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Dual Regulation of Glucocorticoid-Induced Leucine Zipper (GILZ) by the Glucocorticoid
Receptor and Phosphatidylinositol 3-Kinase/AKT Pathways in Multiple Myeloma

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In memory of

Lewis F. Norwood, Jr.,

Ethel K. Norwood,

J. Clinton Davies,

Herbert F. Ramsdell,

and

Thomas Lawson,

and in honor of all who have loved and missed them.

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ABBREVIATIONS

ALL	acute lymphoblastic leukemia
ATRA	all trans retinoic acid
BMME	bone marrow microenvironment
CLL	chronic lymphocytic leukemia
Dex	dexamethasone
DNA	deoxyribonucleic acid
ECM	extracellular matrix
FHRE	forkhead responsive element
FOXO	forkhead box O
GC	glucocorticoid
GILZ	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
GRE	glucocorticoid response element
IgH	immunoglobulin heavy chain
IGF1	insulin-like growth factor-1
IL	interleukin
MM	multiple myeloma
mRNA	messenger ribonucleic acid
NFκB	nuclear factor κ B
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline

PI3-kinase   phosphatidylinositol 3-kinase

RTK   receptor tyrosine kinase

RT-PCR   reverse transcriptase polymerase chain reaction

siRNA   small interfering ribonucleic acid

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ABSTRACT

Dual Regulation of Glucocorticoid-Induced Leucine Zipper (GILZ) by the Glucocorticoid Receptor and Phosphatidylinositol 3-Kinase/AKT Pathways in Multiple Myeloma

Katharine M. Davies

Multiple Myeloma (MM) is an incurable plasma cell malignancy occurring within the bone marrow. Glucocorticoids (GCs) are widely used in the treatment of MM patients due to potent induction of apoptosis. Though known to occur via the glucocorticoid receptor (GR), the molecular details of GC-induced apoptosis in MM cells remain largely undefined. Glucocorticoid-induced leucine zipper (*GILZ*) was identified in a micro-array screen as a GC-regulated gene. Compelling data from the literature suggested a potential functional role for GILZ in apoptosis. These experiments were designed to determine the importance of GILZ in GC-induced apoptosis and to gain an understanding of how other signaling molecules in MM cells could regulate GILZ expression and impact GC-induced apoptosis. The up regulation of GILZ by GCs was confirmed in a panel of MM cell lines and clinical patient samples and the GR was shown to be necessary for GILZ induction in these cells. Reducing the levels of GILZ with siRNA constructs decreased the ability of GCs to induce apoptosis of MM cells suggesting that GILZ contributes functionally to MM cell death. Regulation of GILZ by the phosphatidylinositol 3-kinase (PI3-kinase)/AKT pathway was revealed following a RT-PCR screen to identify other regulators of *GILZ*. Activators of PI3-kinase/AKT (IL-6 and IGF1) down regulate *GILZ* and block GC-induced up regulation. Inhibitors of PI3-kinase/AKT (LY294002, wortmannin, triciribine, AKT inhibitor VIII) up regulate GILZ at both the mRNA and protein level. This effect was observed in a panel of MM cell lines and clinical patient samples. The combination of GCs and PI3-kinase/AKT inhibitors dramatically increased GILZ

levels as measured by real time PCR and enhanced MM cell killing as shown with multiple

apoptosis assays. This effect could be inhibited by the addition of exogenous IL-6 or IGF1 or

upon co-culturing MM cells with immortalized human bone marrow stromal cell lines. The

combination of the synthetic GC, Dexamethasone, and the PI3-kinase inhibitor, LY294002,

caused synergistic cell killing of MM cells. This observation suggests cross-talk between the

two pathways occurring at the level of *GILZ* regulation and warrants further investigation into

the therapeutic benefit of using GCs and PI3-kinase/AKT inhibitors together to treat MM

patients.

CHAPTER 1

INTRODUCTION

Multiple Myeloma

Multiple myeloma (MM) is the second most commonly diagnosed hematologic malignancy in the US with an estimated 16,570 new cases diagnosed and 11,310 deaths in 2006 (1, 2). At present, MM is a uniformly fatal disease with a median survival time of about 3-4 years (3). MM is often inaccurately classified as a rare malignancy because of its short survival time after diagnosis resulting in decreased prevalence compared to other hematologic malignancies. It is a malignant plasma-cell tumor that accumulates at multiple sites within the bone marrow. The hallmarks of the disease include osteolytic bone destruction and accumulation of monoclonal immunoglobulin (Ig) protein in the serum and urine (4). Other disease symptoms include anemia, diffuse osteoporosis, hypercalcemia, renal dysfunction due to deposition of Ig protein, and impaired hematopoiesis resulting in an increased risk of infection. In the early stages, myeloma is a slowly proliferating disease with only 2 percent of tumor cells in S phase (4). The myeloma cells remain localized in the bone marrow until late stages when malignant plasma cells can be detected in the circulation as a plasma cell leukemia (2).

MM cells are the transformed counterparts of genetically-unstable post-germinal center B cells known as bone marrow plasma cells (5). MM evolves through a multistep transformation process resulting in the accumulation of oncogenic mutations (6). Five recurrent primary chromosomal translocations involving the immunoglobulin heavy chain (IgH) gene (14q32) have been identified in MM tumors linking an oncogene to the transcriptional regulatory regions of IgH. These translocations include 11q13, 6p21, 4p16, 16q23, and 20q12 resulting in deregulation of CYCLIN D1, CYCLIN D3, fibroblast growth factor receptor (FGFR3)/MMSET, c-MAF, and MAFB respectively. These translocations are found in 40% of MM cases and are the result of errors in immunoglobulin heavy chain switch recombination (7). Secondary

translocations and other genetic mutations not involving the immunoglobulin genes also occur during disease progression increasing the genetic heterogeneity of the disease. Common examples include deregulation of c-MYC, N-RAS, K-RAS, and FGFR3 (4). The biologic significance of each individual genetic abnormality is unclear due to difficulties separating the combined effects of numerous mutations and the increasing genetic complexity as the disease progresses (2).

Importance of Bone Marrow Microenvironment in Myeloma

In 1889, Stephen Paget published his "seed and soil" hypothesis to explain non-random patterns of cancer metastasis. He was the first to suggest that interactions between the tumor and host cells of the microenvironment are critical in regulating tumor growth (8). The importance of the tumor microenvironment in the bone marrow is well established for myeloma disease progression. The bone marrow microenvironment (BMME) comprised of extracellular matrix (ECM) proteins, fibroblastic stromal cells, osteoblasts, osteoclasts, vascular endothelial cells and phospholipids plays a crucial role in enhancing tumor cell growth, survival, migration, angiogenesis and the development of therapeutic resistance. MM cells interfere with normal skeletal homeostasis resulting in osteolytic bone destruction while a multitude of interactions and paracrine factors from components of the BMME provide protective and growth promoting effects in return to the MM cells (2). This "vicious circle" where the tumor cells help destroy the environment that encourages their growth is caused by signaling effects resulting from the direct adhesion to other cells and ECM proteins and indirectly by paracrine factors secreted in the bone marrow (9). The ever-expanding list of paracrine factors in the BMME includes interleukin (IL)-6, insulin-like growth factor 1 (IGF1), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-8, IL-21, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), stromal derived

factor (SDF)-1, tumor necrosis factor (TNF)-α, Notch, stem cell factor (SCF), macrophage

inhibitory protein (MIP)1α, fibroblast growth factor (FGF)2, and platelet derived growth factor

(PDGF) (2, 6). Both the oncogenic mutations and the BMME activate a complex signaling

network in the MM cells promoting survival, progression, and drug resistance (6). The activated

pathways shown to be important include PI3-kinase/AKT, Ras/MAPK, Jak/STAT3, IKKα/NF-

κB, Notch, and WNT. Recently it was reported that a common oncogenic mutation in MM cases

(c-MAF, 50%) results in the up regulation of integrin β7 enhancing MM adhesion to bone

marrow stromal cells and VEGF production (10). This is one example where MM cells are more

dependent on the BMME than normal plasma cells. Targeting the components of the BMME

that contribute to MM pathogenesis has emerged as a new area of therapeutic development (2,

11)

Phosphatidylinositol-3-kinase/AKT Pathway

Components of the BMME activate the phosphatidylinositol-3-kinase (PI3-kinase)/AKT

pathway in MM cells stimulating cell survival and growth. Two paracrine growth factors IL-6

and IGF1 secreted by the BMME have been shown to promote MM cell proliferation and protect

against drug-induced killing. Both cytokines activate a number of cellular signaling pathways

including PI3-kinase/AKT, Ras/MEK/MAPK, and Jak/STAT3. Evidence in the literature has

shown that the PI3-kinase/AKT pathway is absolutely required to mediate the IL-6 and IGF1

effects on MM proliferation and apoptosis (12, 13). The ever-expanding list of other growth

factors within the BMME that have also been shown to activate PI3-kinase/AKT include PDGF,

VEGF, EGF, IL-2, IL-3, IL-4, IL-5, and IL-8 via corresponding growth factor receptor tyrosine

kinase receptors (RTK) (13, 14).

The class IA PI3-kinase enzyme has been linked to tumorigenesis. In normal mammalian cells, class IA PI3-kinase regulates glucose homeostasis, cell migration, growth, and proliferation. The enzyme is a heterodimer of catalytic p110 (α, β, or δ) and regulatory p85 (α, β, or γ) subunits in the cytoplasm. Extracellular ligand binding and subsequent phosphorylation of cytoplasmic tails of RTKs recruits p85:p110 PI3-kinase complex via the SH2 domain of p85. Adaptor proteins such as insulin receptor substrate-1/2 (IRS1/2) and Ras can also recruit and activate PI3-kinase at RTKs. Activated PI3-kinase catalyses the conversion of the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3). The formation of the lipid second messenger PIP_3 recruits signaling molecules with pleckstrin-homology domains to membrane sites of activated PI3-kinase including serine/threonine kinases phosphoinositide-dependent kinase 1 (PDK1) and AKT. AKT (also known as protein kinase B) is phosphorylated by PDK1 at Thr308 and by the recently identified PDK2 (TORC2, rapamycin-insensitive SIN1/rictor/mTOR complex) at Ser473. Phosphorylation at both sites is necessary to fully activate AKT (14-16). AKT has 3 family members (AKT1, AKT2, and AKT3) which are broadly expressed, but have some isoform specific functions and expression patterns. There are many downstream targets of AKT that effect metabolism, cell growth and proliferation including glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin (mTOR), TSC2, glucose transporters (GLUT1/GLUT4), phosphofructokinase-2 (PFK2), IKK/NF-κB and proteins that play a role in apoptosis including BAD, ASK1, MDM2 and FOXO family members (2, 12-14, 17-22). PI3-kinase/AKT signaling can be terminated by the phosphatase PTEN which dephosphorylates PIP_3 back to PIP_2 (16, 17, 23). The PI3-kinase/AKT pathway is summarized in Figure 1.
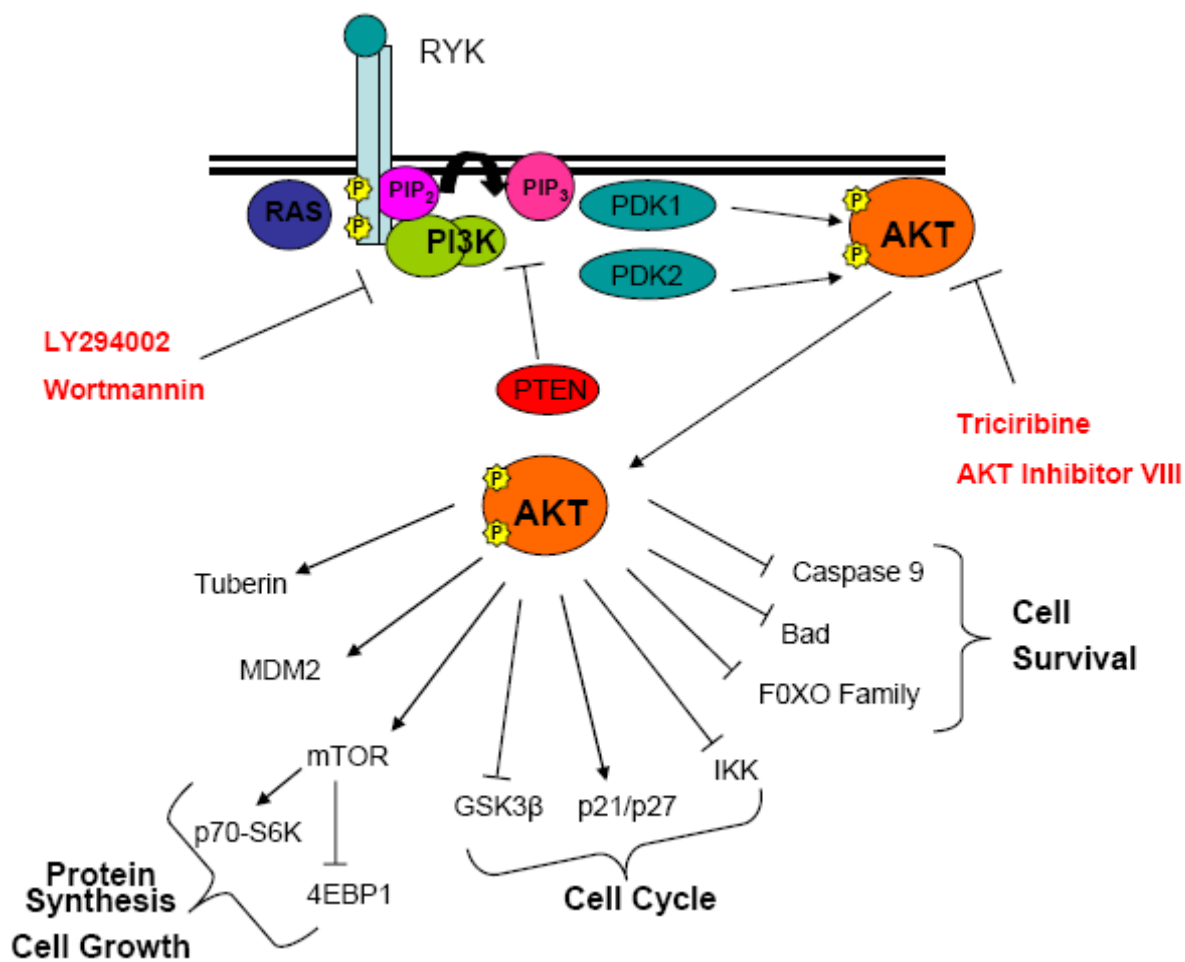
Figure 1.   **Schematic of the PI3-kinase/AKT pathway**

Activation of the PI3-kinase/AKT pathway plays a pivotal role in essential cellular functions including survival, proliferation, migration, and differentiation. Both PI3-kinase and AKT were originally identified as components of retroviral oncogenes (24). Mutation of components of PI3-kinase/AKT accounts for greater than 30% of human cancers with the loss of PTEN occurring most frequently (17, 21, 23). Mutations or amplification in p110α (PIK3CA) and AKT2 have also been reported in a variety of cancers. Mutations in Ras family members and many receptor tyrosine kinases can also lead to aberrant PI3-kinase/AKT signaling (14). The net result of mutations in components of this signaling pathway is loss of growth control checkpoints and promotion of cell survival in nutrient poor conditions (21). Blockade of the PI3-kinase/AKT signaling pathway has been suggested as a potential target area for novel therapeutics in a variety of cancers including MM where mutations upstream of PI3-kinase/AKT including Ras and various RTKs have been reported (20, 25-27). Many laboratories are attempting to develop inhibitors to components of the PI3-kinase/AKT pathway that have therapeutic benefit. Identifying the primary mechanism by the which the PI3-kinase pathway is activated in a given tumor will help guide the choice of therapy and isoform specific inhibitors to PI3-kinase, AKT, and many of the receptor tyrosine kinases are currently being evaluated (14).

Treatment of Multiple Myeloma

MM patients are frequently treated with combination chemotherapy involving anthracylines, alkylating agents, and glucocorticoids. Many patients under the age of 65 also receive autologous or allogeneic hematopoietic stem cell transplants. High dose chemotherapy followed by autologous bone marrow transplantation increases overall survival rate, but ultimately tumor cell resistance develops to all therapeutic approaches and the disease remains fatal (4, 11, 28-30). The complexity and variety in MM cells and the surrounding BMME

greatly limits the likelihood of a curative outcome with any single therapy (2). Recently, investigators have used genetic micro-array analyses to further define disease classifications and sub-groups to predict therapeutic responses to various treatments and ultimately improve therapeutic outcomes (4). Recent advances in understanding the molecular pathogenesis of these tumors and the importance of the BMME to drug resistance have resulted in the development and use of biologically targeted therapeutics including thalidomide and its derivatives and the proteasome inhibitor Velcade (31-34). These advances indicate that improved approaches for treatment and survival should be possible in the near future (4, 11).

Glucocorticoids and the Glucocorticoid Receptor

The first observations that glucocorticoids could kill leukemia and lymphoma cells and had potent anti-inflammatory properties were made in the 1940-50s (35, 36). This has resulted in their widespread use in the treatment of MM, leukemias, lymphomas, and inflammatory diseases making GCs one of the most widely prescribed drugs worldwide (37-39). Physiologically GCs are synthesized by the adrenal gland and are necessary for normal growth, development and homeostasis, liver and immune functions, and in mediating stress responses via the hypothalamic-pituitary-adrenal axis (40, 41). The process of GC-induced apoptosis is important during normal thymocyte maturation and lymphocyte homeostasis (42, 43).

Despite widespread pharmacologic use, the molecular details of GC-induced apoptosis remain largely undefined. GCs are small hydrophobic molecules that diffuse through the plasma membrane and bind the cytosolic glucocorticoid receptor (GR). Most of the effects of GCs are mediated through interaction with the GR and it has been shown that an intact functional receptor is required for cytotoxicity (44-49). Orginally cloned in 1985, the GR belongs to the nuclear steroid hormone receptor family and is a ubiquitously expressed, ligand-dependent

transcription factor that affects growth, development, metabolic functions, and stress responses (29, 50-52). In its unliganded resting state, the intracellular GR protein exists in the cytoplasm as a heterooligomeric complex containing one steroid binding protein and a multi-subunit nonsteroid binding chaperone complex which maintains GR in a conformation compatible with ligand binding. The release of inhibitory molecules upon ligand binding allows the GR to translocate to the nucleus where it homodimerizes and binds to a glucocorticoid response element (GRE), a region of DNA containing two inversely symmetrical GR-binding elements. Classical GREs regulate GC-inducible genes that play roles in metabolism and cell growth (29, 51). The GR regulates gene expression through either transactivation or transrepression of gene targets. Apart from direct GR:DNA binding, ligand bound GR is known to repress the transcription of cytokines and other mediators of immune and inflammatory responses in part due to direct interactions between GR and the transcription factors NF-κB, AP-1, NFAT, CREB, and STAT5 (48, 53-63). The various mechanisms of GC:GR regulation of gene expression are summarized in Figure 2.

