ROLE OF MANGANESE SUPEROXIDE DISMUTASE IN CHEMOTHERAPY-INDUCED OXIDATIVE STRESS

By

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ABSTRACT

Existing treatments for mantle cell lymphoma (MCL) are non-curative, demonstrating a need for a refined treatment approach. Recent clinical trials have shown promising results with the use of mammalian target of rapamycin inhibitors. I hypothesize that the anti-tumor effect of mTOR inhibitors in mantle cell lymphoma is mediated by an increase in manganese superoxide dismutase (MnSOD) protein expression and accumulation of hydrogen peroxide (H_2O_2). Findings indicate that the rapamycin-induced cytostatic effect is characterized by increased levels of MnSOD and H_2O_2, and is necessary for the full growth inhibitory effect of rapamycin. Furthermore, over-expression of MnSOD elevated the level of H_2O_2 and increased sensitivity to MnSOD. Treatment with rapamycin resulted in a loss of serine 473 phosphorylation of AKT and increased levels of MnSOD were found to be due to inhibition of the mTORC2 complex. These results are the first to suggest that long term treatment of MCL cells with rapamycin inhibits the mTORC2 complex. By understanding the key signaling molecules and affected pathways in the anti-tumor effects of mTOR inhibitors, we may be able to identify additional predictive markers to improve the therapeutic value, or study drug combinations that will enhance the effect of ROS-induced cytotoxicity.

A retrospective study utilizing samples from lymphoma patients receiving standard anthracycline-based therapies, identified single nucleotide polymorphisms in oxidative stress-related genes associated with survival. Individuals carrying minor allele SNPs in myeloperoxidase (*MPO*) and an aldo-keto reductase (*AKR1C3*) were found to be associated with shorter time to disease progression and death. This data suggest that some patients may benefit from a different therapy than the current standard of care and that regulation of the redox environment plays a role in aggressive lymphoma treatment response.

CHAPTER ONE: INTRODUCTION

Aggressive Non-Hodgkin Lymphomas

Non-Hodgkin lymphomas (NHL) are a diverse group of cancers that originate from malignant transformation of lymphocytes, a type of white blood cell involved in the immune system (Nogai, 2011). These malignant cells can form tumors in lymph nodes and other sites of lymphatic tissue throughout the body. NHL is recognized as the 7th most common form of cancers in men and women in the US, accounting for approximately 4% of all cancer diagnosed in the United States (http://www.cancer.org). The American Cancer Society estimates that 66,360 new patients will be diagnosed with a form of NHL, and there will be 19,320 deaths in 2011 (http://www.cancer.org). Despite the general improvement in cancer therapies, the rate of incidence of NHL has been steadily increasing in the past three decades [Based upon findings from the National Cancer Institute's SEER (Surveillance, Epidemiology, and End Results) program, 1987 to 1991].

There are now over thirty unique subtypes of NHLs classified by the World Health Organization, each categorized due to their cell of origin, phenotype, and distinct molecular or cytogenetic features (Harris, 1997). These subtypes can be further characterized as "aggressive" or "indolent" diseases. Aggressive NHLs account for 60% of the lymphoma cases in the US (Nogai, 2011) and are fast growing malignancies involving B or T cells. The most common aggressive forms of NHL include diffuse large

B-cell (DLBCL), grade 3 follicular, mantle cell (MCL), Burkitt and anaplastic large cell lymphoma (ALCL).

Diffuse large B-cell lymphoma. The most common form of aggressive NHL among adults in the United States is DLBCL, accounting for roughly 40% of the diagnosed lymphomas (Schneider, 2011). Important insights into the molecular characteristics of this disease were discovered through the use of DNA microarray technology, which allows for a gemone-wide analysis of mRNA expression. This type of analysis has allowed for further identification of DLBCL subtypes: activated B-cell like (ABC), germinal-center B-cell like (GBC), and primary mediastinal B-cell lymphoma (PMBL). These subtypes are molecularly different, arise from B-cells at various stages of differentiation, and respond differently to standard treatment (Lenz, 2008; Lenz, 2010).

The standard treatment for all DLBCL subtypes is a multi-agent therapy using cyclophosphamide, hydroxydaunorubicin (also known as doxorubicin or Adriamycin), oncovin and prednisone, referred to as CHOP (Fisher, 1993; Gordon, 1992). Recently, the CD-20 monoclonal antibody rituximab was added to the standard of treatment based on the improved responses seen with its addition to CHOP (Coiffier, 2002; Habberman, 2006; Pfreundschuh, 2006). However, there is a clear heterogeneous response to treatment. With current treatment, DLBCL has a response rate of approximately 58%, with the remaining not responding to initial treatment or relapsing (Molina, 2008). Drugs in clinical trials to help improve treatment of specific DLBCL subtypes include new agents such as proteasome inhibitors, bcl-2 targeting drugs, or NF-κB targeting drugs (Lenz, 2010).

Mantle cell lymphoma. A rare but incurable form of aggressive NHL is MCL. This subtype of lymphoma accounts for approximately 6% of diagnosed lymphomas among adults in the United States. The 10-year survival rate of patients is only 8%, with a median survival time of 3-4 years (Zhou, 2008). MCL is a malignant transformation of naïve mature B lymphocytes along the edge of a lymph node follicle, in a region called the mantle zone. The majority of transformations are due to an (11;14) translocation resulting in over-expression of the cell cycle protein Cyclin D1 (Williams, 1994; Zukerberg, 1995). Cyclin D1 is involved in movement of cells past the G_1 checkpoint to the S phase of cell growth (Sherr, 1999). MCL has also been found to have an activated PI3K-Akt pathway (Rizzatti, 2005; Rudelius, 2006). MCL frequently displays a loss of the INK4a/ARF gene locus (Rosenwald, 2003), which encodes $p16^{INK}$ and $p14^{ARF}$ proteins involved in tumor suppressor pathways (Sherr, 2004).

Current MCL treatment approaches utilize multi-agent chemotherapy such as CHOP, MCP (melphalan, chlorambucil, prednisone), or hyper-CVAD (hyperfractionated doses of cyclophosphamide, vincristine, adriamycin, dexamethasone). However, improvements in treatment are necessary due to poor response. Novel treatment options including the proteasome inhibitor bortezomib and mammalian target of rapamycin (mTOR) inhibitors are currently being investigated and take into account the specific molecular characteristics of the disease.

Redox environment

The redox environment within a cell is a reflection of the sensitive balance between reactive oxygen species (ROS) generated oxidative stress and ROS removal systems (Jones, 2006). When the rate of ROS generation and ROS removal via anti-oxidant defense enzymes is balanced, cells are at redox equilibrium. When changes occur and there is an accumulation of ROS within the cells, they are said to be under oxidative stress conditions. This imbalance can be attributed to either an increase in the production of ROS or a decrease in removal of ROS due to alterations to anti-oxidant levels or function. This imbalance can lead to a disruption within the cellular environment.

Major sources of ROS can be found naturally occurring within the cellular environment. For example, ROS is formed as a normal by-product of the electron transport chain (ETC) in the mitochondria. An electron leak in the ETC results in production of the free radical superoxide ($O_2^{\bullet -}$) (Valco, 2007). Other sources of superoxide within the cell include synthesis by enzymes such as myeloperoxidase (Wientjes, 1995). A second example of ROS that can be generated by normal enzymatic reactions in the cells is hydrogen peroxide (H_2O_2). Enzymes that participate in reactions to produce H_2O_2 include various cellular oxidases and superoxide dismutases (Beuttner, 2011).

ROS attacks numerous targets in oxidation reactions, including lipids, proteins, and nucleic acids (Valco, 2007). Damage to DNA includes depurination, depyrimidination, strand breaks, sugar modifications, and DNA-protein crosslinks (Barzilai, 2004; Bartsch, 2006). Due to the high reactivity of superoxide, it is a short lived form of ROS that does not diffuse from the source of generation. (Freinbichler, 2011). Unlike superoxide, H_2O_2 is longer lived and readily diffuses through membranes (Veal, 2007; Wood, 2003). Therefore, H_2O_2 can travel throughout the cellular environment and participate in oxidation reactions in multiple locations. Oxidative stress conditions lead to altered functions within the cell due to damage or signaling. ROS, namely H_2O_2, has been shown to act as a signaling molecule to regulate cell growth and apoptosis (Iverson, 2004; Simon, 2000). Specifically, H_2O_2 is thought to react with and oxidize highly specific deprotonated cysteine residues on cellular proteins, which act as sensors of oxidative stress to regulate damage signaling and induction of apoptosis (Kannan, 2007).

Anti-oxidant enzymes in the cell include the enzymes superoxide dismutates, catalase, and glutathione peroxidases. Manganese and copper/zinc superoxide dismutase (MnSOD; Cu/ZnSOD) are the primary anti-oxidant defense enzymes involved in the removal of superoxide. Although SODs are cellular antioxidants, the dismutation of superoxide results in the formation of H_2O_2 (Beuttner, 2011). Catalase and glutathione peroxidases are then involved in removal of H_2O_2 by converting it to H_2O. The removal of ROS can be effected by changes in the expression of anti-oxidant proteins due to cellular signaling pathways. Mutations or single nucleotide polymorphisms within the encoding genes that alter transcription or protein function would also affect anti-oxidant protein levels or activity (Miao, 2009).

Redox environment in cancer. The redox environment plays a role in numerous aspects of cancer including, initiation, progression/growth, and treatment response (Dreher, 1996). The damage that ROS is known to cause plays a key role in the onset of numerous diseases and particularly, the development and progression of cancer (Burhans, 2007; Fearon, 2009; Okamura, 2009; Reddy, 2009; Valko, 2006; Wells, 2009). Generally, a pro-oxidant redox state is associated with cancer growth (Cerutti, 1985; Davies, 1995; Kinnila, 2004). Inhibition of ROS production in pancreactic (Vaquero, 2006), gastrointestinal (Vizan, 2009), and colon cancer (Inokuma, 2004) lines has been shown to inhibit further cancer progression.

Anti-oxidants are regulated in the cell in response to ROS levels in order to counter-balance genomic instability caused by significant DNA damage. If damage to DNA caused by ROS is not quickly and accurately repaired it can result in permanent modifications of genetic material – a primary step in the transformation of normal cells to cancer (Dreher, 1996; Jones, 2008). The BRCA1 gene shows how carcinogenesis can be mediated by ROS. It is responsible for up-regulation of multiple antioxidants with a cytoprotective role (Bae, 2004). Mutation or loss of BRCA1 confers susceptibility to DNA damage and is known to be the major cause of hereditary breast cancer (Rosen, 2003). BRCA1 null mice also display a high frequency of spontaneous lymphomas (Xu, 2001).

ROS induction can also have an anti-tumor effect. Changes to the redox environment can regulate normal physiological processes such as induction of apoptosis or cell cycle arrest via the signaling molecule H_2O_2 (Valko, 2006). For example, treatment with H_2O_2 induces apoptosis in several hematologic cell lines via activation of the caspase cascade (Kannan, 2000; Simon, 2000). This data suggests that changing the redox state of cancer cells by increasing baseline levels of H_2O_2 with treatment can promote cell death instead of maintaining cancer cell growth.

Chemotherapy and oxidative stress. A number of chemotherapeutic drugs used to treat NHLs rely on induction of ROS to promote apoptosis. Vinblastine, doxorubicin, campthothecin, and inostamycin exhibit anti-cancer activity via H_2O_2-dependent mechanisms (Minotti, 2004; Fang, 2007; Simizu, 1998). A novel approach to treat cancer may be to administer pharmacological agents to significantly increase ROS generation and by-pass the cellular anti-oxidant system (Fang, 2007; Jones, 2008; Numsen 2008). This is possible since cancer cells, in general, have higher baseline levels of ROS (Fang, 2007). While cancer cells have developed a resistance to these slightly elevated levels of ROS, it can be postulated that a further increase in ROS via generation by chemotherapeutic treatment would have a greater negative effect on cancer cells than normal cells. For example, studies have found that treatment with a novel NF-κB inhibitor, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), leads to rapid accumulation of H_2O_2 and selectively targets lymphoma cells with minimal toxicity to normal cells (Guzman, 2007).

Role of MnSOD as a tumor suppressor. The anti-oxidant enzyme, MnSOD, has been shown, in multiple studies, to have a cyto-protective role in cancer formation and progression. A potential role for MnSOD in tumor formation was first indicated in studies showing that MnSOD activity was attenuated in transformed cells compared to their normal counterparts (Yamanaka, 1974) and numerous cancer cell lines appear to have decreased MnSOD expression (Oberley, 1979; Oberley, 1982; Oberley, 1986; Oberley, 1988; Oberley, 1994). Manipulation studies with MnSOD demonstrated the importance of MnSOD expression on normal regulation of cell growth and cancer initiation. MnSOD cDNA transfection in colorectal (Lars, 2005), melanoma (Church, 1993), breast cancer (Li, 1995), lung cancer (Yan, 1996), glioma (Zhong, 1997), oral squamous cell carcinoma (Liu, 1997), and prostate cancer (Li, 1998) cell lines resulted in growth suppression and loss of malignant phonotype. SOD mimetics have been found to inhibit malignant transformation (Cerutti, 1985; Oberley, 1986; Oberley, 1994) and over-expression of MnSOD leads to inhibition of radiation-induced transformation in mouse fibroblasts (St. Clair, 1992). Transgenic mice with reduced MnSOD activity are at a higher risk of developing cancer (Van Remmen, 2003).

The tumor suppressive effect of MnSOD is largely attributed to an accumulation of H_2O_2 within the cell. Over-expression of MnSOD in a number of cancer cell lines, including a lymphoma model, increases the cellular levels of H_2O_2 (Davies, 2004; Jaramillo, 2009; Venkataraman, 2005). The growth of oral squamous carcinoma cells is inversely proportional to the level of measured MnSOD activity and production of H_2O_2 (Liu, 1997). When various tumor cell lines over-expressing MnSOD are transfected with glutathione peroxidase 1, an ROS scavenging enzyme, there is a reversal of the tumor suppressive effect of MnSOD (Li, 2000). Similar results are found with the transfection of another antioxidant protein, catalase (Rodriquez, 2000).

A biological significance of MnSOD levels can also be seen when analyzing single nucleotide polyporphisms (SNPs) found in *SOD2*, the gene encoding MnSOD. Two SNPs resulting in amino acid changes are found to alter the enzymatic activity of MnSOD (Ambrosone, 1999; Borgstahl, 1996). One SNP results in an isoleucine to threonine change at amino acid 58 that confers increased MnSOD activity; the variant is associated with a higher tumor suppressive effect when transfected into MCF-7 cells (Zhang, 1999). The second SNP codes for a valine residue in place of alanine at amino acid 9. This results in decreased MnSOD activity, and is associated with an increased risk of breast cancer (Ambrosone, 1989). Thus, it is possible that SNPs resulting in lower activity of MnSOD effect the redox environment and increase the risk of cancer formation and progression due to a loss of the MnSOD tumor suppressive effect.

Recently, our lab utilized gene expression profiling data from DLBCL patients to identify associations between anti-oxidant defense enzyme expression and treatment response (Tome, 2005). The study indicates that expression of MnSOD is decreased in patients with a poor prognosis. Additionally, over-expression of MnSOD in WEHI7.2 cells, a murine thymic lymphoma model, increases sensitivity to dexamethasone-induced apoptosis (Jaramillo, 2009). This data confirms that the status of the redox environment is an important element of NHL treatment. Collectively, the data also suggest that H_2O_2 producing MnSOD can play a role in modulating the redox environment.

