ModFit software.

Activation of Bax and Bak

Cell pellets were lysed in CHAPS lysis buffer (1% CHAPS, 150 mM NaCl, 1 mM Hepes pH7.4) containing protease and phosphatase inhibitors (see above for details) for one hour on ice. Lysates were centrifuged and protein concentration was determined by Bradford assay. 500 μg of protein was precleared with 20 μL of anti-Mouse IgG (whole molecule)-Agarose beads (Sigma# A6531) for one hour at 4°C with rotation. The beads were pelleted and the supernatant was transferred to a new tube. Activated Bax or Bak was immunoprecipitated with 2 μg of anti-Bax 6A7 (Sigma# B8429) or anti-Bak Ab-1 (Calbiochem# AM03) antibodies and 20 μL of anti-Mouse IgG-Agarose beads and incubated overnight at 4°C with rotation. The beads were pelleted and washed three times in CHAPS lysis buffer before being subjected to western blot analysis with rabbit anti-Bax (N-20, #sc-493) and rabbit anti-Bak (G-23, #sc-832), respectively (Santa Cruz).

RNA Interference

Small interfering RNA (siRNA) for Mcl-1 (#sc-35877), Bcl-xL (#43630), and Control-A (#sc-37007) was purchased from Santa Cruz; Signalsilence siRNA for Atg5 (#6345), Beclin-1 (#6222) and Control (#6568) were purchased from Cell Signaling. Cells were transfected with RNAiMax (Invitrogen) with a reverse transfection protocol according to the manufacturer's protocol. Briefly, siRNA and RNAiMax were incubated in serum-free RPMI 1640 for at least 20 minutes at room temperature. After siRNA: RNAiMax complexes were allowed to form, diluted cells in full serum media (RPMI 1640 + 10% FBS) were added and the mixture plated out.

CHAPTER THREE: GAMMA-SECRETASE INHIBITION ATTENUATES OXALIPLATIN-INDUCED APOPTOSIS THROUGH INCREASED MCL-1 AND/OR BCL-XL IN HUMAN COLON CANCER CELLS

Note to Reader

These results have been previously published (Timme et al., 2013) and are utilized with permission of the publisher.

Abstract

The Notch signaling pathway plays a significant role in differentiation, proliferation, apoptosis, and stem cell processes. It is essential for maintenance of the normal colon crypt and has been implicated in colorectal cancer oncogenesis. Downregulation of the Notch pathway through gamma-secretase inhibitors (GSIs) has been shown to induce apoptosis and enhance response to chemotherapy in a variety of malignancies. In this study, we analyzed the effect of MRK-003 (Merck), a potent inhibitor of gamma-secretase, on oxaliplatin-induced apoptosis in colon cancer. Unexpectedly, gamma-secretase inhibition reduced oxaliplatin-induced apoptosis while GSI treatment alone was shown to have no effect on growth or apoptosis. We determined that the underlying mechanism of action involved an increase in protein levels of the anti-apoptotic Bcl-2 family members Mcl-1 and/or Bcl-xL, which resulted in

reduced Bax and Bak activation. Blocking of Mcl-1 and/or Bcl-xL through siRNA or the small molecule inhibitor obatoclax restored the apoptotic potential of cells treated with both oxaliplatin and MRK-003. Moreover, obatoclax synergized with MRK-003 alone to induce apoptosis. Our findings warrant caution when treating colon cancer with the combination of GSIs and chemotherapy, whereas other drug combinations, such as GSIs plus obatoclax, should be explored.

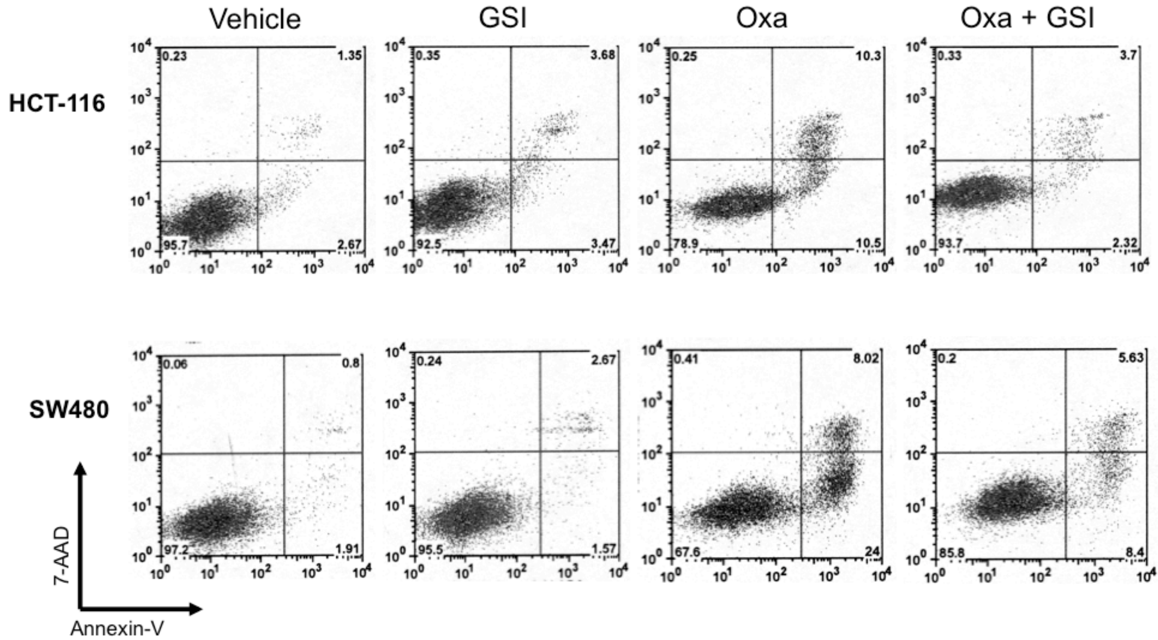
Results

Gamma-Secretase Inhibitors Attenuate Chemotherapy-induced Apoptosis of Colon Cancer Cells

Inhibition of gamma-secretase has shown antitumor effects in a variety of cancers *in vitro* and is thus considered an attractive pharmacological target. Pre-clinical reports using cancer cell lines, patient samples *in vitro*, and *in vivo* mouse models on the activity of MRK-003, a potent and selective GSI, have reported Notch pathway inhibition and antitumor activity in multiple myeloma (Ramakrishnan et al. 2012), Non-Hodgkin's leukemia (Ramakrishnan et al. 2012), T-cell acute lymphoblastic leukemia (Lewis et al. 2007), breast cancer (Grudzien et al. 2010), pancreatic cancer (Plentz et al. 2009; Vo et al. 2011), lung cancer (Konishi et al. 2007), and glioblastoma (Chen et al. 2010).

Oxaliplatin is the first line of chemotherapy for colon cancer. We hypothesized that combining oxaliplatin with gamma-secretase inhibitors would enhance chemosensitivity in colon cancer cell lines, as others have shown using alternative gamma-secretase inhibitors (Akiyoshi et al. 2008; Aleksic et al. 2008; Meng et al. 2009).

A)



B)

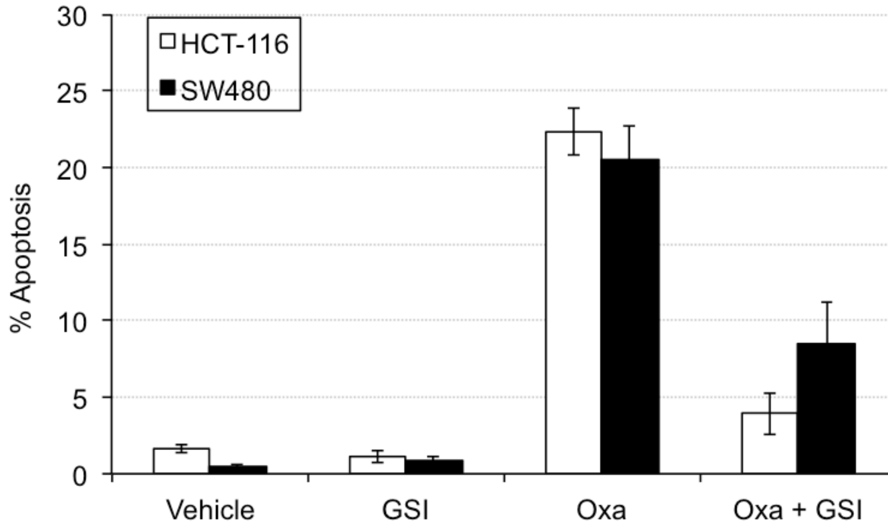


Figure 8: Gamma-secretase Inhibition Attenuates Oxaliplatin-induced Apoptosis
HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30μM (SW480) oxaliplatin (Oxa) for 48 hours in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003 (GSI). Apoptosis rates were measure by flow cytometry following Annexin-V/7-AAD staining (A) and active caspase-3 labeling (B). (C) Apoptosis rates were measured by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of three independent experiments.

To explore the role of gamma-secretase inhibition on oxaliplatin-induced apoptosis, two colon cancer cell lines – HCT-116 and SW480 – were treated with oxaliplatin in the absence or presence of MRK-003 (Merck, Inc), a potent gamma-secretase inhibitor. The doses of MRK-003 used (10 μM for HCT-116 and 5 μM for SW480) were determined, after dose response experiments, to be the maximum dosage with no quantifiable toxicity (data not shown). Apoptosis was measured by flow cytometry using either Annexin-V with 7-AAD double staining or measuring active caspase-3. Annexin-V binds to the membrane phospholipid phosphatidylserine (PS) upon exposure to the external cellular environment in apoptotic cells while 7-AAD is a vital dye that is excluded from cells with an intact plasma membrane. The combination of oxaliplatin and MRK-003 resulted in a reduction in apoptosis by at least 50% as measured by either Annexin-V & 7-AAD positivity (Figure 8a) or activated caspase-3 (Figure 8b) in both cell lines compared to oxaliplatin alone.

To confirm that the results were likely due to gamma-secretase inhibition and not an off target effect of MRK-003, the effect of alternative gamma-secretase inhibitors on apoptosis of oxaliplatin treated cells were repeated using DAPT, GSI-XII, and GSI-XX (DBZ), widely used and commercially available GSIs that are structurally distinct from MRK-003. Both cell lines treated with DAPT and GSI-XX showed apoptosis rates approximately half that of oxaliplatin-treated cells (Figure 9a,c). The gamma-secretase inhibitor GSI-XII showed a slight decrease in oxaliplatin-induced apoptosis in SW480 but a slight induction was observed in HCT-116 (Figure 9b). MRK-003, DAPT, GSI-XII, or GSI-XX alone did not induce apoptosis in either HCT-116 or SW480.

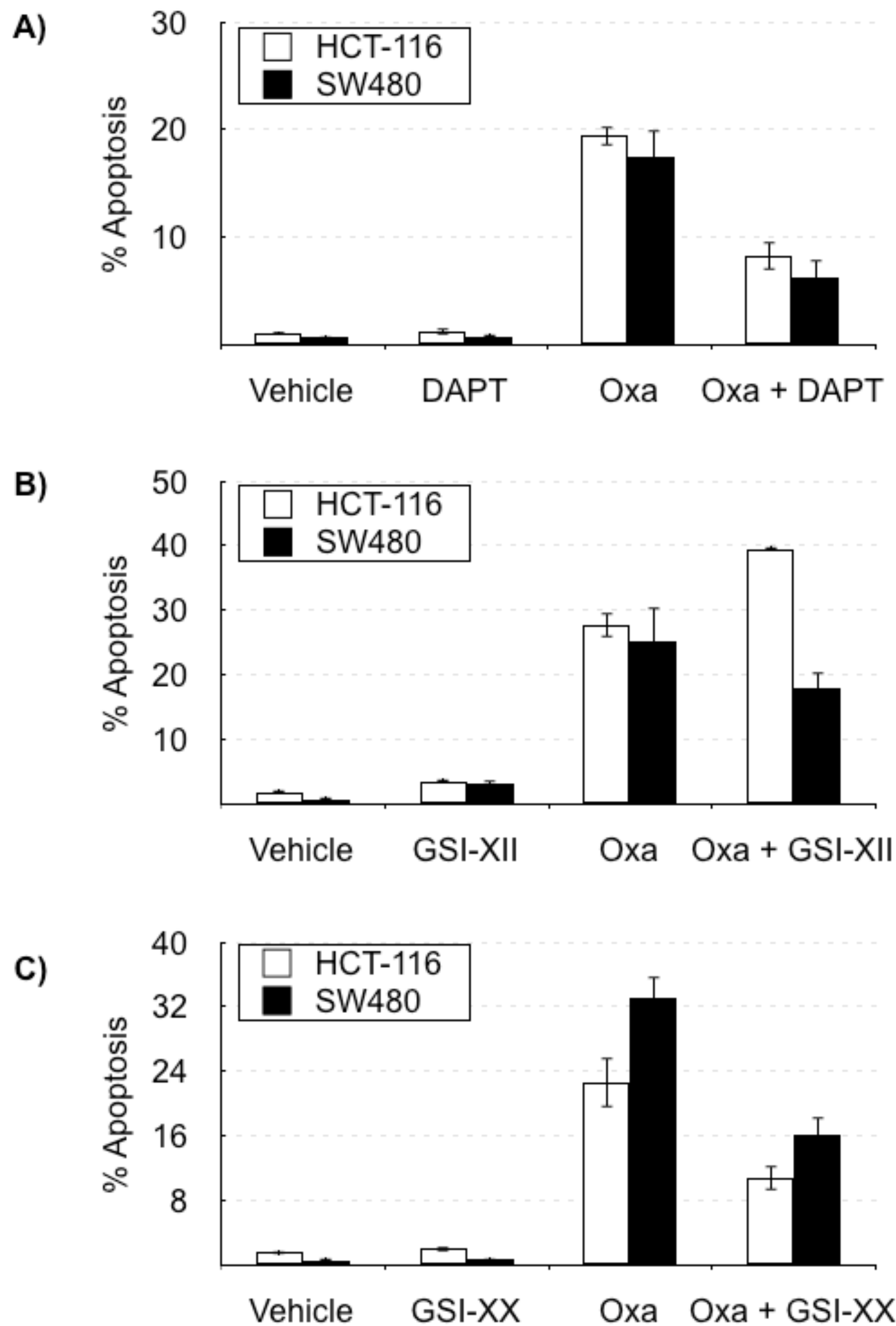


Figure 9: Effects of Alternative GSIs on HCT-116 and SW480 Colon Cancer Cell Lines

HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30μM (SW480) oxaliplatin (Oxa) for 48 hours in the absence or presence of 25 μM DAPT (A), 5 μM (HCT-116) or 10 μM (SW480) GSI-XII (B), or 2 μM GSI-XX (C). Apoptosis rates were measured by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of two independent experiments.

We further tested the effect of MRK-003 on oxaliplatin-induced apoptosis in three additional colon cancer cell lines to determine whether the reduction in apoptosis was cell line specific. We found greater than 50% reduction of apoptosis in oxaliplatin-treated HT-29, HCT-15, and Colo205 cells in the presence of MRK-003 (Figure 10).

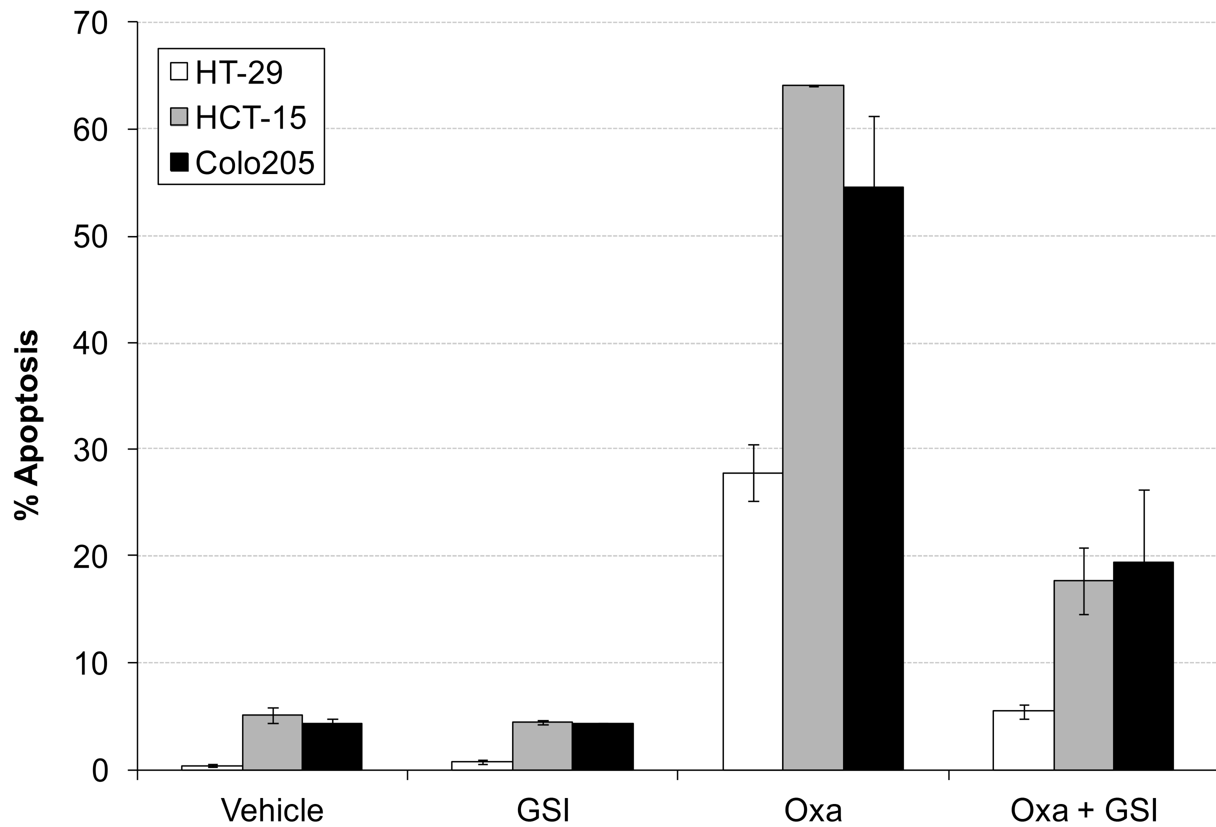


Figure 10: Effects of MRK-003 on Oxaliplatin-Induced Apoptosis in HT-29, HCT-15, and Colo205 Colon Cancer Cell Lines
HT-29, HCT-15, and Colo205 were treated with either 30 (HCT-15) or 50 μM (HT-29 and Colo205) Oxa in the absence or presence of 5 μM (HT-29) or 10 μM MRK-003 (HCT-15 and Colo205) for 48 hours. Apoptosis rates were measured by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of at least two independent experiments

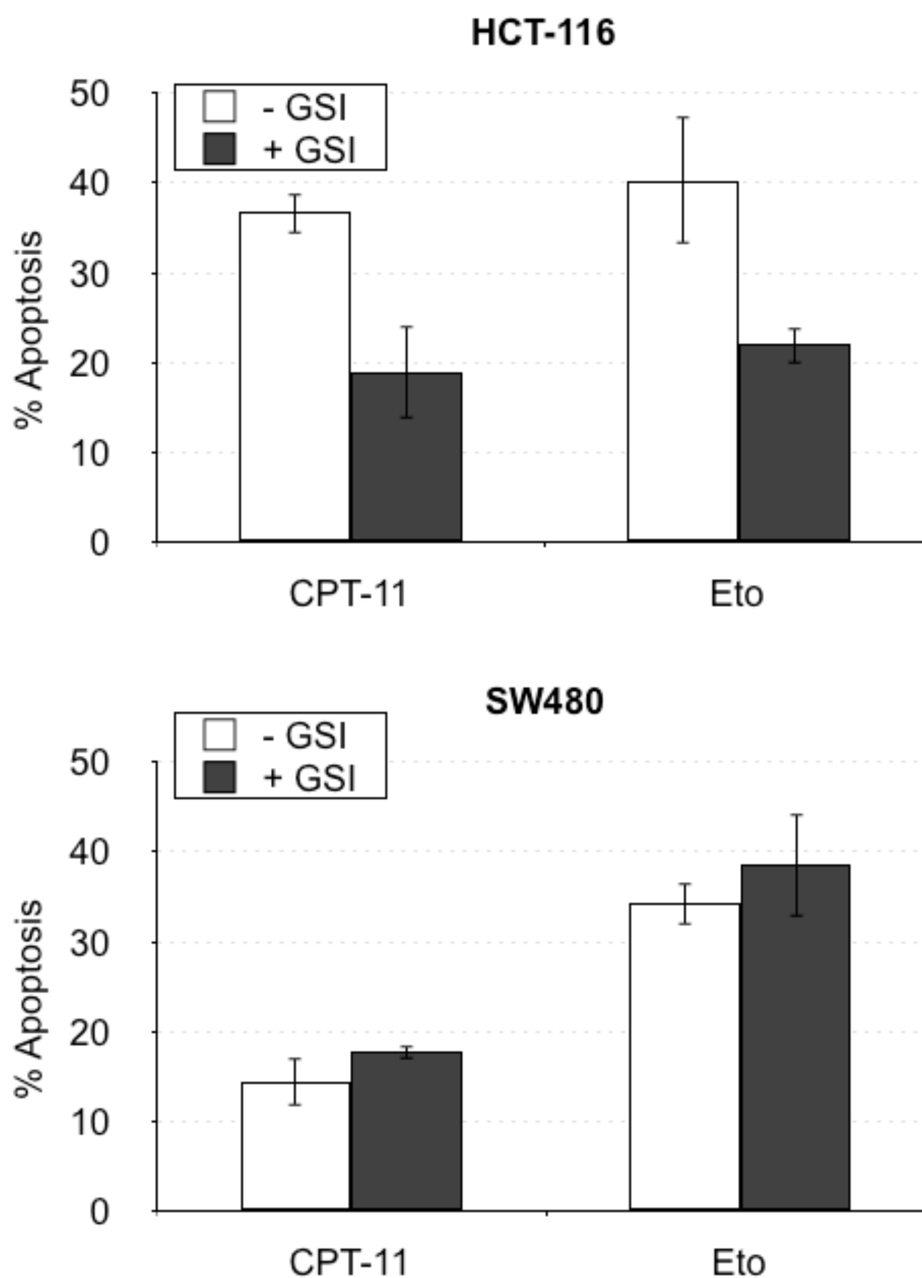


Figure 11: Gamma-secretase Inhibition Attenuates Irinotecan- and Etoposide-induced Apoptosis
HCT-116 and SW480 cells were treated with either 50 μM Etoposide or 50 μM Irinotecan in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003 for 48 hours. Apoptosis rates were measured by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of two independent experiments.