Glucocorticoid-Induced Apoptosis

Even though GCs have long been used as anti-cancer and anti-inflammatory agents due to their ability to induce cell cycle arrest and cell death, the molecular details of GC-induced apoptosis are still being elucidated. The specifics vary greatly in different cell types, developmental stages and disease states which has added to the complexity. GC-induced apoptosis has been reported to involve the mitochondrial instrinsic apoptotic pathway including perturbations in the ratio of pro- and anti-apoptotic Bcl-2 family members, repression of pro-survival signaling pathways, activation of the proteasome, lysosomal release of cathepsins, increased intracellular calcium levels, generation of reactive oxygen species, and activation of
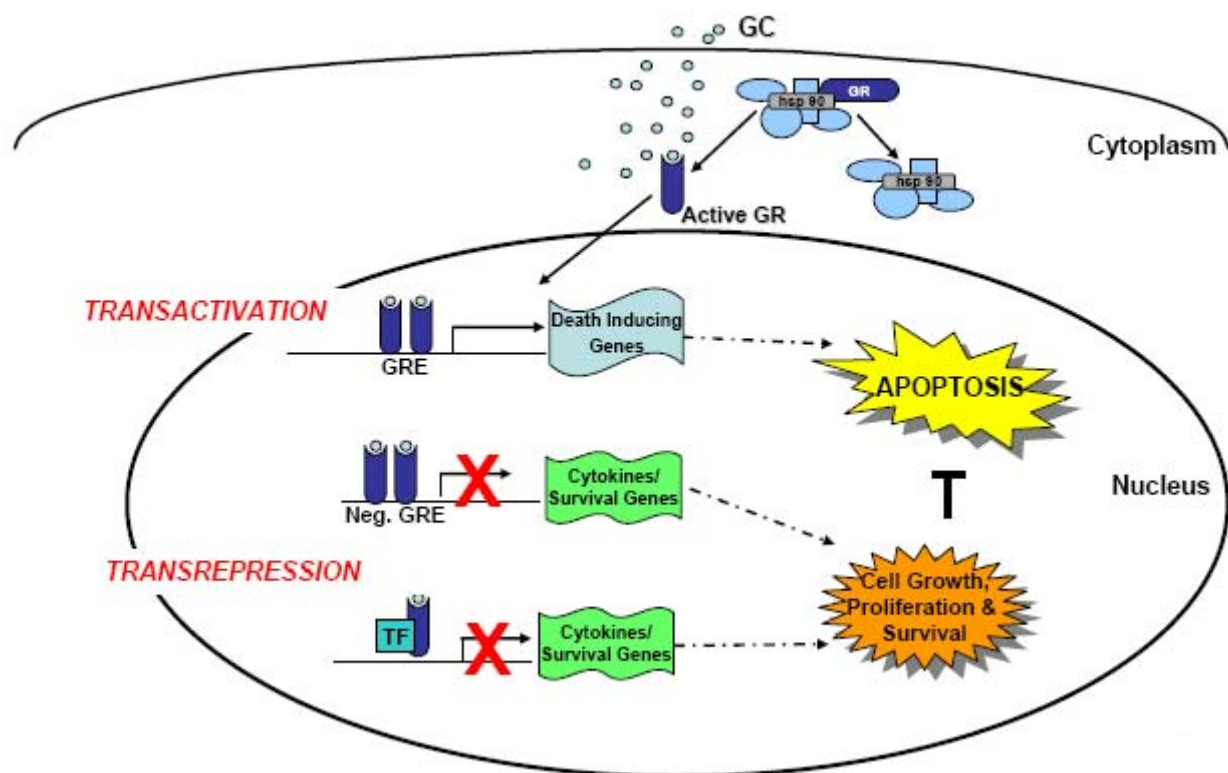
Figure 2.   Schematic of GC regulation of gene expression.

caspases (29, 38, 43, 64). In the case of MM, activation of related adhesion focal tyrosine kinase (RAFTK), release of mitochondrial SMAC/DIABLO, and activation of caspases 9 and 3 have been reported to occur during GC-induced apoptosis (65, 66).

Experiments done with mice expressing a mutant GR which cannot dimerize and bind to GREs have shown that most of the GC effects are mediated by the transrepressive functions of the GR independent of DNA binding. As stated, GR can interfere with the transcription factors NF-κB, AP-1, NFAT, CREB, and Stat5 thus blocking transcription of downstream gene targets important for cell proliferation and growth.   However, multiple pieces of evidence also support the requirement of GR-induced gene activation in lymphocytes for GC-induced apoptosis to occur. De-novo RNA and protein synthesis are necessary as inhibition of transcription or translation blocks thymocyte apoptosis by GCs (37, 67, 68). Using transgenic mice expressing mutant DNA binding deficient GR, it was observed that GC-induced thymocyte apoptosis was defective in the absence of GR DNA binding. This suggests that GR transactivation is necessary for GC-induced apoptosis of lymphocytes (69, 70).

Long term treatment with GC results in a number of adverse side effects including GC-induced osteoporosis, growth retardation, redistribution of fat, muscle degeneration, and skin atrophy. Evidence suggests that many of these side-effects are mediated by GR transactivation. Many companies have attempted to develop selective GC agonists which separate the transrepression or transactivation functions of the GR (71). Work using these compounds will help delineate the importance of GR-dependent gene activation and GR transcription factor inhibition in the process of GC-induced apoptosis.

In order to gain more information on changes in gene regulation that contribute to GC-induced apoptosis, many laboratories have conducted micro-array analysis to screen for

alterations in gene expression upon treatment with GC in a variety of hematological cell lines (72-80). The top genes reported up and down regulated by 6 hr GC treatment in MM.1S cell lines in our micro-array is shown in Table 1 (80). Over 900 different genes have been reported to be GC-regulated in these screens, but only about 70 have appeared in more than one publication (38). A few of the GC-regulated genes identified in the screens that have been found with further study to be important for GC-induced apoptosis include Bim (74), thioredoxin-interacting protein (TXNIP) (81), E4BP4 (82) and dexamethasone induced gene 2 (DIG2) (83). Due to the difficulties in identifying a single primary death-inducing gene, it has been suggested that multiple, cell type dependent mechanisms may exist, rather than one conserved canonical pathway that leads to GC-induced cell death and many GC-gene products may contribute to cell death induction (38).

Glucocorticoid-Induced Leucine Zipper

Glucocorticoid-induced leucine zipper (GILZ) has been identified in a number of cell types as a GC-induced gene (72, 77, 79). In our screen, GILZ was rapidly up regulated by dexamethasone (5.9 fold) in the GC-sensitive MM.1S multiple myeloma cells (Table 1). This 137 amino acid leucine zipper protein was originally isolated in mouse thymocytes as a GC-induced gene and is characterized by a leucine zipper domain and a C-terminal PER domain rich in proline and glutamic acid residues important for homodimerization (84, 85). GILZ is a member of the TSC-22 family of proteins which share homology in leucine zipper domain, but have different N- and C-terminal domains (86). GC treatment rapidly up regulates GILZ expression in T cells (CD4+ and CD8+), B cells, dendritic cells and macrophages suggesting a role in the control of immune cell compartment growth and death (49, 87-91).

Table 1.   **Top 5 up and down regulated genes from the results of c-DNA micro-array of MM.1S cells treated for 6 hours with 1 µM Dex.** (Preliminary Data from Rosen Lab)

(+) Fold Increase	Gene	Function
7.0	FK506-binding protein 51 (FKBP51)	Intracellular receptor binds immunosuppressive drugs
5.9	GC-induced leucine zipper (GILZ)	Transcriptional Regulator
4.4	IL-6 signal transducer (gp130)	Involved in IL-6 signal transduction
3.3	GRB2-associated binding protein 1	Adaptor molecule for signaling by cytokine and growth factor receptors
2.9	Proline 4-hydroxylase	Collagen Synthesis
2.8	Sialyltransferase 1	Regulatory roles in lymphocyte function
(-) Fold Decrease	**Gene**	**Function**
-3.8	Activating Transcription Factor 4	DNA binding protein
-3.5	Bcl2-like 1 (Bcl-xL)	Anti-apoptotic protein; blocks Dex-induced cell death
-3.3	Farnesyl-diphosphate farnesyltransferase 1	Cholesterol Synthesis
-3.2	Insulin-induced Gene 1	Plays a role in growth and differentiation
-2.7	Cell division cycle 2 (CDC 2)	Control of cell cycle progression from G1 to S and G2 to M

Most of the research on the molecular functions of GILZ has been conducted in T cells where it has been reported to block the function of the transcription factors NF-κB and AP-1 and the kinase Raf-1 and downstream MAPK signaling (51, 86, 92, 93). In a very recent report, GILZ was also identified to interact directly with the master regulator Ras and inhibit the activation of downstream targets including ERK and AKT. In these studies, GILZ overexpression inhibited Ras-induced NIH-3T3 cell transformation and GILZ silencing blocked the GC antiproliferative effects in T cells (94). From the structural data reported, there is no readily apparent DNA binding domain within GILZ which suggests that transcriptional repression occurs via protein-protein interactions, though one report of DNA binding to an element of the PPAR-γ2 promoter exists (86, 95). GILZ has also been reported to inhibit NF- κB activation in macrophages and airway epithelial cells and MAPK activity in kidney epithelial cells (90, 93, 96). In most cases studied, GILZ function mirrored that of GCs. Like GCs, GILZ overexpression promoted thymocyte apoptosis and protected T cells from cell death induced by TCR/CD3 triggering. T cell-specific GILZ over-expressing transgenic mice had increased thymocyte apoptosis, reduced Bcl-xl expression, increased caspase 3 and 8 expression, decreased number of CD4+/CD8+ thymocytes, and down regulated Th-1 response and increased Th-2 response (97, 98). Interestingly, the effects on the MAPK pathway observed *in vitro* were not recapitulated in this transgenic model (97). Recently, GILZ counteracted GC effects in a mouse cytotoxic IL-2 dependent T cell line where IL-2 withdrawal and GC treatment up regulated GILZ and induced apoptosis. In these cells, overexpression of GILZ blunted GC-induced cell killing whereas reduction of GILZ levels with siRNA enhanced GC-killing. In this case, GILZ is induced by GCs and functions to counteract the pro-apoptotic function of GCs (99, 100).

The promoter of GILZ has been reported (Figure 3) and contains 6 GREs and binding sites for Forkhead box O (FOXO), signal transducer and activator of transcription-6 (STAT6), nuclear factor of activated T cell (NFAT), Octamer, and c-myc (99-102). The forkhead responsive elements (FHRE) sites were shown to be important for both GC and IL-2 withdrawal-induced up regulation of GILZ and involve the forkhead box O family member FOXO3. Overexpression of FOXO3 in the absence of GC also enhanced GILZ levels (99, 100).

The data on the role of GILZ in B cells is limited. Up regulation of GILZ is observed in resting and tolerant B cells when compared to activated B cells (89). It has been hypothesized that GILZ maintains quiescence while down-regulation of GILZ facilitates B cell activation (89). The role of GILZ in myeloma is unknown. Based on the compelling data in other cell lines, **it was hypothesized that GILZ is a key component of the GC signaling pathway in MM cells, acting as a mediator of GC-induced apoptosis.**

Rationale

GCs are widely used to treat MM patients due to potent induction of apoptosis of MM cells. Though it is know to occur via the GR, the molecular details of GC-induced apoptosis in MM remains largely undefined. This interaction between GC and GR results in the activation and repression of gene transcription in myeloma cells. The crucial downstream targets of GC:GR interaction that result in MM apoptosis had not been fully identified. These studies were undertaken to gain insight into the molecular details of apoptosis. *GILZ* was identified in our laboratory as a highly up regulated gene upon GC treatment using micro-array technologies. Compelling preliminary data in T cells from the literature suggested a

potential functional role for GILZ in apoptosis as it was reported to interfere with NF-κB and AP-1 mediated gene transcription. These experiments were designed to determine the

Figure 3. **GILZ promoter sequence (100).**

importance of GILZ in GC-induced apoptosis. The regulation of GILZ by GCs and other signaling molecules in MM cells was investigated in order to further define the molecular details of GC-induced apoptosis in MM. The aims that tested these questions are detailed below.

AIM 1: Characterization of GC regulation of GILZ

GILZ was identified as a GC-induced gene in the MM.1S cell line using cDNA micro-array technologies. In order to confirm these results, the regulation of GILZ by GCs was studied in MM cell lines and patient samples. The importance of the GR in GILZ regulation was also investigated to confirm that GILZ regulation was the result of GR gene activation.

AIM 2: Importance of GILZ as a marker and mediator of GC-induced apoptosis

GILZ is rapidly and dramatically up regulated by GC treatment in MM cells. However, the function of GILZ in B cells and MM cells is unknown and its importance to GC-induced killing has not been shown. The induction of GILZ in a panel of MM cells with varying sensitivities to GCs was determined to see if a correlation between GILZ up regulation and MM cell death could be made. The ability of GCs to kill MM cells that had reduced levels of GILZ by siRNA was also measured to determine if GILZ is a necessary component of GC-induced apoptosis.

AIM 3: Elucidation of PI3-kinase/AKT regulation of GILZ and synergism with GCs

The molecular details of GC-induced apoptosis are not well defined. In order to gain insight into this pathway and the potential involvement of GILZ, a panel of cytokines and cytotoxic agents were screened to identify other regulators of GILZ. The initial screen identified GILZ regulation by PI3-kinase/AKT pathway. The up regulation of GILZ by PI3-kinase and AKT inhibition was further characterized in MM cell lines and patient samples. The combination treatment of GC and PI3-kinase/AKT inhibitors was tested and the effect on GILZ

regulation and MM cell killing was measured. Attempts to elucidate the mechanism behind PI3-kinase/AKT regulation of GILZ were undertaken.

AIM 4: Effect of Bone Marrow Microenvironment on GILZ regulation and GC-induced apoptosis

The bone marrow microenvironment is important for MM disease progression and drug resistance. The effects on GILZ regulation of co-culturing MM cells with immortalized bone marrow stromal cell lines was studied to determine if the microenvironment could alter the up regulation of GILZ by GCs or PI3-kinase/AKT inhibitors. The mechanism of this inhibition of GILZ expression was further characterized.

CHAPTER 2

MATERIALS AND METHODS

MM.1 Tissue Culture Model System of Multiple Myeloma

The MM.1S, MM.1R$_e$, and MM.1R$_L$ cell lines were originally developed in our laboratory (103). The cells are widely used as a model of myeloma disease progression and resistance to glucocorticoids. The original line (MM.1) was established from the peripheral blood of a myeloma patient in the leukemic phase of MM. A GC-sensitive cell line (MM.1S) and two GC-resistant lines (MM.1R$_e$ & MM.1R$_L$) were isolated from the heterogeneous MM.1 population. The MM.1S cells express functional GR-α and are potently killed by GCs. The MM.1R$_e$ express decreasing levels of GR-α and increasing levels of a truncated GR-α splice variant called GR-P. Both GR-α and GR-P expression is lost in the MM.1R$_L$ cell line (44, 104). The MM.1 cell lines contain the t(14;16)(q32;q23) translocation linking the oncogene c-Maf to IgH (105). The MM.1S and MM.1R$_L$ cells lines express the same cell surface markers and are immunocytochemically identical. They differ only in sensitivity to GC killing (80) (Figures 4-5). This set of cell lines is one of the most frequently used by the myeloma research community and has greatly contributed to the understanding of myeloma biology and therapeutic development (80).

Cell Culture

All cell culture medium, serum and antibiotics were purchased from GIBCO/Invitrogen unless otherwise noted (Carlsbad, CA). The U266 cell line was purchased from ATCC (Manassas, VA). The RPMI-8226 and MDR10V lines were a gift from the laboratory of William Dalton (106). The OPM-II cell line was a gift from the laboratory of Brad Thompson (107). MM.1S, MM.1R$_e$, MM.1R$_L$, U266, RPMI-8226, and MDR10V cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL
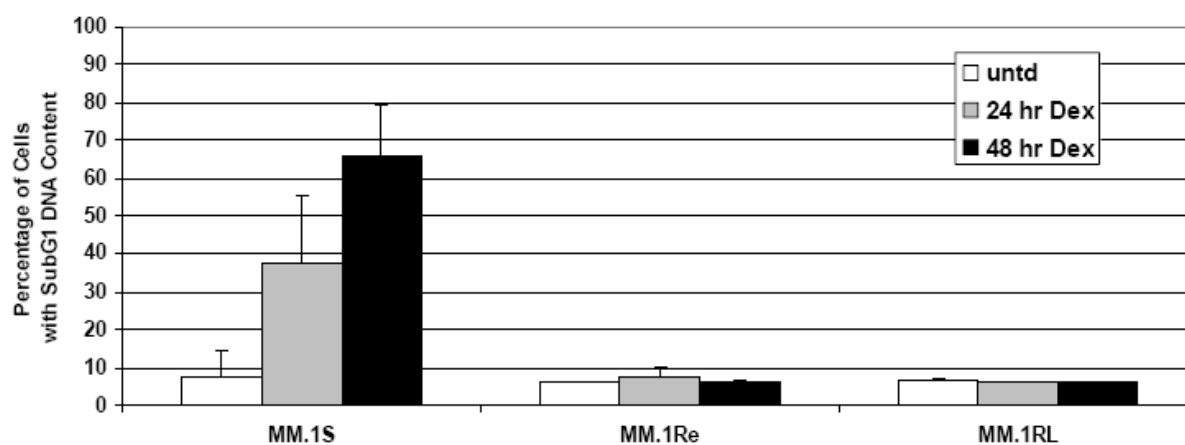
Figure 3.   MM.1 cell lines have varying sensitivity to GC.   MM.1S, MM.1R$_e$, and MM.1R$_L$ cells were treated with 10 μM Dex for 24-48 hours.   Cells were harvested, fixed overnight with ethanol, stained with propidium iodide, and analyzed by flow cytometry.   The percentage of apoptotic cells with sub-G1 content of DNA are presented.
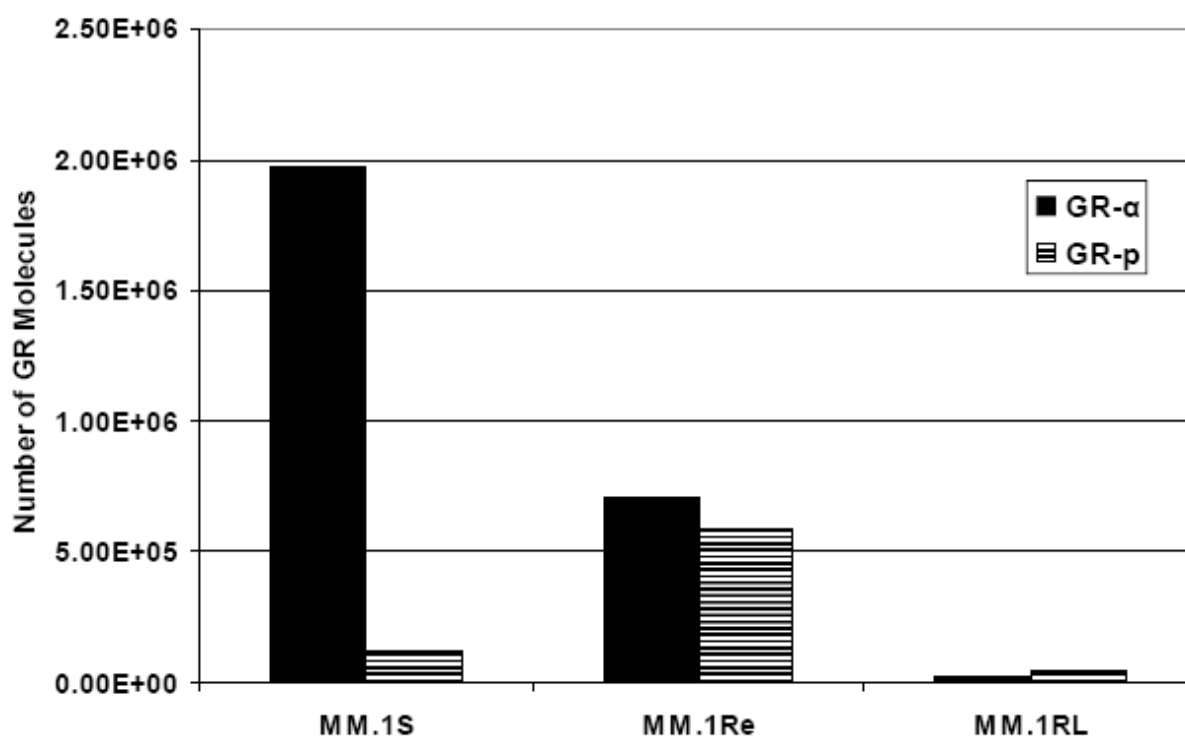
Figure 4. **Levels of GR-α and GR-P in MM.1 cell lines.** Total RNA was harvested from MM.1S, MM.1R$_e$, and MM.1R$_L$ cell lines. The number of GR molecules was measured in 20 ng RNA of each cell line using absolute quantification real time PCR following conversion to cDNA. The number of molecules was calculated following normalization to a standard curve generated using GR-α and GR-P plasmid cRNA.

penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 5 μg/mL Plasmocin (Invivogen, San Diego CA) in a 37°C incubator with 5% CO_2. The MDR10V are maintained with 0.1 μM Doxorubicin and 20 μM Verapamil in order to maintain the resistance phenotype. The OPM-II cells were cultured as above except the 10% fetal bovine serum was replaced with 15% Defined Premium Fetal Bovine Serum from Hyclone (Logan, UT).