Regulation of MnSOD by C/EBPβ and FOXO transcription factors

MnSOD gene expression can be regulated by multiple transcription factors, including the C/AACT enhancer binding protein β (C/EBPβ) (Ranjan, 2006) or forkhead box class O (FOXO) family members (Birkenkam, 2003; Kops, 2002). Along with being involved in regulation of MnSOD, both C/EBPβ and FOXO have been associated with cancer prognosis (Blenk, 2008; Gu, 2004; Rosenwald, 2003; Zhao, 2008). Recent data from our lab shows a strong correlation between expression levels of C/EBPβ and MnSOD in DLBCL patients (Tome, 2005). Disregulated C/EBPβ mRNA expression is also associated with MCL prognosis (Blenk, 2008; Rosenwald, 2003). High expression of FOXO family members has been correlated with patient survival and initiation of cell cycle arrest in ALCL and other NHLs (Gu, 2004; Zhao, 2008). C/EBPβ and FOXO family members may play an important role in regulation of MnSOD and induction of MCL tumor suppression.

C/EBPβ is a member of the leucine zipper transcription factor family. It is transcribed as a single mRNA species but can be translated into three distinct proteins due to multiple in-frame translational start sites (Descombes, 1991). The resulting proteins, LAP1(38 kDa), LAP2 (36 kDa), and LIP (20 kDa) contain the same DNA binding domain and leucine-zipper domain. The truncated isoform, C/EBPβ-LIP, lacks the transactivation domain. The C/EBPβ-LIP isoform plays an important role in modulating transcriptional activity by antagonizing C/EBPβ-LAP1 and LAP2

(Descombes, 1991; Ossipow, 1993). The relative level of isoforms within the cells likely dictates the expression of regulated genes, such as MnSOD.

FOXO is a family of three transcription factors, FOXO1 (Galili, 1993), FOXO3a (Andersen, 1998), and FOXO4 (Borkhardt, 1997). These are regulated at the post-translational level by phosphorylation (Burnet, 1999; Hu, 2004), deacetylation (Brunet, 2004), and degradation (Yang, 2008). Phosphorylation at Ser253 (FOXO1), Thr32 (FOXO4), or Ser315 (FOXO3a) (Biggs, 1999; Brunet, 1999; Kops, 1999; Tang, 1999) via activated PI3K-Akt signaling cascades leads to disruption of the nuclear localization signal. Phosphorylated FOXO is targeted for degradation by the ubiquitin-proteasome system (Yang, 2008). Thus, the phosphorylation status of PI3K and Akt would affect the transcriptional activity of FOXO family members on MnSOD. FOXO3a is the family member most commonly associated with regulation of MnSOD (Li, 2006; Shukla, 2009; Zrelli, 2011).

Mammalian target of rapamycin pathway

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase regulated by a wide variety of cellular signals, including mitogenic growth factors, hormones, nutrients, cellular energy levels, and stress conditions (Hay, 2004). In turn, mTOR functions as a central element in signaling pathways involved in the control of cell growth, proliferation, ribosome biogenesis, and transcription (Hay, 2004). Multiple pathways that signal through mTOR are dysregulated in numerous cancer types (Choo, 2006). With the role of mTOR as an important regulator of growth, a necessary component of tumor development, it is not surprising that it has been pinpointed as a therapeutic target in cancer.

mTOR assists in controlling the production of proteins via its role in formation of two active complexes known as mTORC1 and mTORC2. mTORC1 is responsible for regulation of protein translation initiation and efficiency. In addition to mTOR, the mTORC1 complex contains the scaffold protein, raptor. The mTORC2 complex contains a different scaffolding protein known as rictor and allows for association with different substrates than mTORC1 (Hay, 2004).

The mTORC1 complex is traditionally known as the rapamycin-sensitive complex. Thus, activation can be inhibited by the mTOR inhibitor drug, rapamycin, or its derivatives (Gingras, 2001). When complexed with its cellular receptor, FK506 Binding Protein-12 (FKBP12), rapamycin binds directly to mTOR and inhibits downstream signaling (Cortot, 2006). Active TORC1 phosphorylates p70S6K (S6K1) and eukaryotic initiation factor 4E-binding protein (eIF4E-BP). Active p70S6K can phosphorylate the ribosomal S6 subunit which enhances the translation of 5'-terminal oligopyrimidine (TOP)-mRNAs (Hannan, 2003). The phosphorylation of eIF4E-BP releases eIF4E, permitting it to participate in the formation of the protein translation complex necessary for capped mRNAs (Hannan, 2003), such as cyclin D1 (Fiano, 2004). Another target of translational control by the mTORC1 complex is C/EBPβ. The abundance of specific isoforms depends on translational control by eIF4E (Calkhoven, 2000). Translation of the truncated isoform, C/EBPβ-LIP, has been shown to be a down-stream target of mTOR inhibitor treatment in ALCL. Treatment with rapamycin or its derivatives results in decreased levels of the truncated C/EBPβ-LIP isoform in ALCL (Jundt, 2005; Quintanilla-Martinez, 2006).

In addition to regulating translation, mTOR can activate numerous kinases and their downstream transcription factors via the mTORC2 complex. For example, mTORC2 controls the phosphorylation and activation of Akt. An active mTORC2 complex can phosphorylate Akt at serine 473, resulting in an active Akt signaling pathway (Stephens, 1998). The mTORC2 complex was originally thought to be insensitive to inhibition by rapamycin. Exposure of multiple cell lines to rapamycin does not immediately affect mTORC2 activity as it does with mTORC1 activity (Sarbassov, 2004). More recent studies have shown that long exposure to rapamycin, in some cell lines, promotes inhibition of complex-independent mTOR molecules, inhibiting the formation of new mTORC2. This affects the phosphorylation status of Akt, thus effecting downstream transcription factors regulated by Akt (Sarbassov, 2006). It has been shown that downstream targets of the phospho-S473 Akt pathway include the FOXO family of transcription factors (Tzivion, 2011).

Use of mTOR inhibitors in treatment of lymphoma.

Rapamycin is the prototype mTOR inhibitor, initially developed as an immune suppressive agent (Thompson, 1989). To improve solubility for clinical use, a number of derivatives have been developed and studied in clinical trials. The first class of derivatives include temsirolimus, everolimus and deforolimus (Dancy, 2010). These derivatives function the same way as rapamycin, by binding to the cellular FKBP12 receptor and then binding to mTOR to interfere with activity (Dancey, 2010). Rapamycin and its derivatives were found to have anti-neoplastic activity with a broad spectrum of cancers, particularly hematologic malignancies (Drakos, 2008). The most promising results were seen in MCL.

Clinical trials show mTOR inhibitors, even when used alone, to be promising (Drakos; 2008). Overall response rates of mTOR inhibitors used as a single agent in relapsed MCL patients range from 25 – 41% (Ansell, 2008; Witzeg, 2005; Yee, 2006). More recent studies have achieved a response of 55% when temsirolimus was used in combination with rituxamab (Ansell, 2011). mTOR inhibitors are also very well tolerated with minimal hypertension, edema, and hyperlipidemia (Drakos, 2008). While options for treatment of MCL have clearly improved, the common problem of variable response and resistance to mTOR inhibitors exist, as with previous treatment options. Pre-clinical models suggests that genetic alterations and abnormalities in protein expression in components of the Akt and mTOR pathway correlate with sensitivity to treatment with mTOR inhibitiors (Faivre, 2006). No biomarkers for treatment response or all of the downstream targets of mTOR inhibitors that contribute to anti-tumor activity have been identified.

To date, laboratory use of mTOR inhibitors results in cell cycle arrest in a number of hematologic cancer models (Panwalker, 2004). In two human mantle cell lymphoma cell lines, Granta519 and NCEB1, a 50 nM rapamycin treatment over 48 hours results in decreased thymidine incorporation and is associated with growth arrest between the G_0/G_1 phase (Hipp, 2001). This data indicate that mTOR inhibition in MCL has a cytostatic effect versus a cytotoxic effect. The exact mechanisms that contribute to this growth suppressive effect are not yet understood.

In Molt-4 human T lymphocytes, a rapamycin-induced cytostatic effect is characterized by generation of ROS (Choi, 2008). Another study has identified a critical role for ROS formation in rapamycin-induced Smad signaling in mesangial cells (Osman, 2009). While the mechanism of intracellular ROS generation was not identified in the latter study, it was determined that the increase in ROS did not come from the major superoxide generating systems: NAD(P)H oxidase, xanthine oxidase, or mitochondrial NAD(P)H dehydrogenases (Osman, 2009). The importance of ROS production and source of ROS due to mTOR inhibition in MCL is not yet identified.

Statement of the problem.

Resistance to treatment is a common problem among aggressive NHL lymphoma patients, particularly in MCL. Differences in the molecular characteristics of various subtypes, and even inter-patient differences, have been found to contribute to variable treatment response and must be taken into account when determining treatment options for individuals. Historically, oxidative stress has been found to play an important role in the progression of cancer. More recent laboratory studies and gene expression profiling have found that components of the oxidative stress regulatory system can effect drug sensitivity in NHLs and may contribute to differences in disease prognosis. Thus, it is important to understand if there is a role for oxidative stress in new drug options that show promise in the clinic, such as mTOR inhibitors being used in treatment of MCL.

To fully exploit the use of novel therapeutic drugs, such as mTOR inhibitors, in MCL or other NHLs, any down-stream targets related to ROS need to be identified to pinpoint possible biomarkers to select patients most likely to respond to treatment. How changes in ROS are regulated are also important to understand, as it will offer insights into options for combination treatments that enhance the effect of mTOR inhibitors. In these studies, my first goal was to determine whether increased ROS played a role in mTOR inhibitor-induced growth suppression, and if levels of ROS affected sensitivity to treatment in MCL. The second goal was to identify the source of ROS that was altered with mTOR inhibitors and determine the mechanism of regulation. I was particularly interested in the role of MnSOD as a source of ROS since it is a known tumor suppressor in a number of cancer cell types. My final studies took a more global approach to understanding the role of the oxidative stress-related genes in treatment response. I looked for SNPs in MnSOD and other oxidative stress-related genes that were associated with aggressive NHL survival after different treatment regimens, all of which included at least one drug that has been shown to rely on ROS for a mechanism of cancer cell death.

CHAPTER 2: METHODS

Reagents and drug treatments. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Rapamycin (CalBioChem, Darmstadt, Germany) and bortezomib (Sellek, Houston, TX) were prepared in DMSO and N-acetyl cysteine was prepared in PBS (pH 7.4). Treatments were continuous and co-treatments added at the same time unless otherwise noted.

Cell culture and transfections.

Cell lines - Granta 519, NCEB-1 and Jurkat cells were maintained in RPMI (Invitrogen, Carlsbad, CA). Cell cultures were supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 1% penicillin/streptomycin and 2 mmol/L L-glutamine (Invitrogen). The mouse thymic lymphoma WEHI7.2 parental cell line was maintained as previously described (Tome, 2001). All cultures were maintained in a humidified, 5% CO_2 incubator at 37°C.

C/EBPβ LIP construction - C/EBPβ-LIP lentiviral plasmid was constructed using a CMV vector containing a rat gene for LIP provided by Dr. Uri Schibler (Universite de Geneve; ref. Descombes, 1991). CMV-LIP and the lentiviral plasmid pLVX were digested with XhoI and EcoRI (New England Biolabs, Ipswich, MA) and fragments ligated with T4 DNA ligase (New England Biolabs). Plasmid construction was confirmed via sequencing at the University of Arizona Genomics Core facility (Tucson, AZ).

***Transfections* -** Stable Granta519 cells over-expressing C/EBPβ-LIP were generated by lentiviral transduction using the Lenti-X system (Clonetech, Mountain View, CA) according to manufacturer's instructions. A vector containing the MnSOD cDNA sequence was a gift from Dr. Larry Oberley (University of Iowa; ref. Manna, 1998). Construction of the pcDNA3.1 expression vector containing the human MnSOD cDNA sequence used in this study was previously described (Tome, 2005). Stable Jurkat and WEHI7.2 cells overexpressing MnSOD were generated by electroporation using the Amaxa Nucleofactor™ II, according to manufacturer's instructions. Clone pools were maintained in 800 μg/ml G418 and removed from selection drug two passages prior to experiments. Transient transfection of Jurkat cells with controls, raptor, and rictor siRNA (Dharmacon, Lafayette, CO), were also generated by electroporation using the Amaxa Nucleofactor™ II.

Protein measurements. Total sample protein was measured in clarified lysates using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Nuclear and cytosolic protein samples were separated using the ProteoExtract subcellular extraction kit (CalBioChem) according to the manufacturer's instructions.

Cell viability and apoptosis measurements.

***Eosin Y staining* -** Viable cell number was measured by adding an aliquot of cells to an equal volume of 500 μg/ml Eosin Y solution. The mixture of cells and dye were allowed to stand 5 minutes at room temperature. Cells that excluded dye were counted as viable.

Caspase-3 activity - Caspase 3 activity was measured using a colorimetric assay dependent on the cleavage of the synthetic caspase 3 specific substrate, Ac-DEVD-*p*-nitroanilide (pNA) (BIOMOL International LTD, Bangkok, Thailand). Cells were lysed by sonication in 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.01% Triton X-100. Samples were clarified by centrifugation at 10,000 x g for 10 minutes. Supernatant was incubated in 10 mmol/L PIPES (pH 7.4), 2 mmol/L EDTA, 0.01% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate, 5 mmol/L DTT, 200 μmol/L Ac-DEVD-pNA for 2 hours, and the absorbance was measured at 405 nm using a Synergy HT plate reader. Caspase 3 activity was normalized to cellular protein.

MTS **–** To determine the IC_{50} of bortezomib, the relative cell number following treatment was measured using the Non-radioactive Cell Proliferation Assay (MTS) according to the manufacturer's instructions (Promega Corp., Madison, WI). Absorbance was read at 490 nm using a Synergy HT plate reader (Bio Tek Instruments, Winooski, VT).

ROS measurements.

DCF measurements **-** To determine overall ROS levels, 2'7'-dichlorodihydrofluorescein diacetate (Invitrogen) measurements were used. The dye is transported across the membrane and deacetylated by esterases, forming the nonfluorescent 2’,7’-dichlorfluorescein (DCFH). This compound remains inside the cell and fluoresces in the presence of ROS. Cells were washed with medium containing 0.5% calf serum, then incubated 2 hours at 37°C in a 5% CO2 humidified environment in medium supplemented with 20 μM DCFH-DA. Thirty minutes before analysis, 5 μg/ml propidium iodide was added to the medium. DCF fluorescence of ten thousand cells per samples were measured using a FACScan flow cytometer with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). Cells staining positive for propidium iodide were excluded from further analysis.

***Amplex Red measurements* -** The Amplex Red® assay was used to estimate changes in H_2O_2 levels (Zhou, 1997). Cells were re-suspended in phenol red-free complete medium (Invitrogen). Samples were incubated for 15 minutes then treated with 50 μM Amplex Red® (Invitrogen), 0.1 units/ml horseradish peroxidase, and read over a four hour time-course to determine the rate of H_2O_2 efflux. Absorbance at Ex: 485 nM / Em: 590 nM was measured using the SynergyHT plate reader. Measurements were normalized for cellular protein.

***MitoSOX measurements*-** Cells were incubated in suspension in a final concentration of 5 μM MitoSOX (Molecular Probes, Eugene, OR) in DMEM with 10% calf serum at 37°C for 3 hours. The rate of increase in MitoSOX fluorescence (Ex: 530/Em: 590) was measured using a Synergy HT plate reader. Rates were normalized for cellular protein.