To test if the attenuation of apoptosis was specific to oxaliplatin, we assessed the effects of gamma-secretase inhibition with either irinotecan or etoposide. Again, we observed a reduction of apoptosis in HCT-116 with MRK-003 in combination with either irinotecan or etoposide; no difference in apoptosis was observed in SW480 (Figure 11). Taken together, these data suggest that gamma-secretase inhibition attenuates apoptosis induced by chemotherapeutic agents in colon cancer.

The Combined Use of Oxaliplatin and MRK-003 Increases Long-Term Survival in vitro

We hypothesized the attenuation of apoptosis seen in cells treated with oxaliplatin and MRK-003 might be due to increased survival, as measured by MTT assay and cell cycle analysis. HCT-116 and SW480 were treated for 48 hours with oxaliplatin with or without MRK-003 and survival was measured by MTT assay. As expected, oxaliplatin treatment decreased viability by approximately 60% for both cell lines; the combination of oxaliplatin and MRK-003 showed no difference compared to oxaliplatin alone (Figure 12a). MRK-003 alone had no effect on viability compared to vehicle in either cell line.

Cell cycle analysis showed oxaliplatin induced a G_2-M arrest in HCT-116 that remained unchanged with the addition of MRK-003; the percentage of cells in G_0/G_1, however, increased (21% to 32%, respectively) (Figure 12b). Oxaliplatin arrested SW480 cells in S phase and no change was observed with the addition of MRK-003 in the cell cycle profile (Figure 12b). MRK-003 alone increased the G_0/G_1 fraction from 34% to 48% in HCT-116.

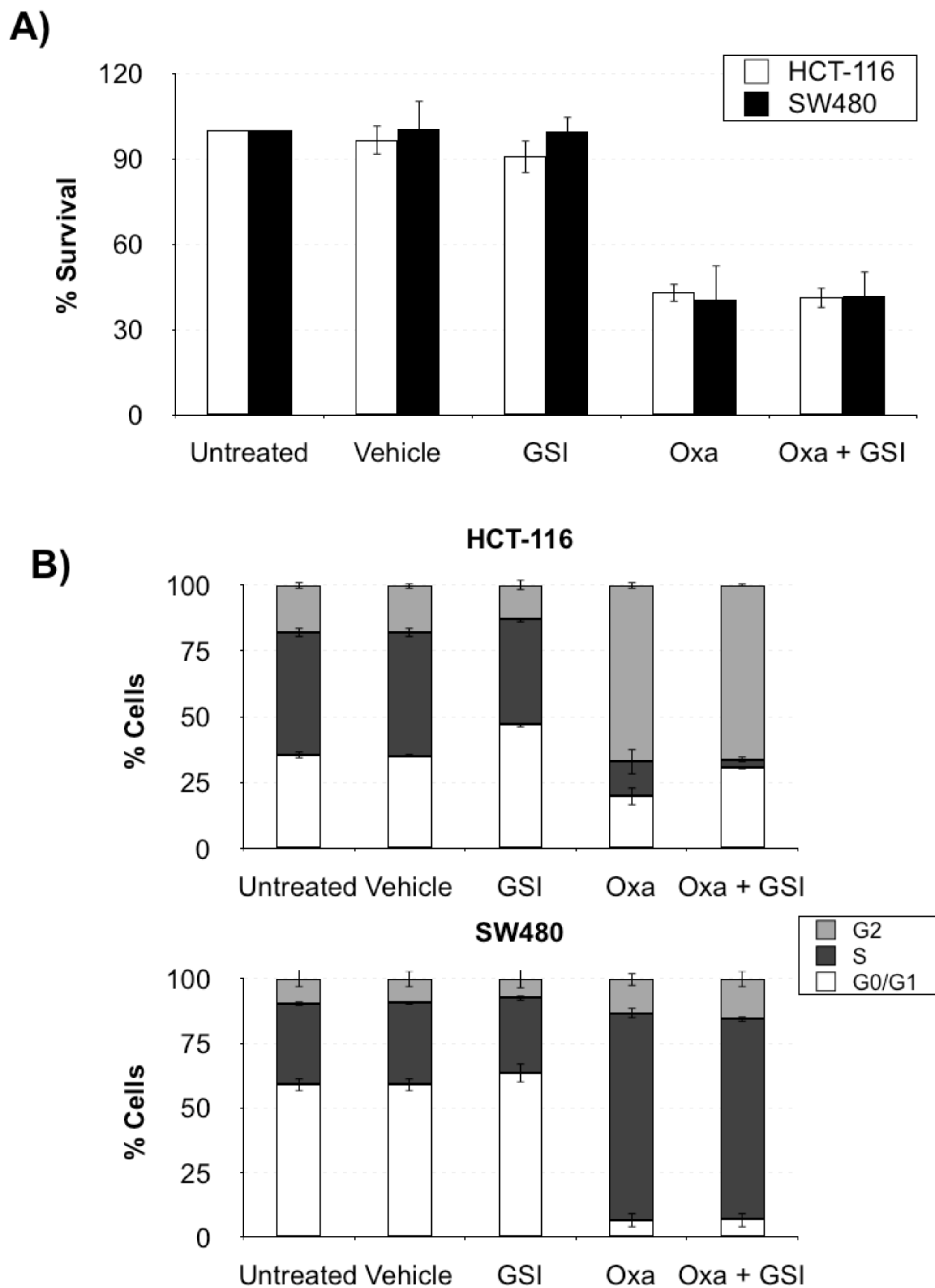


Figure 12: Oxaliplatin and MRK-003 Does Not Affect Short-Term Survival
HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30μM (SW480) Oxa for 48 hours in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003. After treatment, MTT for viability (A) or cell cycle analysis (B) was performed. Represented data are mean +/- SEM of three independent experiments.

Colony forming assays are a measure of long-term survival. Cell death may be mediated through non-apoptotic mechanisms or may be delayed. The combination of oxaliplatin and MRK-003 remarkably enhanced colony formation in both HCT-116 and SW480 when compared to oxaliplatin-treated alone (Figure 13). Collectively, the data suggests that GSI enhances the survival of cells treated with oxaliplatin.

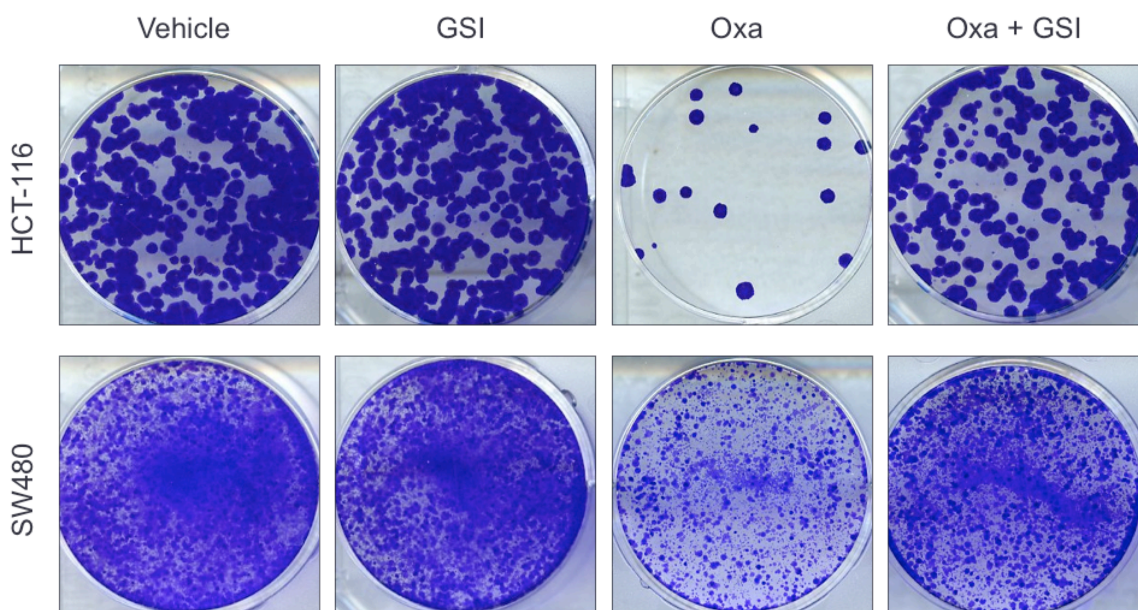


Figure 13: Oxaliplatin and MRK-003 Does Not Affect Short-Term Survival
HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30μM (SW480) Oxa for 48 hours in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003. After treatment, MTT for viability (A) or cell cycle analysis (B) was performed. Represented data are mean +/- SEM of three independent experiments.

Attenuation of the Apoptotic Signaling Cascade

The mechanisms underlying gamma-secretase inhibition on oxaliplatin-induced apoptosis was examined by evaluating changes in the classical apoptotic signaling

cascade. Cells treated with oxaliplatin and MRK-003 blocked cleavage of PARP (Figure 14) and caspase-3 (Figure 8) in both HCT116 and SW480 as measured by western blot and flow cytometry, respectively. Caspase activity can be blocked through direct interaction with the inhibitor of apoptosis protein (IAP) family members c-IAP1, c-IAP2 and XIAP, but no differences in protein levels were detected by western blot (data not shown). Second, oxaliplatin-induced cytochrome c release in both HCT-116 and SW480 was blocked by the addition of MRK-003 (Figure 15).

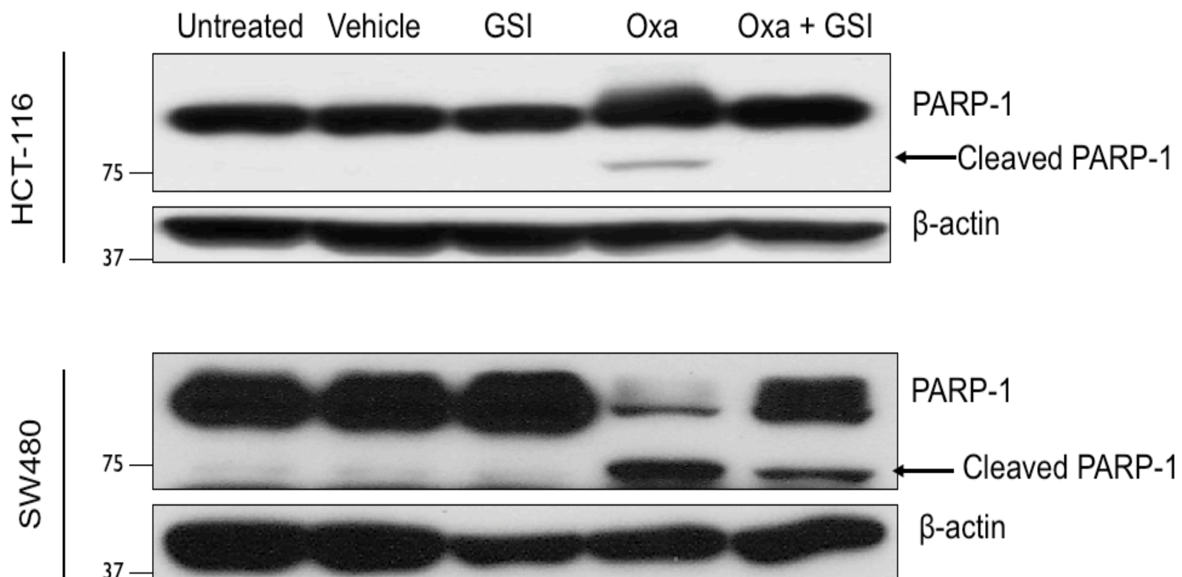


Figure 14: The Combination of Oxaliplatin and MRK-003 Reduces PARP Cleavage
HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30μM (SW480) for 48 hours in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003. Full-length (110 kDa) and cleaved (80 kDa) PARP was analyzed by western blotting of whole cell lysates. Equal protein loading was shown by probing for beta-actin.

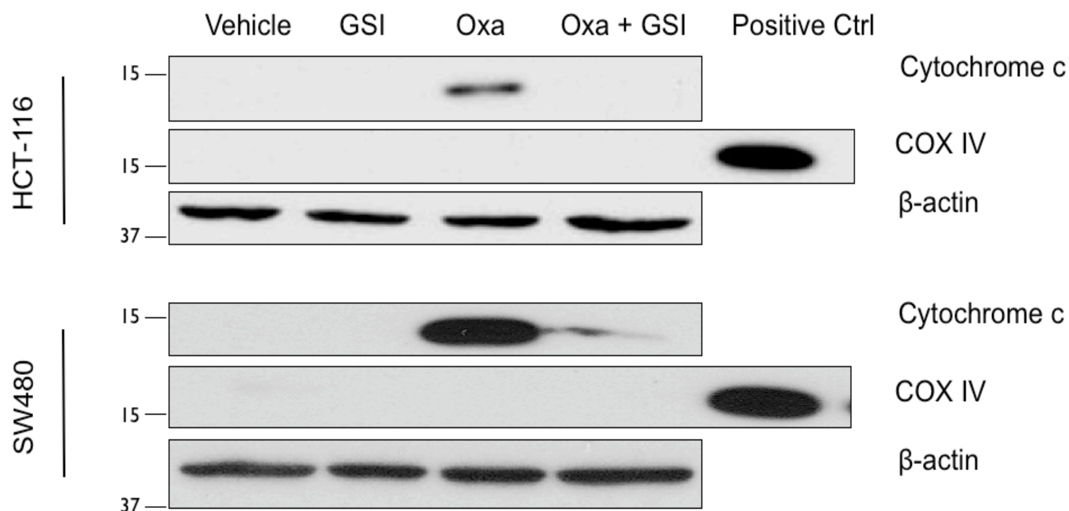


Figure 15: The Combination of Oxaliplatin and MRK-003 Reduces Cytochrome c Release
HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30μM (SW480) for 48 hours in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003. Release of cytochrome c into cytosolic fraction was analyzed by western blot. Potential mitochondrial contamination was detected by COX-IV (positive control lane is a whole cell extract). Beta-actin was used as a loading control.

Bax and Bak are the two key pro-apoptotic proteins that regulate cytochrome c release. Bax and Bak are activated by conformational changes to promote homo-oligomerization to create pore-like channels responsible for cytochrome c release (reviewed in (Tait et al. 2010)). The activation of Bax and Bak can be assessed using conformation specific antibodies (6A7 for Bax and Ab-1 for Bak) (Hsu et al. 1998). To determine if MRK-003 altered the activation of Bax and Bak in oxaliplatin-treated cells, Bax and Bak were immunoprecipitated with their active conformation specific antibodies and western blotted. In response to oxaliplatin, both HCT-116 and SW480 show activation of Bax and Bak, which was markedly inhibited in the presence of MRK-003 (Figure 16).

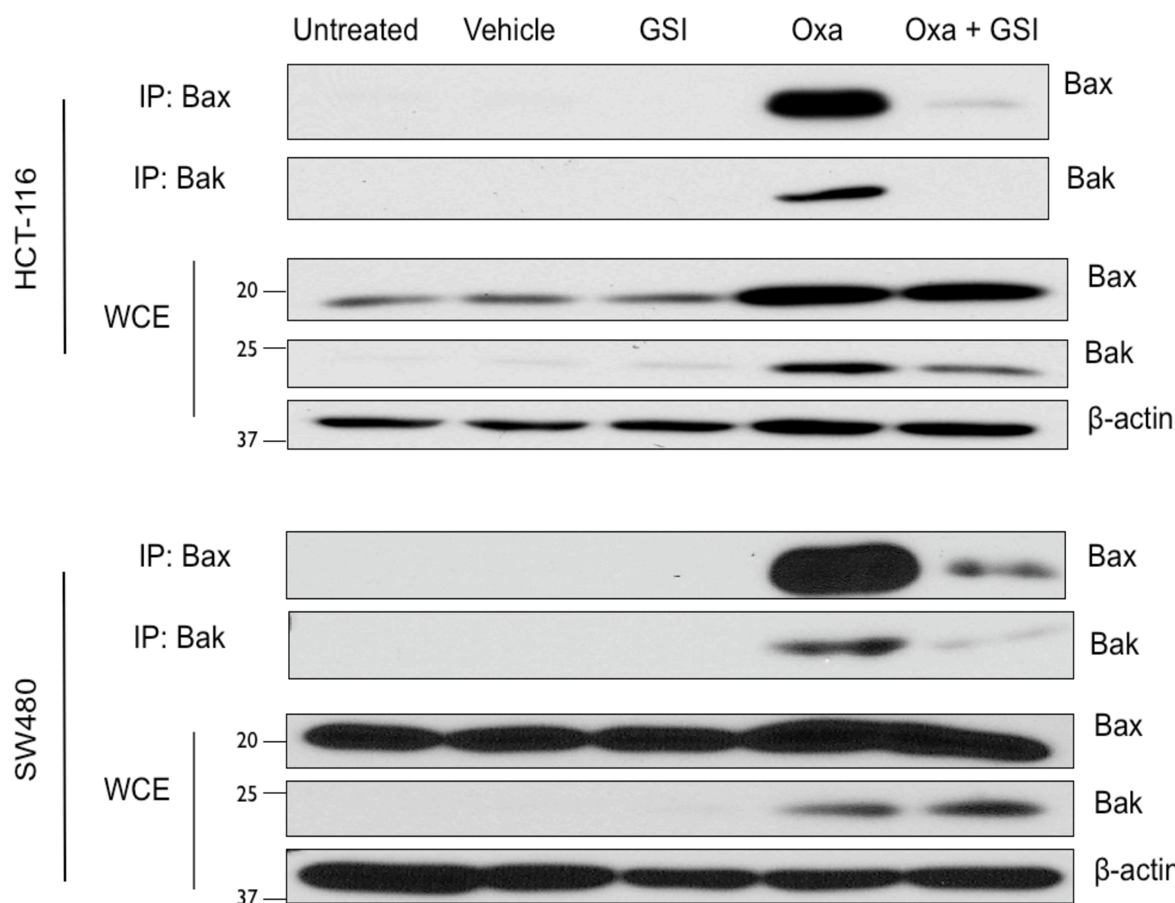


Figure 16: The Combination of Oxaliplatin and MRK-003 Reduces Bax and Bak Activation
HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30μM (SW480) for 48 hours in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003. Immunoprecipitation of active Bax and Bak. Whole cell extracts were probed for total Bax, total Bak, and beta-actin.

Mcl-1 and Bcl-xL levels are Elevated in Oxaliplatin + GSI treated Cells

The activation of Bax and Bak can be prevented by the anti-apoptotic Bcl-2 family member proteins Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A1 (Brunelle et al. 2009). Gamma-secretase inhibition has been shown to regulate protein expression of the Bcl-2 family members (Wang et al. 2010; Ramakrishnan et al. 2012). We tested whether or

not GSI treatment altered total protein levels of Mcl-1 or Bcl-xL (neither cell line expressed detectable levels of Bcl-2, data not shown). Cells treated with MRK-003 alone increased Mcl-1 protein levels without seemingly affecting Bcl-xL levels in both HCT-116 and SW480 (Figure 17). The combination of oxaliplatin and MRK-003 lead to enhanced protein levels of both Mcl-1 and Bcl-xL as compared to oxaliplatin alone in both cell lines.