HPV E6/E7 immortalized bone marrow stromal cell lines Hs5 and Hs27 were obtained from ATCC (108). Hs5 cells were cultured in DMEM (ATCC) media supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 5 μg/mL Plasmocin. Hs27 cells were cultured in modified RPMI-1640 (ATCC) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 5 μg/mL Plasmocin. Both lines were maintained in a 37°C incubator with 5% CO_2.

Clinical Patient Samples

Multiple myeloma patient cells were isolated from fresh bone marrow samples after informed consent. The bone marrow samples were diluted with media 1:1 and mononuclear cells were isolated with Ficoll/Histopaque 1077 (Sigma, St. Louis, MI). The population of myeloma cells was enriched by positive selection using CD138+ microbeads and automated magnetic cell sorting using an AutoMacs cell sorter (Miltenyi Biotec, Auburn, CA). The myeloma cells were maintained in short term culture ($0.1-0.4 \times 10^6$ cells per mL) in similar media conditions as MM.1S cells for 6 hour drug treatments before RNA isolation.

Chronic lymphocytic leukemia cells (CLL) and healthy donor peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples received after informed consent. The blood sample was diluted 1:1 with RPMI media and the mononuclear cells were isolated with Ficoll/Histopaque 1077. The cells were maintained in short term culture

(10x10^6 cells per mL) in similar media conditions to MM.1S cells for 6 hour drug treatments before RNA isolation.

Reagents

Dexamethasone, beclomethasone, beclomethasone DP, hydrocortisone, prednisolone, triamcinolone acetonide, triamcinolone, methylprednisolone, prednisone, wortmannin, mifepristone (RU486), thalidomide and ATRA were obtained from Sigma. LY294002, triciribine, AKT inhibitor VIII, SB203580, U0126, and PD98059 were purchased from Calbiochem (San Diego, CA). Recombinant proteins IL-6, IGF1, IL-2, IL-7, IL-10, TGFβ, and sonic hedgehog were purchased from R&D Systems, (Minneapolis, MN). Enzastuarin was obtained from Eli Lilly (Indianapolis, IN). The PARP antibody was obtained from BD Biosciences (San Jose, CA), GAPDH antibody from Chemicon (Billerica, MA), GR-α (H-300) antibody from Santa Cruz Biotechnologies (Santa Cruz, CA), FOXO3 antibody from Upstate (Lake Placid, NY), phospho-AKT antibody (Ser 473) from R&D, total AKT antibody from Cell Signaling (Beverly, MA) and the GILZ antibodies were obtained from Dr. Xu Cao (Figure 7 left panel only) (95) and Dr. Jane Eddleston (all GILZ blots except Figure 7 left panel) (96). All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Within this thesis project, a number of pharmacologic inhibitors to PI3-kinase and AKT were used. The PI3-kinase inhibitor LY294002 is a morpholino derivative of the broad spectrum kinase inhibitor quercetin and acts on the ATP binding site of PI3-kinase (109). Wortmannin, another PI3-kinase inhibitor, was originally isolated from soil bacteria and is an irreversible inhibitor of all PI3-kinase isoforms (110). Both LY294002 and wortmannin have been widely used in research studies to determine the molecular details of this important pathway, however due to lack of selectivity, instability, and solubility issues, both have limited use as clinical

therapeutics (25). Also used in these studies were two inhibitors of AKT, triciribine (also known as API-2 or NSC 154020) and AKT inhibitor VIII (also known as AKTi-1/2). Triciribine targets phosphorylation of all 3 AKT isoforms via an effector molecule other than PI3-kinase or PDK1 (111). AKT inhibitor VIII selectively inhibits AKT1/2 over AKT3 and is dependent on the pleckstrin homology domain of AKT (15).

Reverse-transcriptase PCR (RT-PCR)

Total RNA was isolated from MM.1S cells using the Qiagen Rneasy Mini Kit (Qiagen, Hilden, Germany). RNA (1 μg) was converted to cDNA using M-MuLV reverse transcriptase and Oligo-d(T)$_{16}$ primers (First Strand cDNA Synthesis Kit, Fermentas, Hanover, MD). GILZ was amplified using 1 μM specific primers with 1 unit Taq DNA Polymerase (Fermentas), in 2.5 mM MgCl$_2$, 0.5 mM dNTPs (Roche) and 1X reaction buffer (Fermentas). Amplification conditions were 94°C 2 minutes, 23 cycles of 94°C 30 seconds, 60°C 30 seconds, 72°C 60 seconds, followed by 72°C for 12 minutes. In each reaction, GAPDH was also amplified to serve as a normalization control. Control experiments were done to ensure that both templates were in the linear phase of amplification. Both cDNA and PCR reactions were run on a PTC-100 Thermal Cycler (MJ Research Inc., Watertown, MA). Sequences of all primers used are provided in Table 2.

Table 2.   Sequences of all RT-PCR and Real Time PCR primers and probes used.
RNaseP primers and probe are sold from ABI as a 20X master mix. Sequence details are not released.

RT-PCR	
GILZ forward primer	5'-CAGCCCGAGCCATGAACACC-3'
GILZ reverse primer	5'-CGCAGAACCACCAGGGGCCT-3'
Product size	410 base pairs
GAPDH forward primer	5'AGGTGAAGGTCGGAGTCAAC-3'
GAPDH reverse primer	5'-CGCTCCTGGAAGATGGTGAT-3'
Product size	232 base pairs
Real Time PCR	
GILZ probe	5'-6FAM-CGTTAAGCTGGACAACAG-3'
GILZ forward primer	5'-CACAATTTCTCCATCTCCTTCTTCT-3'
GILZ reverse primer	5'-TCAGATGATTCTTCACCAGATCCAT-3'
GR-α probe	5'-6FAM-AACTCTTGGATTCTATGCATGAA-3'
GR-α forward primer	5'-GCAGCGGTTTTATCAACTGACA-3'
GR-α reverse primer	5-AATGTTTGGAAGCAATAGTTAAGGAGAT-3'
GR-P probe	5'-6FAM-TTCAGGTTGGTAGAACAC-3'
GR-P forward primer	5'-CTCTGTATGAAAACCTTACTGCTTCTCT-3'
GR-P reverse primer	5'-CGAAAACTGTATTCCACTTTT-3'

Quantitative Real Time PCR

Total RNA was isolated from myeloma cell lines (0.25 – 2 million cells per sample depending on cell line and experiment) after 6 hr drug treatments using the Qiagen Rneasy Mini Kit (Qiagen). The samples were converted to cDNA using Multiscribe reverse transcriptase and random hexamers (TaqMan Reverse Transcription Reagents, Applied Biosystems, Foster City, CA) on a Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). Expression of *GILZ* was quantitatively determined using *GILZ* specific TaqMan MGB probe and primers (Table 2). The probe was designed using Primer Express (Applied Biosystems, Foster City, CA) to cross the junction of exon 1 and 2 to rule out genomic DNA contamination. 20 ng of cDNA was amplified using 900 nM primers, 250 nM probe in 1X TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Reactions were run in triplicate on the Applied Biosystems 7500 Fast Real-Time PCR System using the universal cycling parameters (20 seconds 95°C, 40 cycles of 3 seconds 95°C, 30 seconds 60°C). Parallel reactions were set up on the same plate analyzing *RNaseP* in each sample as an endogenous control (TaqMan RNaseP Detection Reagent, Applied Biosystems). The fold change in gene expression was calculated using the Relative Standard Curve Method (112). Briefly, on each plate a standard curve of MM.1S cDNA was run (5-80 ng cDNA per reaction) for *GILZ* and *RNaseP*. For each sample, the amount of *GILZ* or *RNaseP* was determined from the standard curve using the measured Ct value. The quantity of *GILZ* was divided by *RNaseP* for each to determine the normalized target quantity. The fold change from untreated for each sample was calculated by dividing the normalized target quantity for the treated sample by the normalized target quantity of the untreated control.

The absolute number of *GR* molecules was determined using specific *GR* Taqman probe and primers (Table 2) with similar reaction conditions as described above. The number of *GR*

molecules in each reaction sample was calculated by generating a standard curve using serial dilutions of cRNA. GR-α or GR-P cRNA of known size was generated by in vitro transcription using T7 RNA polymerase (Promega, RiboMax) according to the manufacturer's protocol. The cRNA was quantified by spectrophotometric measurement employing a NanoDrop. The number of RNA molecules was calculated and a dilution curve generated to span the range from 1×10^{11} molecules to 1×10^5 molecules. The standard curve cRNA was converted to cDNA by reverse transcriptase in parallel to the cDNA synthesis for the experimental samples. The isoform GR-α is often referred to as GR unless being compared directly to GR-P when it is called GR-α.

Western Blotting

Myeloma cells (5×10^6) were harvested post treatment, washed with 1X PBS and lysed with 35 μL of RIPA (100 mM sodium chloride (NaCl), 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride (NaF), 20 mM tetrasodium pyrophosphate ($Na_4P_2O_7$), 2 mM sodium orthovanadate (Na_3VO_4), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 60 μg/mL aprotinin, 1 μg/mL pepstatin, 10 μg/mL leupeptin) or PLB (0.5% Triton X-100, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1 mM EDTA, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM magnesium chloride ($MgCl_2$), 10% glycerol, 2.5 mM PMSF) lysis buffers. Lysates were incubated on ice for 90 minutes or subjected to 3 freeze-thaw cycles and centrifuged at 10,000 rpm for 10 minutes. Protein concentration of the supernatants was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) using a BSA standard curve of known protein concentration. 30 μg of total protein diluted with sample buffer (125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.05% bromophenol blue) was separated on a precast 8-16 % Tris-glycine gel (Invitrogen/Novex). Proteins were transferred to

a polyvinylidene difluoride (PVDF) membrane (Immobilion-P, Millipore, Bedford, MA). Following transfer, membranes were blocked with 5% nonfat milk in TBS with 0.1% Tween-20 before incubation with primary antibody (overnight 4°C or 1 hr room temperature). Blots were subsequently incubated with horseradish peroxidase-linked secondary antibodies (Amersham Biosciences/GE Healthcare) and developed using Enhanced Chemiluminescence Plus Western Blotting Detection reagent (Amersham Biosciences/GE Healthcare). Blots were stripped with Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL) in order to reprobe. When blotting for GILZ, 100 mM DTT was added to the 3X sample buffer, a specialized PVDF for low molecular proteins was used (Immobilon-P^{SQ}, Millipore), the proteins were transferred from gel to membrane overnight at 4°C at 10 volts, and the blots developed using SuperSignal West Femto ECL (Pierce Biotechnology, Rockford, IL).

Propidium Iodide Staining

Propidium iodide (PI) intercalates within double-stranded nucleic acid and can be used to stain the DNA content of cells. DNA fragmentation occurs in the nuclei of cells undergoing apoptosis and when stained with PI will have a "sub-G1" population of DNA (less than 2N) (113). MM.1S cells (1-2 x 10^6) were treated for 15 - 48 hours with Dex (1 μM). After harvesting, cells were washed with PBS and fixed overnight with 40% ethanol in PBS to permeabilize the cell membrane. On the day of staining, the cells were washed with PBS, incubated for 30 minutes at 37°C with 50 μg/mL RNaseA in PBS, and then stained with 43 μg/mL propidium iodide in 38 mM sodium citrate buffer. The cells were analyzed using an Epics Profile II flow cytometer (Coulter Electornics, Inc., Hialeah, FL) and the percentage of cells with a sub-GI DNA content identified.

Annexin V Staining

The loss of plasma membrane asymmetry is an early event in the apoptotic cascade. The membrane phospholipid phosphatidylserine translocates from the inner leaflet of the plasma membrane to the outer leaflet. Annexin V is a calcium-dependent phospholipid binding protein with a high affinity for phosphatidylserine and when conjugated to a fluorochorme is used to detect cells undergoing this early apoptotic event (114). Myeloma cells were harvested after drug treatment and washed twice with PBS. Cells were resuspended at 1 x 10^6 cells/mL in 1X Annexin V Binding Buffer. 1 x 10^5 cells were stained with 5 µL of AnnexinV-PE and 5 µL of 7-amino actinomycin D (7-AAD) for 15 minutes at room temperature in the dark. 400 µL of 1X Annexin V Binding Buffer was added and the cells were analyzed using an Epics Profile II flow cytometer (Coulter Electronics, Inc, Hialeah, FL). 7-AAD is similar to PI and can permeate the membrane of dying cells to intercalate and stain DNA. Cells that stain positive for Annexin V-PE and negative for 7-AAD are undergoing apoptosis. Cell that stain positive for both are either in late state apoptosis, necrosis, or already dead (BD Biosciences). Where noted, Annexin V-FITC and propidium iodide was substituted for Annexin V-PE and 7-AAD.

Synergy Analysis

The combination treatment of Dex and LY294002 was analyzed for synergism using the commercially available software CalcuSyn and the median effect plot equation (BIOSOFT, Ferguson MO) (115). Combinatorial indexes (CI) of less than 1.0 indicate synergism, greater than 1.0 antagonism, and equal to 1.0 additive. MM.1S cells were exposed to increasing concentrations of Dex and LY294002 at a fixed ratio (1:25) for 24 hours. Apoptosis was quantified by AnnexinV-PE/7-AAD staining as described above and the apoptotic values used to determine synergism.

siRNA Knockdown

ON-TARGET plus SMARTpool siRNA to GILZ (Human TSC22D3) and FOXO3 (Human FOXO3A) was purchased from Dharmacon (Lafayette, CO) along with ON-TARGET plus siCONTROL non-targeting pool to be used as a control. MM.1S cells were transfected using nucleofection technology (amaxa biosystems, Cologne, Germany). 5 million cells were transfected with $1 - 2$ µM siRNA oligomers per amaxa cuvette using program O23 and solution V following the manufacturers instructions. Following transfection, the cells were aliquoted to 1 T25 flask (entire cuvette) or 1 well of 6 well plate (half of cuvette) and allowed to recover for 24 hours before drug treatment and subsequent apoptosis assays. If multiple cuvettes of the same siRNA construct were used, the samples were pooled prior to aliquoting in order to normalize the uptake efficiency among cuvettes. Gene knock-down was monitored by real time PCR (*GILZ)* and western blotting (GILZ/FOXO3). Using a FITC-labeled dsRNA oligomer (BLOCK-iT^{TM} Fluorescent Oligo, Invitrogen), update of siRNA oligomers by MM.1S was consistently measured to be greater than 90%. The siRNA sequences used are given in Table 3.

MTS Assay

To measure the effect of IL-6 and IGF1 on Dex-induced killing, MM.1S cells were counted and diluted to 500,000 cells per mL with media. The cells were mixed 1:1 with solutions containing increasing concentrations of Dex, IL-6, and/or IGF1 resulting in final concentrations of $0 - 1$ µM Dex, $100 - 250$ ng/mL IGF1, and 5 -100 ng/mL IL-6. The cell solutions were aliquoted (100 µL, 25,000 cells/well) to 4 wells of a 96 well plate and incubated for 72 hours before measuring the number of live cells using the MTS Cell Titer AQ$_{ueous}$ assay (Promega, Madison, WI). The MTS assay uses a 20:1 reagent mixture of a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymehoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Table 3. **Sequences of siRNA constructs used in these studies.**

GILZ SMARTpool siRNA	Dharmacon L-021438-01-0005
Sense	5'-GCUUUGGGAUGACCGCUUAUU
Antisense	5'-PUAAGCGGUCAUCCCAAAGCUU
Sense	5'ACUCCCAGCUAGAGCGUGAUU
Antisense	5'-PUCACGCUCUAGCUGGGAGUUU
Sense	5'-GUAUCUUAGUGUAGCGAUGUU
Antisense	5'-PCAUCGCUACACUAAGAUACUU
Sense	5'-GCUUGUCUGUAGCGGGUUUUU
Antisense	5'-PAAACCCGCUACAGACAAGCUU
FOXO3A SMARTpool siRNA	Dharmacon L-003007-00-0005
Sense	5'-GCACAGAGUUGGAUGAAGUUU
Antisense	5'-PACUUCAUCCAACUCUGUGCUU
Sense	5'-GUACUCAACUAGUGCAAACUU
Antisense	5'-PGUUUGCACUAGUUGAGUACUU
Sense	5'-CGAAUCAGCUGACGACAGUUU
Antisense	5'-PACUCUCGUCAGCUGAUUCGUU
Sense	5'-UAACUUUGAUUCCCUCAUCUU
Antisense	5'-PGAUGAGGGAAUCAAAGUUAUU

(MTS) and an electron coupling reagent, phenazine methosulfate (PMS). Dehydrogenase enzymes in metabolically active cells convert MTS to formazan. 20 µL of the mixture is added to each well of the 96 well plate and incubated for 4 hours in the dark at 37°C. The accumulation of formazan is measured by reading the absorbance at 490 nM using a Synergy HT plate reader (BioTek, Winooski, VT). The amount of formazan product is directly proportional to the number of metabolically active cells in culture. The data is normalized to the amount of formazan in the untreated control cells and expressed as a percentage.