Glutathione measurements. Glutathione (GSH) levels were measured using the Bioxytech GSH/GSSG 412 kit (Oxis Research, Portland, OR) according to the manufacturer's protocol. Measurements were normalized for cellular protein.

Immunoblots. Cells were lysed in 20 mM HEPES, 300 mM NaCl, 100 mM KCl, 10 mM EDTA, 0.1% IGEPAL and clarified by centrifugation unless otherwise specified. Samples were separated by SDS-Page and protein levels were quantified by immunoblotting using the following antibodies: 1:500 dilution of C/EBPβ primary (Santa Cruz Biotechnology, Santa Cruz, CA), 1:2000 dilution of MnSOD primary(Upstate, Lake Placid, NY), 1:1000 dilution of glutathione peroxidase 1 primary (Lab Frontier, Seoul Korea), 1:1000 dilution of AKT phospho–S473 primary (Cell Signaling, Danvers, MA), 1:1000 dilution of FOXO3a primary (Abcam, Cambridge, MA), 1;10000 dilution of β-actin primary or 1:1000 dilution of catalase primary (AbCam), and 1:1000 dilution of anti-rabbit immunoglobulin or anti-mouse immunoglobulin secondary (Cell Signaling) and visualized by a chemiluminescent reagent. To visualize multiple bands on the same blot, blots were stripped with Restore Western Blot Stripping Buffer (Pierce) before being probed with a new antibody. Bands were quantified using AlphaView software (Alpha Innotech, San Leandro, CA) when indicated.

Nuclear run-on assay and Real-Time PCR. A nonradioactive nuclear run-on assay was carried out as previously described (Patrone, 2000), but with the following modifications. Nuclei were isolated from 5 x 10^7 cells by sucrose gradient centrifugation. Biotin-labeled RNA was purified with NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA) and isolated by magnetic Dynabeads M-280 covalently linked to streptavidin (Dynal Biotech, Milwaukee, WI). Beads were re-suspended in PCR grade water and 1µg of isolated RNA was used to generate cDNA for analysis with the iScript cDNA synthesis kit (BioRad, Berkeley, CA) according to manufacturer's protocol. Real-time PCR was carried out with SYBR Green I Master reagents and protocol (Roche, Madison, WI) using a Lightcycler 480 (Roche) for detection and analysis. Primers used in real-time PCR were as follows; SOD2 primer #1: TTGGCCAAGGGAGATGTTA, SOD primer #2: TGGTGGTCATATCAATCATAGCA, GAPDH primer #1: GAGGTGAAGGTCGGA GTC, and GAPDH primer #2: GAAGATGGTGATGGGATTTC.

Statistics. Sample means were compared using Student's *t* tests assuming unequal variances with the algorithms provided by Excel (Microsoft Corp., Redmond, WA). Means were considered significantly different when $p \leq 0.05$.

SNP analysis.

Study population - The study was conducted with clinical data and available diagnostic tissue from seven completed Phase II or Phase III Southwest Oncology Group (SWOG) clinical trials (S8516, S8736, S9125,S9240, S9349, S9704, S0014). Samples included in the study were those from patients diagnosed with a biopsy proven, CD20-positive, aggressive NHL based on the current World Health Organization guidelines (Jaffe, 2001). Samples collected before implementation of the most recent World Health Organization classification were stained for CD-20 using the Benchmark XT (Ventana, Tucson, AZ) and examined by a hematopathologist to confirm diagnosis of aggressive

NHL. Tissue samples were available to us from the lymphoma SWOG tissue bank located at the University of Arizona, Department of Pathology. Details regarding the clinical trial criteria, treatment regimens, outcomes, and patient characteristics were previously reported (Fisher, 1993; Miller, 1998; Gaynor, 2001; Blayney, 2003; Persky, 2008).

Genotyping – Samples were collected from three 5 micron cuts of paraffin-embedded tissues. Genomic DNA was extracted from samples using the QIAamp DNA FFPE Tissue Kit as described in the manufacture's protocol (Quiagen, Valencia, CA). DNA samples were plated in a blind fashion for genotyping, with duplicates from 5% of the samples and DNA from in-house CEPH trio included for controls. Genes involved in oxidative stress related pathways were chosen by Dr. Song Yao (Roswell Park cancer Institute, Buffalo, NY) via a Medline search of SNPs implicated in previous cancer related publications as reported in Gustafson et. al. (submitted, 2011). Genotyping of 71 SNPs was performed in the Genomics Shared Resource Core at Roswell Park Cancer Institute using the MassARRAY technology and iPLEX Gold assay (Sequenom, San Diego, CA). Samples with call rates lower than 80% were excluded from further analysis. Of the initial set of 71 SNPS, 18 SNPs failed for various reasons and were excluded from further analysis, to give a final set of 53 SNPs.

Statistical analysis - The primary end points assessed in this study were the associations between genotype on progression-free (PFS) and overall survival (OS). Survival was defined as the time from the date of registration to the date of recurrence or death due to any cause. Treatment outcome analysis was limited to 10 years post enrollment, with the exception of S9704 and S0014 which closed in 2007 and 2002, respectively. Statistical analysis was performed by SWOG statisticians (Bryan Goldman, Micheal LeBlanc) and has been described (Gustafson, 2011; submitted). Briefly, Kaplan-Meier (Kaplan, 1958) estimates of survival for the subgroups of patients by genotype were generated, and differences were determined using cox-regression models. Associations between SNPs and the occurrence of grade 4 or 5 hematologic toxicity (using Common Toxicity Criteria) were assessed by logistic regression. All regression analyses were stratified be disease stage and adjusted for IPI risk score.

CHAPTER THREE: RESULTS

Hydrogen Peroxide as a Mediator of Rapamycin-induced Growth Inhibition

mTOR inhibitors induce growth arrest. Inhibition of the mTOR pathway with rapamycin, or its derivatives, is known to result in cell cycle arrest. In MCL cell lines, 50 nM rapamycin treatment over 48 hours results in decreased thymidine incorporation and is associated with growth arrest between the G_0/G_1 phase (Hipp, 2008).To confirm the effect of rapamycin on MCL cells in our lab, I measured the number of viable Granta519 and NCEB1 cells, by eosin Y staining, after 48 hours of rapamycin treatment. Granta519 cells displayed a dose-dependent decrease in cell viability (Figure 3.1A). NCEB1 cells showed a greater decrease in viable cell number than the Granta519 line at the lowest rapamycin concentration, indicating that they are more sensitive to rapamycin. NCEB1 viability showed minimal further decrease with higher concentrations of rapamycin, but rather, maintained a steady level of cell viability (Figure 3.1A). A clinically used rapamycin derivative, everolimus, produced similar results, showing a decrease in the number of viable cells with the same treatment doses at 48 hours (Figure 3.1B). As expected from previous studies (Panwalker, 2004; Hipp, 2005), rapamycin did not induce apoptosis, as determined by a lack of significant caspase-3 activation in our studies (Figure 3.1C). These results confirm that rapamycin has a cytostatic effect on these MCL cell lines, and demonstrates how they differ in sensitivity to rapamycin.

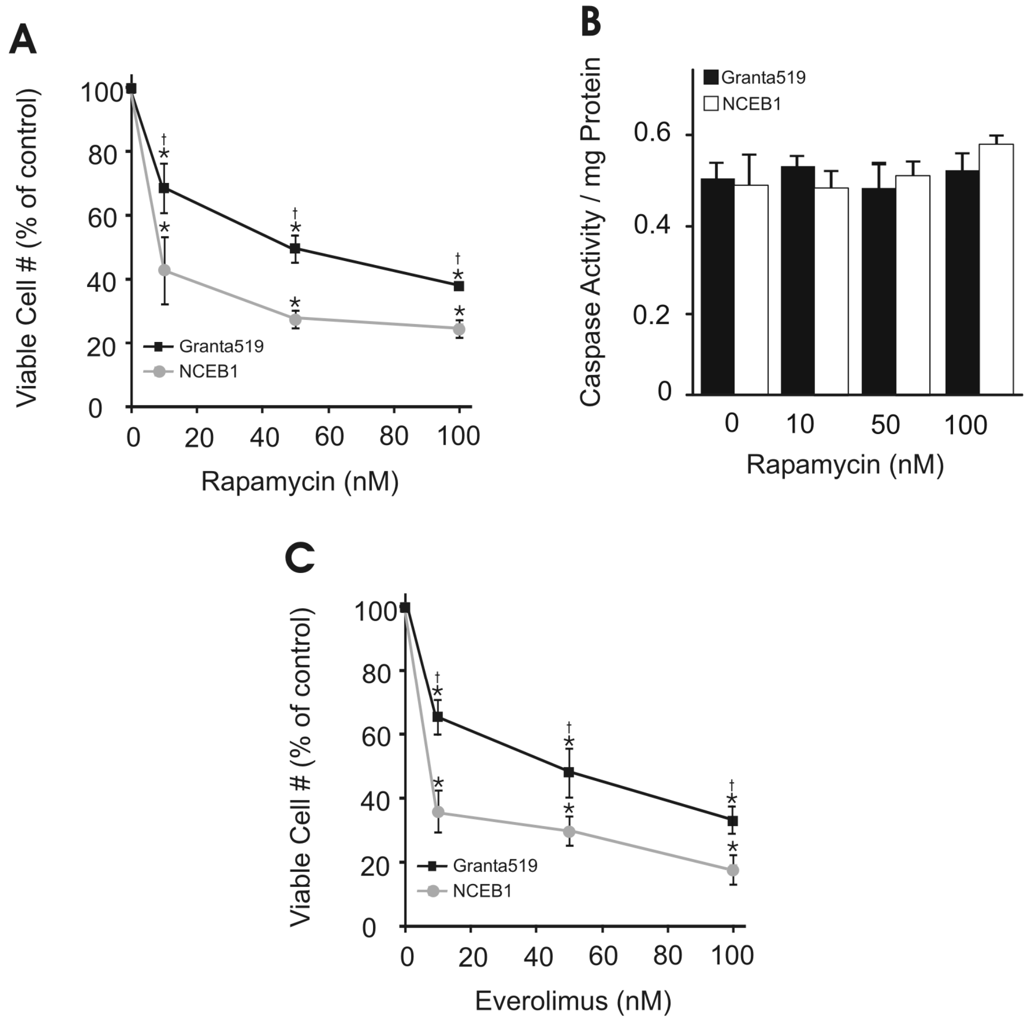

Figure 3.1: mTOR inhibitors induce growth arrest. (A) Relative percentage of viable Granta519 or NCEB1 cells compared to untreated controls after 48 hours treatment with rapamycin (mean + SEM, *n=3*). **(B)** Comparison of the caspase activity in Granta519 and NCEB1 cells treated with varying doses of rapamycin after 48 hours (mean + SEM, *n=3*). **(C)** Relative percentage of viable Granta519 or NCEB1 cells remaining in culture compared to untreated controls after 48 hours treatment with the rapamycin derivative, everolimus (mean + SEM, *n=3*). *denotes significantly different from untreated controls ($p \leq 0.05$). †denotes significantly different from other treatment doses ($p \leq 0.05$).

Rapamycin treatment increases overall oxidative stress and H_2O_2 levels. To investigate the role of ROS in the growth suppressive effect of mTOR inhibitors in MCL, I first measured ROS levels by DCF fluorescence. DCFH is a probe that can be non-specifically oxidized to a fluorescent state by intracellular ROS. To specifically measure H_2O_2 levels I used the fluorescent probe Amplex Red®. Amplex Red® is a cell-impermeable probe that allows for measurement of the efflux of H_2O_2 through the cell membrane; it fluoresces in proportion to the intracellular levels of H_2O_2 (Zhou, 1997). Treatment with rapamycin in Granta519 cells significantly increased overall ROS (Figure 3.2A) and H_2O_2 efflux (Figure 3.2B) in a dose-dependent manner. NCEB1 cells displayed a greater increase in ROS (Figure 3.2A) and H_2O_2 efflux (Figure 3.2B) at the lowest rapamycin concentration, as compared to Granta519 cells, with no significant further increase at higher concentrations. In addition, mitochondrial ROS measured by MitoSOX, a fluorescent probe that localizes to the mitochondria, increased in both cell lines with rapamycin treatment (Figure 3.2C). This supports the idea that ROS generated at the mitochondria is a down-stream effect of rapamycin treatment.

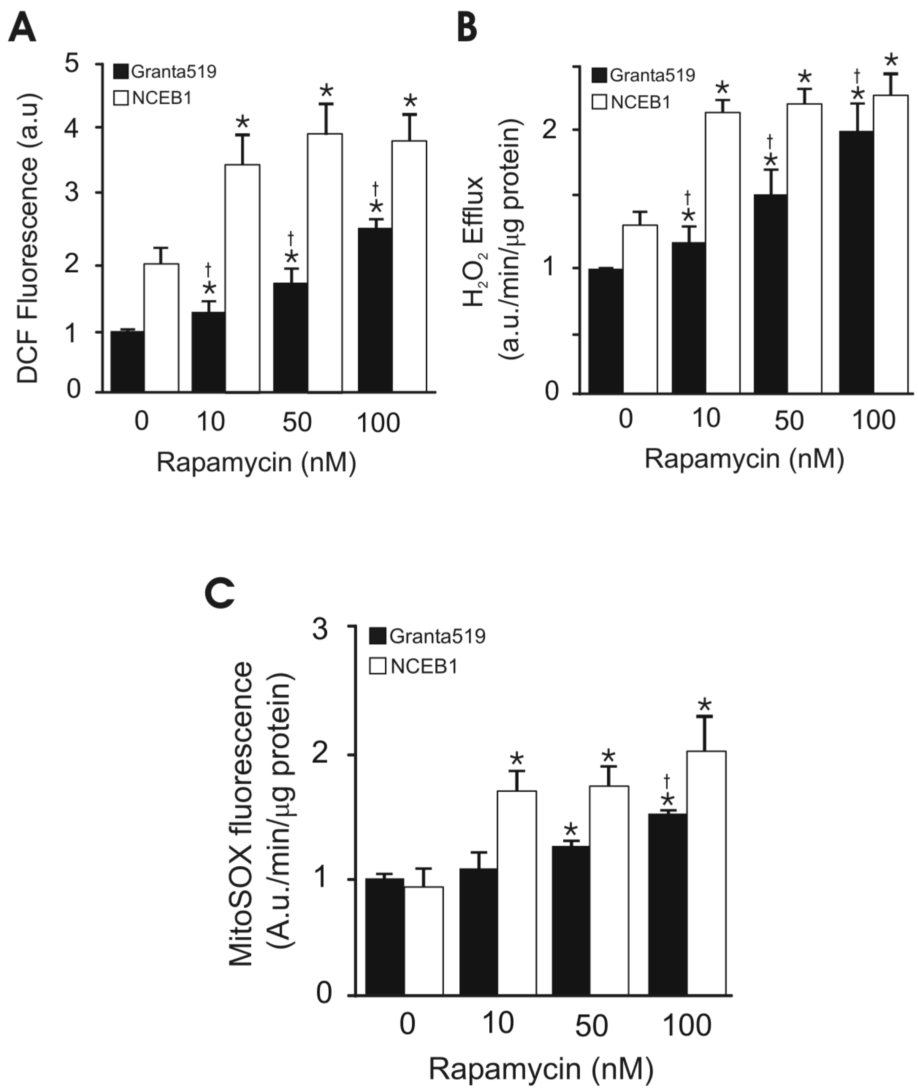

Figure 3.2: Rapamycin treatment increases overall oxidative stress and H_2O_2. Comparison of the **(A)** overall ROS production and **(B)** hydrogen peroxide efflux rate measured in Granta519 or NCEB1 cells after 48 hours treatment with rapamycin (normalized to 1; mean + SEM, *n=3*). **(C)** Comparison of mitochondrially located ROS measured in Granta519 or NCEB1 cells after 48 hours treatment with rapamycin (normalized to 1; mean + SEM, *n=3*). *denotes significantly different from untreated controls ($p \leq 0.05$). †denotes significantly different from other treatment doses ($p \leq 0.05$).