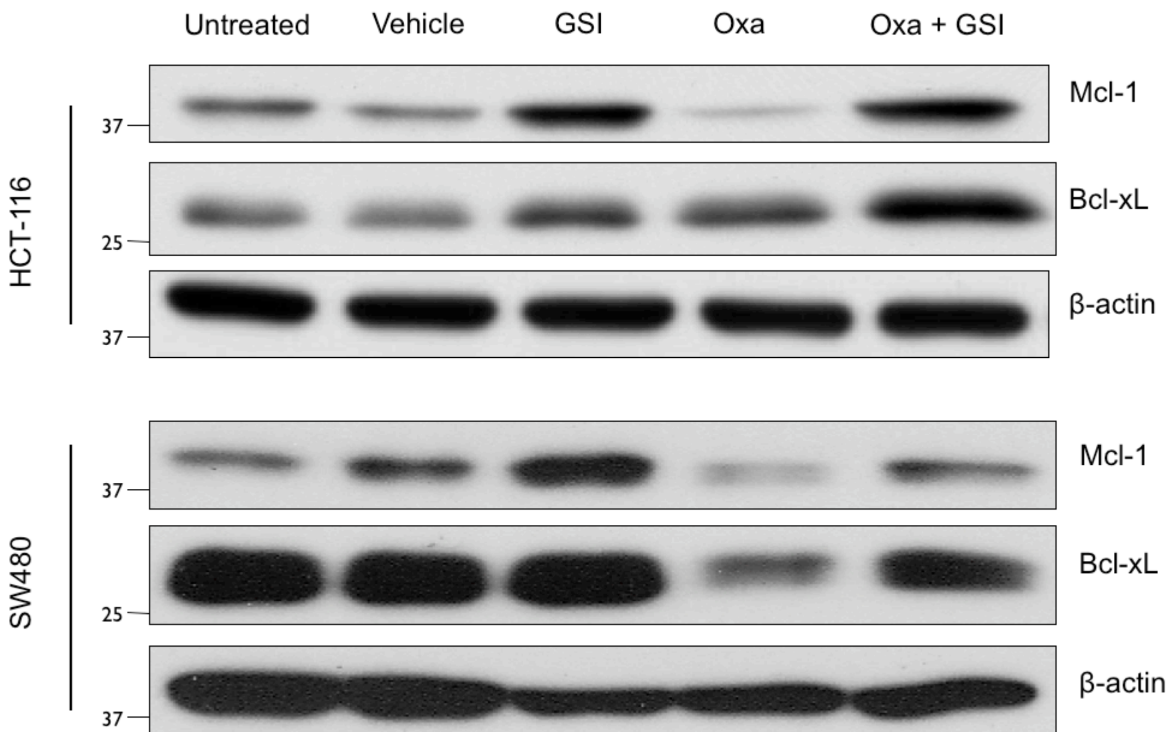
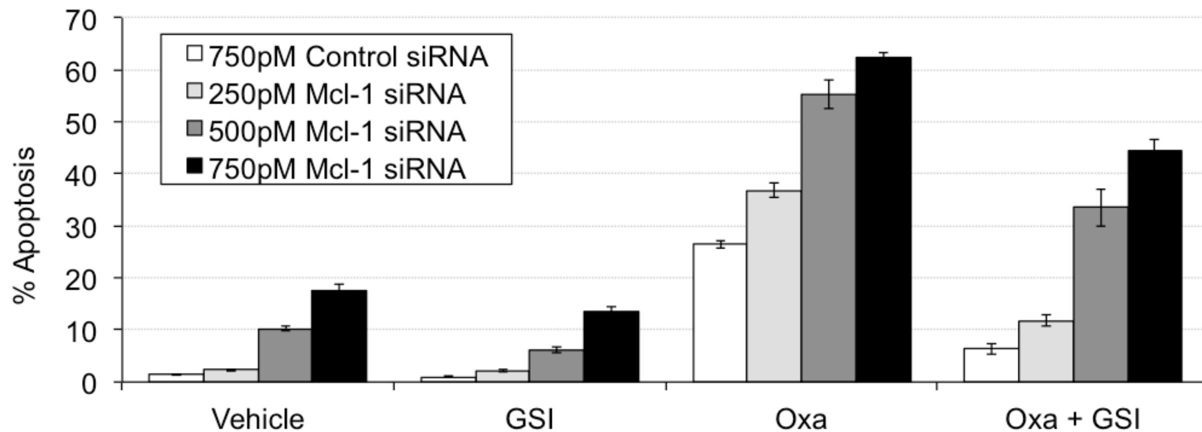


Figure 17: MRK-003 Increases Protein Expression of Mcl-1 and Bcl-xL
HCT-116 and SW480 cells were treated with 15 µM (HCT-116) or 30µM (SW480) for 48 hours in the absence or presence of 10 µM (HCT-116) or 5 µM (SW480) MRK-003. Total levels of Mcl-1 and Bcl-xL were assessed by immunoblotting. Beta-actin was used as a protein loading control.

A)



B)

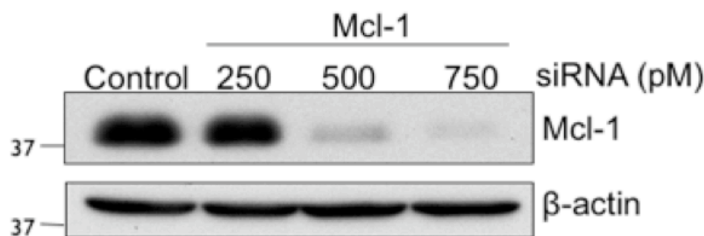
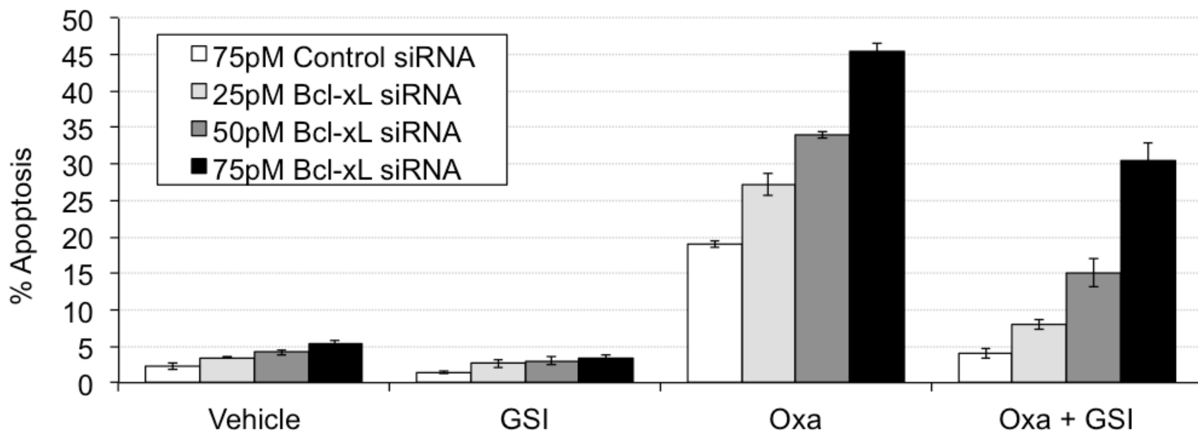


Figure 18: Knockdown of Mcl-1 and/or Bcl-xL Rescues MRK-003 Attenuation of Oxaliplatin-induced Apoptosis
(A) HCT-116 was transfected with increasing amounts of Mcl-1 siRNA as indicated for 24 hours. Cells were then treated with 15 μM Oxa in the absence or presence of 10 μM MRK-003 for 48 hours. Apoptosis rates were measure by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of two independent experiments. (B) Western blot shows efficiency of Mcl-1 knockdown.

Knockdown or Pharmacologic Inhibition of Mcl-1 and/or Bcl-xL Restores Apoptosis in Cells Treated with Oxaliplatin and MRK-003

To examine whether the MRK-003 induced increase in the anti-apoptotic proteins Mcl-1 and/or Bcl-xL is responsible for decreased sensitivity to oxaliplatin treatment, we used RNAi interference (siRNA) assays to silence Mcl-1 and/or Bcl-xL.

A)



B)

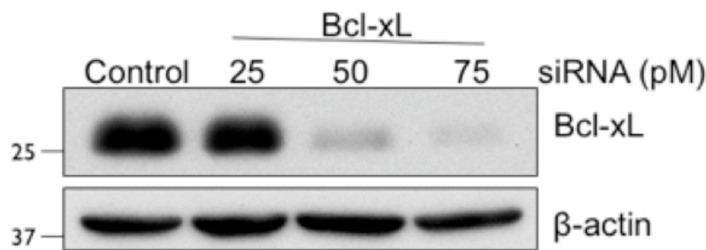
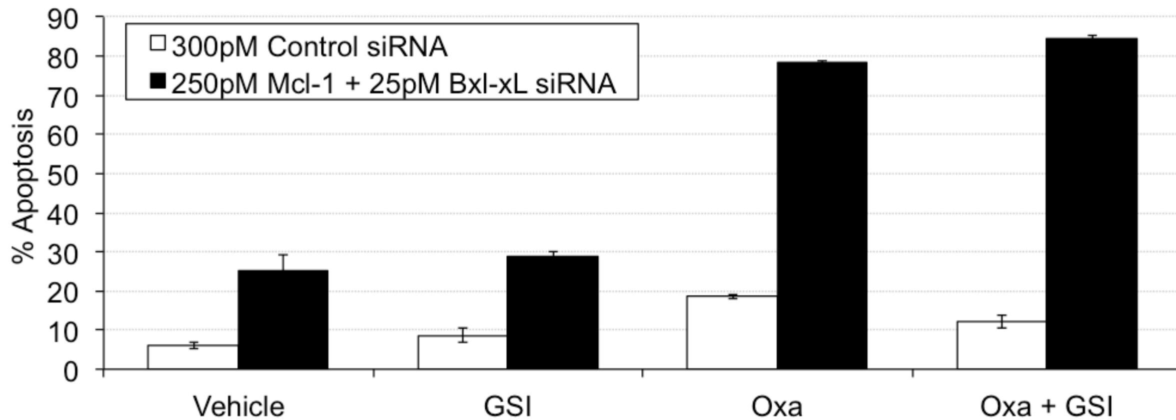


Figure 19: Knockdown of Bcl-xL Rescues MRK-003 Attenuation of Oxaliplatin-induced Apoptosis
(A) HCT-116 was transfected with increasing amounts of Bcl-xL siRNA as indicated for 24 hours. Cells were then treated with 15 μM Oxa in the absence or presence of 10 μM MRK-003 for 48 hours. Apoptosis rates were measure by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of two independent experiments. (B) Western blot shows efficiency of Bcl-xL knockdown.

A dose-dependent knockdown of Mcl-1 resulted in a dose-dependent increase in apoptosis in HCT-116 cells treated with oxaliplatin and MRK-003 but had no effect with MRK-003 alone as measured by flow cytometry for active caspase-3 (Figure 18a). Reduction of Bcl-xL levels in HCT-116 produced a similar dose-dependent increase in apoptosis in cells treated with both oxaliplatin and MRK-003 (Figure 19Figure 18a).

A)



B)

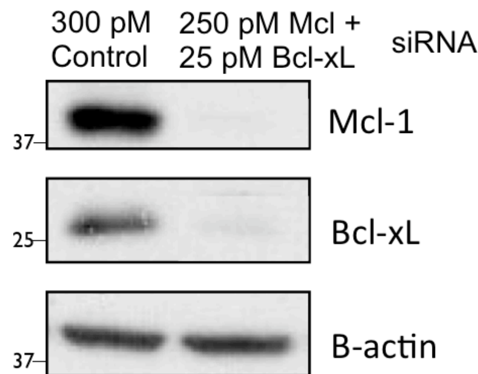


Figure 20: Simultaneous Knockdown of Mcl-1 and Bcl-xL Rescues MRK-003 Attenuation of Oxaliplatin-induced Apoptosis
(A) HCT-116 was transfected with 250 pM Mcl-1 and 25 pM Bcl-xL siRNA as indicated for 24 hours. Cells were then treated with 15 μM Oxa in the absence or presence of 10 μM MRK-003 for 48 hours. Apoptosis rates were measure by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of two independent experiments. (B) Western blot shows efficiency of Mcl-1 and Bcl-xL knockdown.

As expected, reducing levels of Mcl-1 or Bcl-xL lead to increased sensitivity to oxaliplatin. Knockdown of Mcl-1 and Bcl-xL levels were confirmed by western blot (Figure 18b, Figure 19b). Reducing the levels of both Bcl-xL and Mcl-1 with low

picomolar doses greatly increased oxaliplatin-induced apoptosis, which was not inhibited with the addition of MRK-003 (Figure 20a). Simultaneous knockdown of Mcl-1 and Bcl-xL levels were confirmed by western blot (Figure 20b)

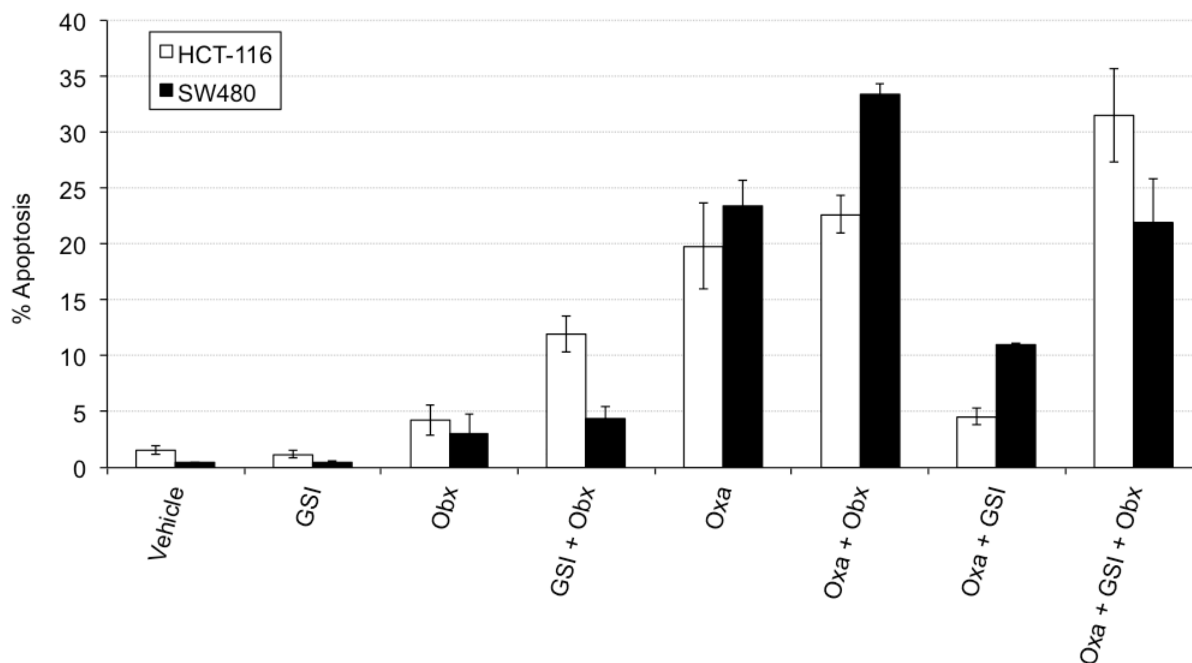


Figure 21: Obatoclax Rescues MRK-003 Attenuation of Oxaliplatin-Induced Apoptosis.
HCT-116 was treated with 15 μM Oxa in the absence or presence of 10 μM MRK-003 and/or 75 nM obatoclax (Obx) for 48 hours. SW480 was treated with 30 μM Oxa in the absence or presence of 5 μM MRK-003 and/or 25 nM obatoclax for 48 hours. Apoptosis rates were measured by flow cytometry following active caspase-3 reactivity. Represented data are mean +/- SEM of at least two independent experiments.

To further confirm the role of Mcl-1 and Bcl-xL in mediating the abrogation of oxaliplatin-induced apoptosis by MRK-003, we used the BH3-peptide mimetic obatoclax (GX15-070) to broadly antagonize Bcl-2 pro-survival family members Mcl-1 and Bcl-xL. Apoptosis inhibition associated with the combination of oxaliplatin and MRK-003 treatment was reversed with the addition of obatoclax in both HCT-116 and SW480

(Figure 21). Of note, the combination of MRK-003 and obatoclax also resulted in an increase in apoptosis in HCT-116 (Figure 21). Thus, inhibiting Mcl-1 and/or Bcl-xL through RNA interference or small molecule inhibitor (obatoclax) lead to a restoration of apoptosis in colon cancer.

Discussion

In this study, we report that the combination of the gamma-secretase inhibitor MRK-003 with oxaliplatin elicited potent chemoresistance in colon cancer cells by greatly decreasing apoptosis and leading to increased long-term survival. We showed the combination of oxaliplatin and MRK-003 reduced apoptosis in HCT-116, SW480, HCT-15, HT-29, and Colo205 colon cancer cell lines by at least half. The reduction in apoptosis could be replicated using alternative GSIs (DAPT and GSI-XX) in HCT-116 and SW480. GSI-XII, a fourth GSI used in this study, slightly increased apoptosis by 10% in HCT-116 and only decreased apoptosis by approximately 7% in SW480. None of the four GSIs used in this study elicited apoptosis by themselves. When MRK-003 was combined with other chemotherapeutic agents (etoposide and irinotecan), decreased apoptosis was also seen in HCT-116. MRK-003, however, had no effect on apoptosis of etoposide- or irinotecan-treated SW480 cells, a finding reported elsewhere using DAPT in combination with various chemotherapeutic agents (Akiyoshi et al. 2008). These data suggest that the effects seen are not cell-context specific, mediated by gamma-secretase inhibition (not an off-target effect), and not unique to oxaliplatin treatment. We observed no change in viability in oxaliplatin-treated cells with or without MRK-003 in either HCT-116 or SW480. Cell cycle analysis showed a slight increase in

G_0/G_1 arrest with the addition of MRK-003 in oxaliplatin-treated HCT-116 cells alone (no change was observed in SW480). These data do not account for the reduction in apoptosis seen with MRK-003 treatment. However, to further support the decrease in apoptosis observed in oxaliplatin and MRK-003 treated cell, long-term survival assays showed increased cell survival in combination treatment compared to oxaliplatin alone.

We identified increased protein levels of the anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-xL in response to MRK-003 as pivotal players responsible for decreased oxaliplatin-induced apoptosis, leading to a reduction of Bax and Bak activation and downstream suppression of the apoptotic cascade (cytochrome c release, caspase-3 cleavage, and PARP cleavage). Restoration of apoptosis was achieved by blocking Mcl-1 and/or Bcl-xL through RNA interference or the Mcl-1/Bcl-xL/Bcl-2 antagonist obatoclax. Knockdown of Mcl-1 and/or Bcl-xL, however, did not induce apoptosis upon GSI treatment alone but the combination of GSI and obatoclax did in HCT-116. From these data, we conclude that increased protein levels of Mcl-1 and/or Bcl-xL are responsible for the attenuation of oxaliplatin-induced apoptosis observed upon MRK-003 treatment.

Several studies have highlighted the role of Notch signaling in the regulation of Mcl-1 and Bcl-xL. Mcl-1 levels are decreased by blocking the Notch pathway via a GSI (Wang et al. 2010) or with small-interfering RNA (Zhao et al. 2010) and increased by overexpression of NICD (Wang et al. 2010). GSIs have been shown to increase Noxa, a pro-survival BH3-only protein that promotes Mcl-1 degradation (Nefedova et al. 2008; Seveno et al. 2012). Likewise, blocking Notch signaling can decrease Bcl-xL levels (Nefedova et al. 2008; Vo et al. 2011). In contrast, our data shows increased protein

levels of Mcl-1 and Bcl-xL upon GSI treatment. This phenomena has been replicated by Liu et. al in T-ALL cell lines when examining the effect of gamma-secretase inhibition on chemosensitivity. One subset of cells exhibited enhancement of apoptosis while another subset showed resistance via upregulation of Bcl-xL (Liu et al. 2009). The mechanism by which this occurs is still not well defined.

Our data also conflicts with other reports in the literature showing synergism between cytotoxic agents and GSIs in colon cancer cell lines. One study showed that blocking Notch-1 signaling using GSI34 (a novel gamma-secretase inhibitor) lead to an enhancement of oxaliplatin sensitivity (Meng et al. 2009). Alternatively, DAPT sensitized colon cancer cell lines SW480 and DLD-1 to paclitaxel only but not 5-FU, camptothecin, TRAIL, or cisplatin (Akiyoshi et al. 2008). A third group tested the effects of GSI-XX (DBZ), another GSI, in combination with platinum compounds (cisplatin, carboplatin, and oxaliplatin) on a panel of 20 human colon cancer cell lines (Aleksic et al. 2008). GSI-XX alone had no effect on cell survival and, in combination with cisplatin, only a subset exhibited some degree of increased cell death; a few cell lines sensitive to the combination of cisplatin and GSI-XX showed no effect with oxaliplatin or carboplatin. In our hands, gamma-secretase inhibition using MRK-003, DAPT, or GSI-XX abrogated oxaliplatin-induced apoptosis via increased Mcl-1 and/or Bcl-xL protein levels, as seen by Liu et al. (Liu et al. 2009). The pleotropic nature of gamma-secretase inhibition on chemotherapy-induced apoptosis is not well understood. It likely could be a reflection of complex context-dependent signaling downstream of the Notch pathway, and thus may only be of clinical utility to a particular (as yet undefined) subtype of colorectal cancer.