<div align="center">**Bone Marrow Stromal Cell Co-culture**</div>

Hs5 or Hs27 stromal cells were plated (0.5×10^6 cells) in 2 mL of respective culture media in 6 well dishes and allowed to adhere overnight. The following day, MM.1S cells were counted and resuspended in Hs5 or Hs27 culture media so that 1×10^6 cells were plated on top of the stromal cells in 0.5 mL media (final culture volume 2.5 mL). After 24 hours of co-culture, Dex and/or LY294002 was added for 6 hr treatments before the MM cells were harvested by gently pipetting and removing the media from the culture dish taking care not to disturb the adherent layer of stromal cells. Cells were washed with PBS and then RNA was isolated from the MM cells and real time PCR analysis for *GILZ* was done as above. As a control, MM cells were also plated into empty wells containing Hs5 or Hs27 media so that the effect of the stromal cells on *GILZ* induction in MM.1S could be measured.

In some cases, the MM cells were plated into a transwell inset with high pore density translucent PET membrane (BD Falcon™ cell culture insert, 0.4 micron pore size, 1×10^8 pores cm^2, BD Biosciences) to separate the two cell types between a porous membrane. The pores in the membrane allow for the passage of media components, but not cells. Prior to adding the MM cells, the transwell inserts were added to the stromal cell cultures to equilibrate the membrane.

The MM cells were added in 1 mL of media and 1 mL of media from the bottom of the well was transferred to the transwell after the MM cells were added to the filter. Cells were treated as above after 24 hours of co-culture. To harvest the MM cells in the transwell insert, the media was gently pipetted up and down to loosen the cells with care taken not to tear the transwell membrane.

Statistical Analysis

All data presented in this dissertation are the result of at least three independent experiments unless otherwise noted. Error bars represent the first standard deviation from the mean. Drug treatments and gene analysis on the clinical patient samples were only done once per patient due to availability. The real time PCR analysis was performed multiple times on the isolated RNA to provide the error bars. The two tailed p value calculated for Figure 20 was determined using a paired t test and the software GraphPad InSTAT3 (San Diego, CA).

CHAPTER 3

CHARACTERIZATION OF GLUCOCORTICOID REGULATION OF GILZ

GILZ is up regulated by GCs in MM cells and requires the GR.

Prior to commencement of these studies, DNA micro-array analysis of the MM.1S cell line following a 6 hour treatment with the synthetic GC, Dexamethasone (Dex), identified *GILZ* as a GC-induced gene (80). In order to confirm these findings, GILZ mRNA levels were measured by quantitative real time PCR following Dex treatment and a potent concentration dependent increase in GILZ levels was observed at 6 hours (Figure 6). The concentration of Dex used to induce GILZ levels is consistent with the concentration required for GCs to induce apoptosis in these cells. In parallel to the GC-induced increase in *GILZ* mRNA, the effect of GC on GILZ protein level was also assessed. A GC-induced increase in GILZ protein level was observed upon 1-10 µM Dex treatment and western blotting with two different GILZ antibodies. Increased protein levels were detected as early as 1 hour after treatment, peaked at 4-6 hours after treatment, and persisted for at least 48 hours (Figure 7).

To test whether the GR is required for the up regulation of *GILZ* expression, the GR antagonist RU486 was utilized. If the receptor is required, the addition of RU486 would inhibit the GC-induced expression of *GILZ*. Using RT-PCR and real time PCR to assess *GILZ* expression, 10 µM of RU486 was able to inhibit Dex-induced up regulation of *GILZ* (25 fold with Dex compared to 10 fold with Dex + RU486 or 7 fold with RU486 alone) (Figures 8-9). RU486 has been reported in the literature to act as a partial agonist in other cell types and a 7 fold increase in *GILZ* in MM.1S cells treated 10 µM RU486 alone was observed (116, 117). A similar effect is observed at the protein level where RU486 alone slightly up regulates GILZ, but can block Dex-induced up regulation of GILZ (Figure 10). The ability of Dex to up regulate *GILZ* was tested in a panel of other myeloma cell lines. These cells have varying levels of GR as
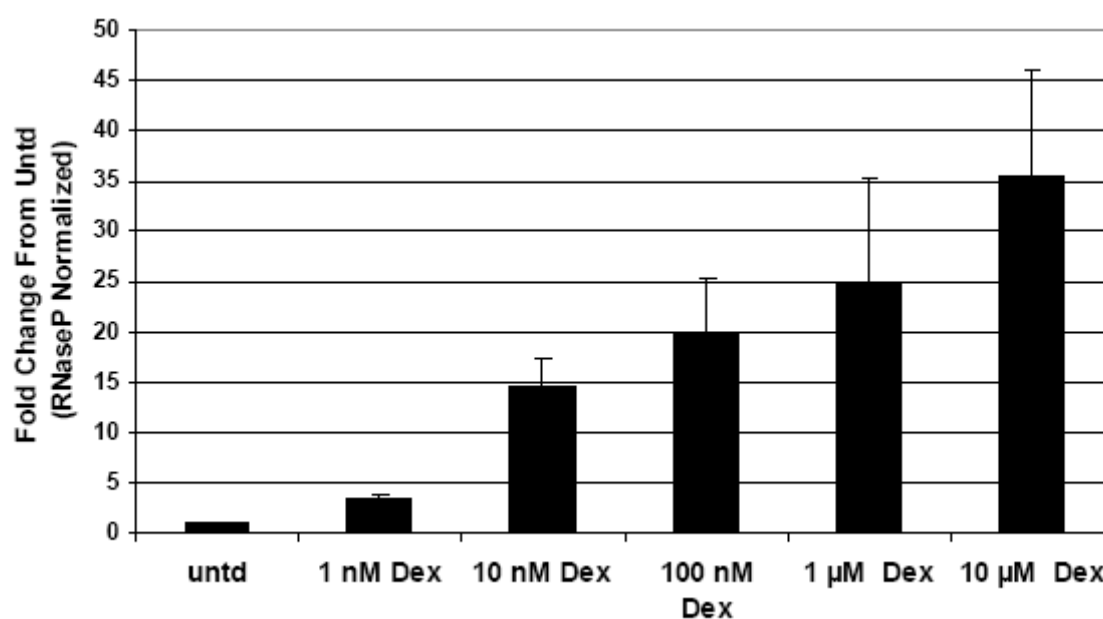
Figure 6. **Dose response increase in *GILZ* expression upon GC treatment.** Real time PCR of total RNA isolated from MM.1S cells after 6 hours of Dex treatment. *GILZ* expression levels were normalized to *RNaseP* levels in each sample and expressed as fold change from untreated sample.
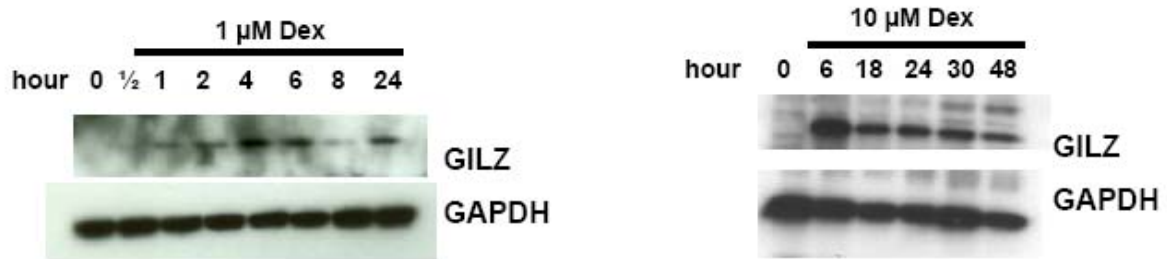
Figure 7. **Time course of induction of GILZ upon GC treatment.** Western blot for GILZ in whole cell lysates of MM.1S cells treated with 1 µM (left blot) or 10 µM (right blot) Dex for indicated time. GAPDH was probed as loading control.
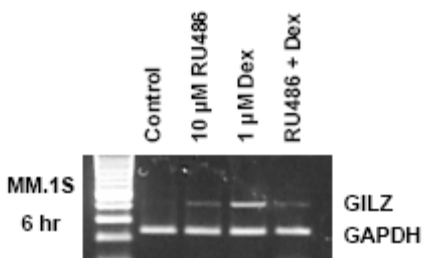
Figure 8.   **RU486 can inhibit Dex-induced up regulation of *GILZ* mRNA.** MM.1S cells

were treated with 10 µM RU486 and/or 1 µM Dex (RU486 was added 1 hour before Dex). Total

RNA was isolated after 6 hour treatment and *GILZ* up regulation was measured by RT-PCR.

GAPDH was used as a normalization control for RT-PCR.
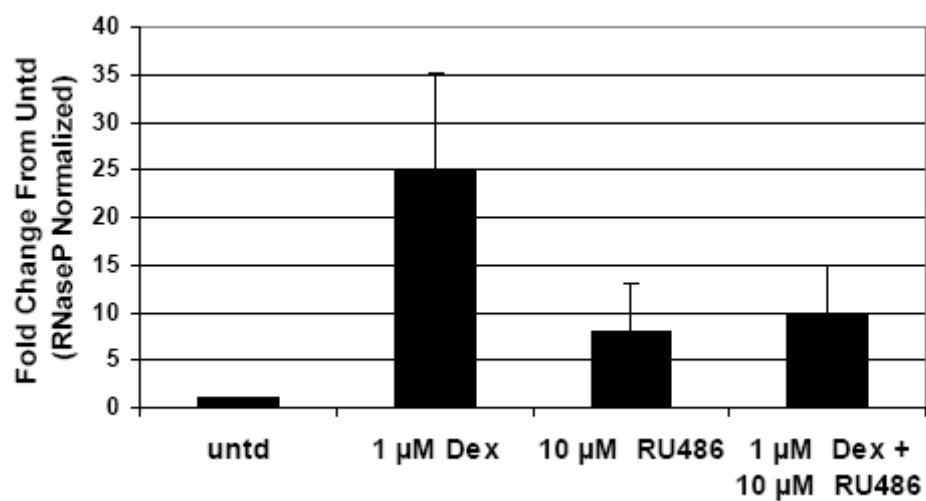
Figure 9.   **RU486 can inhibit Dex-induced up regulation of *GILZ* mRNA.** MM.1S cells

were treated with 10 μM RU486 and/or 1 μM Dex (RU486 was added 1 hour before Dex). Total

RNA was isolated after 6 hour treatment and *GILZ* up regulation was measured by real time

PCR. *RNaseP* was used as a normalization control for real time PCR.
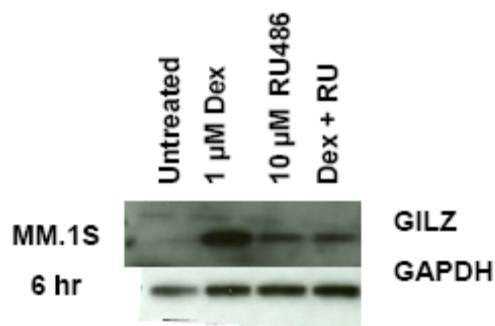
Figure 10. **RU486 can inhibit Dex-induced up regulation of GILZ protein.** Western blot of whole cell extracts of MM.1S cells treated with 10 µM RU486 and/or 1 µM Dex (RU486 was added 1 hour before Dex) for 6 hours. GILZ protein induction was measured using a GILZ specific antibody and GAPDH levels were measured to ensure equal protein loading.

shown with absolute quantification real time PCR in Figure 11. MM.1S cells were treated with

1 μM Dex for 6 hours and the extent of GILZ up regulation was measured with real time PCR.

A fold change increase 10 fold or greater in *GILZ* levels upon Dex treatment was observed in

MM.1S, OPM-II, MDR10V, and RPMI-8226 cells lines, but not in the MM.1R$_e$, MM.1R$_L$, and

U266 cells lines (Figure 12). As shown in Figure 11, MM.1R$_e$ and U266 have a decreased

number of GR molecules compared to the other cell lines tested and MM.1R$_L$ cells have almost

no measurable GR message. This cell line data in combination with the experiments using the

GR antagonist RU486 suggest that the GR is necessary for GC regulation of GILZ. The

correlation between level of GR expression and extent of *GILZ* up regulation is not exact as the

greatest up regulation of *GILZ* is observed in the RPMI-8226 cells, whereas the highest level of

GR message is observed in the MDR10V line. However the trend observed with this data

supports the conclusion that the GR is required for *GILZ* up regulation by GCs.

To assess the glucocorticoid specificity of *GILZ* up regulation, the ability of additional

glucocorticoids other than Dex to induce *GILZ* transcription in MM.1S cells was examined.

GILZ expression was also up regulated by treatment with beclomethasone, beclomethasone DP,

hydrocortisone, prednisolone, methylprednisolone, triamcinolone acetonide, and triamcinolone

(Figure 13). Treatment with all of these GCs resulted in an increase in the subG1 DNA content

of MM.1S cells as measured with PI staining, one hallmark of cell death (Figure 14). The only

GC tested that did not up regulate *GILZ* was prednisone which requires conversion to its active

form by the liver and cannot cause the death of MM.1S cells *in vitro*.
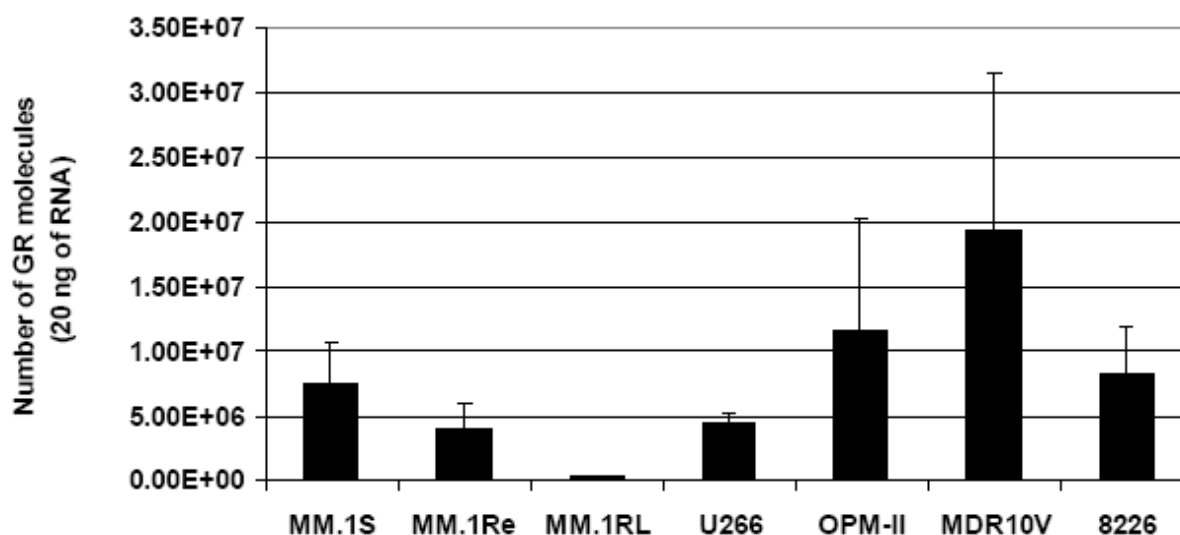
Figure 11. **MM cell lines have varied levels of GR molecules.** Total RNA was harvested from MM.1S, MM.1R$_e$, MM.1R$_L$, U266, OPM-II, MDR10V, and RPMI-8226 MM cell lines. The number of GR- α molecules was measured in 20 ng RNA of each cell line using absolute quantification real time PCR following conversion to cDNA. The number of molecules was calculated following normalization to a standard curve generated using GR-α plasmid cRNA.
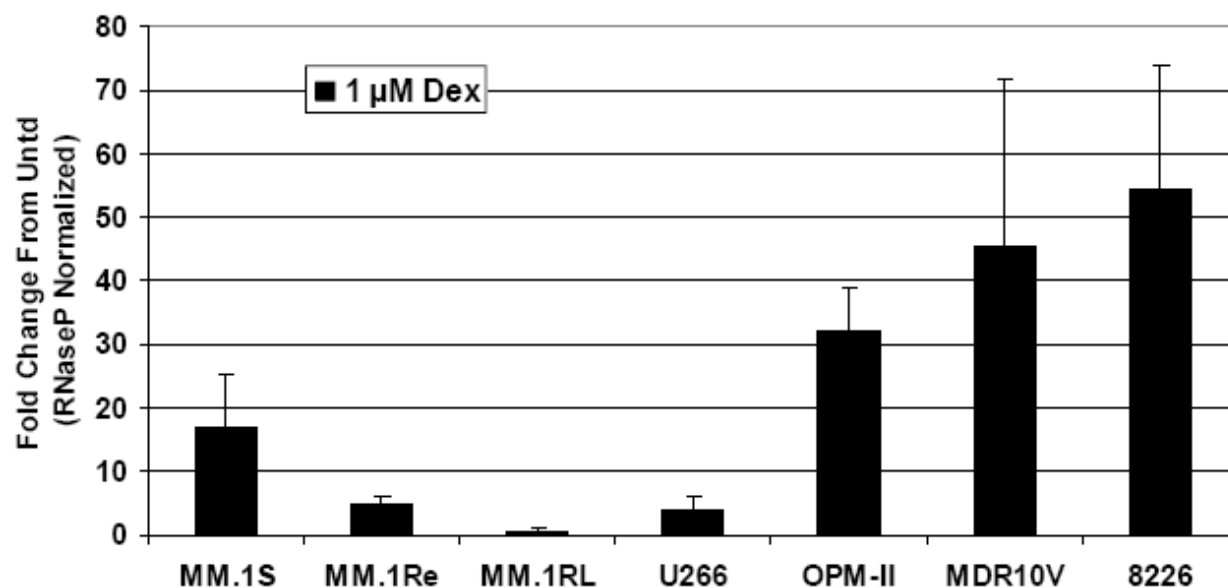
Figure 12. **Induction of *GILZ* by Dex treatment in MM cell line panel.** MM cells (0.25 x 10^6) were treated with 1 µM Dex for 6 hours and total RNA was isolated following harvesting. Up regulation of *GILZ* was measured using real time PCR and expressed as fold induction from the untreated sample of each cell type following normalization to *RNaseP*.
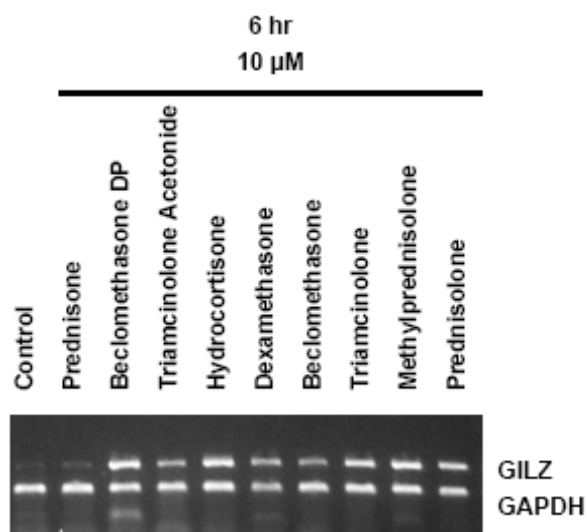
Figure 13. **GILZ is up regulated by a panel of GCs.** MM.1S cells were treated with 10 µM of indicated GCs for 6 hours. Following treatment, the cells were harvested and total RNA isolated. GILZ induction was measured using RT-PCR and GAPDH was used as a normalization control.
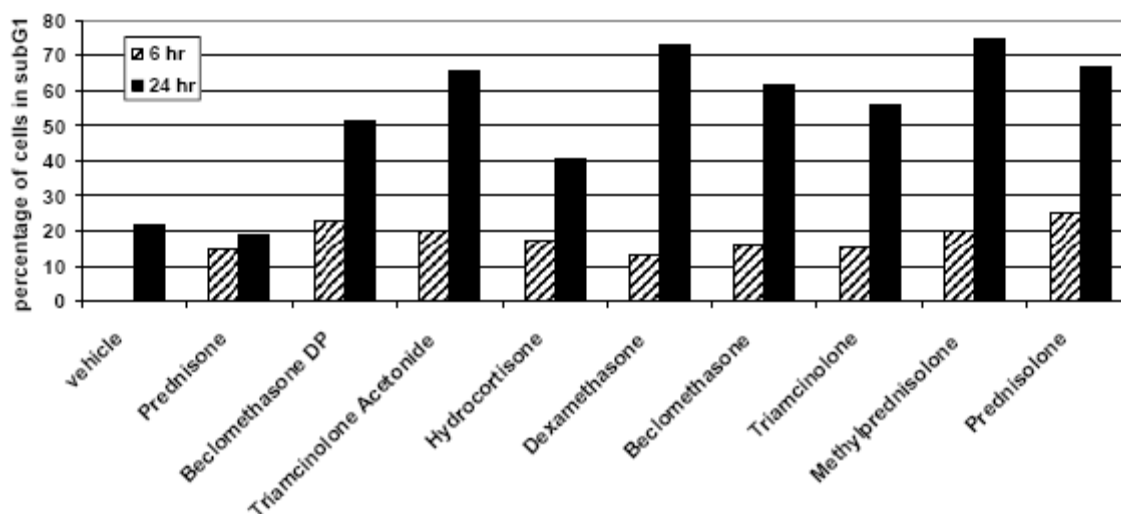
Figure 14. **Cytotoxicity of various GCs to MM.1S cells.** MM.1S cells were treated with 10 μM of indicated GC or vehicle (10 μL ethanol) for 6 or 24 hours. Cells were harvested, fixed overnight with ethanol, and stained with propidium iodide. The percentage of apoptotic cells with sub-G1 content of DNA are presented.