Rapamycin-induced increase in H_2O_2 is not due to decreased removal. To determine if the change in H_2O_2 is due to a decrease in H_2O_2 removal, I used immunoblots to screen for changes in two H_2O_2 removal enzymes, catalase and glutathione peroxidase 1 (GPx1). The levels of these two proteins did not significantly change with rapamycin treatment (Figure 3.3). This suggests that the increase in ROS, and specifically H_2O_2, seen with rapamycin treatment is not due to a decrease in the cells ability to remove H_2O_2. Thus, the increase in H_2O_2 is likely due to a change in the rate of generation.

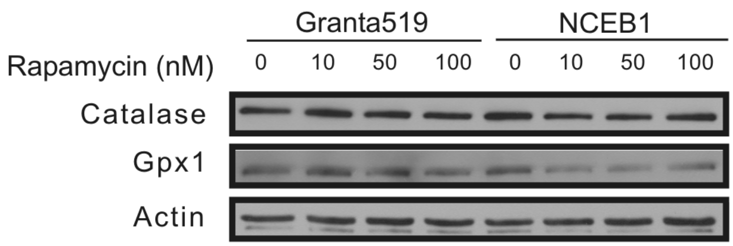

Figure 3.3: Rapamycin-induced increase in H_2O_2 is not due to decreased removal. Representative western blot of Granta519 and NCEB1 cells at 48 hours post treatment, displaying levels of catalase or glutathione peroxidase 1, enzymes that remove hydrogen peroxide. Actin was used as a loading control.

NAC co-treatment attenuates growth inhibitory effect of rapamycin. To establish to what degree increased oxidative stress is essential for rapamycin-induced growth inhibition, Granta 519 and NCEB1 cells were treated with rapamycin only, or rapamycin plus 20 mM N-acetyl cysteine (NAC). NAC is a cell permeable source of L-cysteine, which is a precursor for the formation of glutathione, which can remove H_2O_2. NAC can also act as a non-specific ROS scavenger. Co-treatment with NAC and rapamycin for 48 hours resulted in a significant attenuation of the growth inhibitory effect of rapamycin at each concentration in both cell lines (Figure 3.4A and C). The addition of NAC also attenuated the increase in H_2O_2 levels (Figure 3.4B and D) seen in the rapamycin only-treated cells.

Interestingly, the increase in H_2O_2 following rapamycin treatment was never completely eliminated, only reduced. NAC likely needs to reach a saturated concentration to completely eliminate the generation of H_2O_2 following rapamycin treatment. However, concentrations of NAC higher that 20mM adversely effected cell growth (data not shown), and would have caused confounding issues when analyzing results. One explanation for needing such high levels of NAC to reverse the increase in H_2O_2 following rapamycin treatment is that rapamycin my bind NAC in culture and reduce its availability. Additional studies would be necessary to test this idea.

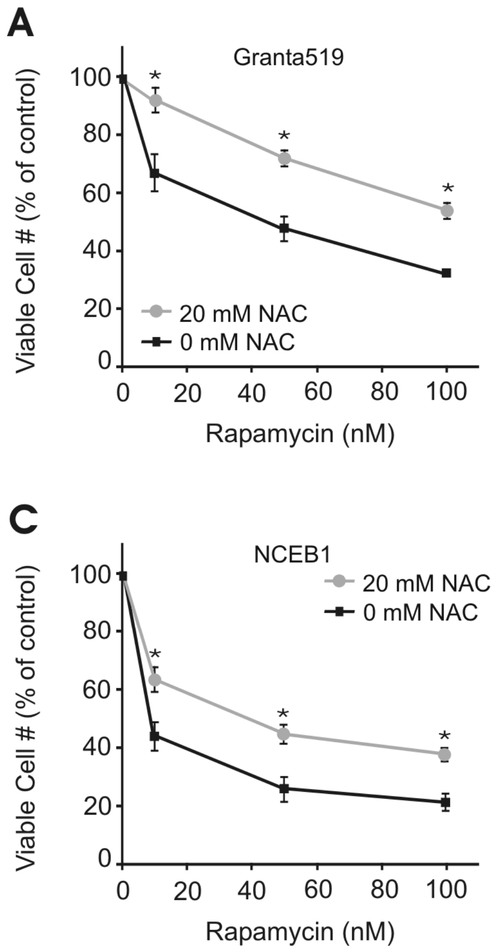

Figure 3.4: NAC co-treatment attenuates growth inhibitory effect of rapamycin. (A) Relative percentage of viable Granta519 cells remaining in culture compared to untreated controls and **(B)** hydrogen peroxide efflux rate in Granta519 cell after 48 hours treatment with rapamycin and N-acetyl cysteine (normalized to 1; mean + SEM, *n=3*). **(C)** Relative percentage of viable NCEB1 cells remaining in culture compared to untreated controls and **(D)** hydrogen peroxide efflux rate in NCEB1 cells after 48 hours treatment with rapamycin and N-acetyl cysteine (normalized to 1; mean + SEM, *n=3*). *denotes significant difference ($p \leq 0.05$).

MnSOD Expression as a Target of mTOR Inhibitors in MCL

MnSOD protein expression increases with rapamycin-induced growth inhibition and H_2O_2. The previous studies show that rapamycin treatment is followed by an increase in H_2O_2 levels. I saw no change in the levels of H_2O_2 metabolizing enzymes, suggesting that the increase is due to production. Finally, there was an increase in the ROS specifically located at the mitochondria. Thus, a source of rapamycin-induced ROS in MCL may be the intracellular production of H_2O_2 via increased MnSOD. The mitochondrially-located MnSOD has been shown to be a growth suppressor in multiple tumor types (Oberley, 1994). This is likely due to MnSODs ability to generate ROS, specifically, H_2O_2 (Oberley, 2001).

Immunoblots showed that, upon rapamycin treatment for 48 hours, protein levels of MnSOD increased (Figure 3.5) and MnSOD expression strongly correlated with rapamycin-induced growth inhibition. The Granta519 cells, which displayed a dose-dependent response to rapamycin, also had a dose-dependent increase in MnSOD protein expression. NCEB1 cells, which were more sensitive to rapamycin than the Granta519 cells (refer back to Figure 3.1), had higher initial MnSOD protein levels than the Granta519 cells in the absence of rapamycin. NCEB1 cells showed a greater increase in MnSOD levels at the lowest rapamycin concentration as compared to Granta519 cells, and then expression remained relatively stable with higher concentrations of rapamycin (Figure 3.5).

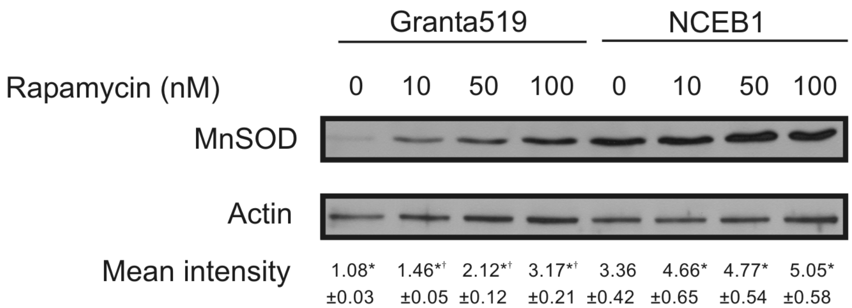

Figure 3.5: Rapamycin treatment increases MnSOD protein. Representative western blots showing levels of MnSOD in Granta519 and NCEB1 cells after 48 hours treatment with rapamycin. Actin is used as a loading control. Mean intensities of MnSOD are derived from an average of 3 experiment ± SEM (untreated Granta519 control normalized to 1). *denotes significantly different from untreated controls ($p \leq 0.05$). †denotes significantly different from other treatment doses ($p \leq 0.05$).

Increase in MnSOD is not in response to increased oxidative stress. The increase in MnSOD seen following rapamycin treatment could be due to a direct effect of rapamycin on transcriptional or translational regulation of MnSOD, or an indirect effect such as a stress response to ROS. To identify if the increase in MnSOD is a stress response to ROS or is being activated via another pathway, cells were co-treated with NAC. If the increased H_2O_2 following rapamycin occurs first and induces a stress response increase in MnSOD expression, then depleting the levels of H_2O_2 with NAC should also results in decreased induction of MnSOD following rapamycin treatment. Utilizing the Granta519 cells, I confirmed that addition of NAC increased glutathione levels in the cells (3.6A) and decreased the overall levels of ROS (3.6B). In addition, I found that MnSOD protein levels increased in cells co-treated with NAC and rapamycin in the same manner as those cells treated with rapamycin only (Figure 3.6C), suggesting the up-regulation of MnSOD is a downstream effect of rapamycin and not a response to oxidative stress.

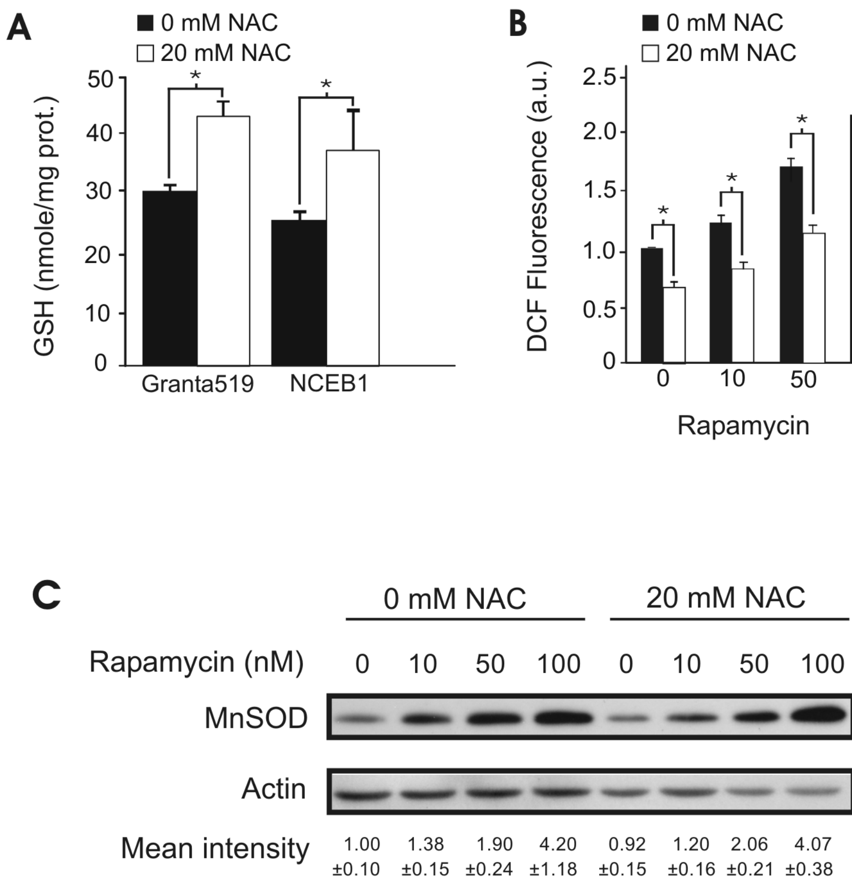

Figure 3.6: Increase in MnSOD is not in response to increased ROS. (A) Levels of glutathione in Granta519 cells in the presence of the glutathione precursor N-acetyl cysteine (NAC). **(B)** Overall ROS levels in Granta519 cells after 48 hours in the presence of the ROS scavenger and glutathione precursor, NAC. *denotes significant difference ($p \leq 0.05$). **(C)** Representative western blot showing the levels of MnSOD in Granta 519 cells after 48 hours treatment with rapamycin and NAC. Actin is used as a loading control. Mean intensities of MnSOD are an average of 3 experiment ± SEM (untreated control normalized to 1).

Pattern of increased MnSOD protein expression is specific to rapamycin. In recent years, production of ROS has been noted when cells were treated with a number of unrelated drugs not usually associated with ROS-induced cell death (Doroshaw, 2006). However, the mechanisms by which these drugs generate ROS has not yet been elucidated. My data showing a strong correlation between MnSOD levels and the effect of rapamycin on cell growth raises the question of whether increased MnSOD expression could be a common response to chemotherapy in MCL.

To briefly test this idea, MnSOD levels were measured after treatment with two other chemotherapeutic known to be active in MCL, vincristine and bortezomib. The mechanism of action of vincristine does not include induction of ROS. Bortezomib is known to generate high levels of ROS in the cells, which would induce a stress response and could be expected to trigger increased production of MnSOD and other antioxidants. An increase in MnSOD and no change to major H_2O_2 detoxifying enzymes, would suggest that changes to MnSOD levels may be component of all active drugs in MCL. I found that treatment with varying concentrations of vincristine (Figure 3.7A) did not significantly increase MnSOD protein levels after a 48 hour treatment, suggesting changes to MnSOD are specific to rapamycin. Treatment with the newer drug, bortizomeb, did increase MnSOD levels. However, upon further investigation, I also found the levels of other antioxidant enzymes such as catalase and glutathione peroxidase 1 (GPx1) were also increased with Bortizomeb treatment (Figure 3.7B), which is not found with rapamycin treatment.

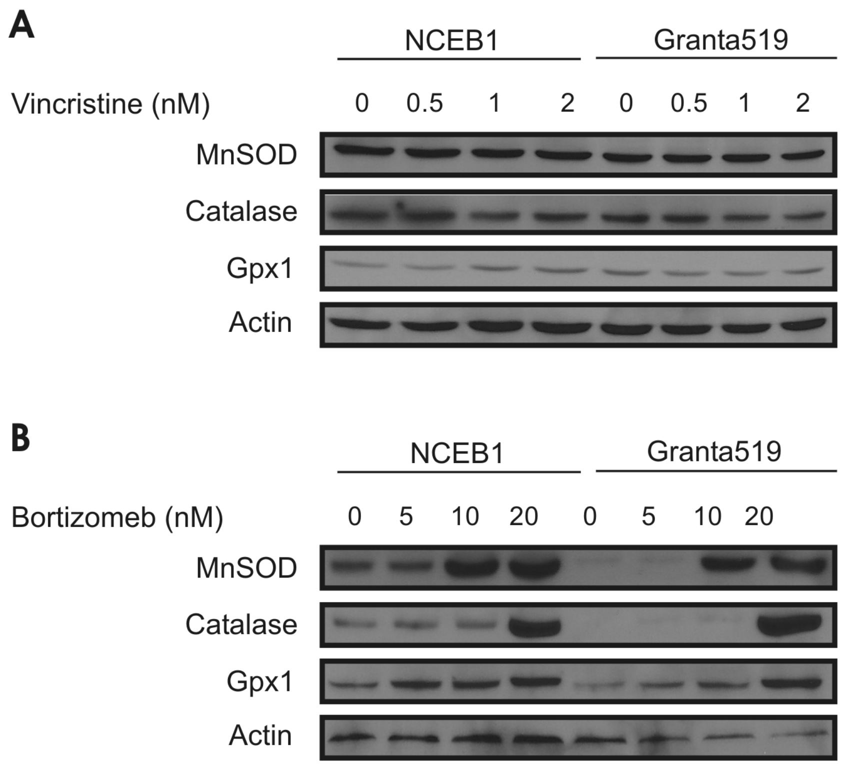

Figure 3.7: Pattern of increase in MnSOD is specific to rapamycin. Representative western blots showing the levels of MnSOD, or two additional ROS responsive proteins in NCEB1 or Granta519 cells after 48 hours treatment with **(A)** vincristine or 36 hours treatment with **(B)** bortizomeb. Actin is used as a loading control.