The observed effects of MRK-003, DAPT, and GSI-XX on attenuating chemotherapy-induced apoptosis may not be caused by Notch inhibition but rather differential targeting of other known gamma-secretase substrates. Gamma-secretase primarily catalyzes the intramembranous cleavage of Notch and APP (amyloid precursor protein). However, it has also been shown to target at least a dozen known type I transmembrane proteins, including E-cadherin, ErbB4, CD44, and c-Met, but their mechanism and function is not well studied (for review, see (Haapasalo et al. 2011)). Also, the specificity of GSIs for different substrates may drive the discrepancies found in the literature, thus it will be important to understand the biochemical differences between specific GSIs, particularly since we showed that GSI-XII did not have the same effect as the other three GSIs. While we used four commercially available inhibitors known to block both Notch cleavage and activity as well as induce apoptosis in multiple tumor cell lines, we cannot rule out the possibility that these GSIs may also indirectly target other molecular pathways, such as pro-apoptotic signaling pathways, which may outweigh or antagonize any pro-apoptotic effects conferred by reduced Notch signaling in colon cancer cells. Therefore, the contribution of single, combinatorial, or complete ablation of all four Notch receptors on chemosensitivity is needed to address these concerns. The newly emerging neutralizing antibodies to individual Notch receptors will surely help to answer this question.

Standard chemotherapy approaches may need to be modified to include other novel inhibitors. Despite the apparent discrepancies of gamma-secretase inhibition on chemosensitivity, we were able to restore the attenuation of oxaliplatin-induced apoptosis seen with MRK-003 by antagonizing Mcl-1 and/or Bcl-xL. Combining GSIs

and chemotherapy with an anti-apoptotic Bcl-2 family member agonist like obatoclax may be a valid therapeutic approach. We also found enhanced apoptosis with MRK-003 and obatoclax combined. Previous studies have shown sensitization with the combination of GSIs and the BH3 mimetic ABT-737 (antagonizes Bcl-2/Bcl-xL/Bcl-w but not Mcl-1 (Oltersdorf et al. 2005)) in GSI-sensitive cell lines where GSI alone increased Noxa levels, leading to subsequent Mcl-1 degradation (Li et al. 2010; Seveno et al. 2012). Our data shows MRK-003 synergism with obatoclax in GSI-resistant colon cancer cell lines where GSI alone increases Mcl-1 and Bcl-xL levels. Therefore, combining GSIs with other targeted therapeutics instead of chemotherapy may be a more effective strategy in the treatment of colorectal cancer.

To our knowledge, the only published Phase II clinical trial studying the efficacy of gamma-secretase inhibitor – RO4929097 – monotherapy in metastatic colorectal cancer patients demonstrated no antitumor effects (Strosberg et al. 2012). Nevertheless, there are multiple clinical trials investigating mono- and combination therapies involving GSIs in a wide variety of tumors (www.clinicaltrials.gov). The results posed here do not diminish the potential use of GSIs in a clinical setting but rather suggest caution when developing drug combinations. First, further detailed mechanistic studies looking at the biochemical differences in substrate specificity and potential off-target effects of the many available GSIs are needed. Also, the effects of gamma-secretase inhibition in relation to cell context and interactions with signaling pathways needs further clarification as there are clear indications that GSIs can have opposing effects even in same tumor types. Third, the potential still exists for using GSIs in combination with other therapeutic agents to elicit anti-tumorigenic responses. Our

results provide a cautionary tale for broad application of gamma-secretase inhibitors. Understanding the actions of this drug when used in combination with standard chemotherapy and/or targeted therapies will provide greater knowledge of its potential use for a personalized approach to cancer treatment.

CHAPTER FOUR: TARGETING AUTOPHAGY-SENSITIZED COLON CANCER CELLS TO GAMMA-SECRETASE INHIBITORS

Abstract

Gamma-secretase has been considered an attractive target for colorectal cancer due to its ability to block Notch signaling. In the previous section, we found that gamma-secretase inhibitors (GSIs) alone had no effect on cell proliferation or apoptosis in human colorectal cancer cell lines. When GSIs were combined with chemotherapy, apoptosis was abrogated and long-term survival increased. The combination of oxaliplatin, the first line of chemotherapy for colorectal cancers, with MRK-003, a specific GSI, resulted in elevated levels of anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-xL. Addition of the Bcl-2 inhibitor obatoclax (Obx) and knockdown of Mcl-1 and/or Bcl-xL were able to, at least partially, restore apoptosis. Interestingly, the combination of MRK-003 and obatoclax induced cell death.

We show here that cell death due to the combination of MRK-003 and obatoclax was only partially due to apoptosis in HCT-116 and SW480 by measuring Annexin-V positivity, Parp-1 cleavage, caspase-3 cleavage, and effects on death using the pan-caspase inhibitor z-VAD-fmk. Obatoclax has been shown to induce autophagy, a cannibalistic cell survival pathway activated upon cellular stress. We examined if

autophagy played a role in increasing cell death due to the MRK-003 + Obx combination.

We measured autophagic flux, the complete process of autophagy initiation and progression leading to the release of degraded substrates, by the bafilomycin A1 clamp assay and total p62 levels. The bafilomycin A1 clamp assay prevents LC3-II degradation, a protein that is generated during autophagic flux initiation and degraded upon completion; p62 is a degraded during autophagic flux. Our data indicates that autophagy is blocked with the combination of MRK-003 and obatoclax. We saw no or little decrease in LC3-II levels with the bafilomycin clamp assay and increased total p62 levels. Our data also indicated that each drug alone induced autophagy. What remains unclear is how blockage of autophagy is attained with two drugs that individually induce autophagy.

We are the first to show that gamma-secretase inhibitors stimulate cytoprotective autophagy; this may be a reason for their insensitivity in our human colon cancer cell lines. To address whether modulation of autophagy can sensitize GSI-treated colon cancer cells, we blocked autophagy by the autophagy inhibitors 3-MA and bafilomycin A1 and by knockdown of the critical autophagy genes Beclin-1 or Atg5. Blocking autophagy with bafilomycin A1 and knockdown with both Beclin-1 and Atg5 increased cell death in GSI-treated cells. The mechanism that stimulates autophagy is GSI-treated cells remains unknown.

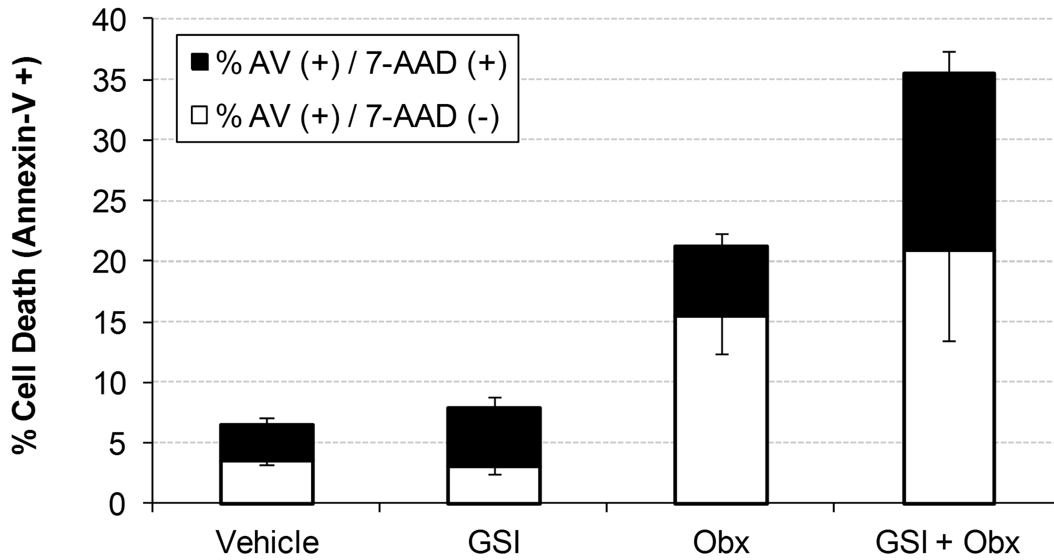
These findings suggest that one possible explanation for the insensitivity of colon cancer cell lines to GSI treatment is the induction of cytoprotective autophagy. This calls into question the validity of using GSIs to treat colon cancer patients

Results

Apoptosis is Partially Responsible for Cell Death Induced by the Combination of MRK-003 and Obatoclax

In the previous chapter, we observed high levels of cell death under the microscope in HCT-116 and SW480 cells when treated with both MRK-003 and obatoclax compared to either drug alone. However, only 12% and 4% of HCT-116 or SW480 cells, respectively, were positive for active caspase-3 (Figure 8b). To further characterize the role of apoptosis in the observable cell death, HCT-116 and SW480 were treated with both drugs and apoptosis was assessed 48- hours post treatment by Annexin-V & 7-AAD staining. HCT-116 cells showed similar levels of early apoptosis [Annexin-V (+) / 7-AAD (-)] and late apoptotic / dead [Annexin-V (+) / 7-AAD (+)] cells in MRK-003 + Obx treated cells (Figure 22a). SW480, however, had almost no early apoptotic positive cells; most labeled positive for late apoptosis / dead (Figure 22b). Similarly, higher Parp-1 cleavage, a marker of apoptosis, was observed in HCT-116 by western blot in MRK-003 + Obx treated cells as early as 24 hours but no detectable levels of Parp-1 cleavage was observed in SW480 by 48 hours (Figure 25a-b). Cell death also assessed using the vital dye propidium iodide (PI), which is excluded from live cells, resulted in similar levels of cell death when compared to the Annexin-V & 7-AAD assay in both HCT-116 and SW480 (Figure 23). Cell death will henceforth be assessed by PI staining for all following experiments.

A)



B)

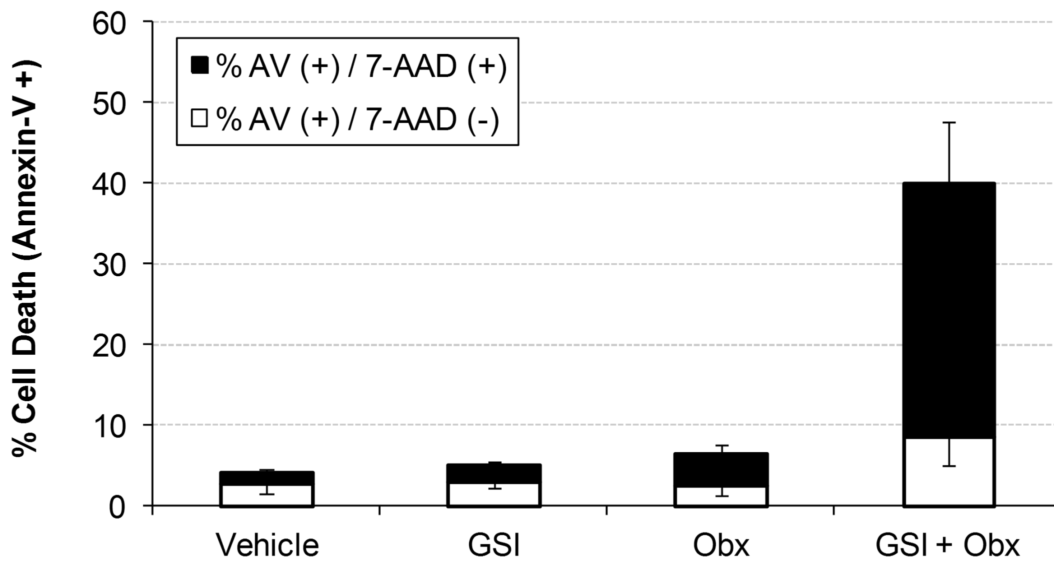


Figure 22: Contribution of Apoptosis in Cell Death Mediated by the Combination of MRK-003 and Obatoclax
HCT-116 (A) and SW480 (B) cells were treated with 10 µM (HCT-116) or 5µM (SW480) MRK-003 (GSI) in the absence or presence of 75 nM (HCT-116) or 25 nM (SW480) obatoclax (Obx) for 48 hrs. Apoptosis rates were measure by flow cytometry following Annexin-V/7-AAD staining. Represented data are mean +/- SD of two independent experiments.

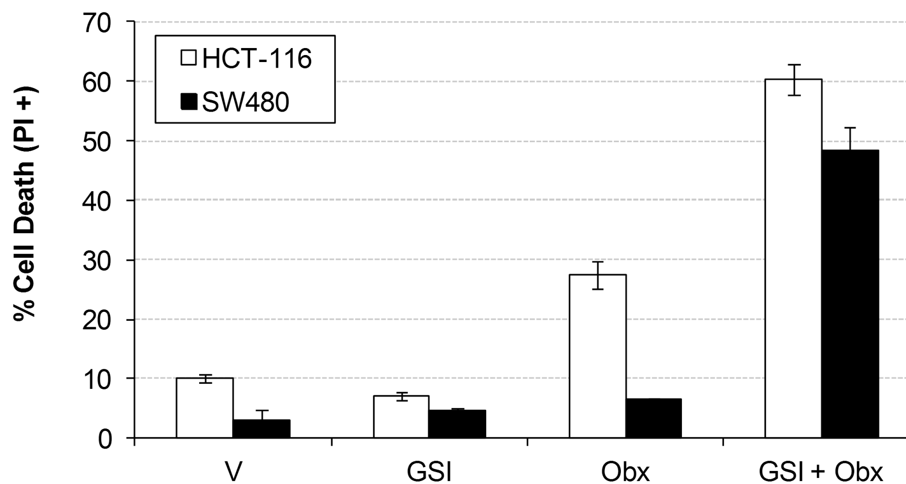


Figure 23: The Combination of MRK-003 and Obatoclax Induces Cell Death in Human Colon Cancer Cell Lines
HCT-116 (A) and SW480 (B) cells were treated with 10 µM (HCT-116) or 5µM (SW480) MRK-003 (GSI) in the absence or presence of 75 nM (HCT-116) or 25 nM (SW480) obatoclax (Obx) for 48 hrs. Cell death rates were measure by flow cytometry following PI staining. Represented data are mean +/- SD of two independent experiments.

The classical markers of apoptosis, viable Annexin-V positivity, caspase-3 cleavage, and Parp-1 cleavage, were present in HCT-116 but not SW480. To determine if apoptosis is the only mechanism responsible for the observed cell death, HCT-116 and SW480 were treated with MRK-003 and Obx in the presence or absence of the pan caspase inhibitor z-VAD-fmk. Oxaliplatin-treated cells were used as a positive control to show greater than 50% suppression of cell death in the presence of z-VAD-fmk. The addition of z-VAD-fmk to MRK-003 + Obx-treated cells suppressed cell death by 50% in HCT-116 and 20% in SW480 (Figure 24). Interestingly, z-VAD-fmk had no effect on cell death on Obx-treated cells in HCT-116. These data suggest an alternative cell death mechanism may be responsible for killing even when apoptosis is inhibited.

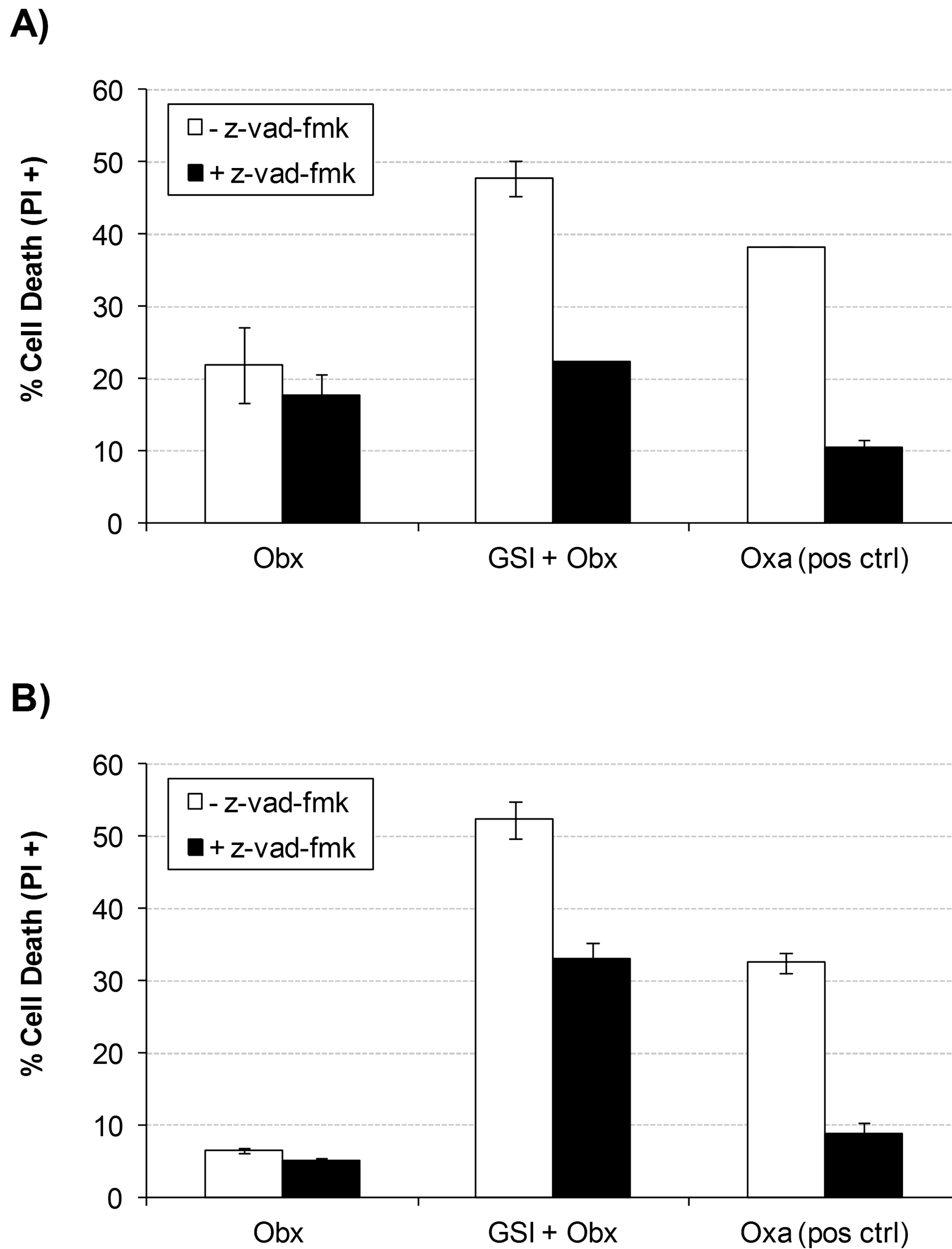


Figure 24: MRK-003 + Obx-induced Cell Death is Partially Inhibited by Z-VAD-FMK
HCT-116 (A) was treated with 10 μM MRK-003 (GSI) and 75 nM obatoclax (Obx) in the presence or absence of 20 μM z-VAD-fmk for 48 hrs. SW480 (B) was treated with 5 μM MRK-003 and 75 nM Obx in the presence or absence of 20 μM z-VAD-fmk for 48 hrs. Cells were treated with 50 μM oxaliplatin (Oxa) as a positive control. Cell death rates were measure by flow cytometry following PI staining. Represented data are mean +/- SD of two independent experiments.

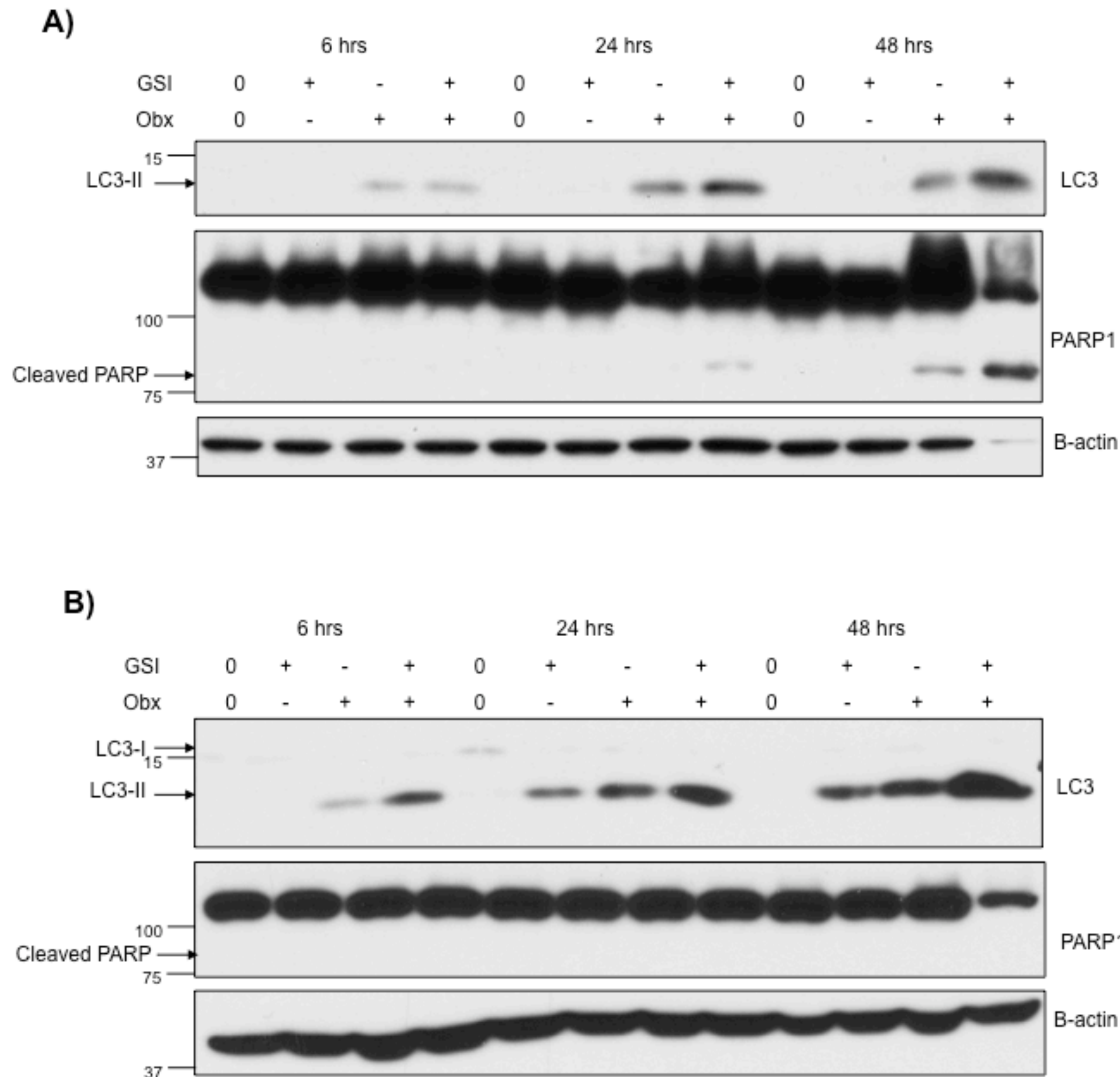


Figure 25: Total Levels of LC3-II are Increased in MRK-003 + Obx Treated Cells
HCT-116 (A) and SW480 (B) cells were treated with 10 µM (HCT-116) or 5µM (SW480) MRK-003 (GSI) in the absence or presence of 75 nM (HCT-116) or 25 nM (SW480) obatoclax (Obx) for 48 hrs. Total levels of LC3-II and PARP-1 were assessed by western blot. Beta-actin was used as a protein loading control.