GILZ is up regulated by GCs in patient cells.

MM cells were isolated from fresh bone marrow biopsies obtained from patients in the clinic. The amount of GR was measured in each patient sample and heterogeneity in GR level was observed (Figure 15). In order to determine if GC treatment resulted in *GILZ* up regulation in MM patient cells, short term *in vitro* cultures were set up following MM cell enrichment from the bone marrow samples. As shown in Figure 16, up regulation of *GILZ* upon Dex treatment occurred in 3 of the 4 samples tested. The extent of up regulation ranged from 4 – 22 fold in the three responding samples. Patient 4 did not respond to GC treatment, however as shown in Figure 15, the level of GR in this sample was almost undetectable supporting the requirement of GR to mediate GC function. There was not enough sample from patient 2 isolated to measure the GR level, however based on patients 1, 3, and 4, a correlation between GILZ upregulation and GR level can be made, though patient 1 had the greatest level of *GILZ* induction while patient 3 had the highest amount of GR.

In order to determine if GC can regulate *GILZ* in another B cell malignancy, samples were also obtained from patients in the clinic with chronic lymphocytic leukemia (CLL). Malignant cells can be purified from the peripheral blood of these patients and short term *in vitro* cultures of purified CLL patient cells were treated with Dex and the extent of *GILZ* up regulation was measured by real time PCR. As shown in Figure 17, the level of GR in the CLL patients varied from sample to sample, but all contained a level of GR lower than the MM patients samples. In order to consider the GC effects on *GILZ* in a normal cell population, peripheral blood mononuclear cells were isolated from a healthy donor and included with this sample set. The level of GR in the PBMC sample was in the same range as the MM cells. *GILZ* levels were increased 2 – 5 fold upon 6 hour incubation with Dex in all of the CLL patient samples and 10
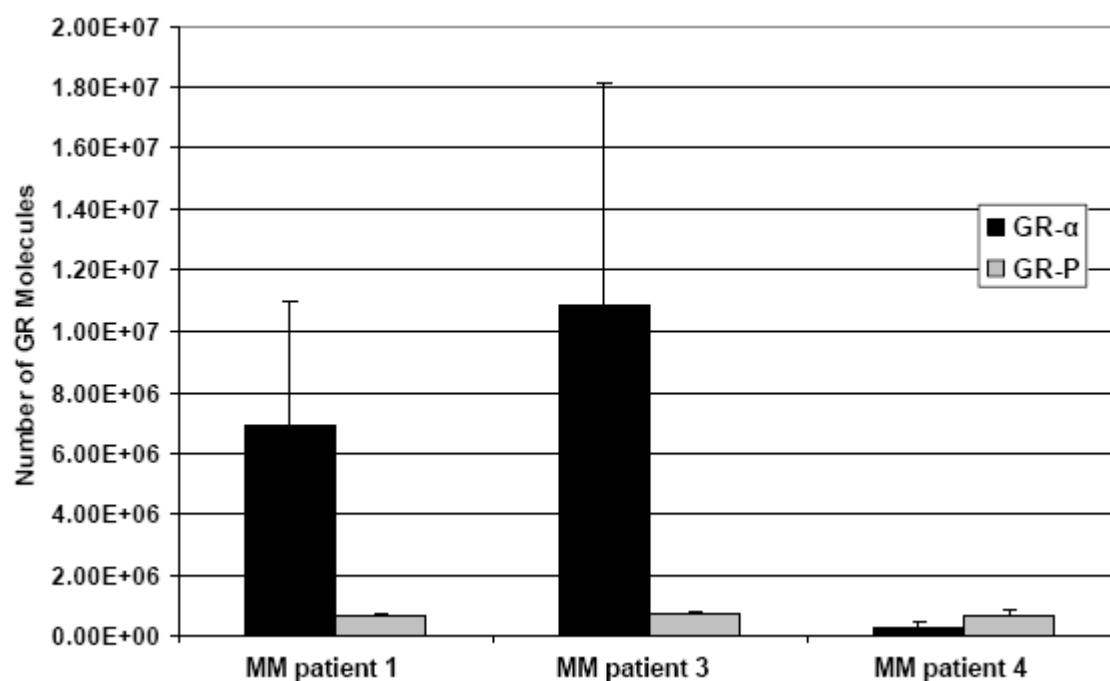
Figure 15. **GR-α and GR-P levels of MM patient samples.** Total RNA was isolated from purified MM patient samples and the levels of GR-α and GR-P in each patient were determined with absolute quantification real time PCR. The number of GR molecules of each isoform were determined by normalizing to a standard curve of cRNA of known size.
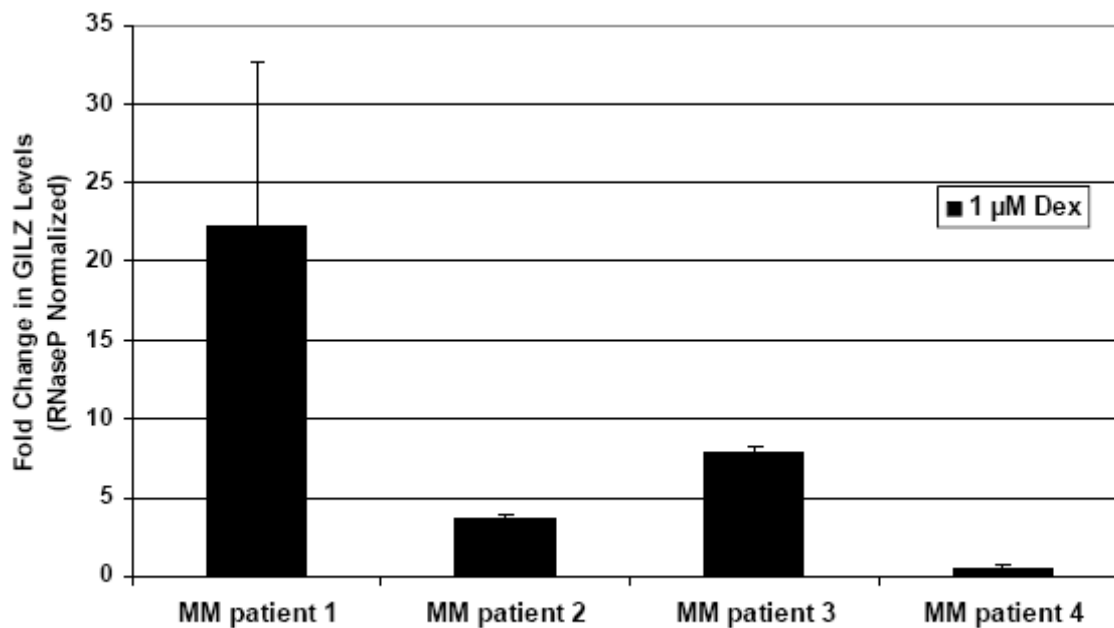
Figure 16. *GILZ induction in MM patient samples.* MM cells isolated from bone marrow patient samples obtained from the clinic were treated for 6 hours with 1 μM Dex. Total RNA was isolated following harvest and the level of *GILZ* induction was measured using real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from the untreated level in each patient sample.
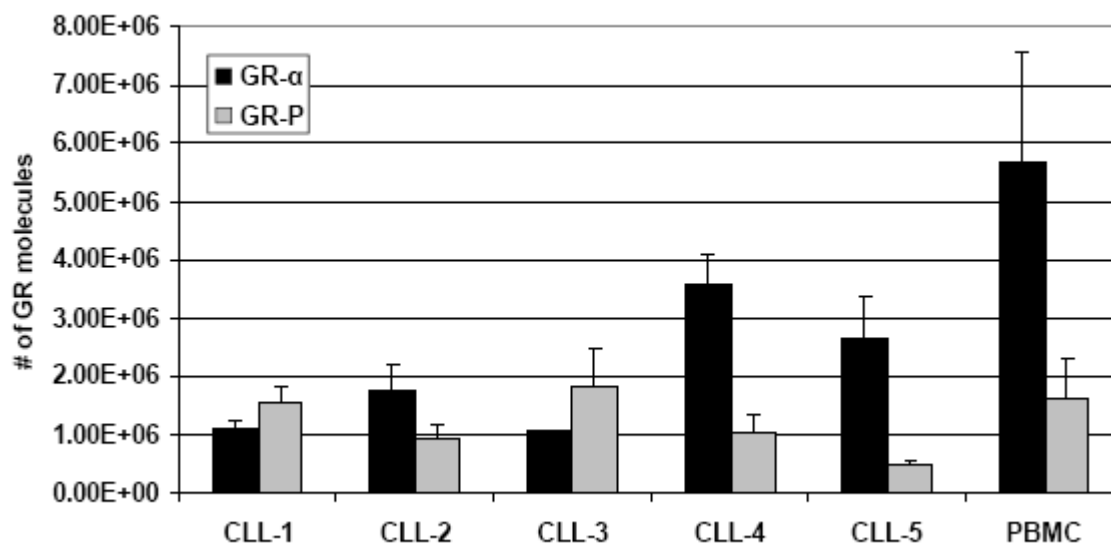
Figure 17. **GR-α and GR-P levels of CLL patient and normal PBMC samples.** CLL cells were isolated from peripheral blood samples obtained from the clinic. PBMCs were isolated from peripheral blood sample of a healthy donor. Total RNA was isolated and the levels of GR-α and GR-P in each patient were determined with absolute quantification real time PCR. The number of GR molecules of each isoform were determined by normalizing to a standard curve of cRNA of known size.

fold in the PBMC sample (Figure 18). The amount of *GILZ* induction in the CLL patient samples roughly parallels the amount of measured GR-α. This is a similar observation to what was observed with the MM patient samples linking the level of GR to the amount of *GILZ* induction.

From the data presented in this chapter, it can be concluded that *GILZ* is a GC-target gene regulated by GC in a variety of MM cell lines and patients samples. This regulation occurs at both the mRNA and protein level and is mediated by the GR. Regulation by GC was also observed in a different B cell malignancy, CLL, and in normal PBMCs. *GILZ* is potently up regulated in MM cells sensitive to GCs (MM.1S), but not in resistant lines (MM.1R$_e$ and MM.1R$_L$) and by GCs which are cytotoxic to MM cell lines, but not by those that are not. This provides additional support for the hypothesis that GILZ is important to the process of GC-induced apoptosis.
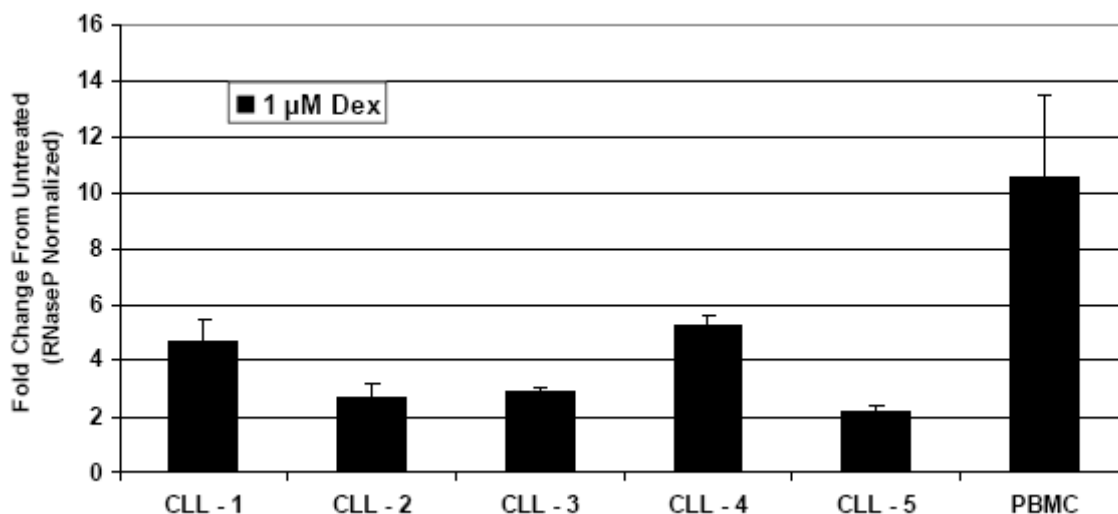
Figure 18. *GILZ* **induction in CLL patients and normal PBMC samples.** CLL cells were isolated from peripheral blood samples obtained from the clinic. PBMCs were isolated from peripheral blood sample of a healthy donor. Isolated cells were set up in short term *in vitro* culture and treated for 6 hours with 1 μM Dex. Total RNA was isolated following harvest and the level of *GILZ* induction was measured using real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from the untreated level in each patient sample.

CHAPTER 4

IMPORTANCE OF GILZ AS A MARKER AND MEDIATOR OF GLUCOCORTICOID-

INDUCED APOPTOSIS

Correlation between GILZ up regulation and GC-induced killing

The studies presented in Chapter 3 reported that GILZ up regulation by GCs occurs in a variety of MM cells lines and in primary cells from MM and CLL patients. This next set of studies were designed to elucidate the functional significance of GILZ up regulation. Specifically the contribution of GILZ to GC-induced apoptosis was considered. The same panel of MM cell lines used in Figure 12 to confirm *GILZ* up regulation in other MM cells lines was tested to observe their sensitivity to GC killing and determine if there was a correlation between level of *GILZ* up regulation and sensitivity to GC killing. The order of *GILZ* up regulation upon 6 hr 1 μM Dex treatment from highest to lowest fold change was RPMI-8226 > MDR10V > OPM-II > MM.1S > MM.1R$_e$ > U266 > MM.1R$_L$ (Figure 12). As shown in Figure 17, MM.1S cells are much more sensitive to GC-killing as measured here with PI staining than the RPMI-8226, MDR10V, and OPM-II. This suggests that while there is a general agreement between higher *GILZ* induction and GC-induced apoptosis, it is not a perfect correlation suggesting that other differences in the cell lines may contribute to sensitivity to GCs.

Knockdown of GILZ reduces GC-induced apoptosis in MM.1S

In order to determine if GILZ participates in GC-induced apoptosis of MM cells, experiments to reduce the levels of GILZ in MM cells were done and the effect on apoptosis was monitored. Baseline *GILZ* levels were reduced at the mRNA level upon transfection with GILZ siRNA to approximately 50% the level of GILZ in control siRNA transfected cells. Treatment with Dex resulted in *GILZ* up regulation even in the GILZ siRNA transfected cells, however the extent of up regulation was reduced (9 fold increase in GILZ siRNA transfected cells, 30 fold increase in control siRNA transfected cells) (Figure 20). Similarly, GILZ protein levels were
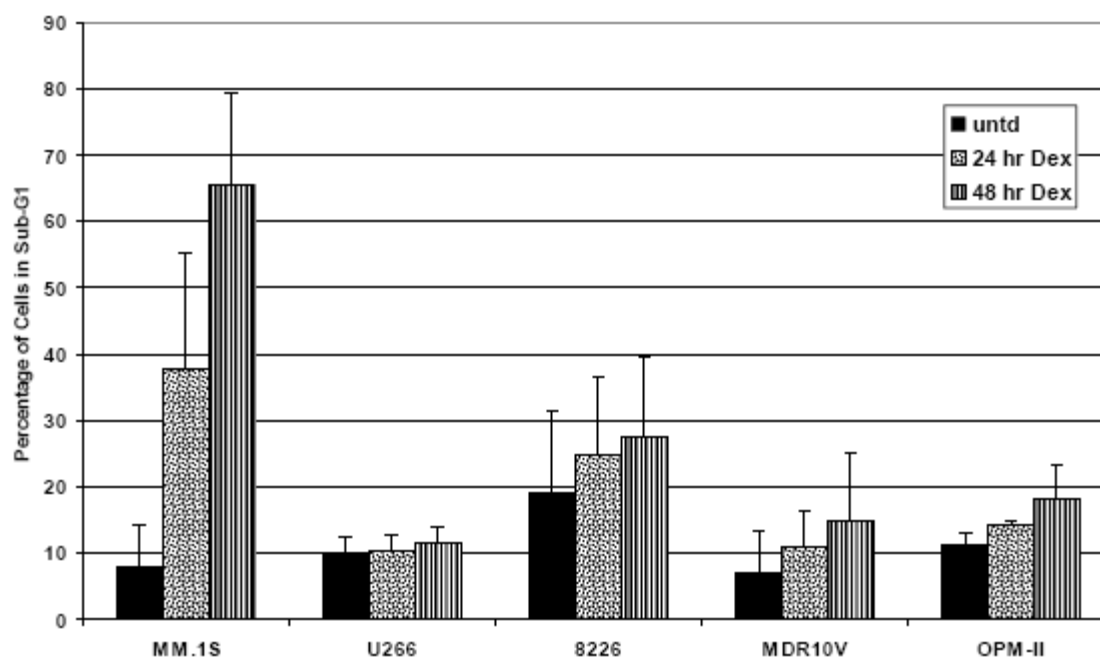
Figure 19.   MM cell line panel has varying sensitivity to GC-induced killing. MM.1S, U266, RPMI-8226, MDR10V, and OPM-II cell lines were treated with 10 µM Dex for 24-48 hours. Cells were harvested, fixed overnight with ethanol, stained with propidium iodide, and analyzed by flow cytometry. The percentage of apoptotic cells with sub-G1 content of DNA are presented.

reduced in the GILZ siRNA cells compared to control siRNA cells where a faint GILZ band can be seen at baseline. Up regulation of GILZ at the protein level occurred in both samples, however the extent of GILZ increase was decreased in the GILZ siRNA cells compared to control siRNA (Figure 21).