Over-expression of MnSOD increases H_2O_2 production and sensitivity to rapamycin. With the strong correlations between sensitivity to rapamycin and elevated levels of MnSOD after treatment, a genetic approach was used to determine a possible mechanistic role of MnSOD in rapamycin sensitivity. Human MnSOD was stably over-expressed in two different systems. Murine thymic (WEHI7.2) and Jurkat lymphoma cells were used due to ease of transfection of these cell lines compared to the Granta519 or NCEB1 cells. The two lines were treated with the same range of rapamycin concentrations as used for the MCL cell lines. This resulted in a similar increase in MnSOD protein expression (data not shown). Over-expression of the human *SOD2* gene (coding for MnSOD) in both WEHI7.2 and Jurkat was verified by western blot (Figure 3.8A). Over-expression in the cell lines results in a 2-fold increase in intracellular levels of H_2O_2 (Figure 3.8B).

Over-expression of MnSOD in both WEHI7.2 (Figure 3.9A) and Jurkat (Figure 3.9B) cells dramatically increased sensitivity to rapamycin at all doses. Unlike the parental cell lines, over-expression of MnSOD had a cytotoxic effect after rapamycin treatment instead of the cytostatic effect seen in control cells as seen by an increase in caspase activity in both WEHI7.2 (Figure 3.9C) and Jurkat (Figure 3.9D) cells. Caspase activity is only shown for 10 nM treatment with rapamycin because higher concentration resulted in too few live cells to allow for measurement.

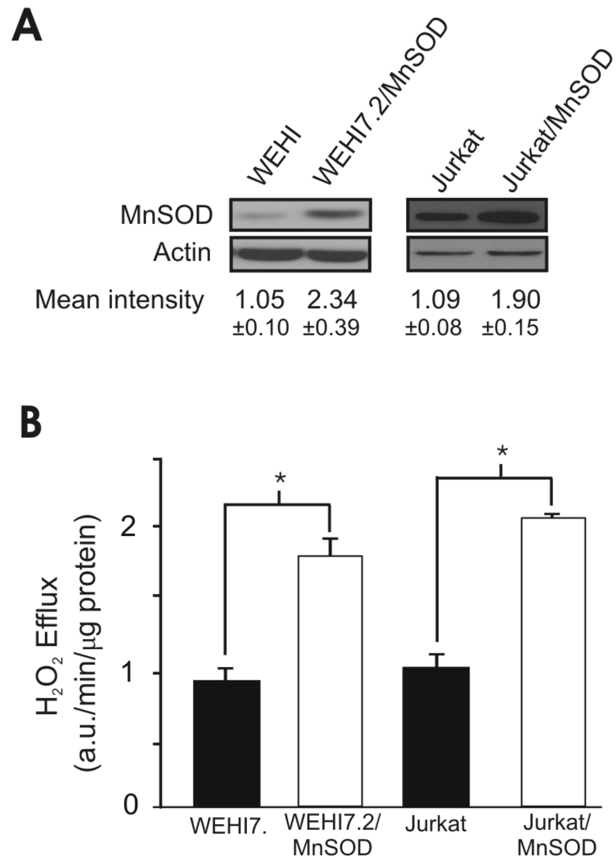

Figure 3.8: Over-expression of MnSOD increases H_2O_2 production. (A) Representative western blots comparing levels of MnSOD in WEHI7.2 or Jurkat cells transfected with MnSOD. Mean intensity of MnSOD is the average of 3 experiments (untransfected controls normalized to 1). Actin was used as a loading control. **(B)** Hydrogen peroxide efflux rate after 12 or 48 hours rapamycin treatment of parental cells compared to those over-expressing MnSOD (normalized to 1), respectively. *denotes significant difference ($p \leq 0.05$).

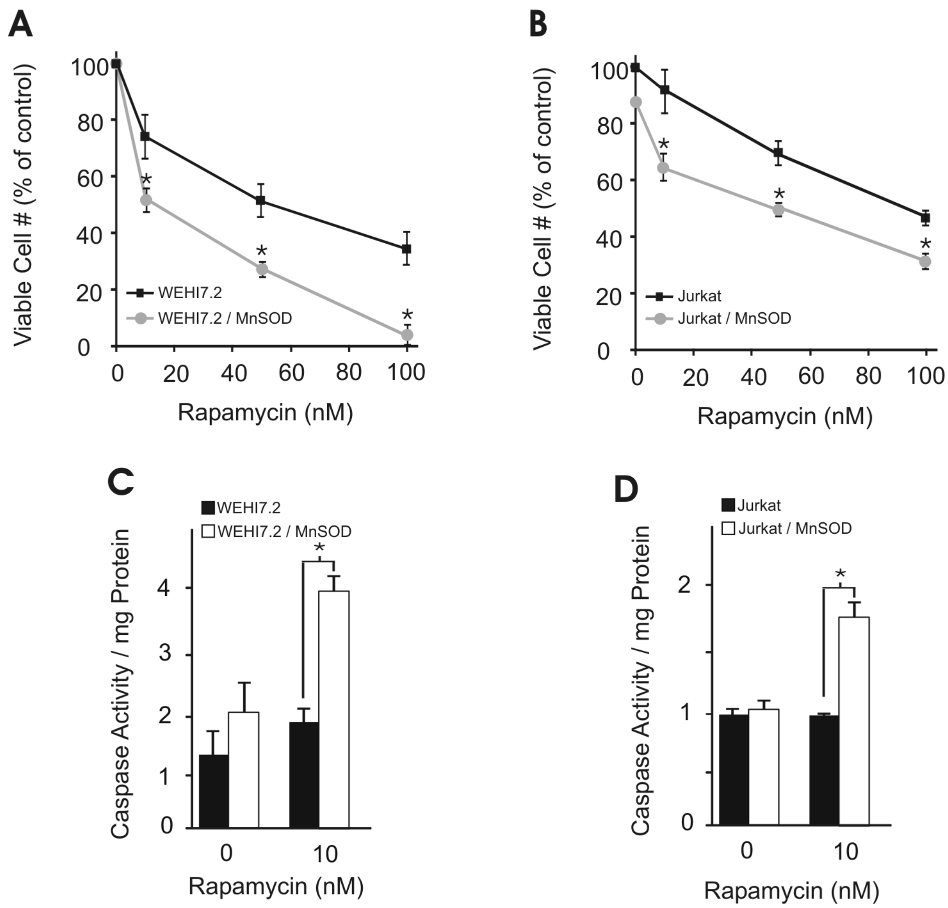

Figure 3.9: Over-expression of MnSOD increases sensitivity to rapamycin. Relative percentage of viable cells remaining in culture compared to untreated controls after 12 or 48 hours treatment with rapamycin in **(A)** WEHI7.2 or **(B)** Jurkat parental cell lines and transfectants over-expressing MnSOD. Comparison of the caspase activity in **(C)** WEHI7.2 and **(D)** Jurkat parental cell lines and transfectants over-expressing MnSOD treated with 10 nM rapamycin. *denotes significant different ($p \leq 0.05$).

Levels of H_2O_2 determine a cytostatic or cytotoxic effect of rapamycin. The induction of caspase activity when MnSOD over-expressing cells are treated with rapamycin suggests that the increase in H_2O_2 by MnSOD results in levels that promote cell death instead of growth inhibition. To test this, the parental Jurkat cells and those over-expressing MnSOD were treated with a range of rapamycin concentrations that included lower doses. If cell death is still found with lower doses that do not generate high levels of H_2O_2, it is likely that MnSOD over-expression may be having other signaling effects that account for increased rapamycin sensitivity. These results (Table 3.1) show that H_2O_2 activity in Jurkat cells ranged from 1 – 2.1 (a.u), and Jurkat cells over-expressing MnSOD ranged from 1.3 – 4.5 (a.u). Caspase activity was not detected in MnSOD over-expressing cells treated with 0 – 5nM of rapamycin, which obtained H_2O_2 levels within the range of 1.3 – 2.0 (a.u.). However, caspase activity was significantly increased at 10 nM rapamycin in MnSOD over-expressing cells, which had H_2O_2 levels past the upper limit of the parental cells which did not display any caspase activity. This suggests that there is a threshold level of intracellular H_2O_2 at which these cells start to undergo cell death in response to rapamycin.

Table 3.1: Levels of H_2O_2 determine a cytostatic or cytotoxic effect of rapamycin.

	Rapamycin (nM)	H_2O_2 Efflux (a.u/min/µg protein)	Caspase Activity (a.u./mg protein)	G_1 (%)
Jurkat Cells	0	1.0 ± 0.00	1.26 ± 0.06	58.3 ± 4.3
	2.5	1.2 ± 0.07	1.34 ± 0.07	61.3 ± 7.0
	5	1.4 ± 0.17	1.32 ± 0.07	67.6 ± 6.8
	10	1.6 ± 0.27*	1.35 ± 0.08	71.1 ± 7.6*
	50	2.1 ± 0.15*	1.39 ± 0.08	86.5 ± 2.4*
Jurkat/MnSOD Cells	0	1.5 ± 0.00†	1.30 ± 0.02	65.2 ± 8.1
	2.5	1.7 ± 0.10†	1.31 ± 0.05	70.6 ± 12.2†
	5	2.0 ± 0.05*†	1.45 ± 0.09*†	74.5 ± 13.1*†
	10	3.4 ± 0.42*†	1.88 ± 0.04*†	89.0 ± 9.0*†
	50	4.5 ± 0.67*†	2.56 ± 0.03*†	94.9 ±1.7*†

*denotes significantly different from untreated control cells ($p \leq 0.05$).
†denotes significantly different from parental cell line ($p \leq 0.05$).

Mechanism of Rapamycin Induced MnSOD Expression

Rapamycin treatment increases MnSOD mRNA transcription. The previous experiments showed that MnSOD is a target of rapamycin. The subsequent increase in H_2O_2 due to the enzymatic activity of MnSOD is necessary for the full growth inhibitory effect of rapamycin. Rapamycin may be increasing MnSOD protein levels via transcriptional or translational regulation. Utilizing the Granta519 cells, real-time PCR of *SOD2* indicated that total mRNA level within the cell started to increase with 100 nM rapamycin treatment compared to untreated controls around 36 hours, and were significantly increased at 48 hours after treatment (Figure 3.10A). Increased MnSOD mRNA could be due to increased transcription or lack of degradation. A nuclear run-on assay can more specifically measure active transcription at time of collection. The nuclear run-on assay demonstrated that in Granta519 cells treated with 100 nM of rapamycin, the amount of MnSOD gene being actively transcribed was significantly increased at 48 hours compared to untreated controls (Figure 3.10B). Thus, the increased MnSOD protein expression is due, at least in part, to a change in transcriptional regulation.

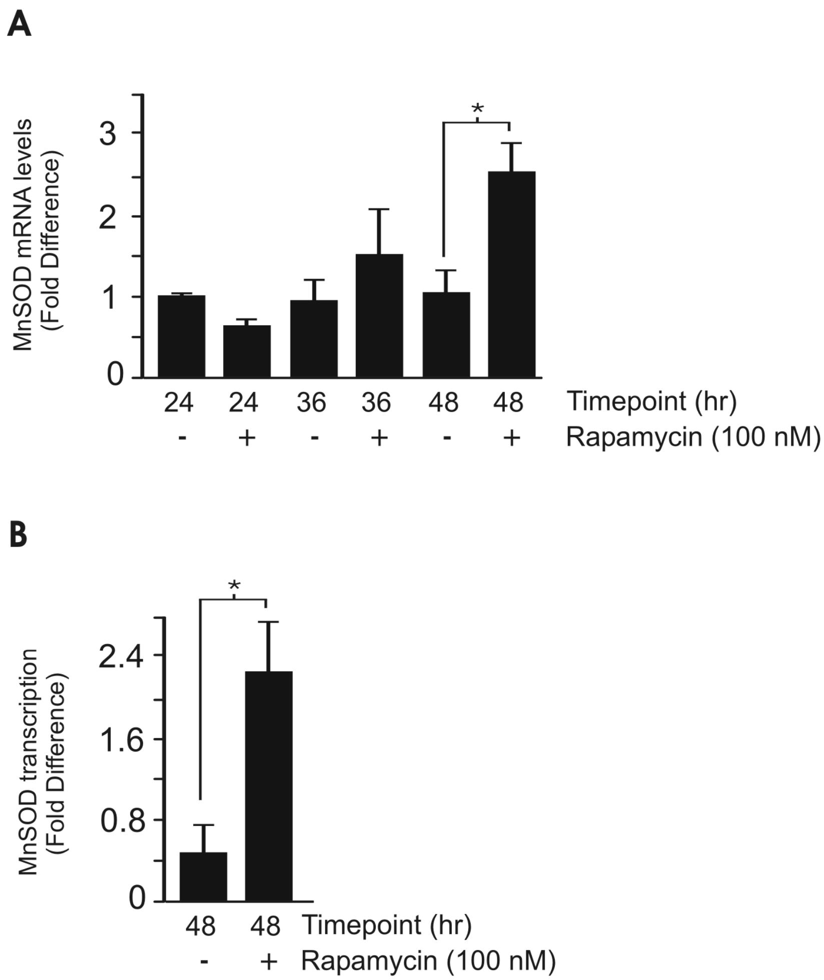

Figure 3.10: Rapamycin treatment increases MnSOD mRNA transcription. (A) Comparison of total MnSOD transcripts after no treatment or 48 hours treatment with 100 nM rapamycin detected by real time PCR (mean + SEM, *n=3)*. **(B)** Nuclear run-on assay shows in vitro transcription of MnSOD in Granta519 cells after 48 hours treatment with 100 nM rapamycin. Active transcripts were detected by real time PCR (mean + SEM, *n=3)*. *denotes significant difference ($p \leq 0.05$).

Over-expression of C/EBPβ transcription factor isoforms does not alter MnSOD protein expression. Gene expression profiling data from our lab (Tome, 2005) showed a significant correlation between MnSOD expression and the transcription factor C/EBPβ. Expression of C/EBPβ isoforms are regulated via downstream components of the mTORC1 signalling pathway, suggesting C/EBPβ expression would be effected by rapamycin treatment. Upon 48 hours of treatment with rapamycin both Granta519 and NCEB1 cell lines show alterations to the protein levels of specific C/EBPβ isoforms. While the two C/EBPβ-LAP isoforms did not appear to significantly change, the LIP isoform decreased with treatment. In fact, the decrease in the LIP isoform was directly proportional to the increase in MnSOD protein levels and sensitivity to rapamycin seen in previous experiments (Figure 3.11A), indicating that the change in the LIP isoform could be responsible for regulation of MnSOD. Over-expression of the LIP isoform in Granta519 cells, however, did not alter MnSOD expression (Figure 3.11B) or sensitivity to rapamycin (Figure 3.11C). The LAP isoform appears to decrease slightly, suggesting changes in LIP are involved in a feedback loop, but this still does not account for changes in the regulation of MnSOD upon rapamycin treatment, despite the very strong correlation in protein levels.