Cytoprotective Autophagy is Blocked in MRK-003 + Obatoclax-Treated Cells

In addition to repressing apoptosis, the anti-apoptotic Bcl-2 family proteins can also directly bind to Beclin-1, preventing autophagy initiation (Rahmani et al. 2012; Tang

et al. 2012; Schwartz-Roberts et al. 2013). Autophagy, a process of recycling cellular material for energy, is thought to act as a survival process during times of stress. Therefore, obatoclax may both promote apoptosis and facilitate autophagy simultaneously. Measuring autophagy must be performed using multiple assays as no single assay provides conclusive information.

Total LC3-II protein levels, a specific autophagic marker, was assessed in HCT-116 and SW480 cells treated with MRK-003 or obatoclax, alone or in combination, by western blot. First, we found slight increases in LC3-II levels in both cell lines with each drug alone compared to untreated controls; levels were further elevated in combination treatment (Figure 25).

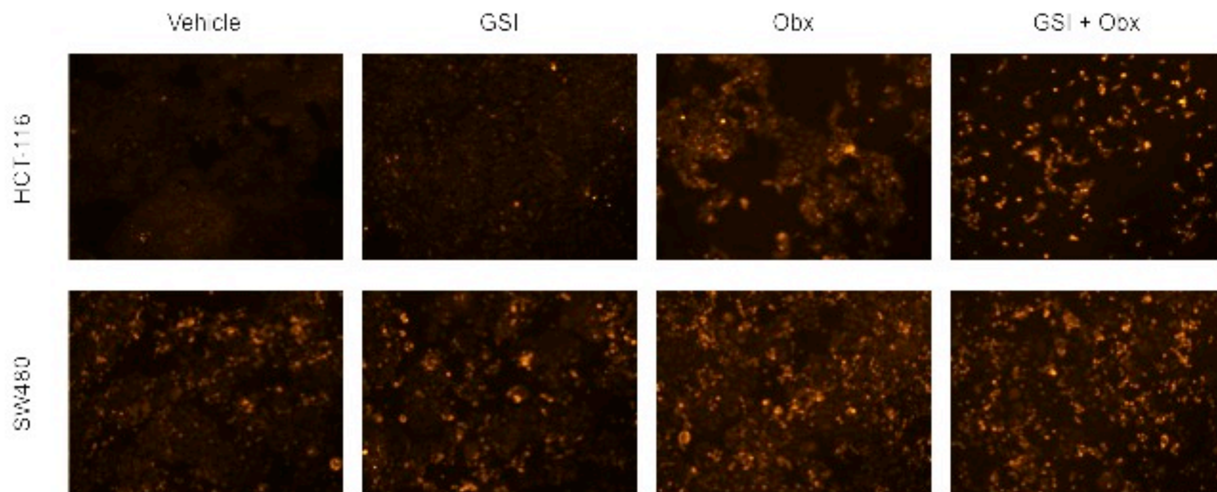


Figure 26: Increased Acidic Vesicular Organelles by Acridine Orange Staining in MRK-003 + Obx Treated Cells
HCT-116 and SW480 cells were treated with 10 μM (HCT-116) or 5μM (SW480) MRK-003 (GSI) in the absence or presence of 75 nM (HCT-116) or 25 nM (SW480) obatoclax (Obx) for 48 hrs. Autophagic vesicles were detected in live cells by acridine orange staining and examined by fluorescence microscopy.

When autophagosomes fuse with the acidic lysosomes, the resultant autolysosome is an acidic compartment. Acridine Orange (AO) is a dye used to detect autophagic vesicles. Acridine orange is an acidotropic (pH sensitive) dye that crosses into lysosomes (and other acidic compartments) within the cell where it becomes protonated and trapped within the organelle and then forms aggregates that emit red fluorescence; at neutral pH, the unprotonated dye emits green fluorescence. Detection of acidic vesicular organelles (AVOs), a morphologic characteristic of autophagy, by acridine orange staining was qualitatively analyzed using an inverted fluorescent microscope. The combination of MRK-003 and obatoclax showed increased staining of AVOs as compared to control or either drug alone in HCT-116; no differences could be seen in SW480 (Figure 26).

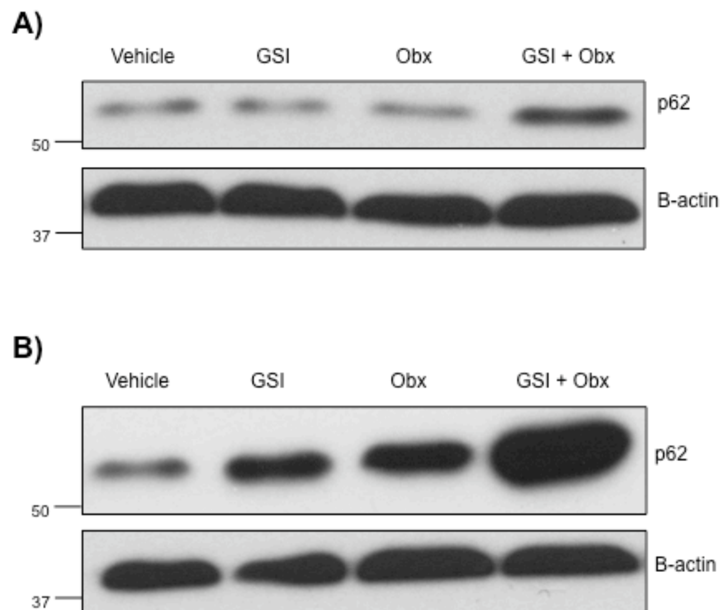


Figure 27: Elevated Levels of p62 in MRK-003 + Obx Treated Cells
HCT-116 (A) and SW480 (B) cells were treated with 10 μM (HCT-116) or 5μM (SW480) MRK-003 (GSI) in the absence or presence of 75 nM (HCT-116) or 25 nM (SW480) obatoclax (Obx) for 48 hrs. Total level of p62 was assessed by western blot. Beta-actin was used as a protein loading control.

The protein p62 is degraded during autophagy induction and serves as another autophagy marker; p62 levels should inversely correlate with LC3-II levels during autophagy promotion. When p62 was measured in treated cells, we found increased protein levels in MRK-003 + Obx-treated cells compared to either drug alone (Figure 27). The increase in p62 levels do not inversely correlate with LC3-II levels, suggesting that autophagy is not being promoted in combined treatment to elicit cell death.

To address this inconsistency, we must reevaluate what information total LC3-II levels provide. Elevated LC3-II protein levels can indicate either: 1) increased autophagy induction or 2) blockage in autophagy. LC3-II has a half-life of approximately 30 minutes. If autophagy is inhibited by blocking the fusion of the autophagosome to the lysosome for a period of time, allowing for accumulation of LC3-II without subsequent degradation, we can compare the levels of LC3-II in these cells to their unblocked counterpoint to determine whether autophagy is being induced or blocked. This feature can be exploited by the bafilomycin A1 clamp assay (Rubinsztein et al. 2009). In this assay, cells are treated with the compound of interest and during the last four hours of treatment, saturating concentration of bafilomycin A1, a drug that blocks the fusion of the autophagosome with the lysosome, is added. When comparing cells treated with bafilomycin A1 to those without, increased levels of LC3-II would indicate induction of autophagy while decreased or no change in LC3-II levels would indicate a block in autophagy. When MRK-03 + Obx treated HCT-116 and SW480 cells were subjected to the bafilomycin A1 clamp assay, we saw a decrease in LC3-II levels in the presence of Baf A1 in HCT-116 (Figure 28a, compare lanes 8 to 4) and no change in LC3-II levels in SW480 (Figure 28b, compare lanes 8 to 4).

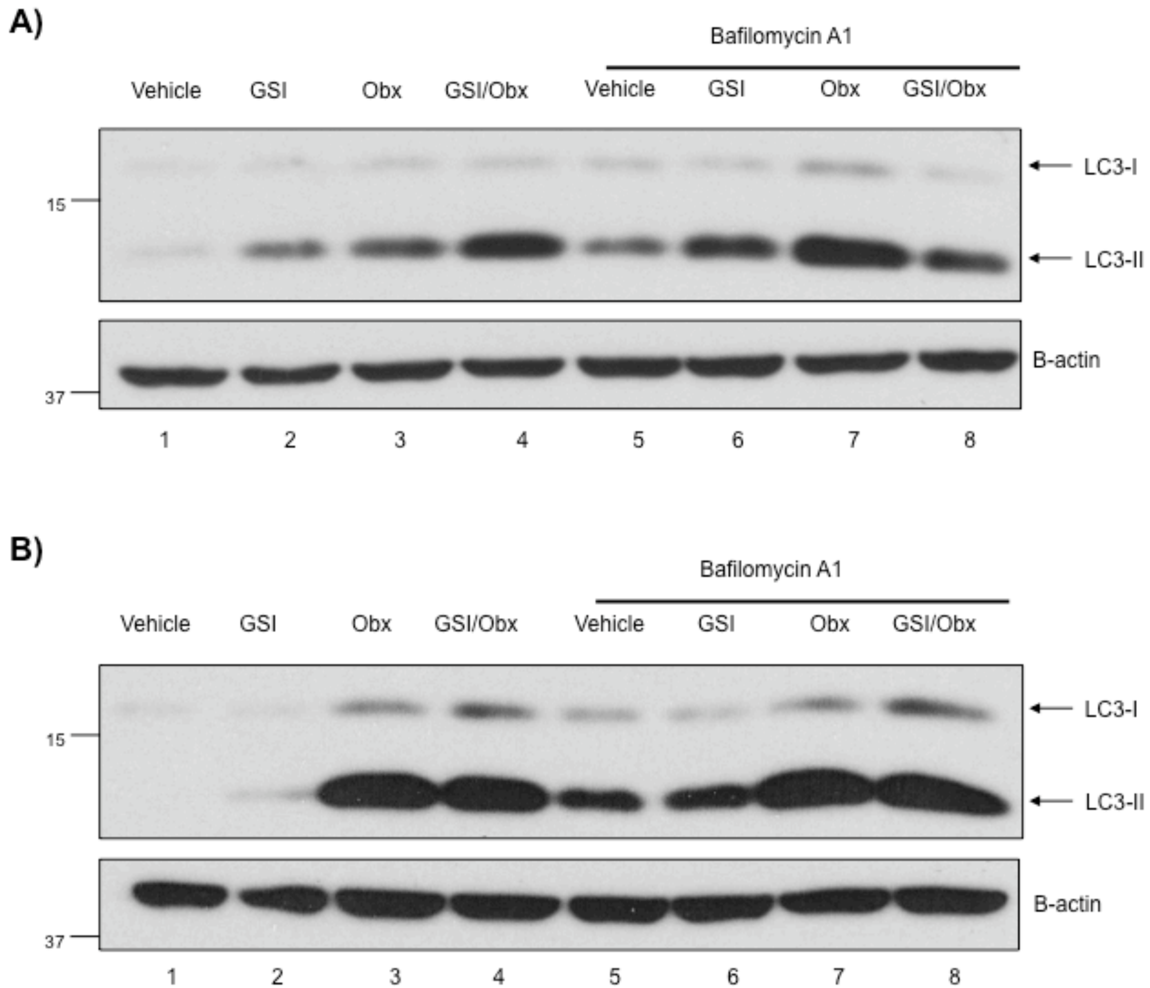


Figure 28: Blocked Autophagic Flux in MRK-003 + Obx Treatment and Increased Autophagic Flux in GSI Treatment
HCT-116 (A) and SW480 (B) cells were treated with 10 μM (HCT-116) or 5μM (SW480) MRK-003 (GSI) in the absence or presence of 75 nM (HCT-116) or 25 nM (SW480) obatoclax (Obx) for 48 hrs. During the last four hours, cells were treated with 400 nM bafilomycin A1 for 4 hours and harvested. Total level of LC3-II was assessed by western blot. Beta-actin was used as a protein loading control.

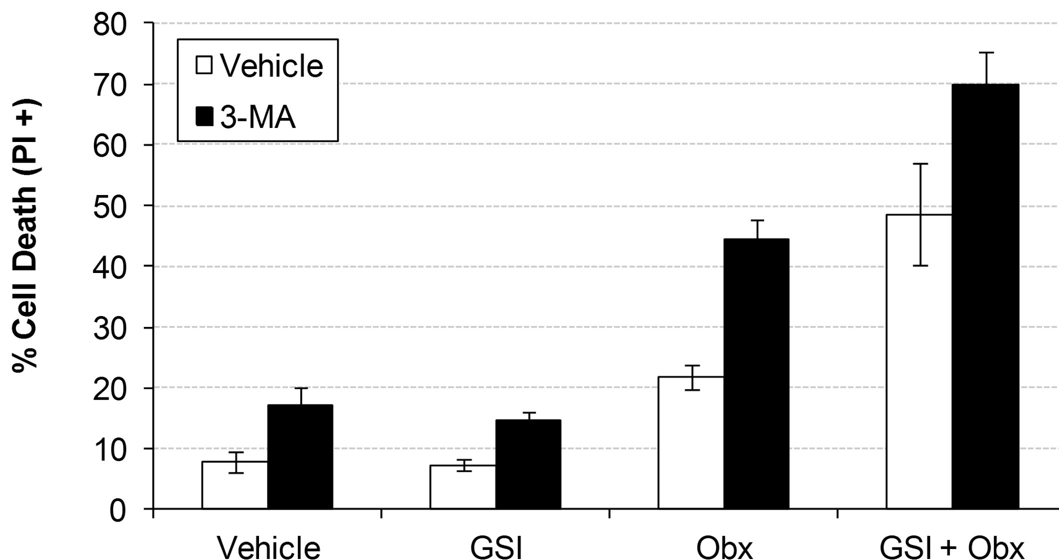
We see an increase in LC3-II levels in the presence of Baf A1 for each drug alone in both HCT-116 (Figure 28a, compare lanes 6 to 2 and lanes 7 to 3) and SW480 (Figure 28b, compare lanes 6 to 2 and lanes 7 to 3). These data indicate that both MRK-003 and obatoclax alone may promote autophagy but when combined, autophagy is

somehow blocked. In summary, we see an absence (with MRK-003 alone) or lower levels (with obatoclax alone) of cell death with simultaneous autophagy induction in our colon cell lines; increased cell death (with MRK-003 + Obx combined treatment) is observed simultaneously with blocked autophagy. Our findings suggest a cytoprotective role for autophagy in this cellular context. It remains unclear how the combination of MRK-003 and obatoclax blocks autophagy when both drugs alone promote it.

Blocking Autophagy Sensitizes Colon Cancer Cells to GSI Treatment

If we instead focus on the data suggesting induction of cytoprotective autophagy in insensitive GSI treated colon cancer cells, we can hypothesize that blocking autophagy may overcome their resistance. To test this, we treated colon cells with two common compounds used to suppress autophagy – 3-MA and bafilomycin A1 (note: neither compound is a specific autophagy inhibitor). 3-MA is a nucleotide derivative that blocks class III PI3 kinases and prevents autophagy initiation (Seglen et al. 1982). When treated with 3-MA, we did not see sensitization of GSI-treated HCT-116 or SW480 cells (Figure 29a-b). As expected, 3-MA did increased cell death in obatoclax-treated cells and in GSI + Obx treated HCT-116. Bafilomycin A1, an ATPase inhibitor, blocks fusion of the autophagosome to the lysosome. When treated with bafilomycin A1, we saw massive increases in cell death in GSI-treated HCT-116 (Figure 30). Bafilomycin A1 had no effect on cell death in Obx-treated or MRK-003 + Obx-treated cells.

A)



B)

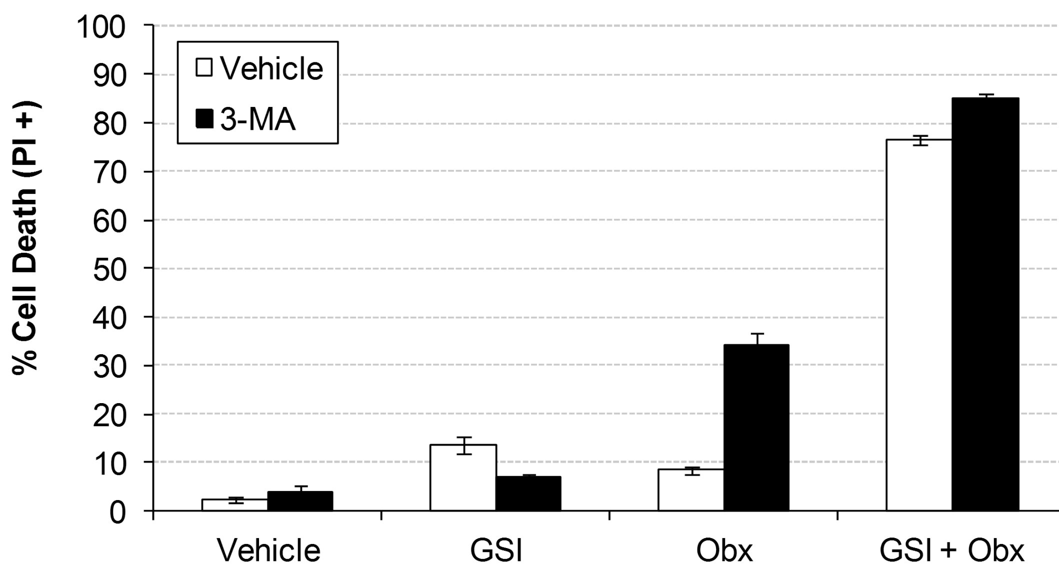


Figure 29: Blocking Autophagy through 3-MA Does Not Sensitize GSI-treated Cells
HCT-116 (A) was treated with 10 μM MRK-003 (GSI) and 75 nM obatoclax (Obx) in the presence or absence of 5 mM 3-MA for 48 hrs. SW480 (B) was treated with 5 μM MRK-003 and 75 nM Obx in the presence or absence of 5mM 3-MA for 48 hrs. Cell death rates were measure by flow cytometry following PI staining. Represented data are mean +/- SD of two independent experiments.