GILZ siRNA transfected cells were treated with 1 μM Dex for 48 hours and the percentage of cells undergoing apoptosis was measured with Annexin-V/PI staining and compared to control siRNA transfected cells. There was a consistent 10% reduction in apoptosis in the cells where *GILZ* levels were reduced 50% compared to the control siRNA cells that was shown to be statistically significant (Figure 22). Whole cell lysates were prepared from these samples and the extent of poly (ADP-ribose) polymerase (PARP) cleavage was compared in the cells with reduced GILZ to normal cells. PARP cleavage is a common marker of apoptotic induction and caspase activation. A visible reduction in full length PARP was observed after 48 hours of Dex treatment in the control siRNA transfected cells that was not observed in cells transfected with GILZ siRNA (Figure 23). Thus it can be concluded that GILZ contributes to GC-induced apoptosis in MM.1S cells. Because there was only a 50% reduction in baseline *GILZ* levels and the fact that GILZ could still be increased upon GC treatment at both the mRNA and protein level albeit to a lower level than the control siRNA cells, it is not surprising that the difference in percentage of apoptotic cells was only 10% as there is still some GILZ protein in the GILZ siRNA transfected cells. However these results are an encouraging beginning to elucidating the functional importance of GILZ in MM cells and GC-induced apoptosis.
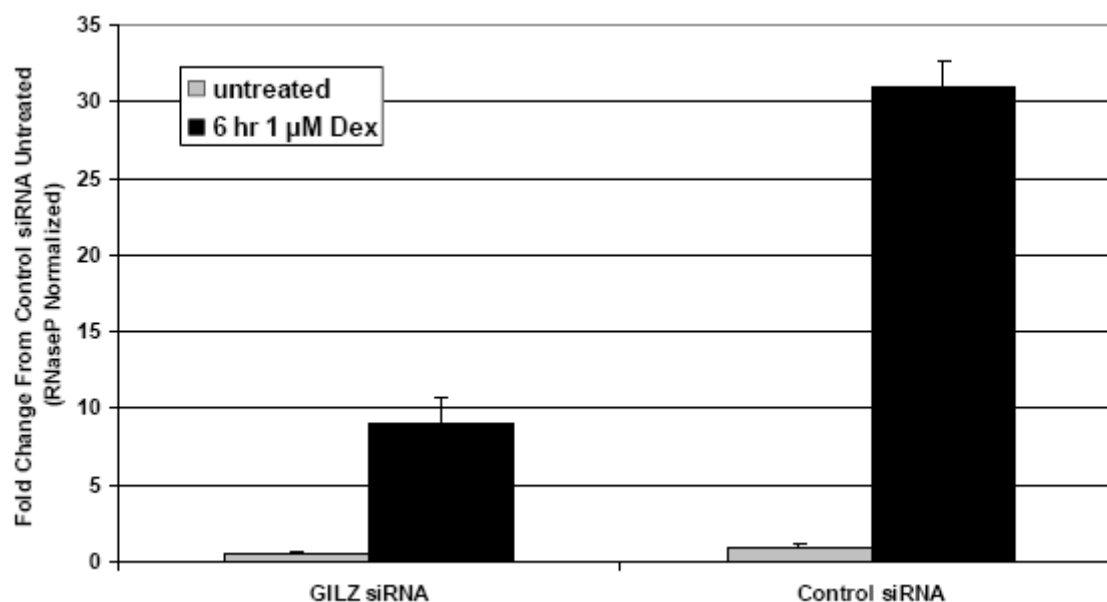
Figure 20. *GILZ* **knockdown with GILZ siRNA.** MM.1S were transfected with GILZ SMARTpool siRNA or control SMARTpool siRNA. 2 days after transfection, cells were treated with 1 μM Dex for 6 hr. Total RNA was isolated and the level of *GILZ* measured by real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from untreated control siRNA transfected cells.
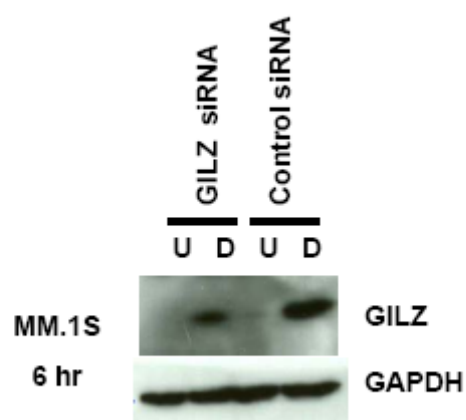
Figure 21. **Extent of GILZ protein knockdown with GILZ siRNA.** MM.1S cells were transfected with GILZ SMARTpool siRNA or control SMARTpool siRNA. 2 days after transfection, cells were treated with 1 μM Dex for 6 hr. Whole cell lysates were prepared following harvesting and GILZ protein levels determined with western blotting. GAPDH was probed for as a corresponding loading control.
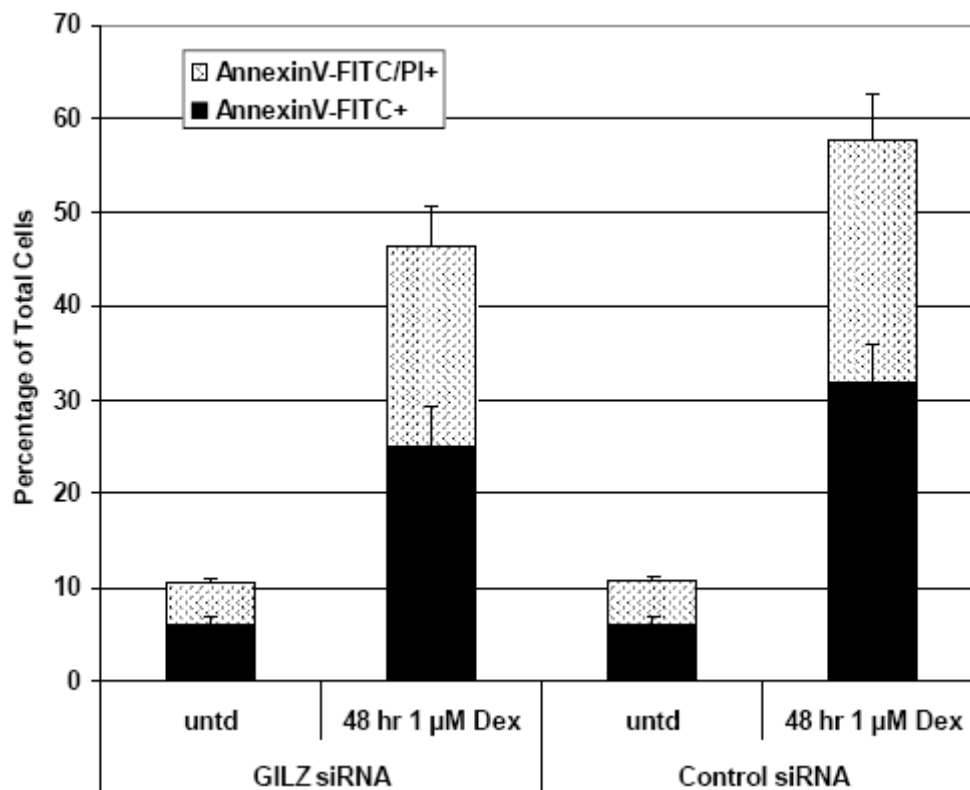
Figure 22.   **GILZ knockdown reduces GC-induced killing of MM cells.**   MM.1S cells were transfected with GILZ SMARTpool siRNA or control SMARTpool siRNA. 24 hours after transfection, cells were treated with 1 μM Dex for 48 hours. The percentage of cells undergoing apoptosis was determined by Annexin-V-FITC/PI Staining.   Cells staining Annexin-V-FITC+/PI- are early stage apoptotic and cells staining Annexin-V-FITC+/PI+ are late stage apoptotic or necrotic.   The difference between the control siRNA and GILZ siRNA treated samples was significant with a two tailed p value < 0.005 using paired t test and n=4 (GraphPad InSTAT3, San Diego, CA).
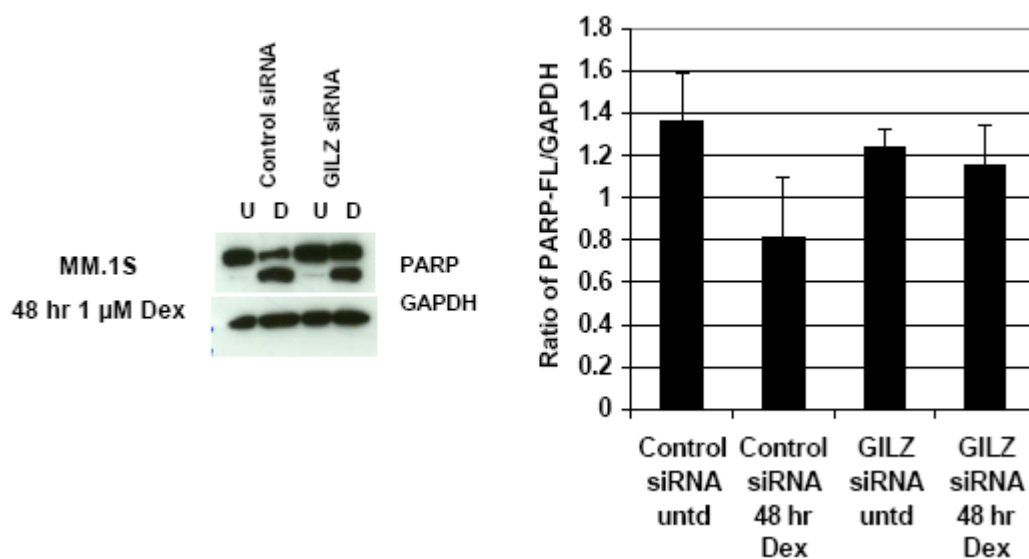
Figure 23. **GILZ knockdown reduces GC-induced PARP cleavage of MM cells.** MM.1S

cells were transfected with GILZ SMARTpool siRNA or control SMARTpool siRNA. 24 hours

after transfection, cells were treated with 1 μM Dex for 48 hours and then harvested. Whole cell

lysates were analyzed by western blotting and probed with antibodies to PARP and GAPDH (left

panel). A representative blot of one experiment is shown. Densitometry was used to measure

the decrease in full length PARP in three replicate experiments (right panel) by normalizing the

band intensity of full length PARP to the band intensity of GAPDH.

CHAPTER 5

ELUCIDATION OF PI3-KINASE/AKT REGULATION OF GILZ AND SYNERGISM

WITH GLUCOCORTICOIDS

IL-6 and IGF1 inhibit GC-induced GILZ up regulation and GC-induced cell death.

It has been previously reported that both IL-6 and IGF1 are important growth factors in MM cells and that apoptosis induced by Dex can be blocked by exogenous IL-6 or IGF1 (12, 13, 20, 118). To determine if these growth factors can affect GC regulation of *GILZ*, the effect of IL-6 and IGF1 on *GILZ* expression levels in MM.1S cells was tested. Pre-treatment with IL-6 or IGF1 partially inhibited the Dex-induced up regulation of *GILZ*. Shown with real time PCR, MM.1S cells treated with 1 µM Dex for 6 hours had a 25 fold increase in *GILZ* while the addition of increasing concentrations of either IL-6 or IGF1 limited Dex-induced up regulation of *GILZ* to only 5-10 fold (Figure 24). These concentrations of IL-6 and IGF1 were sufficient to block Dex-induced killing in MM.1S cells as measured by MTS assay (Figure 25). The addition of IL-6 and IGF1 alone also reduced the basal level of *GILZ* expression in MM.1S cells as much as 50% suggesting that these cytokines can regulate *GILZ* independent of the presence of GCs (Figure 26). These results provide evidence to support the hypothesis that *GILZ* up regulation is a component of GC-induced apoptosis in myeloma cells as the concentrations of IL-6 and IGF1 which block *GILZ* up regulation also inhibit GC killing.

RT-PCR screen to identify other regulators of GILZ.

In order to gain a better understanding of the regulation of *GILZ* and insight into GR signaling pathways in MM.1S cells, a panel of cytokines and drugs was screened for effect on *GILZ* expression levels using RT-PCR. The results of the screen are summarized in Table 4. This panel of cytokines, growth factors, and growth conditions was selected based on previous reports indicating regulation of GILZ or its related family members in other cell lines or systems and included IL-2, IL-7, IL-10, TGF-β, β-estradiol, sonic hedgehog, progesterone, EGF and serum starvation (76, 90, 100, 119-123). None of the cytokines or growth conditions tested was
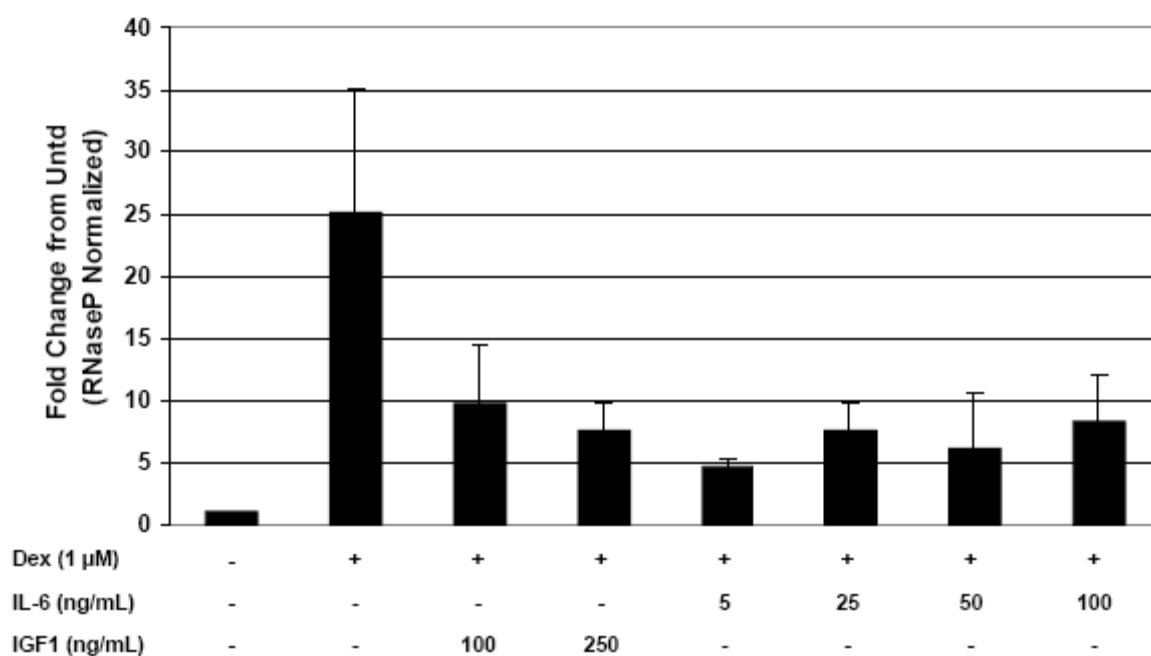
Figure 24. **IL-6 and IGF1 inhibit Dex-induced up regulation of *GILZ*.** MM.1S cells were treated with 1 μM Dex for 6 hours. Where indicated, 100-250 ng/mL IGF1 and 5-100 ng/mL IL-6 was added 30 minutes prior to Dex. Total RNA was isolated and *GILZ* levels measured in each sample with real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from untreated sample.
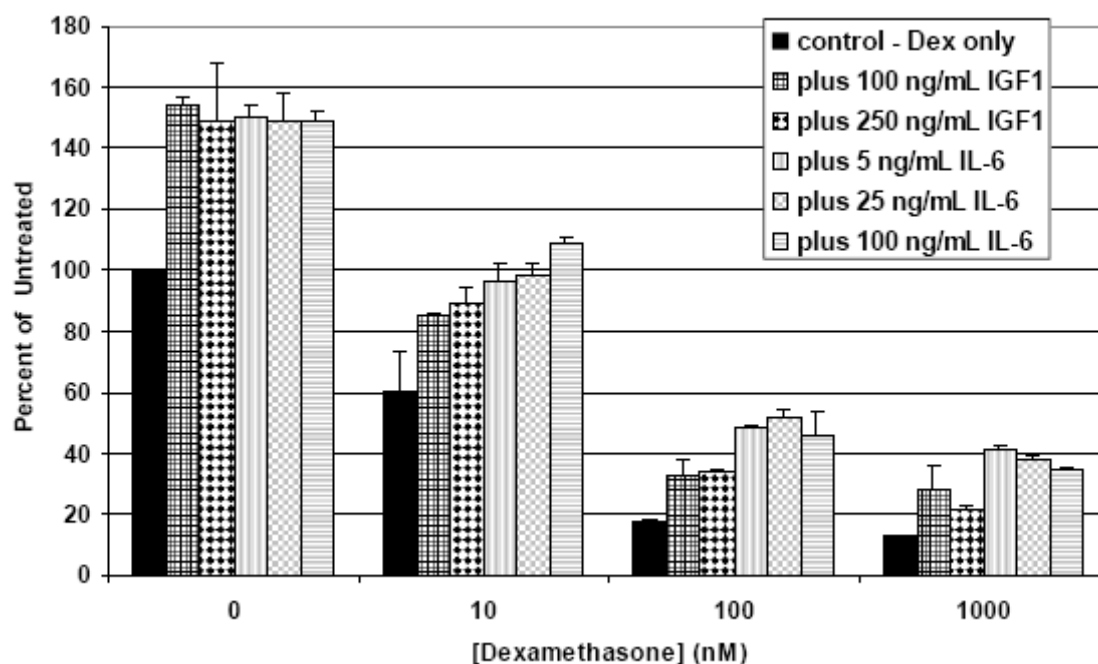
Figure 25.   IL-6 and IGF1 reduce Dex-induced MM.1S growth inhibition. MM.1S cell

were incubated with increasing concentrations of Dex (0-1 µM) for 72 hours. Where indicated,

100-250 ng/mL IGF1 and 5-100 ng/mL IL-6 was added to the cells. Cell viability was measured

using MTS assay and expressed as a percentage of the untreated control cells.