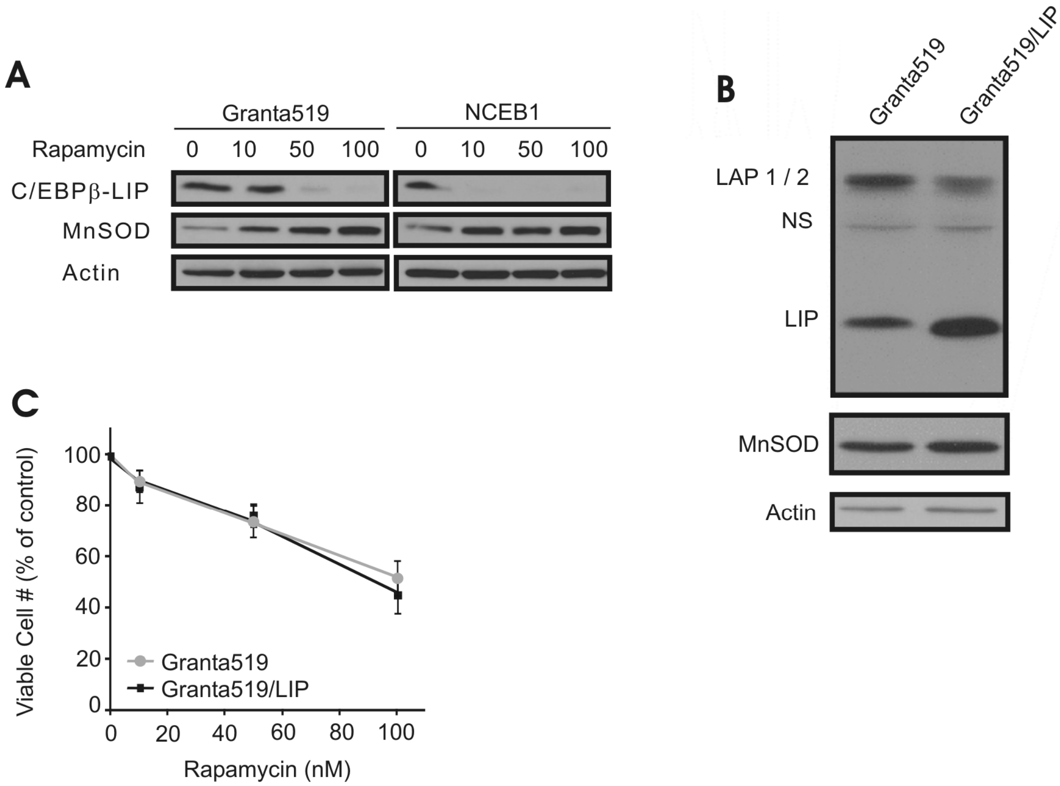

Figure 3.11: Over-expression of C/EBPβ-LIP isoform does not alter MnSOD protein expression. (A) Representative western blot showing levels of MnSOD and the truncated C/EBPβ-LIP isoform after 48 hours treatment of rapamycin in MCL cells. Actin was used as a loading control. **(B)** Western blot showing increased levels of the truncated C/EBPβ-LIP isoform in LIP transfected Granta519 cells compared to no change in MnSOD protein expression. **(C)** Relative percentage of viable cells remaining in culture compared to untreated controls after 48 hours treatment with rapamycin in Granta519 parental cell line and transfectants over-expression LIP.

Rapamycin effects signaling downstream of mTORC2 pathway. With the up-regulation of MnSOD being a transcriptional event and a lack of regulation by C/EBPβ, a transcription factor downstream of the mTORC1 pathway, I choose to look at the possibility of transcriptional regulation via the mTORC2 pathway. Phosporylation of Akt at serine 473 is regulated by an active mTORC2 pathway and has been shown to be inhibited with long-term rapamycin treatment extending past 24 hours (Sarbassov, 2009). Short term (1 h) treatment of Granta519 and NCEB1 cells with 100 nM rapamycin had no effect on phosphorylation status on serine 473 of Akt, but levels were decreased by 24 hours, and completely gone by 48 hours in both cell types (Figure 3.12).

A direct substrate of phospho-Akt is a member of the forkhead family of transcription factors, FOXO3A (Burnet, 1999; Hu, 2004), which is also known to regulate transcription of MnSOD (Li, 2006; Shukla, 2009; Zrelli, 2001). Phospho-Akt phosphorylates FOXO3A, preventing it from translocating to the nucleus (Tang, 1999). I found that decreased phospho-Akt due to 100 nM treatment of rapamycin also corresponded with increased levels of total FOXO3A. However, treatment with rapamycin and the subsequent changes to the phosphorylation status of Akt did not result in translocation of FOXO3A to the nucleus. Western blot analysis show that upon rapamycin treatment FOXO3A remains in the cytosol (Figure 3.13). The presence of lamin A/C only in the nuclear fraction indicates there was no cross-over of the cytosolic fraction into the nuclear sample.

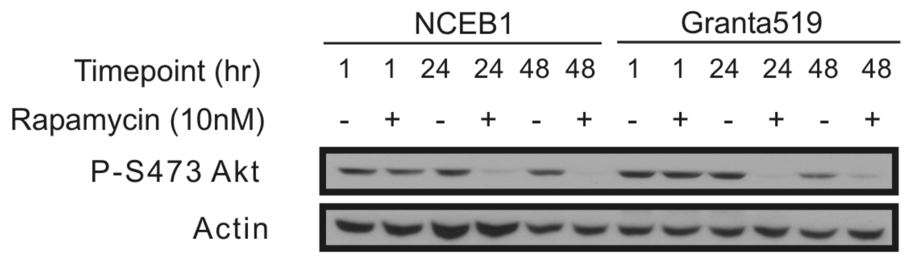

Figure 3.12: Akt phosphorylation is decreased by rapamycin treatment in MCL cells. Representative western blot showing the levels of phospho-S473 in NCEB1 and Granta519 cells after a timecourse treatment with or without 100 nM rapamycin.

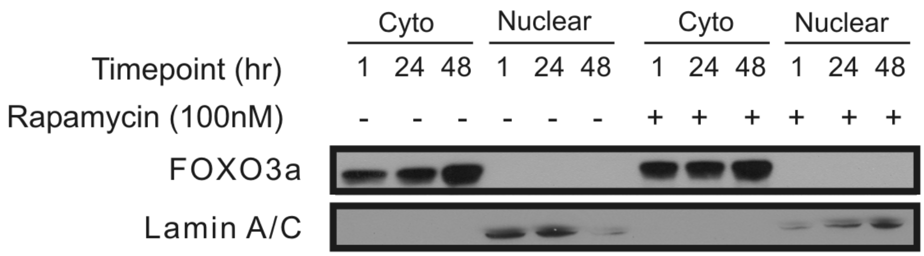

Figure 3.13: Rapamycin does not alter FOXO3a nuclear translocation. Western blot showing the total levels of transcription factor FOXO3a in cytosolic versus nuclear fractions of Granta519 and NCEB1 cells treated with a timecourse treatment of 100 nM rapamycin. Lamin A/C was used as a control for contamination of nuclear fraction into cytosolic fraction.

siRNA knock-down of mTORC2 component increases MnSOD protein expression. To further analyze the effect of inhibition of the mTOR complexes on regulation of MnSOD expression, siRNA was used to knock-down either raptor (component of mTORC1 complex) or rictor (component of mTORC2 complex), to determine if inhibition of either complex mimics the effect of rapamycin. Transient transfections were again done in the Jurkat cell line due to ease of use. Previous publications have shown Jurkat cells to also have loss of AKT S473 phosphorylation with a 48 hour treatment with rapamycin (Sarbossov, 2006), similar what I saw in the MCL cell lines. Transient transfection of Jurkat cells with raptor siRNA after 48 hours resulted in no change in MnSOD protein levels. However, transfection with rictor siRNA results in a significant increase in MnSOD protein levels (Figure 3.14), mimicking the increase in MnSOD seen with rapamycin treatment. This data suggests that regulation of MnSOD expression resulting from mTOR inhibitor rapamycin is due to the inhibitory effect of the drug on the mTORC2 pathway.

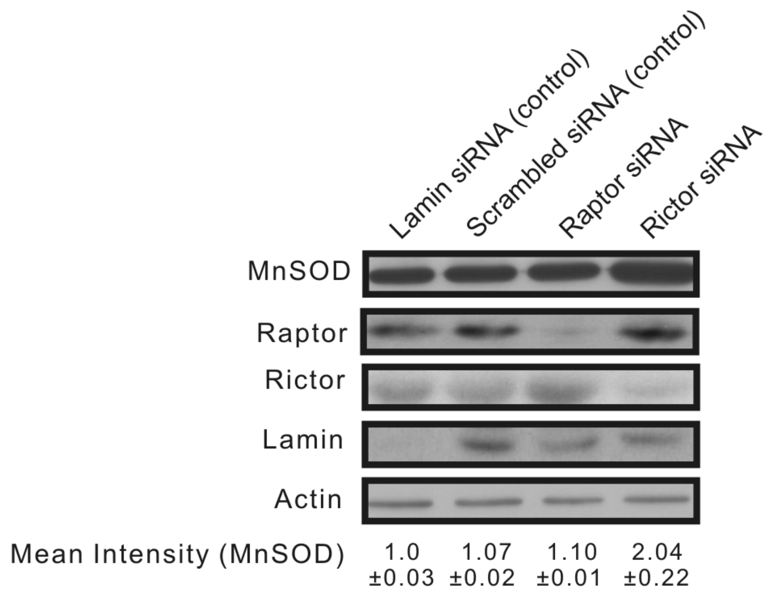

Figure 3.14: siRNA knock-down of the mTORC2 component, Rictor, increases MnSOD protein expression. Representative western blot showing the levels of siRNA controls, MnSOD, Raptor and Rictor 48 hours after a transient transfection with siRNA in Jurkat cells. Mean intensities for MnSOD are an average of three blots.

GENETIC POLYMORPHISMS IN OXIDATVE STRESS RELATED GENES AND TREATMENT RESPONSE

Previous studies in our lab support the idea that oxidative-stress related gene expression is associated with survival in the aggressive NHL, DLBCL (Tome, 2005). Recent studies in breast cancer (Choi, 2006) and DLBCL (Rossi, 2007) have found SNPs in genes encoding protein involved in oxidative stress to be associated with outcome following treatment We hypothesize that unfavorable polymorphisms may affect the clinical response of patients to ROS dependent drugs when MnSOD or other stress related proteins are a critical component of the treatment response. There were no large sets of clinical samples available of MCL treated with rapamycin available for us to study association of SNPs in genes encoding MnSOD (*SOD2)*, or other oxidative stress-related genes with treatment outcome. However, aggressive NHLs are treated with combination chemotherapy that includes drugs which increase ROS. Thus, these could be used to test for associations between SNPs in oxidative stress-related genes and clinical outcome.

Long-term storage of FFPE diagnostic tissue associate with more quality control failures. In these studies, we analyzed 53 SNPs in genes encoding for MnSOD (*SOD2)* and 32 other oxidative stress-related genes for associations with survival after treatment (Table 3.2). This retrospective study included an initial set of 410 patient samples, with sufficient tissue for DNA extraction, from a collection of 7 SWOG Phase II or II clinical trials. These trials are now closed and allowed for up to 10 years follow-up. All patients received combination treatments that included an anthracycline (doxorubicin). Addition of rituximab was included in the two most recent trials, S9704 and S0014. The trials and number of cases derived from each trial are outlined in Table 3.3, as are the treatment regimens for each trial. While we initially started with a set of 410 patients samples that were confirmed aggressive B-cell lymphomas with sufficient tissue, survival analysis was only performed on 337 samples due to a loss of samples during SNP sequencing. Seventy-three samples were dropped from analysis due to SNP call rates lower than 80%, suggesting that those DNA samples were degraded or otherwise unsuitable for sequencing. Interestingly, the majority of samples that did not pass the quality threshold were from the earliest studies between 1985 and 1992.

Table 3.2: SNPs included in analysis

Gene	SNP	Chromosome	Coordinate	Rare allele	Common allele	Minor allele frequency	Function
PRDX1	rs6667191	1	45982844	A	G	0.29	Intronic
AKR1A1	rs2088102	1	46032974	G	A	0.47	Intronic
GCLM	rs3170633	1	94351328	A	G	0.38	5' near gene
	rs2064764	1	94358343	G	A	0.47	Intronic
	rs2301022	1	94372878	A	G	0.31	Intronic
PPARG	rs3856806	3	12475557	T	C	0.11	His477His
GPX1	rs1800668	3	49395757	T	C	0.27	5' UTR
SOD3	rs2536512	4	24801315	G	A	0.38	Ala58Thr
NQO2	rs1143684	6	3010390	C	T	0.21	Leu47Phe
SOD2	rs4880	6	160113872	C	T	0.48	Val16Ala
NOS3	rs1799983	7	150696111	T	G	0.30	Glu298Asp
GSR	rs4628224	8	30556809	T	A	0.24	Intronic
TXN	rs2776	9	113006288	A	G	0.18	3' near gene
	rs4135221	9	113007237	T	C	0.41	Intronic
	rs4135165	9	113017562	T	C	0.17	Intronic
AKR1C3	rs12529	10	5136651	G	C	0.38	His5Gln
	rs10508293	10	5141137	C	T	0.18	Intronic
CAT	rs769214	11	34459717	G	A	0.37	5' UTR
	rs7943316	11	34460472	A	T	0.38	5' near gene
GSTP1	rs1695	11	67352689	G	A	0.37	Ile105Val
	rs1138272	11	67353579	T	C	0.09	Ala114Val
	rs4891	11	67353970	C	T	0.36	Ser185Ser
TXNRD1	rs1128446	12	104680842	G	C	0.12	Arg92Pro
	rs35009941	12	104682528	G	C	0.27	Intronic

Table 3.2: continued

Gene	SNP	Chromosome	Coordinate	Rare allele	Common allele	Minor allele frequency	Function
GPX2	rs4902346	14	65407160	G	A	0.19	Intronic
	rs2737844	14	65408509	T	C	0.32	Intronic
NQO1	rs12232410	16	69736851	A	G	0.22	Pro187Ser
	rs1800566	16	69745145	T	C	0.21	Pro187Ser
CYBA	rs1049254	16	88709828	T	C	0.38	Val174Ala
	rs4673	16	88713236	T	C	0.32	His72Tyr
NOS2	rs8068149	17	26088855	A	G	0.44	Intronic
	rs1137933	17	26105932	T	C	0.23	Asp385Asp
MPO	rs2243828	17	56358884	G	A	0.23	5' near gene
GPX4	rs713041	19	1106615	T	C	0.38	3' UTR
PRDX2	rs10427027	19	12911285	C	T	0.14	Intronic
	rs12151144	19	12912396	C	A	0.14	Intronic
SOD1	rs4998557	21	33034892	A	G	0.18	Intronic
	rs2070424	21	33039320	G	A	0.12	Intronic
CBR1	rs9024	21	37445313	A	G	0.08	Intronic
G6PD	rs8133052	21	37507501	A	G	0.43	Cys4Tyr
	rs881712	21	37507769	T	C	0.43	Val93Val
	rs1056892	21	37518706	A	G	0.34	Val244Met
TXNRD2	rs1139793	22	19868218	A	G	0.22	Ile370Thr
	rs1548357	22	19878362	C	T	0.19	Intronic
	rs5992495	22	19882984	G	T	0.31	Arg299Ser
	rs756661	22	19905802	C	T	0.34	Intronic
	rs5748469	22	19907099	A	C	0.24	Ser66Ala
TXN2	rs2281082	22	36872750	T	G	0.47	Intronic