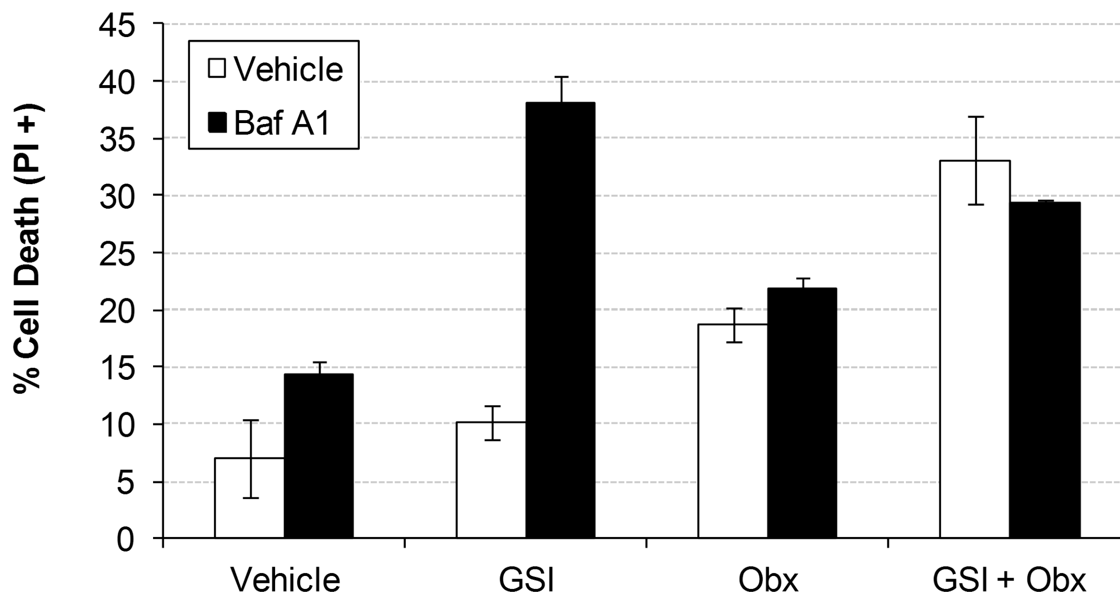
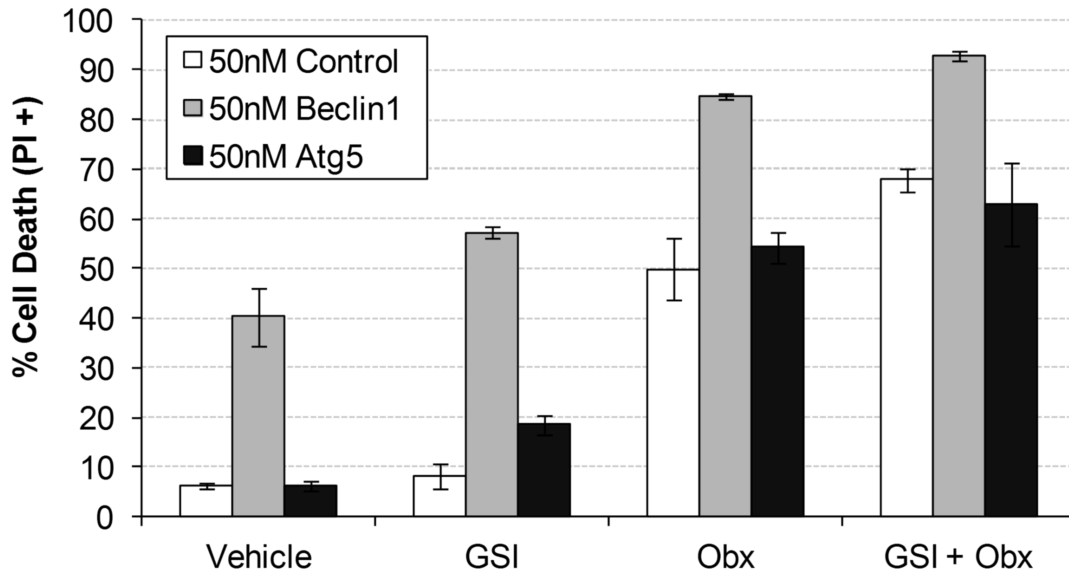


Figure 30: Blocking Autophagy with Bafilomycin A1 Induces Cell Death in GSI-treated Cells
HCT-116 was treated with 10 µM MRK-003 (GSI) and 75 nM obatoclax (Obx) in the presence or absence of 10 nM bafilomycin A1 for 48 hrs. Cell death rates were measure by flow cytometry following PI staining. Represented data are mean +/- SD of two independent experiments.

To further characterize the cytoprotective role of autophagy mediating GSI insensitivity, we knocked down two essential autophagy proteins – Beclin-1 and Atg5 – by RNAi techniques. Beclin-1 is required during the vesicle nucleation step of autophagy. Partial knockdown of Beclin-1 resulted in 40% cell death; treatment with MRK-003 increased cell death to ~60% (Figure 31a). Atg5 is required to convert LC3-I to LC3-II during autophagosome formation. Partial knockdown of Atg5 sensitized cells to MRK-003, increasing cell death by ~15% (Figure 31a). Knockdown was verified by western blot (Figure 31b). These data further support the theory that inhibition of autophagy may sensitize colon cells to gamma-secretase inhibitors.



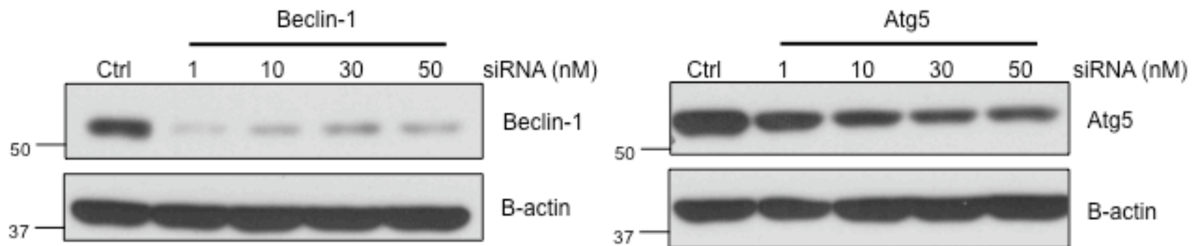


Figure 31: Knockdown of Beclin-1 or Atg5 Increases Cell Death in GSI-treated Cells
HCT-116 was transfected with 50 nM siRNA (Control, Beclin-1, or Atg5) for 24 hours and then treated with 10 μM MRK-003 (GSI) and 75 nM obatoclax (Obx) 48 hrs. Cell death rates were measure by flow cytometry following PI staining. Represented data are mean +/- SD of two independent experiments.

Discussion

Here, we show that the GSI MRK-003 in combination with obatoclax induces cell death in colon cancer cell lines. Others have shown increased killing with the combination of GSI and anti-apoptotic Bcl-2 agonists but we are the first to report a role

for autophagy in mediating cell death with combination treatment. Apoptosis seems to only have a partial role in this cell death induction. HCT-116 exhibited features of apoptosis by Annexin-V positivity and Parp-1 cleavage, both absent in SW480. Neither cell line showed significant caspase-3 cleavage. Inhibition of apoptosis by z-VAD-fmk blocked MRK-003 + Obx induced cell death by 50% in HCT-116 and 20% in SW480. Therefore, we concluded that apoptosis was not sufficient in mediating cell death induced by the combination of MRK-003 and obatoclax.

Obatoclax also induces autophagy, likely through antagonizing the anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-xL, which in addition to blocking apoptosis, can also block autophagy via direct binding to Beclin-1 (see above). We decided to assess the modulation of autophagy with this combination to examine its role in mediating the cell death observed. Alone, MRK-003 and obatoclax both promote autophagic flux. However, in combination, autophagy is inhibited. Our data showed no change or decreased levels of LC3-II during the bafilomycin clamp assay and increased p62 protein levels, all indicators of blocked autophagic flux. The mechanism for this remains unknown.

Only one report has looked at the effect of GSIs on modulation of autophagy in mouse embryonic fibroblasts; they found no differences in autophagy (Neely et al. 2011). We show are the first to show that the GSI MRK-003 increases autophagic flux in both HCT-116 and SW480. It remains unclear how this occurs as MRK-003 also increases protein levels of Mcl-1 and to some extent Bcl-xL, which in theory should block autophagy. Cytoplasmic localization of these proteins may explain this inconsistency, as only ER-localized, not mitochondria-localized, anti-apoptotic Bcl-2

protein can inhibit autophagy (Pattingre et al. 2005). Therefore, if Mcl-1 and/or Bcl-xL are not translocated to the ER even with increased total protein levels, autophagy will not be blocked. Induction of autophagy in GSI-treated cells may be due to a general stress response or due to specific mechanism of action by GSI. This is an area that needs to be further explored.

As mentioned, we are the first to show that cytoprotective autophagy may contribute to GSI-insensitivity in human colon cancer cell lines. We targeted autophagy via two chemical inhibitors – 3-MA and bafilomycin A1 – or through RNAi to examine its role in mediating GSI sensitivity. We found that bafilomycin A1, but not 3-MA, dramatically induced cell death in GSI-treated cells. Why both compounds did not have similar effects is unknown but this phenomenon has been reported in the literature (Shingu et al. 2009; Kanematsu et al. 2010). To address this, other autophagy inhibiting compounds, like chloroquine, need to be utilized. In addition to pharmacologically inhibiting autophagy, we knocked down two critical autophagy proteins – Beclin-1 and Atg5 – via RNAi. Knockdown of both Beclin-1 and Atg5 enhanced cell death in GSI-treated cells by ~20%. Our data supports a cytoprotective role of autophagy in sensitivity to GSIs in human colon cancer cell lines. Other GSI-insensitive cell types, i.e. breast cancer cell lines, need to be examined to determine if autophagy induction by GSI is universal. Therefore, we concluded that targeting autophagy in GSI-insensitive human colon cancer cell lines might be a rational approach to render cells sensitive to GSI treatment.

As mentioned previously, GSIs have yet to show any clinical significance in the treatment of colorectal cancer. We are the first to show that GSI insensitivity is due to

cytoprotective autophagy induction, though other mechanisms, like increased anti-apoptotic Bcl-2 family proteins, may also play a role. Our data supports the theory of targeting autophagy to modulate GSI sensitivity and therefore, combination of GSIs with autophagy inhibitors may be clinically useful.

CHAPTER 5: CONCLUSION

In conclusion, we show two potential drug resistance mechanisms to gamma-secretase inhibitors when given in combination with chemotherapy or as a single agent in colorectal cancer cells.

In the first scenario, combination of the GSI MRK-003 with oxaliplatin, the first line of chemotherapy for colorectal cancer patients, resulted in attenuation of apoptosis in the colon cancer cell lines HCT-116, SW480, HT-29, HCT-15, and Colo205. Abrogation of chemotherapy-induced apoptosis by GSIs was further supported by using alternative GSIs (DAPT and GSI-XX) and by other chemotherapeutic agents (etoposide and irinotecan). Decreased apoptosis seen in the combination of oxaliplatin and MRK-003 resulted in increased colony formation (long-term survival) compared to oxaliplatin treatment alone, refuting the possibility of delayed apoptosis. A decrease in the entire apoptotic cascade, from caspase-3 cleavage, cytochrome c release, and Bax and Bak activation, was observed in the combination of oxaliplatin and MRK-003. We found an increase in protein levels of the anti-apoptotic protein Mcl-1 with MRK-003 treatment alone and in combination with oxaliplatin compared to control and oxaliplatin-treated cells, respectively. We also observed elevated levels of the anti-apoptotic protein Bcl-xL with the combination of oxaliplatin and MRK-003 compared to oxaliplatin treatment alone. We knocked-down both proteins, individually and simultaneously, and

pharmacologically inhibited them with their agonist obatoclax to show that Mcl-1 and/or Bcl-xL played a causative role in the attenuation of oxaliplatin-induced apoptosis by the GSI MRK-003.

In the second scenario, we have shown that the GSI MRK-003 has little to no effect on cell death as a single agent in colon cancer cell lines. However, we did observe high levels of cell death in the combination of MRK-003 and obatoclax that was only partially (in HCT-116) or not (in SW480) due to apoptosis. Literature has supported a role of the anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 in preventing autophagy through binding and sequestering the autophagy protein Beclin-1. As autophagy is primarily a survival mechanism of the cell during times of stress, degrading proteins and cellular material for recycling and energy production, we assessed the modulation of autophagy in the combination of MRK-003 and obatoclax. Both MRK-003 and obatoclax promoted autophagic flux as single agents but inhibited autophagy in combination. Why two agents that individually promote autophagy block it in combination remains unknown. However, we were intrigued by the fact that the GSI MRK-003 induced autophagy and hypothesized that blocking autophagy could sensitize colon cells to GSIs. We blocked autophagy via two chemical inhibitors – 3-MA and bafilomycin A1 – and through knockdown of the autophagy proteins Beclin1 and Atg5 to show sensitization to MRK-003 in autophagy-deficient colon cancer cell lines.

The results of this work have important implications for the clinical use of GSIs. While this work provides a cautionary tale for the use of GSIs alone or in combination with chemotherapy in the treatment of colorectal cancer, the apoptosis inhibition or autophagy induction seen may be off-target effects of GSIs and not due to

downregulation of Notch signaling. Newly developed monoclonal Notch receptor antibodies will need to be tested *in vitro* and *in vivo* to address this issue. Furthermore, because Notch signaling can be both oncogenic and tumor suppressive, we need to determine what tumor types will potentially benefit from GSIs. Multiple ongoing Phase I and II clinical trials in both liquid and solid tumors are underway utilizing a variety of GSIs (MRK-0752 Merck, RO4929097 Roche, BMS-906024 Bristol-Meyers Squibb, and PF-03084014 Pfizer) as single agents or in combination with chemotherapy or other targeted drugs to address this issue. One small Phase II clinical trial using RO4929097 in refractory metastatic colorectal patients showed no radiographic response; *in vitro* experiments using GSIs in colon cancer cell lines have resulted in conflicting data (Strosberg et al. 2012). It is clear likely that the indicated study dosage and schedule are not optimal to achieve measureable responses. It may also be possible that only certain subsets of a tumor type may respond to GSI therapy and therefore biomarkers identifying these subtypes will be useful.

We may instead need to rethink how targeting Notch signaling using GSIs can be of clinical utility. There are conflicting reports of the role of Notch signaling in tumorigenesis; evidence exists supporting both oncogenic and tumor suppressive functions within tumor types. On one hand, the Notch pathway has been shown to be upregulated in colorectal tumors compared to normal (Reedijk et al. 2008); a different group has shown upregulation of the Notch pathway in low-grade adenomas but not adenocarcinomas (Fre et al. 2009). In pancreatic cancer, recent work has also shown differential effects of Notch pathway inhibition on precursor lesions (pancreatic intraepithelial neoplasia, PanIN) and pancreatic ductal adenocarcinomas (PDAC).

Treatment of mice PanIN lesions with GSIs lead to decreased progression whereas treatment of PDAC lesions with GSIs had no effect on progression. Further studies have revealed opposite effects of individual Notch receptors on pancreatic cancer progression in mice – embryonic deletion of Notch1 produces advanced stage PanIN lesions while embryonic deletion of Notch2 inhibits PanIN progression (Avila et al. 2013). Therefore, Notch signaling, and each individual receptor, may have differential roles in tumor initiation and progression.

Additionally, drugs targeting developmental pathways like Notch may not work like cytotoxic agents to kill cells but rather function to modulate cell fate programs to sensitize cells to cytotoxic or growth arrest signals. Or, their main target might be cancer-initiating cells rather than bulk tumor cells. Significant evidence exists for the role of Notch signaling in the maintenance of a variety of tumor-initiating cells in the colon (Sikandar et al. 2010), brain (Fan et al. 2006), breast (Wong et al. 2012), and T-cell leukemia (Armstrong et al. 2009). Therefore, the main therapeutic target of GSIs may be stem-like tumor-initiating cells. In a recent study using Her2/Neu positive breast xenographs in mice, the effect of two GSIs (MRK-003 and LY411,575) alone and in combination with Herceptin (trastuzumab) was studied (Pandya et al. 2011). Herceptin treatment alone lead to complete tumor regression; neither GSI had an effect alone or in combination with Herceptin on tumor size. However, approximately 50% of Herceptin-treated mice showed tumor recurrence; few (using LY411,575) to no (using MRK-003) mice treated with the combination of Herceptin and GSI showed tumor recurrence. Thus, GSIs may be clinically significant in limiting recurrence more than tumor shrinkage.

In conclusion, a comprehensive understanding of the role of Notch signaling, especially the contribution of individual Notch receptors, in tumor initiation, progression, metastasis, and recurrence needs further clarification. This will help determine whether or not GSIs, or more targeted Notch receptor agonists, will be a valid therapeutic approach to combating specific tumor subtypes.

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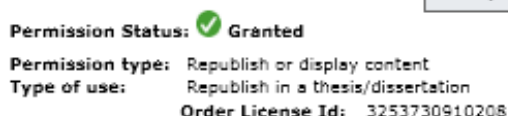

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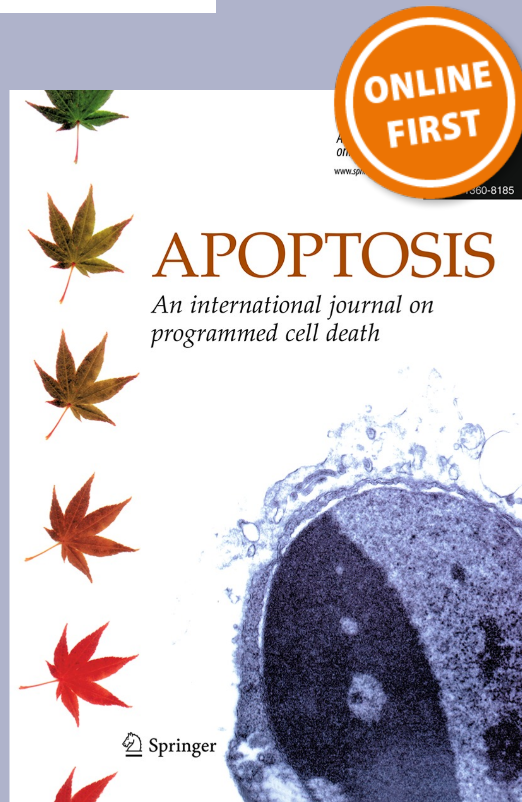
Apoptosis
An International Journal on Programmed Cell Death

ISSN 1360-8185

Apoptosis
DOI 10.1007/s10495-013-0883-x



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ORIGINAL PAPER

Gamma-secretase inhibition attenuates oxaliplatin-induced apoptosis through increased Mcl-1 and/or Bcl-xL in human colon cancer cells

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Abstract The Notch signaling pathway plays a significant role in differentiation, proliferation, apoptosis, and stem cell processes. It is essential for maintenance of the normal colon crypt and has been implicated in colorectal cancer oncogenesis. Downregulation of the Notch pathway through gamma-secretase inhibitors (GSIs) has been shown to induce apoptosis and enhance response to chemotherapy in a variety of malignancies. In this study, we analyzed the effect of MRK-003 (Merck), a potent inhibitor of gamma-secretase, on oxaliplatin-induced apoptosis in colon cancer. Unexpectedly, gamma-secretase inhibition reduced oxaliplatin-induced apoptosis while GSI treatment alone was shown to have no effect on growth or apoptosis. We determined that the underlying mechanism of action involved an increase in protein levels of the anti-apoptotic Bcl-2 family members Mcl-1 and/or Bcl-xL which resulted in reduced Bax and Bak activation. Blocking of Mcl-1 and/or Bcl-xL through siRNA or the small molecule inhibitor obatoclax restored the apoptotic potential of cells treated with both oxaliplatin and MRK-003. Moreover, obatoclax synergized with MRK-003 alone to induce apoptosis. Our findings warrant caution when treating colon cancer with the combination of GSIs and chemotherapy, whereas other drug combinations, such as GSIs plus obatoclax, should be explored.

Keywords Gamma-secretase inhibitor · Oxaliplatin · Mcl-1 · Bcl-xL · Colon

Background

Colorectal cancer accounts for almost 50,000 deaths each year in the United States and remains the third leading cause of cancer-related mortality [1]. While surgical resection remains the gold standard of treatment for localized disease, chemotherapy combinations with oxaliplatin, irinotecan, 5-fluorouracil, the VEGF inhibitor bevacizumab, and EGFR inhibitors have led to significant improvements in survival at all stages [2, 3]. However, most metastatic colorectal cancer patients eventually develop drug resistance to current therapies so new strategies are needed for treatment and improved survival of these patients.

The Notch pathway is an evolutionarily conserved pathway important in cell differentiation, proliferation, apoptosis, cell fate decisions, and stem cell renewal during development and cancer. The Notch family consists of four trans-membrane receptors (Notch1–4) that are activated upon binding to their ligands (Jagged 1–2 and Delta-like 1, 3, and 4) located on adjacent cells, triggering two proteolytic processing events. The first cleavage by TACE/ADAM metalloproteases releases the extracellular domain followed by gamma-secretase mediated cleavage to release the Notch intracellular domain (NICD) [4]. The activated form of Notch, NICD, translocates to the nucleus and binds to the

Electronic supplementary material The online version of this article (doi:10.1007/s10495-013-0883-x) contains supplementary material, which is available to authorized users.

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Published online: 26 July 2013

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transcriptional repressor CSL (CBF-1/suppressor of hairless/Lag-1) and recruits MAML-1 (mastermind-like-1) and p300/CBP to induce transcription of Notch target genes such as the Hes (hairy/enhancer of split) and Hey family members [5], c-myc, p21, and cyclin D1 (reviewed in [6]).

Aberrant activation of Notch signaling has been implicated in a variety of solid tumors, including breast [7, 8], melanoma [9], ovarian [10], and prostate [11, 12]. It was first linked to T cell acute lymphoblastic leukemia (T-ALL), where >50 % of tumors harbored activating mutations within *Notch1* [13, 14]. In contrast, reduced Notch signaling has been implicated in HPV-positive cervical cancer [15], neuroendocrine tumors [16], small cell lung cancer [17], hepatocellular carcinoma [18], and skin squamous cell carcinoma [19]. Indeed, the use of gamma-secretase inhibitors (GSIs) in clinical trials for Alzheimer's Disease (the amyloid precursor protein—APP—implicated in this disease is a gamma-secretase target) showed an increase risk of skin cancer development [20]; decreased gamma-secretase activity is also associated with increased skin cancer occurrence in mice [21]. Therefore, the biological outcome/function of Notch signaling as oncogenic or tumor-suppressive is highly cell context specific (reviewed in [22]).