IL-6 (ng/mL)	-	-	-	-	5	50	100
IGF1 (ng/mL)	-	100	250	500	-	-	-
% decrease	-	68	50	53	74	61	60
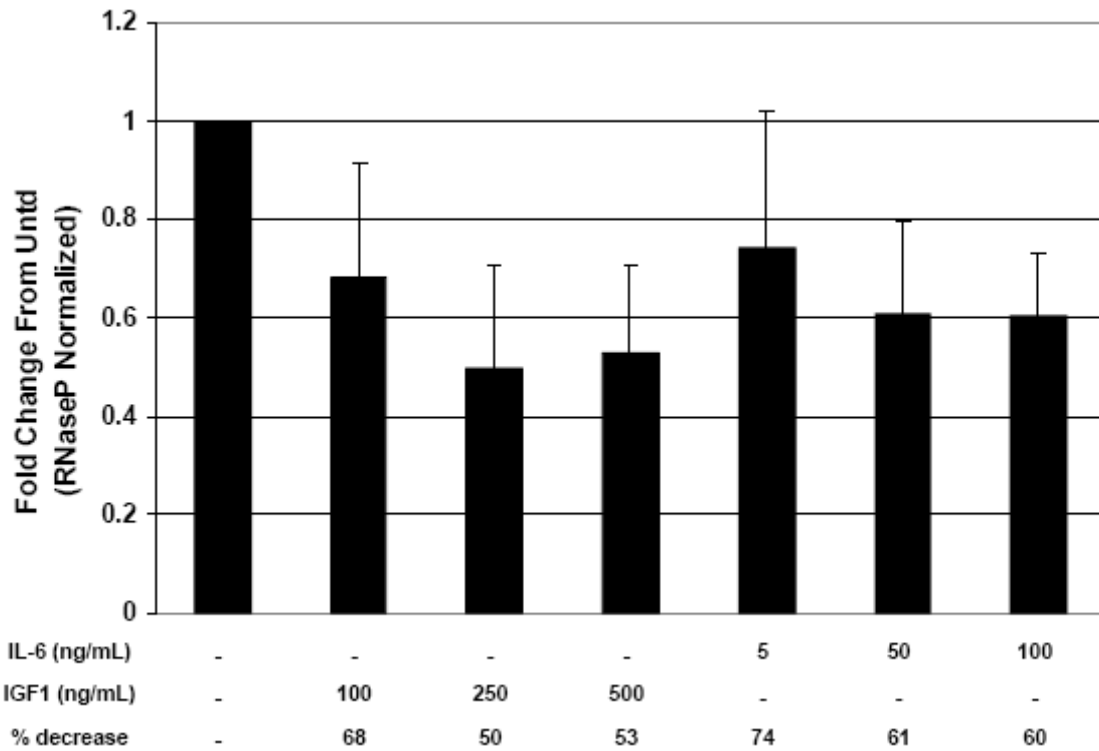
Figure 26.   IL-6 and IGF1 decrease *GILZ* baseline levels in MM.1S. MM.1S cells were treated for 6 hours with 100-500 ng/mL IGF1 or 5-100 ng/mL IL-6. *GILZ* levels were measured in total RNA isolated from each sample using real time PCR.   Levels were normalized to *RNaseP* in each sample and expressed as fold change from untreated control sample.

Table 4.   Panel of various agents that were screened in MM.1S cells for effect on

GILZ expression. GILZ has been reported to be up regulated in other cell lines by IL-2, IL-10,

sonic hedgehog, β-estradiol, and upon serum starvation. IL-7, IL-2, TGF-β, progesterone and

EGF have all been reported to affect the expression of GILZ family member TSC-22. ATRA, 2-

methoxyestradiol, enzastaurin, rapamycin, and thalidomide all have been reported to kill MM

cells. IL-15 and the PI3-kinase/Akt inhibitors were hypothesized to affect the GILZ promoter.

As reported in the text, GILZ upregulation was shown using additional cytotoxic glucocorticoids

including beclomethasone, beclomethasone DP, hydrocortisone, prednisolone, methyl

prednisolone, triamcinolone, and triamcinolone acetonide. GILZ was not upregulated in our

screen by prednisone which is unable to kill MM.1S cells *in vitro* because it requires conversion

to its active form by liver enzymes.

Cytokines/Growth Factors	Upregulate GILZ?	Cytotoxic Drugs/ Kinase Inhibitors	Upregulate GILZ?
IL-6	NO[*]	ATRA	NO
IGF1	NO[*]	2-methoxyestradiol	NO
IL-2	NO	Rapamycin (mTOR)	NO
IL-7	NO	Enzastaurin (PKC-β)	NO
IL-10	NO	Thalidomide	NO
IL-15	NO	SB203580 (p38)	NO
TGF-β	NO	U0261 (MEK)	NO
Sonic hedgehog	NO	PC98059 (MEK)	NO
EGF	NO	LY294002 (PI3-K)	YES
β-Estradiol	NO	Wortmannin (PI3-K)	YES
Progesterone	NO	Triciribine (AKT)	YES
Serum starvation	NO	AKT inhibitor VIII	YES

[*]IL-6 and IGF1 were shown to down regulate GILZ expression (Figure 23).

found to up regulate *GILZ* in MM.1S cells. Because glucocorticoids are potent inducers of

apoptosis in myeloma cells, additional MM chemotherapuetic agents were screened including 2-

methoxyestradiol, all-trans retinoic acid (ATRA), enzastaurin, rapamycin, and thalidomide to

determine if *GILZ* up regulation was observed upon induction of apoptosis in myeloma cells by a

variety of agents other than GCs (31, 124-127). Despite inducing apoptosis in MM.1S cells,

none of the drugs tested up regulated *GILZ* in this screen (Table 4). Therefore *GILZ* up

regulation is specific to GC-induced apoptosis in MM cells. A representation of the negative

results from the screen is shown in Figure 27.

GILZ is up regulated by inhibiting the PI3-kinase/AKT pathway.

Due to previous reports highlighting the importance of the forkhead responsive elements

(FHRE) in the *GILZ* promoter (99, 100) and the regulation shown with IL-6 and IGF1 (Figures

24-26), the effect of inhibitors of the PI3-kinase/AKT pathway on *GILZ* was also tested in the

screen. These proteins were targeted because both PI3-kinase and AKT are upstream of the

forkhead box family O (FOXO) members and can be activated by IL-6 and IGF1 (12, 13, 22,

128). Interestingly, the PI3-kinase inhibitors LY294002 and wortmannin up regulated *GILZ*

levels in MM.1S cells as shown with RT-PCR after 6 and 24 hours (Figure 28). The results

identifying *GILZ* up regulation upon inhibition of PI3-kinase was investigated further.

Regulation of *GILZ* by PI3-kinase inhibitors LY294002 and wortmannin was confirmed using

real time PCR after 6 hours of treatment (Figure 29). Two AKT inhibitors, triciribine and AKT

Inhibitor VIII, also up regulated *GILZ* in MM.1S after 6 hours (Figure 29). The up regulation of

GILZ by inhibitors of PI3-kinase or AKT was tested in additional multiple myeloma cell lines to

ensure that this effect was not limited to the MM.1S cells. In OPM-II, U266, RPMI-8226,

MM.1R$_e$, and MM.1R$_L$ cell lines, *GILZ* expression was increased 5-27 fold by either 25 µM
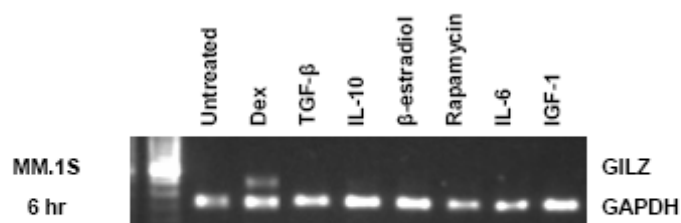
Figure 27.   Representative result from *GILZ* regulation RT-PCR screen. MM.1S cells were treated with 1 µM Dex, 2 ng/mL TGF-β, 20 ng/mL IL-10, 200 nM β-estradiol, 100 nM rapamycin, 100 ng/mL IL-6 and 250 ng/mL IGF1 for 6 hours. Total RNA was isolated and up regulation of *GILZ* measured by RT-PCR. GAPDH levels were measured in each sample as a normalization control.
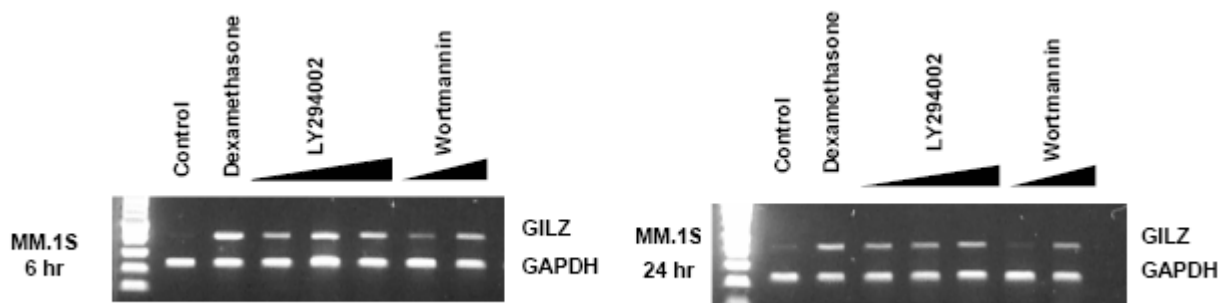
Figure 28.   LY294002 and wortmannin increase *GILZ* levels in MM.1S Cells. MM.1S cells were treated for 6 and 24 hours with Dex (1 µM), LY294002 (25, 50, & 75 µM), and wortmannin (0.1 & 1 µM). Following harvest, total RNA was isolated and levels of *GILZ* and *GAPDH* were analyzed with RT-PCR.
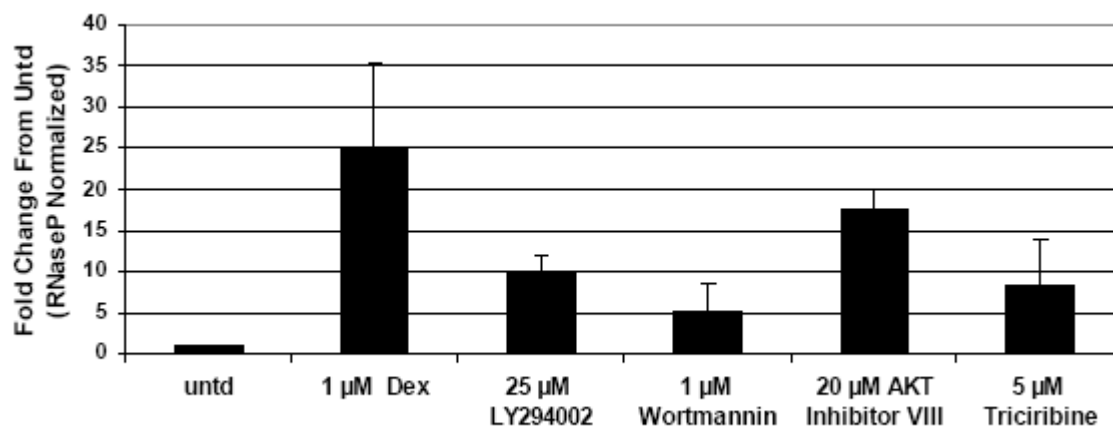
Figure 29. **Inhibitors of PI3-kinase and AKT up regulate *GILZ*.** MM.1S cells were treated for 6 hours with 1 µM Dex, 25 µM LY294002, 1 µM wortmannin, 20 µM AKT inhibitor VIII, or 5 µM triciribine. Total RNA was isolated following treatment and *GILZ* levels were measured with real time PCR. *GILZ* levels were normalized to RNaseP and expressed as fold change from untreated control.

LY294002 or 20 μM AKT Inhibitor VIII. The multi-drug resistant myeloma cell line MDR10V was the only line tested where inhibitors of PI3-kinase and AKT did not increase *GILZ* by at least 5 fold over untreated control levels (Figure 30). The extent of *GILZ* up regulation by PI3-kinase and AKT inhibition in the GC-sensitive MM.1S was similar to the GC-resistant MM.1R$_L$ cell line and is therefore independent of the level of the GR. *GILZ* up regulation by PI3-kinase and AKT inhibitors was also measured in human multiple myeloma patient samples where a 1.7-5 fold increase in *GILZ* expression with LY294002 and AKT inhibitor VIII was observed in 2 of the 4 samples tested (Figure 31). A 2 fold or greater increase in *GILZ* levels was observed in only 2 of the 5 CLL patients samples and also in the PBMC control (Figure 32). Taken together, *GILZ* is regulated by inhibitors of PI3-kinase and AKT in a panel of MM cell lines and clinical samples and this regulation appears to be independent of GR level.

When combined, GCs and PI3-kinase/AKT inhibitors dramatically enhance GILZ.

To further investigate the ability of PI3-kinase and AKT inhibitors to up regulate *GILZ*, the effect of simultaneous addition of GCs and inhibitors to PI3-kinase and AKT on *GILZ* expression was explored. When GC and PI3-kinase/AKT inhibitors were used in combination, *GILZ* levels were dramatically enhanced 100 - 200 fold from untreated control values in MM.1S cells (Figure 33). In a similar manner to the effect on GC-induced up regulation of *GILZ,* pretreatment with IL-6 or IGF1 blunted the enhanced up regulation of *GILZ* upon Dex and LY294002 combination treatment 50-75% (Figure 34). This enhanced up regulation of *GILZ* reported at the mRNA level with real time PCR translated to a similar enhancement at the protein level. Treatment with LY294002, wortmannin, and AKT Inhibitor VIII alone resulted in an increase in detectable GILZ protein. The combination of Dex with any of the four inhibitors
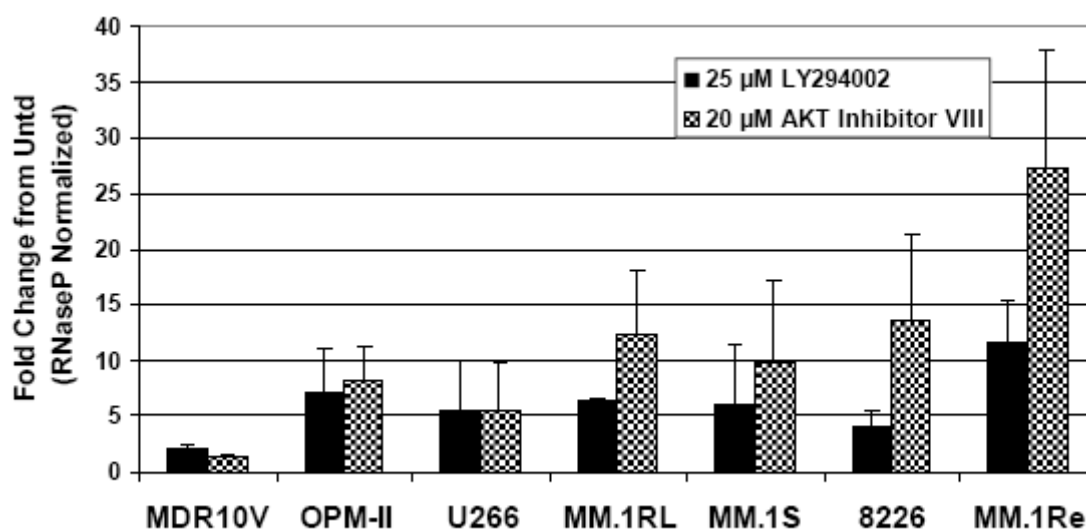
Figure 30. **PI3-kinase and AKT inhibitors up regulate *GILZ* in a panel of MM cell lines.**

MDR10V, OPM-II, U266, MM.1R$_L$, MM.1S, RPMI-8226, and MM.1R$_e$ cells were treated with 25 uM LY294002 or 20 μM AKT inhibitor VIII for 6 hours. The extent of *GILZ* up regulation was measured with real time PCR. *GILZ* levels were normalized to RNaseP and expressed as fold change from untreated control.
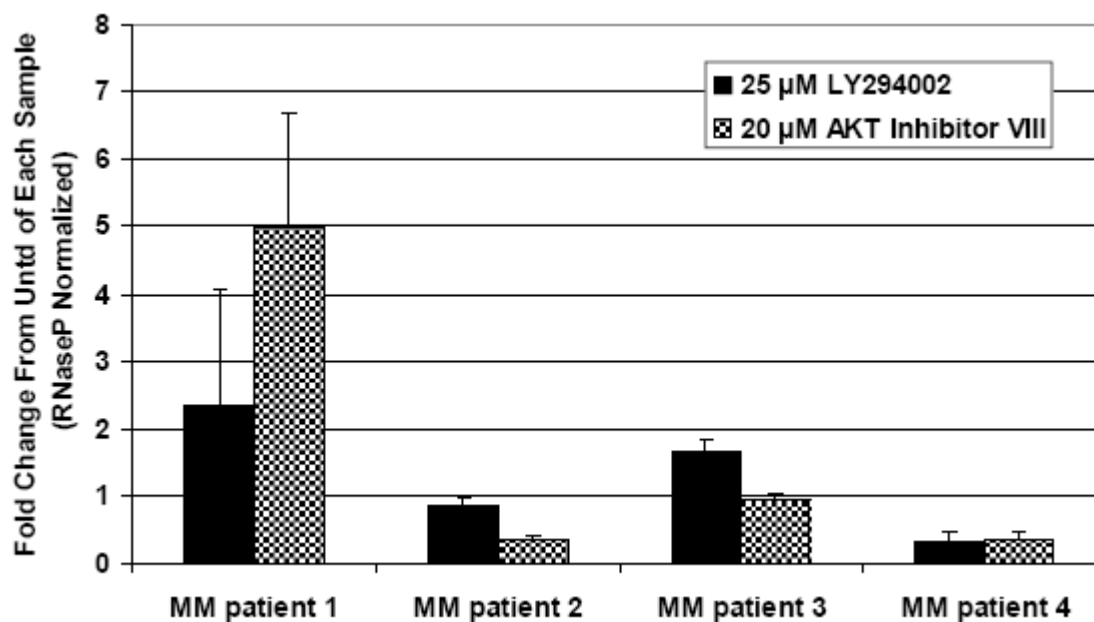
Figure 31. *GILZ* **regulation by PI3-kinase and AKT inhibitors in MM patient samples.**

MM cells isolated from bone marrow patient samples obtained from the clinic were treated for 6 hours with 25 μM LY294002 and 20 μM AKT inhibitor VIII. Total RNA was isolated and the level of *GILZ* in each sample was measured using real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from the untreated level in each patient sample.
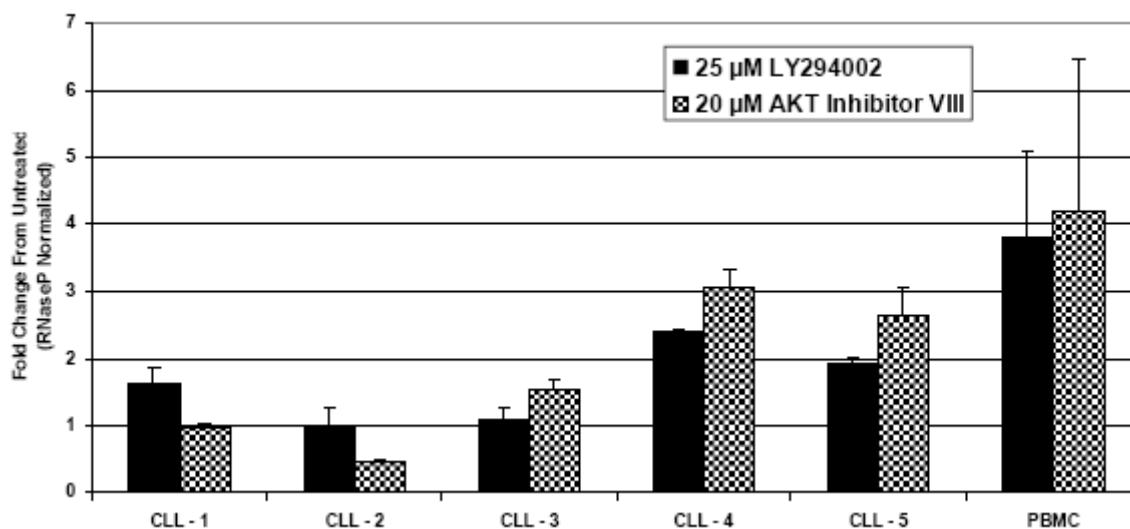
Figure 32. **Up regulation of *GILZ* in CLL patient cells and normal PMBCs by PI3-kinase and AKT inhibitors.** CLL cells were isolated from peripheral blood samples obtained from the clinic. PBMCs were isolated from peripheral blood sample of a healthy donor. Isolated cells were set up in short term *in vitro* culture and treated for 6 hours with 25 µM LY294002 or 20 µM AKT inhibitor VIII. Total RNA was isolated following harvest and the level of *GILZ* induction was measured using real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from the untreated level in each patient sample.