Table 3.2: continued

Gene	SNP	Chromosome	Coordinate	Rare allele	Common allele	Minor allele frequency	Function
NCF4	rs1883112	22	37256846	A	G	0.40	5' near gene
	rs729749	22	37263846	T	C	0.24	Intronic
	rs4821554	22	37276084	A	G	0.23	Intronic
RAC2	rs1476002	22	37630788	T	C	0.17	Intronic
	rs13058338	22	37632770	A	T	0.25	Intronic

Table 3.3: SWOG trials and final samples used in SNP study

SWOG Trial	Study Title	Initial Samples	Number Analyzed
S8516	A Phase III Comparison of CHOP Versus m-BACOD Versus Pro-MACE-CytaBOM Versus MACOP-B in Patients with Intermediate or High-Grade Non-Hodgkin's Lymphoma	952	31
S8736	Treatment of Localized Non-Hodgkin's Lymphoma: Comparison of Chemotherapy (CHOP) to Chemo-therapy Plus Radiation Therapy	401	71
S9125	A Phase II Trial of CVAD/Verapamil/Quinine for Treatment of Non-Hodgkin's Lymphoma	89	14
S9240	A Phase II Trial of CVAD for Treatment of Non-Hodgkin's Lymphoma	89	36
S9349	A Randomized Phase II Trial of CHOP with G-CSF Support or ProMACE-CytaBOM with G-CSF Support for Treatment of Non Hodgkin's Lymphoma	186	95
S9704	A Randomized Phase III Trial Comparing Early High Dose Chemoradiotherapy and an Autologous Stem Cell Transplant to Conventional Dose CHOP Chemotherapy Plus Rituximab for $CD20^{+}$ B Cell Lymphomas for Patients with Diffuse Aggressive Non-Hodgkin's Lymphoma in the High-Intermediate and High Risk International Classification Prognostic Groups	388	60
S0014	Evaluation of CHOP Plus Rituximab Plus Involved Field Radiotherapy for Stages I, IE, and Non-Bulky Stages II and IIE, CD20 Positive, High Risk Localized Aggressive Histologies of Non-Hodgkin's Lymphoma	61	30
Total		2166	337

SNP association with outcome after anthracycline-based therapy in NHL. Among the 53 SNPs analyzed, associations between progression-free survival (PFS) and overall survival (OS) were identified in gene encoding aldo-ketose reductase 1C3 (*AKR1C3*, rs10508293) and myeloperoxidase (*MPO*, rs2243828). There was no significant association found between survival outcomes and a SNP in the gene encoding MnSOD (*SOD2*). Table 3.4 indicates the adjusted hazard ratios and 95% confidence intervals determined by Cox regression analysis for PFS and OS. In the case of *AKR1C3* and *MPO*, we found that the homozygous recessive genotype was associated with a 2 fold increase in hazard of death after treatment compared to the more common reference genotype. The significant association between genotype of *AKR1C3* (Figure 3.15A) and *MPO* (Figure 3.15B) can be seen by Kaplan-Meier curves.

Table 3.4: Associations between *SOD2*, *AKR1C3* and *MPO* genotypes and survival

Genotype	PFS			OS		
	HR (95% CI)†	P	Adj. P†	HR (95% CI)*	P	Adj. P†
SOD2 (rs4880)						
TT/CT	1.00			1.00		
CC	1.22 (0.89-1.68)	0.23	1.00	1.24 (0.88-1.75)	0.22	1.00
AKRIC3 (rs1058293)						
TT/CT	1.00			1.00		
CC	2.09 (1.28-3.41)	0.0032	0.17	2.14 (1.29-3.55)	0.0032	0.20
MPO (rs2243828)						
AA/AG	1.00			1.00		
GG	1.87 (1.14-3.06)	0.013	0.50	2.34 (1.40-3.90)	0.0011	0.066

*All models adjusted for IPI risk score and stratified by treatment trial.

†p values adjusted for multiple comparisons.

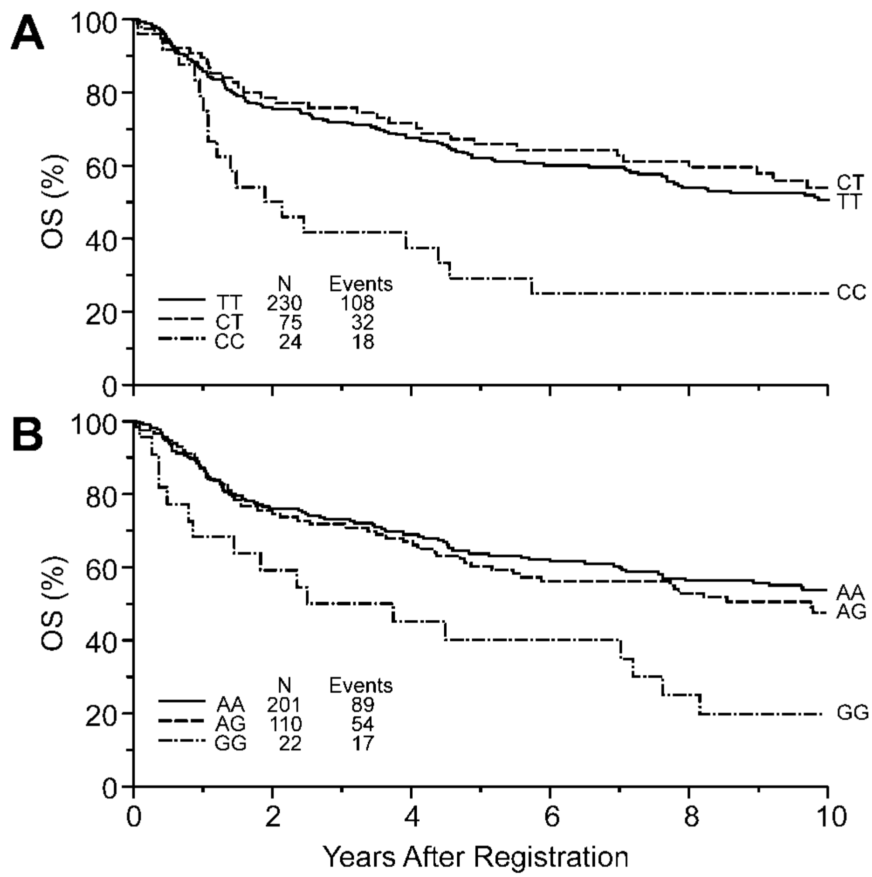

Figure 3.15: KM curve of OS for *AKR1C3* and *MPO* genotypes in NHL patients. Estimated overall survival for patients with different **(A)** *AKR1C3* and **(B)** *MPO* genotypes.

SNP association with hematologic toxicity following anthracyline-based therapy for NHL. In additional analysis of the 53 SNPs, statistically significant associations between increased risk of Grade 4-5 hematologic toxicity were not identified. However, SNPs in the gene encoding MnSOD (*SOD2*) and neutrophil factor 4 (*NCF4*, rs1883112) were borderline significant (Table 3.5). I found that the homozygous recessive genotype for both genes were associated with a 1.7 or 1.8 fold increase in risk of Grade 4-5 hematologic toxicity after treatment compared to the more common reference genotype, respectively.

Table 3.5: Associations between *SOD2* and *NCF4* genotypes and hematologic toxicity

Genotype	Hematologic Toxicity		
	HR (95% CI)*	P	Adj. P†
NCF4 (rs1883112)			
GG/GA	1.00		
AA	1.81 (0.86-3.77)	0.02	1.00
SOD2 (rs4880)			
TT/CT	1.00		
CC	1.70 (0.92-3.14)	0.09	0.99

*All models adjusted for IPI risk score and stratified by treatment trial.

†p values adjusted for multiple comparisons.

CHAPTER FOUR: DISCUSSION

Aggressive lymphomas are currently one of the leading causes of cancer death in the United States (Nogai, 2011). This is especially true for MCL, which has no curative treatment options. A suggested approach to treat cancer has been treatment with pharmacological agents to significantly increase ROS generation and by-pass the cellular anti-oxidant system (Fang, 2007; Jones, 2008; Numsen 2008). A promising new class of drugs that may employ such an approach are mTOR inhibitors. Inhibition of mTOR is the major mechanism of action of the mTOR inhibitor rapamycin and its derivatives; however, the downstream targets of mTOR inhibition that contribute to the anti-cancer activity are not clear. To best utilize this new treatment option, it is important to understand the molecular mechanisms to determine which patients will respond to therapy. With studies citing a role for ROS in mTOR inhibitor-induced growth suppression in leukemia cells (Choi, 2008), my research focused on investigating the importance of ROS in rapamycin treated MCL cells. Single nucleotide polymorphisms in oxidative stress related genes that alter the protein's enzymatic activity may affect response to ROS-dependent treatments. Additional studies focused on survival outcomes after treatment of aggressive lymphoma patients (DLBCL, MCL, grade 3 follicular lymphoma, and Burkitt) according to genotypes for polymorphisms in oxidative stress related genes.

Role of ROS and MnSOD in rapamycin-induced cytostasis. My data showed that distinct changes in MnSOD expression, via rapamycin treatment or genetic manipulation, were proportional to the cytostatic effect of rapamycin, pinpointing MnSOD as a target of mTOR inhibitors. An increase in oxidative stress, namely H_2O_2, was essential for the full growth inhibitory effect of rapamycin, and all studies supported the role of MnSOD as a source of rapamycin-induced H_2O_2. Based on these studies, I conclude that the effect of rapamycin is dependent upon the level of MnSOD and cytotoxicity can be obtained depending on the increase in H_2O_2 levels.

A previously unstudied aspect of rapamycin treatment in MCL is the effect of rapamycin on the redox environment, including the effect of H_2O_2, an important component of cell growth regulation. As reviewed in chapter 1, H_2O_2 has been shown to act as a signaling molecule to regulate both cell growth and cell death (Iverson, 2004; Simon, 200). Cellular response to H_2O_2 is influenced by the cell type and dosage level (Finkel, 2000; Temple, 2005). For example, osteoblasts treated with a high level of exogenous H_2O_2 resulted in cell death after 72 hours, but lower doses had no effect on cell viability (Li, 2009). While low doses of exogenous H_2O_2 did not induce cell death, cells arrested in the cell cycle (Li, 2009). Similar results have also been found in fibroblast and glioma cells (Arnold, 2001; Byun, 2009). These studies, however, did not test the effect of endogenously produced H_2O_2. My results support the dual role of endogenously produced H_2O_2 as a signaling molecule that can inhibit growth at low levels, but can induce cell death at higher levels. These data pinpoint H_2O_2 as an important target to consider and exploit to increase cell kill during cancer treatment.

The mechanism of H_2O_2-induced growth inhibition or cell death is likely cell type specific, as a number of studies have identified different signaling pathways affected by H_2O_2 treatment. In osteoblasts, low doses of H_2O_2 decreased cyclin B1 expression, contributing to cell cycle arrest (Li, 2001). High doses of H_2O_2 have been found to inhibit the mTOR/p706SK pathway, contributing to induction of apoptosis, necrosis, or autophagy in osteoblasts and glioma cells (Byun, 2009; Li, 2009). In my studies, higher baseline levels of H_2O_2 clearly enhanced the effect of mTOR inhibitors. This may be due to a greater inhibition of the mTOR pathway that increases the effect and pushes that cell towards cell death. Elevated H_2O_2 may also be targeting proteins and pathways not affected by mTOR inhibitors, resulting in a synergistic effect to push cells toward death. H_2O_2 treatment had been shown to effect the gene expression of a number of proteins (Arnold, 2001), including several involved in cell cycle maintenance. Further investigation into the genes affected by elevated H_2O_2 in MCL cells in the presence if mTOR inhibitors would help to identify the mechanism leading to cell death instead of cell cycle arrest commonly found with mTOR inhibitors alone.

Elucidating the role of ROS in the anti-cancer activity of mTOR inhibitors demonstrates the importance of the redox environment in modulation of MCL cell growth. Another novel hematologic chemotherapeutic in clinical trials for MCL is bortezomib. Bortezomib is a proteosome inhibitor found to induce high levels of ROS in multiple hematologic cancers (Bhalla, 2009; Berges, 2009; Ling, 2009; Miller, 2007; Rushworth, 2011). The increase in ROS has been attributed to inhibition of multiple pathways, including NF-κB (Bhalla, 2009; Nakata, 2011), NOXA (Ohshima-Hosoyama, 2011), and Nrf-2 (Rushworth, 2011). Bortezomib has shown synergy with other drugs that increase ROS (Bhalla, 2009). Combination therapies of these drugs with mTOR inhibitors may be synergistic if they enhance the production of ROS and promote ROS-induced cell death; particularly, if they increase ROS via different mechanisms than MnSOD regulation, such as bortezomib does.

The efficacy of mTOR inhibitors could also be enhanced with other drugs that increase H_2O_2 production. Vitamin C and N^1,N^{11}-bis(ethyl)nonspermine (BENSpm) are two molecules that have been found to generate H_2O_2 and have been evaluated in clinical trials (Chen, 2005; Padayatty, 2006; Pledgie, 2005). Ideally, drug combinations should attack different cellular processes. These combinations may increase H_2O_2 enough to force rapamycin treated MCL cells past the threshold of a cytostatic effect to a cytotoxic effect. Other aggressive lymphomas such as ALCL and DLBCL have constitutively activated PI3K and Akt pathways (both upstream of mTOR), which should make them sensitive to mTOR inhibitors (Coiffier, 2009). These lymphomas rarely display overall response rates above 15% in clinical trials (Teachey, 2009). Combinations that enhance the ROS-induced cell death effect of rapamycin may also improve the clinical effect in hematologic malignancies that previously displayed only moderate activity.

The distinct role of MnSOD in ROS production after rapamycin treatment clearly highlights that sensitivity to rapamycin is dependent upon the amount of MnSOD in the cells. Previous research from our lab indicates that low expression of MnSOD in diffuse large B-cell lymphoma, another aggressive B-cell lymphoma, is associated with a poor prognosis (Tome, 2005). In additional studies, utilizing a murine thymic lymphoma cell line, over-expression of MnSOD sensitized cells to dexamethasone-induced apoptosis (Jaramillo, 2009). These data support the idea of manipulating MnSOD, and subsequent H_2O_2 levels, to improve cancer treatment. Additionally, MnSOD may serve as a predictive marker for patient response to mTOR inhibitors. Mutations that result in poor expression or loss of ability to up-regulate the expression of MnSOD may not allow for the increase in H_2O_2 necessary for the full effect of rapamycin. Patients with these mutations or low baseline levels of MnSOD may not respond as well to mTOR inhibitors. Clinical studies evaluating correlations between survival outcome of MCL patients treated with mTOR inhibitors and gene expression of MnSOD are necessary to further evaluate the use of MnSOD as a biomarker for treatment response.

Regulation of MnSOD by mTOR inhibition. Despite the mTOR pathway being well known as a major controller of protein translation, gene expression profiling of breast (Akcakanat, 2009) and prostate (Majumder, 2004) cancer cells have shown that it is not uncommon for some genes to be transcriptionally up-regulated in response to rapamycin treatment. While I did not exclude the possibility of translational regulation, I found MnSOD to be transcriptionally up-regulated in response to rapamycin at 36-48 hours. In addition, these studies showed MnSOD to be a direct target of rapamycin. Upon further investigation, it did not appear that the regulation of MnSOD was due to a transcription factor downstream of the mTORC1 pathway, but in fact was via inhibition of the mTORC2 pathway as shown in Figure 4.1.