Inhibition of gamma-secretase has shown antitumor effects in a variety of cancers in vitro and is thus considered an attractive pharmacology target. Pre-clinical reports using cancer cell lines, patient samples in vitro, and in vivo mouse models on the activity of MRK-003, a potent and selective GSI, have reported Notch pathway inhibition and antitumor activity in multiple myeloma [23], Non-Hodgkin's leukemia [23], T-cell acute lymphoblastic leukemia [24], breast cancer [25], pancreatic cancer [26, 27], lung cancer [28], and glioblastoma [29].

Notch signaling plays a vital role in intestinal homeostasis, self-renewal, and cell fate decisions during post-development [30–34] and is activated in colorectal adenocarcinomas [35, 36]. In theory, blocking Notch activation using GSIs might enhance chemotherapy effects for the treatment of colon cancer. Here, we tested the capacity for GSIs to synergize with oxaliplatin in colon cancer cell lines and evaluated the underlying molecular mechanisms. Surprisingly we show that GSIs blocked oxaliplatin-induced apoptosis through increased protein levels of Mcl-1 and/or Bcl-xL. Restoration of apoptosis was achieved by blocking Mcl-1 and/or Bcl-xL with obatoclax or siRNA.

Materials and methods

Cell lines and chemicals

The human colorectal cancer cell lines HCT-116, HCT-15, HT-29, and Colo205 were obtained from NCI; SW480 was obtained from ATCC. All cell lines were grown in RPMI 1640 supplemented with 10 % FBS and L-glutamine and grown in a 37 °C humidified camber 5 % CO_2. Cells were maintained for no more than 30 passages after thawing and tested as mycoplasma-free. HCT-116 and SW480 cell lines were authenticated using short tandem repeat (STR) DNA typing according to ATCC's "Authentication of Human Cell Lines: Standardization of STR Profiling" (2012).

Oxaliplatin (Sigma) was suspended in 5 % dextrose. The GSIs MRK-003 (a generous gift from Merck Inc), DAPT (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)-S-phenylglycine]-t-butyl ester; Santa Cruz), GSI-XII (Calbiochem), and GSI-XX (DBZ; Calbiochem) and the Bcl-2 inhibitor obatoclax (GX15-070; Selleck) were suspended in DMSO.

Apoptosis assays via flow cytometry

Apoptosis was measured by flow cytometry by detecting activated Caspase-3 or by detecting Annexin-V. Cleaved Caspase-3 was measured using the active caspase 3-FITC Apoptosis Assay Kit (BD# 550480) according to the manufacturer's protocol. Data were acquired on a FACS Scan or Calibur and analyzed with FlowJo software.

Apoptosis was also measured by AnnexinV-FITC (or AnnexinV-APC) (BD# 556420 or 550475) and 7-AAD staining according to the manufacturer's protocol. Data were acquired on a FACS Scan or Calibur and analyzed with FlowJo software. Dot plots with the percentages of each quadrant were reported for each sample.

Western blotting

Cell pellets were lysed in standard RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 % Igepal CA-630, 0.5 % sodium deoxycholate, and 0.1 % SDS) containing protease and phosphatase inhibitors—1 µg/ml each of aprotin, leupeptin, and pepstatin and 1 mM each of sodium fluoride, sodium vanadate, phenylmethylsulfonyl fluoride, and sodium pyrophosphate—for 30 min on ice. Cell extracts were clarified by centrifugation at 15,000×*g* for 10 min at 4 °C. Protein concentration was determined via the Bradford Assay and lysates were mixed 1:1 in 2× Laemmeli Sample Buffer and heated at 95 °C for 5 min. 30 µg of protein was resolved onto SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked in 1× TBST (Tris buffered saline with 0.05 % Tween-20) supplemented with 5 % milk and primary antibodies were incubated overnight at 4 °C in 1 % BSA or 5 % milk dissolved in TBST. The primary antibodies used for western blot were: rabbit anti-Mcl-1 (#5453), rabbit anti-Bcl-xL (#2764), and rabbit anti-PARP (#9542) from

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Cell Signaling; mouse anti-β-actin (#A5441) from Sigma. Membranes were washed and incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Jackson ImmunoReasearch Laboratories). The signals were visualized using SuperSignal West Pico Chemiluminescent detection reagent (Pierce/ThermoScientific).

Cytochrome C release

Adherent and non-adherent cells were harvested by scrapping and pelleted by centrifugation. For preparation of cytosolic extracts, the cell pellet was immediately suspended in a cytochrome c permeabilization buffer (20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM Sucrose) containing 200 μg/ml Digitonin (Sigma# D141) supplemented with protease and phosphatase inhibitors (see above in Western blotting) and incubated on ice for 5 min. Lysates were clarified by centrifugation at 1,000×*g* for 5 min at 4 °C to remove unlysed cells, cell debris and nuclei. The supernatant transferred to a new tube and centrifuged at 20,000×*g* for 20 min at 4 °C to remove mitochondria and any remaining debris. The supernatant was transferred to a new tube and 20–30 μg of protein was subjected to western blot analysis using anti-cytochrome c (BD# 556433) antibody. An anti-COX IV (Cell Signaling# 4580) antibody was used as a control to measure mitochondrial contamination of cytosolic extracts.

Cell survival and colony formation assays

Short-term cell survival was measured by MTT assay. Cells were seeded into 96-well plates in quadruplicate and drug treated the next day for 48 h. MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma# M2128) was added at a final concentration of 0.5 μg/uL per well and incubated for 3–4 h in a humidified chamber at 37 °C in the dark. The plate was centrifuged, supernatant aspirated, 100 μl/well of DMSO added, and the absorbance measured at 540 nm. Data were normalized relative to untreated controls, which were taken as 100 % survival.

Colony/foci formation assays were used to assess long-term cell survival. Cells were seeded into 12-well plates, allowed to attach overnight, and drug treated for 24 h. Attached cells were then trypsinized, counted, and 1,000 cells were plated in triplicate in 6-well plates and allowed to grow for 8 or 12 days. Cells were fixed with 4 % paraformaldehyde for 15 min with 0.1 % crystal violet.

Cell cycle analysis

Adherent and non-adherent cells were collected, fixed in ice-cold 70 % ethanol, and kept at 4 °C overnight. Cells were then stained with 20 μg/ml propidium iodine, 200 μg/ml RNase A, and 0.1 % Triton X-100 in PBS for 30 min in the dark at room temperature. Data was acquired on a FACS Scan or Calibur and analyzed with ModFit software.

Activation of Bax and Bak

Cell pellets were lysed in CHAPS lysis buffer (1 % CHAPS, 150 mM NaCl, 1 mM Hepes pH 7.4) containing protease and phosphatase inhibitors (see above for details) for 1 h on ice. Lysates were centrifuged and protein concentration was determined by Bradford assay. 500 μg of protein was precleared with 20 μl of anti-mouse IgG (whole molecule)-agarose beads (Sigma# A6531) for 1 h at 4 °C with rotation. The beads were pelleted and the supernatant was transferred to a new tube. Activated Bax or Bak was immunoprecipitated with 2 μg of anti-Bax 6A7 (Sigma# B8429) or anti-Bak Ab-1 (Calbiochem# AM03) antibodies and 20 μl of anti-mouse IgG-agarose beads and incubated overnight at 4 °C with rotation. The beads were pelleted and washed three times in CHAPS lysis buffer before being subjected western blot analysis with rabbit anti-Bax (N-20, #sc-493) and rabbit anti-Bak (G-23, #sc-832), respectively (Santa Cruz).

RNA interference

Small interfering RNA (siRNA) for Mcl-1 (#sc-35877), Bcl-xL (#43630), and Control-A (#sc-37007) was purchased from Santa Cruz. Cells were transfected with RNAiMax (Invitrogen) with a reverse transfection protocol according to the manufacturer's protocol. Briefly, siRNA and RNAiMax were incubated in serum-free RPMI 1640 for at least 20 min at room temperature. After siRNA: RNAiMax complexes were allowed to form, diluted cells in full serum media (RPMI 1640 + 10 % FBS) were added and the mixture plated out.

Results

Gamma-secretase inhibitors attenuate chemotherapy-induced apoptosis of colon cancer cells

Oxaliplatin is the first line of chemotherapy for colon cancer. We hypothesized that combining oxaliplatin with GSIs would enhance chemosensitivity in colon cancer cell lines, as others have shown using alternative GSIs [36–38]. To explore the role of gamma-secretase inhibition on

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oxaliplatin-induced apoptosis, two colon cancer cell lines—HCT-116 and SW480—were treated with oxaliplatin in the absence or presence of MRK-003 (Merck, Inc.), a potent gamma-secretase inhibitor. Apoptosis was measured by flow cytometry using either Annexin-V with 7-AAD double staining or measuring active caspase-3. The combination of oxaliplatin and MRK-003 resulted in a reduction in apoptosis by at least 50 % as measured by either Annexin-V and 7-AAD positivity (Fig. 1a) or activated caspase-3 (Fig. 1b) in both cell lines compared to oxaliplatin alone. To confirm that the results were likely due to gamma-secretase inhibition and not an off target effect of MRK-003, the effect of alternative GSIs on apoptosis of oxaliplatin treated cells were repeated using DAPT, GSI-XII, and GSI-XX (DBZ), widely used and commercially available GSIs that are structurally distinct from MRK-003. Both cell lines treated with DAPT and GSI-XX showed apoptosis rates approximately half that of oxaliplatin-treated cells (Supplemental Fig. 1a, c). The gamma-secretase inhibitor GSI-XII showed a slight decrease in oxaliplatin-induced apoptosis in SW480 but a slight induction was observed in HCT-116 (Supplemental Fig. 1b). MRK-003, DAPT, GSI-XII, or GSI-XX alone did not induce apoptosis in either HCT-116 or SW480 (Fig. 1a, b, Supplemental Fig. 1a–c). We further tested the effect of MRK-003 on oxaliplatin-induced apoptosis in three additional colon cancer cell lines to determine whether the reduction in apoptosis was cell line specific. We found greater than 50 % reduction of apoptosis in oxaliplatin-treated HT-29, HCT-15, and Colo205 cells in the presence of MRK-003 (Supplemental Fig. 2). To test if the attenuation of apoptosis was specific to oxaliplatin, we assessed the effects of gamma-secretase inhibition with either irinotecan or etoposide. Again, we observed a reduction of apoptosis in HCT-116 with MRK-003 in combination with either irinotecan or etoposide; no difference in apoptosis was observed in SW480 (Fig. 1c). Taken together, these data suggest that gamma-secretase inhibition attenuates apoptosis induced by chemotherapeutic agents in colon cancer.

The combined use of oxaliplatin and MRK-003 increases long-term survival in vitro

We hypothesized the attenuation of apoptosis seen in cells treated with oxaliplatin and MRK-003 might be due to increased survival, as measured by MTT assay and cell cycle analysis. HCT-116 and SW480 were treated for 48 h with oxaliplatin with or without MRK-003 and survival was measured by MTT assay. As expected, oxaliplatin treatment decreased viability by approximately 60 % for both cell lines; the combination of oxaliplatin and MRK-003 showed no difference compared to oxaliplatin alone (Fig. 2a). MRK-003 alone had no effect on viability compared to vehicle in either cell line.

Cell cycle analysis showed oxaliplatin induced a G_2-M arrest in HCT-116 that remained unchanged with the addition of MRK-003; the percentage of cells in G_0/G_1, however, increased (21–32 %, respectively) (Fig. 2b). Oxaliplatin arrested SW480 cells in S phase and no change was observed with the addition of MRK-003 in the cell cycle profile (Fig. 2b). MRK-003 alone increased the G_0/G_1 fraction from 34 to 48 % in HCT-116.

Colony forming assays are a measure of long-term survival. Cell death may be mediated through non-apoptotic mechanisms or may be delayed. The combination of oxaliplatin and MRK-003 remarkably enhanced colony formation in both HCT-116 and SW480 when compared to oxaliplatin treated alone (Fig. 2c). Collectively, the data suggests that GSI enhances the survival of cells treated with oxaliplatin.

Attenuation of the apoptotic signaling cascade

The mechanisms underlying gamma-secretase inhibition on oxaliplatin-induced apoptosis was examined by evaluating changes in the classical apoptotic signaling cascade. Cells treated with oxaliplatin and MRK-003 blocked cleavage of PARP (Fig. 3a) and caspase-3 (Fig. 1b) in both HCT116 and SW480 as measured by western blot and flow cytometry, respectively. Caspase activity can be blocked through direct interaction with the inhibitor of apoptosis protein (IAP) family members c-IAP1, c-IAP2 and XIAP, but no differences in protein levels were detected by western blot (data not shown). Second, oxaliplatin-induced cytochrome c release in both HCT-116 and SW480 was blocked by the addition of MRK-003 (Fig. 3b).

Bax and Bak are the two key pro-apoptotic proteins that regulate cytochrome c release. Bax and Bak are activated by conformational changes to promote homo-oligomerization to create pore-like channels responsible for cytochrome c release (reviewed in [39]). The activation of Bax and Bak can be assessed using conformation specific antibodies (6A7 for Bax and Ab-1 for Bak) [40]. To determine if MRK-003 altered the activation of Bax and Bak in oxaliplatin-treated cells, Bax and Bak were immunoprecipitated with their active conformation specific antibodies and western blotted. In response to oxaliplatin, both HCT-116 and SW480 show activation of Bax and Bak, which was markedly inhibited in the presence of MRK-003 (Fig. 3c).

Mcl-1 and Bcl-xL levels are elevated in oxaliplatin+GSI treated cells

The activation of Bax and Bak can be prevented by the anti-apoptotic Bcl-2 family member proteins Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A1 [41]. Gamma-secretase

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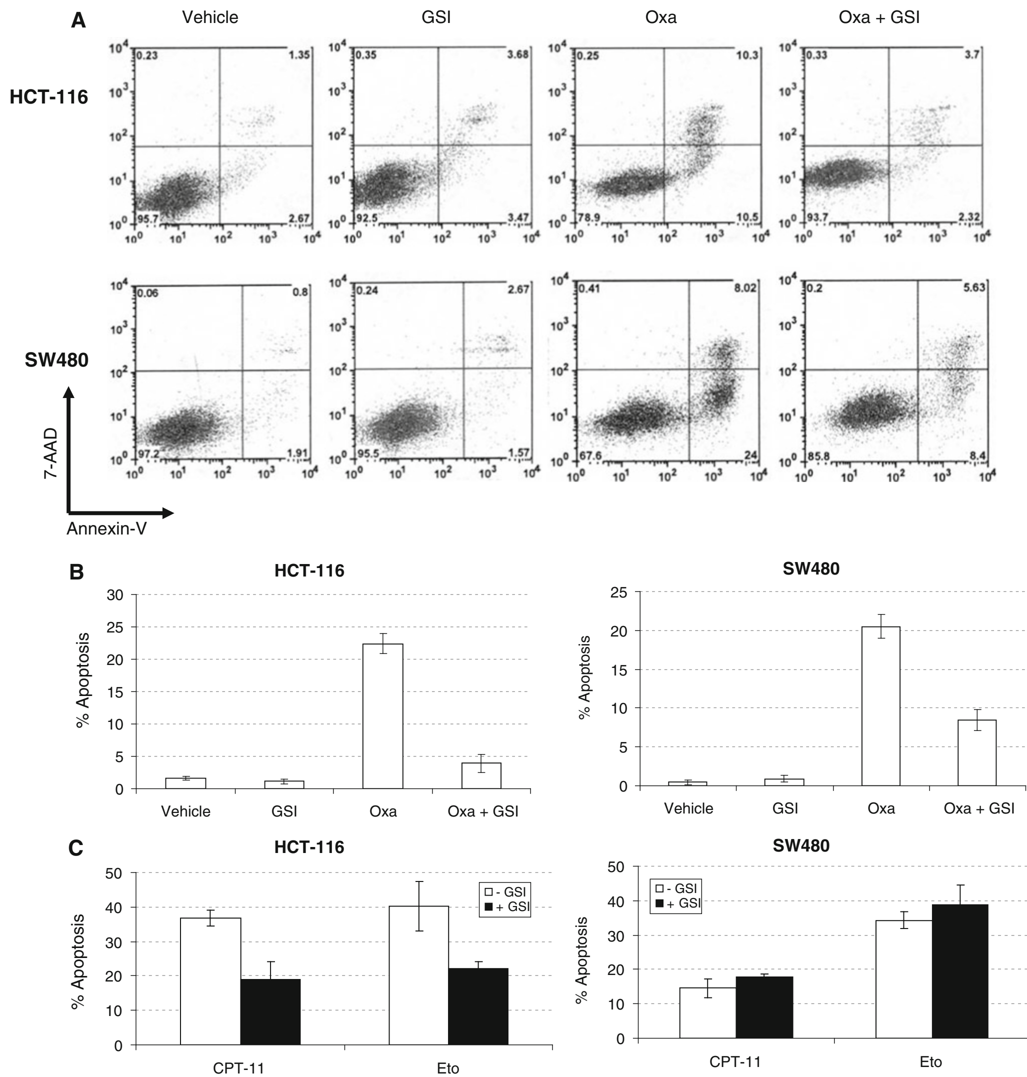

Fig. 1 Gamma-secretase inhibition attenuates oxaliplatin-induced apoptosis. HCT-116 and SW480 cells were treated with 15 µM (HCT-116) or 30 µM (SW480) oxaliplatin (Oxa) for 48 h in the absence or presence of 10 µM (HCT-116) or 5 µM (SW480) MRK-003 (GSI). Apoptosis rates were measure by flow cytometry following Annexin-V/7-AAD staining (**a**) and active caspase-3 labeling (**b**). **c** HCT-116 and SW480 were treated with either 50 µM Etoposide or 50 µM Irinotecan in the absence or presence of 10 µM (HCT-116) or 5 µM (SW480) MRK-003 for 48 h. Apoptosis rates were measured by flow cytometry following active caspase-3 reactivity. Represented data are mean ± SEM of three (**a**, **b**) or two (**c**) independent experiments

inhibition has been shown to regulate protein expression of the Bcl-2 family members [23, 42]. We tested whether or not GSI treatment altered total protein levels of Mcl-1 or Bcl-xL (neither cell line expressed detectable levels of Bcl-2, data not shown). Cells treated with MRK-003 alone increased Mcl-1 protein levels without seemingly affecting Bcl-xL levels in both HCT-116 and SW480 (Fig. 4). The combination of oxaliplatin and MRK-003 lead to enhanced protein levels of both Mcl-1 and Bcl-xL as compared to oxaliplatin alone in both cell lines.

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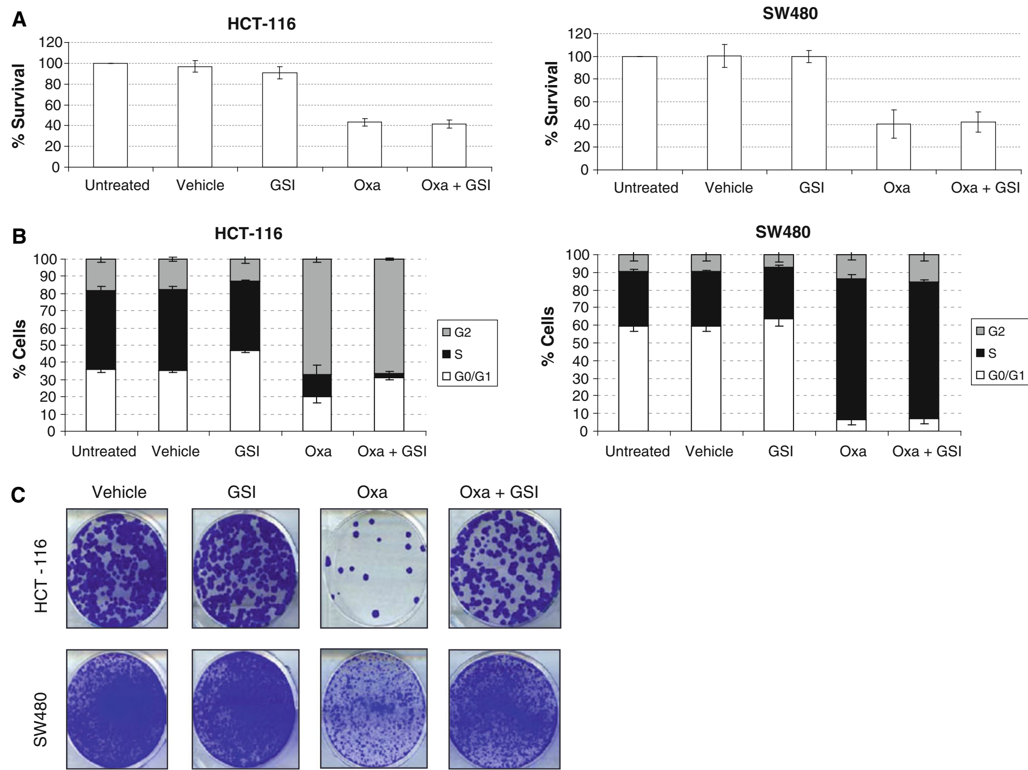

Fig. 2 Oxaliplatin and MRK-003 increases long-term survival. HCT-116 and SW480 cells were treated with 15 µM (HCT-116) or 30 µM (SW480) Oxa for 48 h in the absence or presence of 10 µM (HCT-116) or 5 µM (SW480) MRK-003. After treatment, MTT for viability (**a**) or cell cycle analysis (**b**) was performed. Represented data are mean ± SEM of three independent experiments. **c** Colony/Foci formation assays on HCT-116 and SW480 cells treated with 1 µM Oxa in the absence or presence of MRK-003 (10 µM for HCT-116, 5 µM for SW480) for 24 h. Results were repeated twice with similar results

Knockdown or pharmacologic inhibition of Mcl-1 and/or Bcl-xL restores apoptosis in cells treated with oxaliplatin and MRK-003

To examine whether the MRK-003 induced increase in the anti-apoptotic proteins Mcl-1 and/or Bcl-xL is responsible for decreased sensitivity to oxaliplatin treatment, we used RNAi interference (siRNA) assays to silence Mcl-1 and/or Bcl-xL. A dose-dependent knockdown of Mcl-1 resulted in a dose dependent increase in apoptosis in HCT-116 cells treated with oxaliplatin and MRK-003 but had no effect with MRK-003 alone as measured by flow cytometry for active caspase-3 (Fig. 5a). Reduction of Bcl-xL levels in HCT-116 produced a similar dose dependent increase in apoptosis in cells treated with both oxaliplatin and MRK-003 (Fig. 5b). As expected, reducing levels of Mcl-1 or Bcl-xL lead to increased sensitivity to oxaliplatin. Knockdown of Mcl-1 and Bcl-xL levels were confirmed by western blot. Reducing the levels of both Bcl-xL and Mcl-1 with low picomolar doses greatly increased oxaliplatin-induced apoptosis which was not inhibited with the addition of MRK-003 (Fig. 5c).

To further confirm the role of Mcl-1 and Bcl-xL in mediating the abrogation of oxaliplatin-induced apoptosis by MRK-003, we used the BH3-peptide mimetic obatoclax (GX15-070) to broadly antagonize Bcl-2 pro-survival family members Mcl-1 and Bcl-xL. Apoptosis inhibition associated with the combination of oxaliplatin and MRK-003 treatment was reversed with the addition of obatoclax in both HCT-116 (Fig. 6a) and SW480 (Fig. 6b). Of note, the combination of MRK-003 and obatoclax also resulted in an increase in apoptosis in HCT-116 (Fig. 6a). Thus, inhibiting Mcl-1 and/or Bcl-xL through RNA interference or small molecule inhibitor (obatoclax) lead to a restoration of apoptosis in colon cancer.

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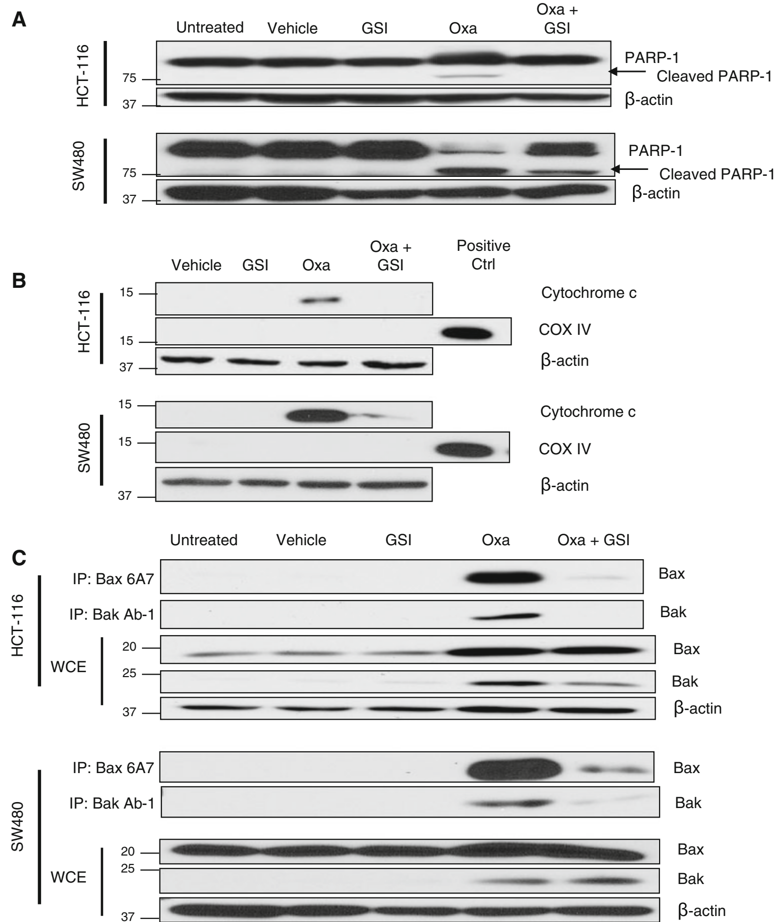

Fig. 3 The combination of oxaliplatin and MRK-003 reduces Bax and Bak activation, cytochrome c release, and PARP cleavage. HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30 μM (SW480) for 48 h in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003. **a** Full-length (110 kDa) and cleaved (80 kDa) PARP was analyzed by western blotting of whole cell lysates. Equal protein loading was shown by probing for beta-actin. **b** Release of cytochrome c into cytosolic fraction was analyzed by western blot. Potential mitochondrial contamination was detected by COX-IV (positive control lane is a whole cell extract). Beta-actin was used as a loading control. **c** Immunoprecipitation of active Bax and Bak. Whole cell extracts were probed for total Bax, total Bak, and beta-actin

Discussion

In this study, we report that the combination of the gamma-secretase inhibitor MRK-003 with oxaliplatin elicited potent chemoresistance in colon cancer cells by greatly decreasing apoptosis and leading to increased long-term survival. We showed the combination of oxaliplatin and MRK-003 reduced apoptosis in HCT-116, SW480, HT-15, HT-29, and Colo205 colon cancer cell lines by at least half. The reduction in apoptosis could be replicated using alternative GSIs (DAPT and GSI-XX) in HCT-116 and SW480. GSI-XII, a fourth GSI used in this study, slightly increased apoptosis by 10 % in HCT-116 and only decreased apoptosis by approximately 7 % in SW480. None of the four GSIs used in this study elicited apoptosis by themselves. When MRK-003 was combined with other chemotherapeutic agents (etoposide and irinotecan), decreased apoptosis was also seen in HCT-116. MRK-003, however, had no effect on apoptosis of etoposide- or irinotecan-treated SW480 cells, a finding reported elsewhere using DAPT in combination with various chemotherapeutic agents [38]. These data suggest that the effects seen are not cell-context specific, mediated by gamma-secretase inhibition (not an off-target effect), and not unique to oxaliplatin treatment. We observed no change in viability in oxaliplatin-treated cells with or without MRK-003 in either HCT-116 or SW480. Cell cycle analysis showed a slight increase in G_0/G_1 arrest with the addition of MRK-003 in oxaliplatin-treated HCT-116 cells alone (no change was observed in SW480). These data do not account for the reduction in apoptosis seen with MRK-003 treatment. However, to further support the decrease in apoptosis

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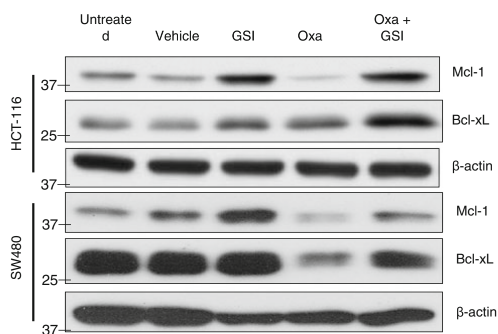

Fig. 4 MRK-003 increases protein expression of Mcl-1 and Bcl-xL. HCT-116 and SW480 cells were treated with 15 μM (HCT-116) or 30 μM (SW480) for 48 h in the absence or presence of 10 μM (HCT-116) or 5 μM (SW480) MRK-003. Total levels of Mcl-1 and Bcl-xL were assessed by immunoblotting. Beta-actin was used as a protein loading control

observed in oxaliplatin and MRK-003 treated cell, long-term survival assays showed increased cell survival in combination treatment compared to oxaliplatin alone.

We identified increased protein levels of the anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-xL in response to MRK-003 as pivotal players responsible for decreased oxaliplatin-induced apoptosis, leading to a reduction of Bax and Bak activation and downstream suppression of the apoptotic cascade (cytochrome c release, caspase-3 cleavage, and PARP cleavage). Restoration of apoptosis was achieved by blocking Mcl-1 and/or Bcl-xL through RNA interference or the Mcl-1/Bcl-xL/Bcl-2 antagonist obatoclax. Knockdown of Mcl-1 and/or Bcl-xL, however, did not induce apoptosis upon GSI treatment alone but the combination of GSI and obatoclax did in HCT-116. From these data, we conclude that increased protein levels of Mcl-1 and/or Bcl-xL are responsible for the attenuation of oxaliplatin-induced apoptosis observed upon MRK-003 treatment.

Several studies have highlighted the role of Notch signaling in the regulation of Mcl-1 and Bcl-xL. Mcl-1 levels are decreased by blocking the Notch pathway via a GSI [42] or with small-interfering RNA [43] and increased by overexpression of NICD [42]. GSIs have been shown to increase Noxa, a pro-survival BH3-only protein that promotes Mcl-1 degradation [44, 45]. Likewise, blocking Notch signaling can decrease Bcl-xL levels [27, 44]. In contrast, our data shows increased protein levels of Mcl-1 and Bcl-xL upon GSI treatment. This phenomena has been replicated by Liu et al. [46] in T-ALL cell lines when examining the effect of gamma-secretase inhibition on chemosensitivity. One subset of cells exhibited enhancement of apoptosis while another subset showed resistance via upregulation of Bcl-xL. The mechanism by which this occurs is still not well defined.

Our data also conflicts with other reports in the literature showing synergism between cytotoxic agents and GSIs in colon cancer cell lines. One study showed that blocking Notch-1 signaling using GSI34 (a novel gamma-secretase inhibitor) lead to an enhancement of oxaliplatin sensitivity [36]. Alternatively, DAPT sensitized colon cancer cell lines SW480 and DLD-1 to paclitaxel only but not 5-FU, camptothecin, TRAIL, or cisplatin [38]. A third group tested the effects of GSI-XX (DBZ), another GSI, in combination with platinum compounds (cisplatin, carboplatin, and oxaliplatin) on a panel of 20 human colon cancer cell lines [37]. GSI-XX alone had no effect on cell survival and, in combination with cisplatin, only a subset exhibited some degree of increased cell death; a few cell lines sensitive to the combination of cisplatin and GSI-XX showed no effect with oxaliplatin or carboplatin. In our hands, gamma-secretase inhibition using MRK-003, DAPT, or GSI-XX abrogated oxaliplatin-induced apoptosis via increased Mcl-1 and/or Bcl-xL protein levels, as seen by Liu et al. [46]. The pleotropic nature of gamma-secretase inhibition on chemotherapy-induced apoptosis is not well understood. It likely could be a reflection of complex context-dependent signaling downstream of the Notch pathway, and thus may only be of clinical utility to a particular (as yet undefined) subtype of colorectal cancer.

The observed effects of MRK-003, DAPT, and GSI-XX on attenuating chemotherapy-induced apoptosis may not be caused by Notch inhibition but rather differential targeting of other known gamma-secretase substrates. Gamma-secretase primarily catalyzes the intramembranous cleavage of Notch and APP. However, it has also been shown to target at least a dozen known type I transmembrane proteins, including E-cadherin, ErbB4, CD44, and c-Met, but their mechanism and function is not well studied (for review, see [47]). Also, the specificity of GSIs for different substrates may drive the discrepancies found in the literature, thus it will be important to understand the biochemical differences between specific GSIs, particularly since we showed that GSI-XII did not have the same effect as the other three GSIs. While we used four commercially available inhibitors known to block both Notch cleavage and activity as well as induce apoptosis in multiple tumor cell lines, we cannot rule out the possibility that these GSIs may also indirectly target other molecular pathways, such as pro-apoptotic signaling pathways, which may outweigh or antagonize any pro-apoptotic effects conferred by reduced Notch signaling in colon cancer cells. Therefore, the contribution of single, combinatorial, or complete ablation of all four Notch receptors on chemosensitivity is needed to address these concerns. The newly emerging neutralizing antibodies to individual Notch receptors will surely help to answer this question.

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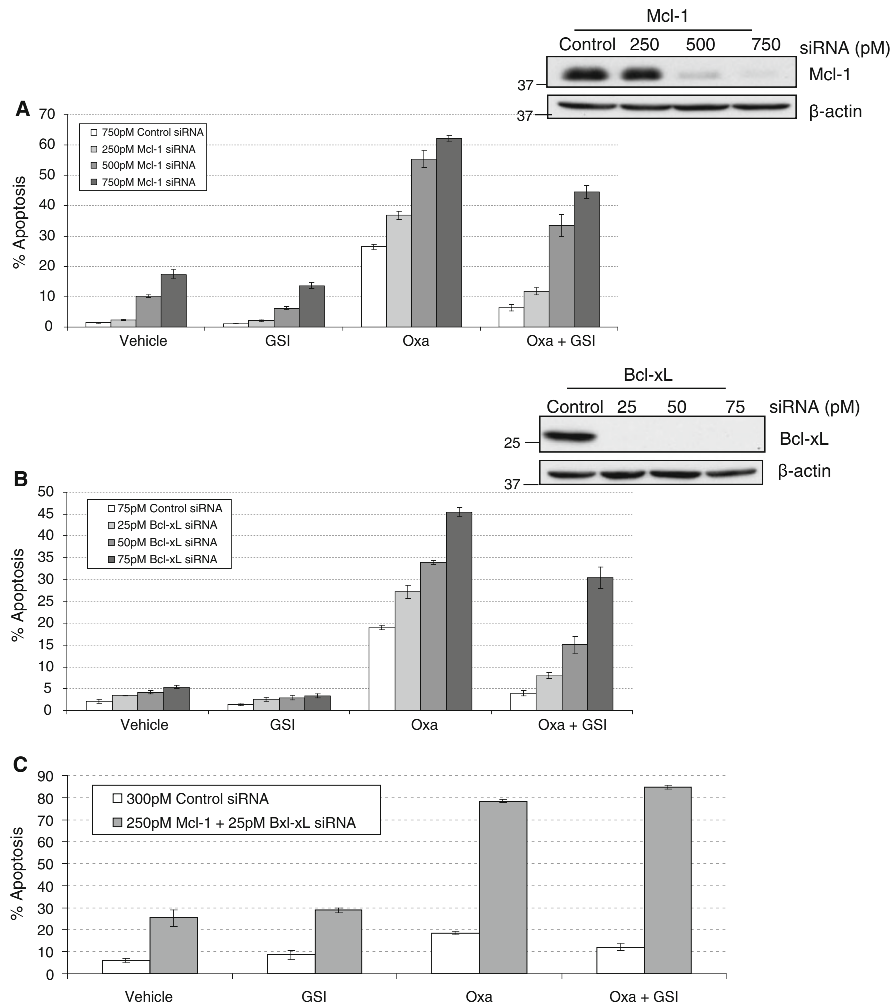

Fig. 5 Knockdown of Mcl-1 and/or Bcl-xL rescues MRK-003 attenuation of oxaliplatin-induced apoptosis. **a** HCT-116 was transfected with increasing amounts of Mcl-1 siRNA as indicated for 24 h. Cells were then treated with 15 µM Oxa in the absence or presence of 10 µM MRK-003 for 48 h. Western blot shows efficiency of Mcl-1 knockdown. **b** HCT-116 was transfected with increasing amounts of Bcl-xL siRNA as indicated for 24 h. Cells were then treated with 15 µM Oxa in the absence or presence of 10 µM MRK-003 for 48 h. Western blot shows efficiency of Bcl-xL knockdown. **c** HCT-116 was transfected with 250 pM Mcl-1 and 25 pM Bcl-xL siRNA as indicated for 24 h. Cells were then treated with 15 µM Oxa in the absence or presence of 10 µM MRK-003 for 48 h. Apoptosis rates were measure by flow cytometry following active caspase-3 reactivity. Represented data are mean ± SEM of two independent experiments

Standard chemotherapy approaches may need to be modified to include other novel inhibitors. Despite the apparent discrepancies of gamma-secretase inhibition on chemosensitivity, we were able to restore the attenuation of oxaliplatin-induced apoptosis seen with MRK-003 by antagonizing Mcl-1 and/or Bcl-xL. Combining GSIs and chemotherapy with an anti-apoptotic Bcl-2 family member agonist like obatoclax may be a valid therapeutic approach. We also found enhanced apoptosis with MRK-003 and obatoclax combined. Previous studies have shown sensitization with the combination of GSIs and the BH3 mimetic ABT-737 (antagonizes Bcl-2/Bcl-xL/Bcl-w but not Mcl-1

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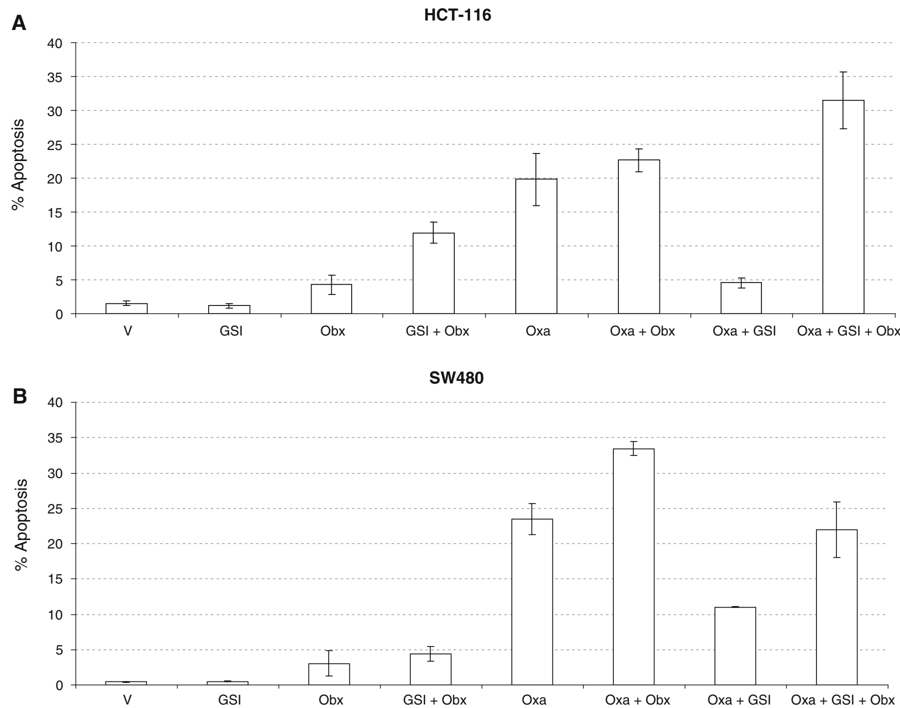

Fig. 6 Obatoclax rescues MRK-003 attenuation of Oxaliplatin-induced apoptosis. **a** HCT-116 was treated with 15 μM Oxa in the absence or presence of 10 μM MRK-003 and/or 75 nM obatoclax (Obx) for 48 h. **b** SW480 was treated with 30 μM Oxa in the absence or presence of 5 μM MRK-003 and/or 25 nM obatoclax for 48 h. Apoptosis rates were measured by flow cytometry following active caspase-3 reactivity. Represented data are mean ± SEM of at least two independent experiments

[48]) in GSI-sensitive cell lines where GSI alone increased Noxa levels, leading to subsequent Mcl-1 degradation [45, 49]. Our data shows MRK-003 synergism with obatoclax in GSI-resistant colon cancer cell lines where GSI alone increases Mcl-1 and Bcl-xL levels. Therefore, combining GSIs with other targeted therapeutics instead of chemotherapy may be a more effective strategy in the treatment of colorectal cancer.

To our knowledge, the only published Phase II clinical trial studying the efficacy of gamma-secretase inhibitor—RO4929097—monotherapy in metastatic colorectal cancer patients demonstrated no antitumor effects [50]. Nevertheless, there are multiple clinical trials investigating mono- and combination therapies involving GSIs in a wide variety of tumors (www.clinicaltrials.gov). The results posed here do not diminish the potential use of GSIs in a clinical setting but rather suggest caution when developing drug combinations. First, further detailed mechanistic studies looking at the biochemical differences in substrate specificity and potential off-target effects of the many available GSIs are needed. Also, the effects of gamma-secretase inhibition in relation to cell context and interactions with signaling pathways needs further clarification as there are clear indications that GSIs can have opposing effects even in same tumor types. Third, the potential still exists for using GSIs in combination with other therapeutic agents to elicit anti-tumorigenic responses. Our results provide a cautionary tale for broad application of GSIs. Understanding the actions of this drug when used in combination with standard chemotherapy and/or targeted therapies will provide greater knowledge of its potential use for a personalized approach to cancer treatment.

Acknowledgments This work has been supported in part by the Flow Cytometry Core Facility at the H. Lee Moffitt Cancer Center & Research Institute, a comprehensive cancer center designated by the National Cancer Institute. This work was supported by Bankhead-

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Coley Biomedical Research Program, Florida Department of Health, Grant number 08BR-02 and by institutional funds from the Moffitt Cancer Center (to T.J.Y.).

Conflict of interest No potential conflicts of interest.

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