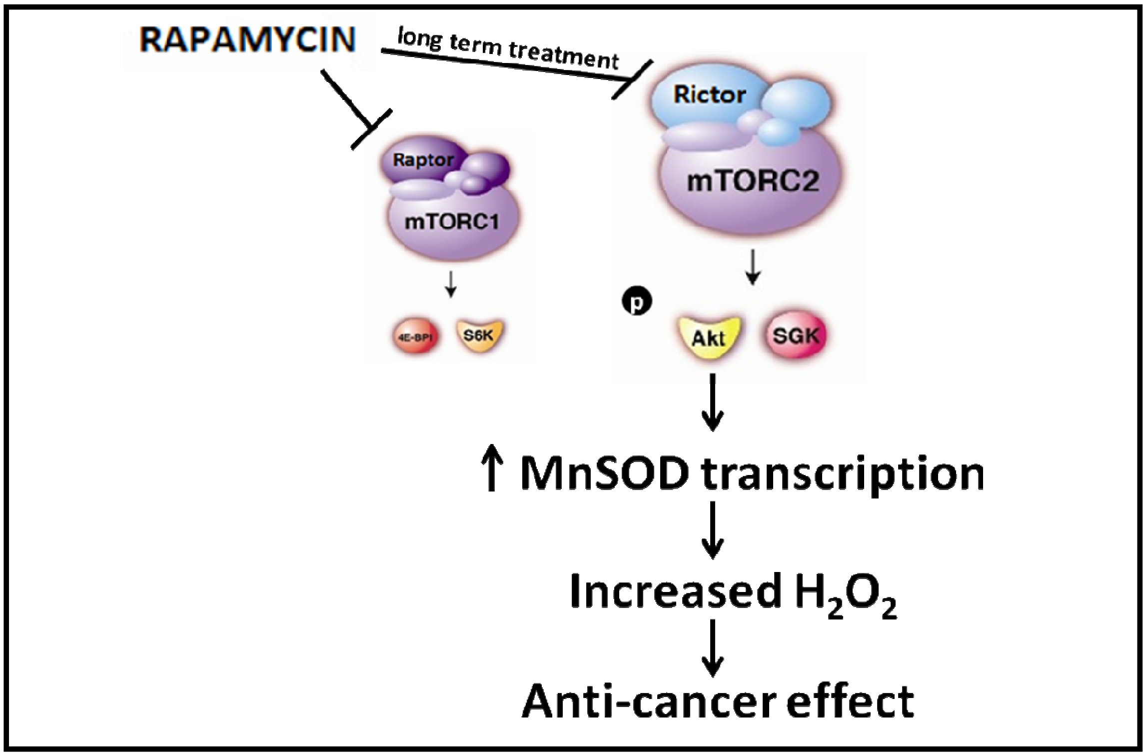

Figure 4.1: Proposed model of MnSOD and H_2O_2 regulation by mTOR inhibitors. Long term treatment of MCL cell lines with the mTOR inhibitor rapamycin results in inhibition of the mTORC2 complex, and affects the phosphorylation state of Akt. De-phosphorylated Akt results in regulation of a currently unknown transcription factor to up-regulate transcription of MnSOD. Increased levels of MnSOD result in elevated levels of H_2O_2 due to the dismutase activity of MnSOD. This increase in H_2O_2 is necessary for the growth inhibitory effect of rapamycin. Results further indicate that the anti-cancer effect can enhanced by further increasing MnSOD and H_2O_2 levels following rapamycin treatment.

Rapamycin is traditionally known for its inhibition of the mTORC1 complex and a downstream effect on translation proteins involved in regulation of cell growth. With this in mind, I postulated that a likely transcription factor regulating MnSOD was C/EBPβ. Previous microarray studies in our lab demonstrated a strong correlation between MnSOD mRNA and C/EBPβ mRNA (Tome, 2005). With the translation of the three possible C/EBPβ isoforms being dependent upon the mTORC1 pathway, it was expected that inhibition of mTOR with rapamycin would affect translation of isoforms and thus effect regulation of MnSOD transcription. While this result was evident in the correlation of increasing MnSOD and a decrease in C/EBPβ-LIP after rapamycin treatment, over-expression of C/EBPβ-LIP had no effect on MnSOD protein levels. It is possible that C/EBPβ-LIP is working in conjunction with other factors that were not present at increased levels to alter MnSOD transcription when C/EBPβ was over-expressed. Multiple studies have found that nuclear localized NF-κB is necessary for regulation of MnSOD by C/EBPβ (Kiningham, 2001; Xi, 1999; Jones, 1997; Ranjan, 2006). Specifically, C/EBPβ is required for induced NF-κB p65 binding to MnSOD's intronic response element (Ranjan, 2006). While NF-κB is constituatively activated in MCL (Pham, 2003), if the protein levels were not increased enough to partner with C/EBPβ to regulate MnSOD, I would see no effect from C/EBPβ-LIP over-expression. Knocking down C/EBPβ-LIP would eliminate this problem, and better replicate the changes seen with rapamycin (ie., decreased C/EBPβ-LIP expression). However, since the three isoforms are translated from multiple in-frame start sites on the messenger RNA, it would currently be impossible to knock-out expression of just one isoform without affecting the other transcriptional active isoforms. Additional studies looking at the role of both C/EBPβ and NF-κB in regulation of MnSOD may bring to light another way to regulate MnSOD and enhance mTOR inhibitor treatment of MCL.

A second possibility for regulation of MnSOD is via transcription factors regulated by the mTORC2 pathway. Recently, the mTORC2-Akt signaling pathway has been shown to be inhibited with long-term treatment with rapamycin in multiple cell lines (Sarbossov, 2006). My studies are the first to show inhibition of the mTORC2-AKT pathway in MCL with rapamycin treatment. Furthermore, MnSOD regulation appeared to be regulated via the mTORC2 pathway. The data showed that while the overall levels of FOXO3a, a downstream target of Akt, increased with rapamycin treatment, there did not appear to be a translocation of the transcription factor into the nuclear environment. This does not rule out the possibility of regulation of MnSOD by other FOXO transcription factors. Another member, FOXO1, has also been implicated as a regulator of MnSOD expression (Birkenkam, 2003) and should be investigated at a possible regulator of MnSOD upon inhibition of mTORC2. Elucidating the mechanism by which MnSOD is being transcriptionally regulated via rapamycin would help us to further understand the downstream effects of mTOR inhibition and treatment response.

The inhibition of Akt phosphorylation (a downstream target of mTORC2) after rapamycin treatment and subsequent increase in MnSOD after 48 hours suggest that immediate inhibition of both mTORC1 and mTORC2 pathways may be a better treatment option for MCL patients. Second generation mTOR inhibitors, such as XL765 and NVP-

BEZ235, are small molecule inhibitors of the mTOR kinase domain that have recently entered clinical trials (Dancey, 2010). By targeting the mTOR kinase domain, these drugs are able to inhibit both the mTORC1 and mTORC2 pathways as an immediate response. In addition, these drugs target PI3K, which is a molecule upstream of Akt (Dancey, 2010). XL765 has been found to have clinical activity in glioblastoma (Prasad, 2011) and pancreatic cancer (Mirzoeva, 2011). Published studies on NVP-BEZ235 show clinical activity of the drug in hematologic malignancies, including acute lymphoblastic leukemia (Chiarini, 2010) and follicular lymphoma (Bhende, 2010). Together, the data suggest that second-generation mTOR inhibitors may be a better treatment option for MCL than the current mTOR inhibitors.

Genetic polymorphisms in oxidative stress related genes and treatment response. While increased levels of ROS can be due to drug treatment, it can also be an intrinsic characteristic of cell function. With an important role of redox biology in the metabolism and function of anti-cancer drugs, inherited variants of oxidative stress-related genes were expected to contribute to inter-patient variability in treatment response. SNPs in genes encoding myeloperoxidase (*MPO*) and an aldo-keto reductase (*AKR1C3*) were associated with both progression-free and overall survival following anthracycline-based therapies. SNPs in the gene encoding MnSOD (*SOD2*) and neutrophil factor 4 (*NCF4)* were marginally associated with risk of Grade 4-5 hematologic toxicity. These are the first studies to link variants of these genes in aggressive NHL patients to outcomes.

The *SOD2* (MnSOD) SNP (rs4880) used in this analysis is the most extensively studied and results in an alanine to valine transition at amino acid 16, affecting MnSODs mitochondrial targeting sequence (Rosenblum, 1996). The valine form, which contains the disrupted targeting sequence, has been associated with decreased activity compared to the alanine form (Sutton, 2006). This SNP may not have displayed an association with survival after treatment for multiple reasons. While the polymorphism alters the localization of MnSOD, it does not affect its ability to dismute superoxide into H_2O_2. Thus, it is possible that it can still participate in this reaction at other locations within the cell and participate in the generation of H_2O_2 necessary for treatment response. Future analysis of a different *SOD2* SNP (Ile58Thr), which results in reduced activity of MnSOD (Borgstahl, 1996), would not have this caveat and may provide a different result. Another possibility is that the SNP does not contribute individually, but may have a combined effect with SNPs in other oxidative stress related genes. Evidence of this is seen in a recent breast cancer study which found the same *SOD2* SNP to contribute to cancer development only when analyzed in combination with other oxidative stress-related SNPs (Bag, 2008). Further statistical analysis looking at the combined effect of SNPs would provide further insight into this possibility.

The SNP identified in *AKR1C3* may influence survival after lymphoma treatment by affecting repair of ROS damage or metabolism of doxorubicin, which is an anthracyline. AKR1C3 is a member of a superfamily of oxidoreductases involved in a diverse number of reactions. Those reactions include reduction of carbonyl-containing drugs, such as anthracyclines (Flynn, 1982), and repair to oxidative damage to proteins, lipids, and DNA (Esterbauer, 1991; Rindgen, 1999). Multiple AKR family members have been linked to drug resistance in cancer treatment as well as increased risk of occurrence. Increased mRNA expression of AKR1B1 and AKR1C2 was identified in a drug resistant human stomach carcinoma (Ax, 2000), and over-expression of AKR1B1 was linked to anthracycline resistance in HeLa cervical carcinoma cells (Lee, 2002). Polymorphisms in the gene encoding the AKR1A1 family member have been significantly associated with increased risk of NHL (Lan, 2007). It has also been noted that the Gln5His SNP in *AKR1C3* is associated with increased risk of polycyclic aromatic hydrocarbon-induced lung cancer (Lan, 2004). The *AKR1C3* SNP that we identified (rs10508293) is located in intron 4 of the gene, and is of unknown functional significance since it does not result in changes to the protein structure. However, these type of non-synonymous SNPs can result in post-translational modifications that effect activity, or alter transcription (Chorley, 2008). These previous studies support the idea that SNPs in *AKR1C3* may be affecting patient response to chemotherapy.

AKR1C3 may also be involved due to the ability to catalyze the conversion of doxorubicin to the inactive alcohol metabolite, doxorubicinol. The role of AKRs in doxorubicin metabolism is supported by the reduced formation of the alcohol metabolite in rat heart and liver cytosols in the presence of an AKR inhibitor (Behnia, 1999). A study by Bains et al (2010) has identified two non-synonymous SNPs in the *AKR1C3* gene that significantly reduce the rate at which doxorubicin is converted to doxoribicinol. However, I predict that this *AKR1C3* genotype is associated with an increased hazard of death for lymphoma patients due to higher levels of the AKR1C3 enzyme. This would result in cells converting more doxorubicin to the inactive doxorubicinol. With the additional involvement of AKR family members in repair of protein cross-links and DNA adducts caused by ROS, higher levels of AKR1C3 may also mean increased repair, once again, hindering the activities of ROS-inducing chemotherapy.

The SNP identified in *MPO* may influence survival after lymphoma treatment due to decreased enzymatic activity. MPO is primarily found in neutrophils and produces the reactive oxygen species, hypochlorous acid. It has also been identified as a participant in a variety of other oxidative reactions (Winterbourn, 2000). The minor allele of the *MPO* SNP has been shown to result in lower expression of the gene in breast cancer (Piedrafita, 1996) and leukemia (Reynolds, 1997), and decreased MPO activity in lung cancer (Van Schooten, 2004). Interestingly, our findings with aggressive NHL patients are in agreement with another study (Ambrosone, 2005) where women with the least common genotype of a concordant *MPO* SNP had a 2-fold increased hazard of breast cancer recurrance after adjuvant chemotherapy. This association was not identified in low-risk breast cancer patients that did not receive adjuvant treatment. With the central role of MPO in regulating oxidative stress via production of hypochlorous acid or participation in oxidation reactions, a potential consequence of decreased MPO due to SNPs could be decreased production of ROS that are needed for complete cell kill by ROS-dependent drugs, like doxorubicin.

The SNPs identified as having a borderline significant association with Grade 4 -5 hematologic toxicity could influence toxicity for multiple reasons. *NCF4* (rs1883112) encodes a regulatory subunit of NAD(P)H oxidase and *SOD2* (rs4880) encodes MnSOD. Both NCF4 and MnSOD are involved in enzymatic reactions that contribute to the production of intracellular ROS at the mitochondria. For both SNPs, the least common genotype was marginally associated with increased risk of hematologic toxicity. The SNP in *NCF4* is of unknown biological significance. The encoded protein is part of the NADPH oxidase, which plays a role in generation of superoxide by transferring electrons from NADPH inside a cell across the membrane of phagosomes. It is possible that the SNP increases activity or levels of NCF4, allowing increased generation of superoxide. The particular SNP in SOD2 results in a A16V switch, and the alanine variant of the enzyme has been shown to have higher enzymatic activity than the valine variant. Since the alanine variant is localized to the inner mitochondrial membrane (Sutton, 2003), it likely results in increased production of H_2O_2 at the mitochondria. If ROS production is at extremely high levels within the cell and not neutralized, it may induce damage in normal cells as opposed to low levels which participate is cellular signaling (Kannan, 2000). Damage to normal cells may be contributing to the source of hematologic toxicity.

Concluding summary. In conclusion, my data demonstrates a critical role for H_2O_2 in treatment and identifies MnSOD as a novel downstream target of mTOR inhibitors that has the potential to be exploited therapeutically in order to improve survival rates of MCL. This opens up a new range of combination treatment options that may best enhance the therapeutic activity of mTOR inhibitors. A study of aggressive lymphoma patients identified single nucleotide polymorphisms in oxidative-related genes associated with survival. The treatments received by these patients all included at least one standard treatment drug that is ROS dependent. For example, the commonly used drug, doxorubicin, partially relies on H_2O_2 for induction of cancer cell death (Fang, 2007), similar to results of my studies utilizing the mTOR inhibitor rapamycin. Individuals carrying minor allele SNPS in *MPO* and *AKR1C3* were found to be associated with shorter time to disease progression and death. Individuals carrying minor allele SNPS in *NCF4* and *SOD2* (MnSOD) were found to be borderline associated with risk of hematologic malignancy. This data suggests that SNPs may alter the redox environment and some patients may not benefit from drugs relying on ROS as a mechanism of function. Collectively, these studies indicate the importance of the redox environment in cancer treatment, and demonstrate how mTOR inhibitors may be utilizing ROS to induce cancer cell death.

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