The chart plots "Fold Change From Untd (RNaseP Normalized)" on the y-axis (0 to 300) with the following treatment conditions indicated below each bar:

Dex	-	+	-	-	-	-	+	+	+	+
LY294002	-	-	+	-	-	-	+	-	-	-
Wortmannin	-	-	-	+	-	-	-	+	-	-
AKT Inhibitor VIII	-	-	-	-	+	-	-	-	+	-
Triciribine	-	-	-	-	-	+	-	-	-	+

Figure 33. *GILZ* **levels are dramatically enhanced by combination of GC and PI3-kinase/AKT inhibitor treatment.** MM.1S cells were treated for 6 hrs with 1 μM Dex, 25 μM LY294002, 1 μM wortmannin, 20 μM AKT inhibitor VIII, or 5 μM triciribine alone or in combination as indicated. *GILZ* levels were measured by real time PCR, normalized to *RNaseP* and expressed as fold change from untreated cells.
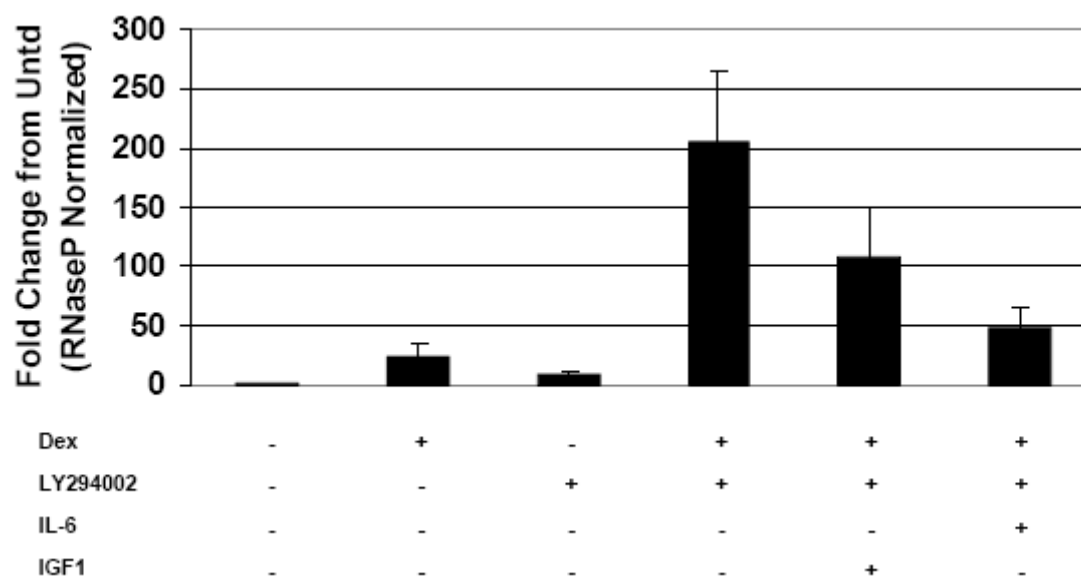
Figure 34. **Exogenous IL-6 and IGF1 partially inhibit *GILZ* up regulation by GC and LY294002.** MM.1S cells were treated for 6 hrs with 1 μM Dex and 25 μM LY294002 with and without 100 ng/mL IL-6 and 250 ng/mL IGF1 (added 30 min prior to Dex and LY294002). *GILZ* levels were measured by real time PCR, normalized to *RNaseP*, and expressed as fold change from untreated cells.

tested (LY294002, wortmannin, triciribine, and AKT Inhibitor VIII) resulted in increased protein expression to a level greater than the level observed with Dex alone (Figure 35).

Pharmacologic inhibitors to other important signaling molecules in MM cells, p38 (SB203580) and MEK (U0126, PD98059), were tested to determine if this effect on *GILZ* expression was the result of global inhibition of myeloma growth stimulatory pathways or specific to PI3-kinase/AKT inhibition in MM.1S cells. Neither inhibition of MEK nor p38 resulted in up regulation of *GILZ*. Also, when combined with Dex, none of these inhibitors tested dramatically up regulated *GILZ* expression to levels higher than what is observed with Dex treatment alone (Figure 36). This is contrary to a recent report in human epidermal keratinocytes where PD98059 and U0126 significantly sensitized GC-induced GILZ up regulation in the presence of TNF-α and wortmannin had no effect on GILZ (129). This suggests that the signaling pathways that modulate GC function and GILZ expression vary in different cell types.

The effect of the combination of Dex with LY294002 or AKT Inhibitor VIII was tested in the panel of other MM cell lines to determine if the combinatorial enhancement was specific to only MM.1S cells or occurred more broadly in myeloma. A similar *GILZ* up regulation was observed in the RPMI-8226 and OPM-II cell lines and to a lesser extent in MM.1R$_e$, but not in MM.1R$_L$, U266, or MDR10V (Figure 37). As shown in Figures 10 and 27, *GILZ* was not up regulated substantially by GCs in MM.1R$_e$, MM.1R$_L$, and U266 cell lines or by LY294002 or AKT Inhibitor VIII in MDR10V cells. Therefore a similar dramatic enhancement in *GILZ* levels with the combination treatment would not be expected in any of these cell lines. The effect of the combination treatment on *GILZ* levels was also investigated in the MM patient samples where dramatic enhancement upon combination treatment was observed in MM patients 2 and 3

Dex	-	+	-	+	-	+	-	+	-	+
Wortmannin	-	-	+	+	-	-	-	-	-	-
Triciribine	-	-	-	-	+	+	-	-	-	-
AKT Inhibitor VIII	-	-	-	-	-	-	+	+	-	-
LY294002	-	-	-	-	-	-	-	-	+	+

GILZ

GAPDH
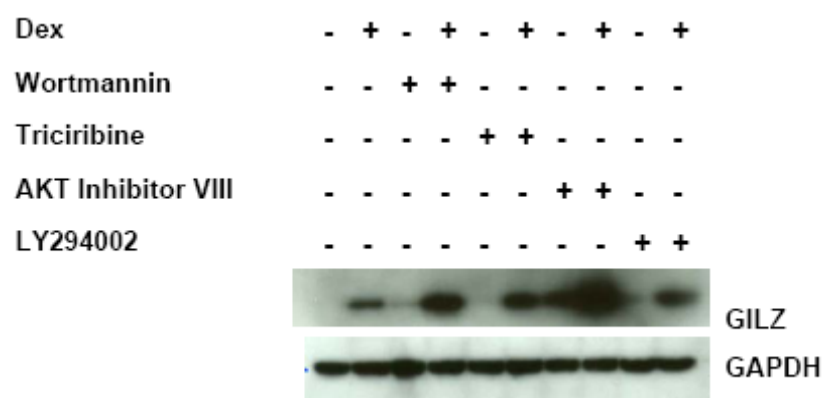
Figure 35. **PI3-kinase/AKT inhibitors alone and in combination with Dex up regulate GILZ protein levels.** MM.1S cells were treated with 1 μM Dex, 25 μM LY294002, 1 μM wortmannin, 20 μM Akt inhibitor VIII, or 5 μM triciribine alone or in combination for 6 hours.

Dex (1 µM)	-	+	-	+	-	+	-	+	-	+
Wortmannin (1 µM)	-	-	+	+	-	-	-	-	-	-
SB203580 (10 µM)	-	-	-	-	+	+	-	-	-	-
U0126 (10 µM)	-	-	-	-	-	-	+	+	-	-
PD98059 (50 µM)	-	-	-	-	-	-	-	-	+	+
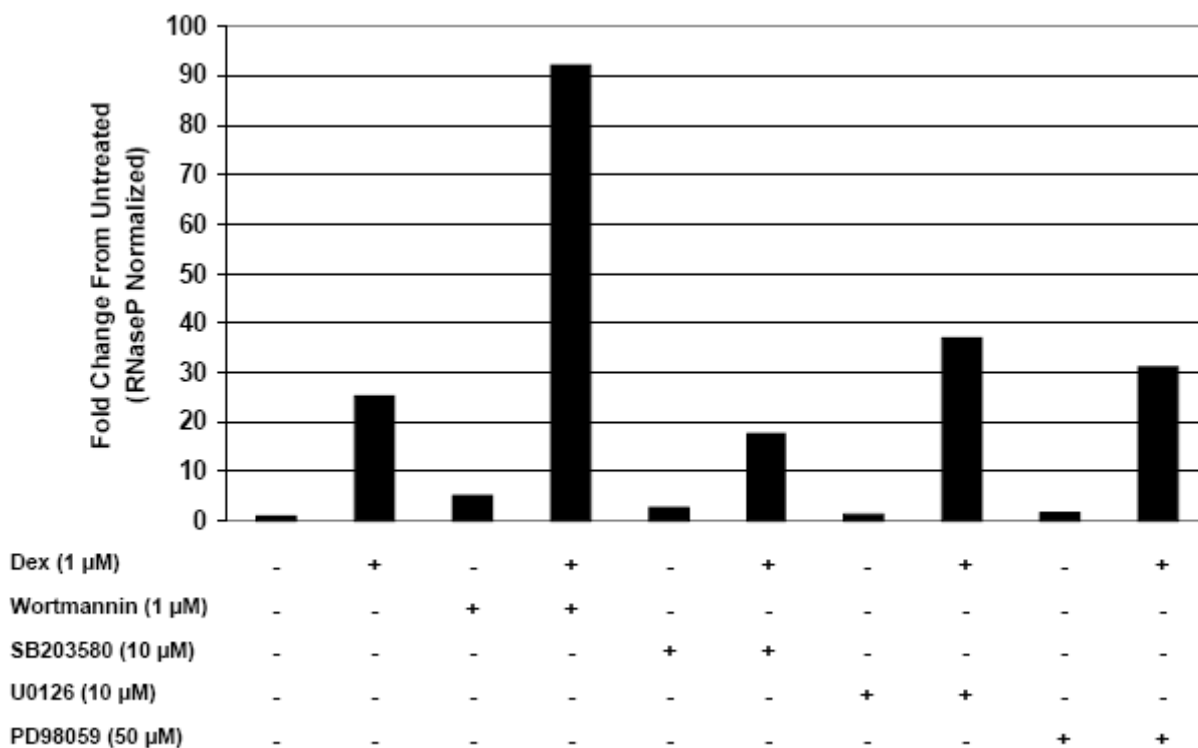
Figure 36.   MEK and p38 inhibition does not up regulate *GILZ* or cooperate with GC.
MM.1S cells were treated with 1 µM Dex, 1 µM wortmannin, 10 µM SB203580, 10 µM U0126, or 10 µM PD98059 alone or in combination as indicated for 6 hours.   *GILZ* levels were measured by real time PCR, normalized to *RNaseP*, and expressed as fold change from untreated cells.
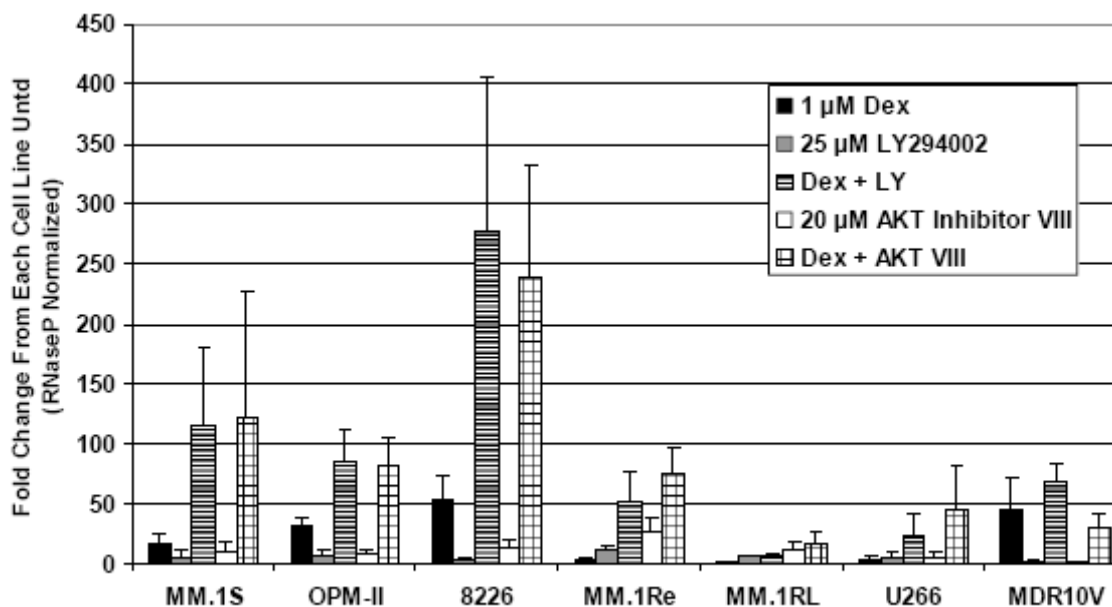
Figure 37. *GILZ* **is dramatically enhanced by PI3-kinase/AKT inhibitor and GC combination treatment in a panel of MM cell lines.** MDR10V, OPM-II, U266, MM.1R$_L$, MM.1S, RPMI-8226, and MM.1R$_e$ cells were treated with 1 μM Dex, 25 uM LY294002 or 20 μM AKT inhibitor VIII alone or in combination for 6 hours. The extent of *GILZ* up regulation was measured with real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from untreated control.

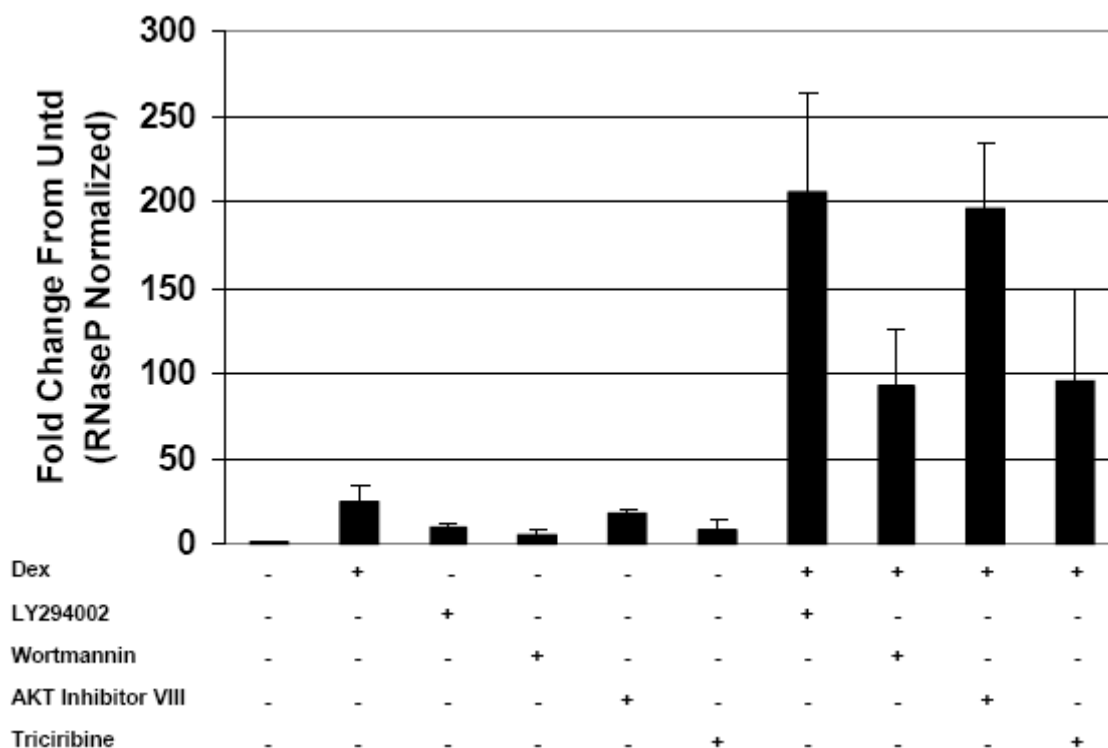compared to the induction of GILZ observed with either agent alone (Figure 38). Similar effects with the combination of Dex and LY294002 was observed in CLL patient #5 and in the normal PBMC sample, but not with the other CLL patients (Figure 39). Collectively the data presented in this section reports dramatic up regulation of GILZ upon treatment with the combination of GCs and PI3-kinase/AKT inhibitors and suggests that the PI3-kianse/AKT and the GR signaling pathways are converging at the regulation of *GILZ* expression.

PI3-kinase inhibitors enhanced GC-induced cell death in myeloma cells.

Because modulators of the PI3-kinase/AKT pathway were shown to affect *GILZ* expression and GILZ was shown to play a functional role in GC-induced apoptosis, the effect of the combination treatment of GCs and inhibitors of PI3-kinase/AKT on MM cell apoptosis was explored next. Lysates of MM.1S cells treated with the combination of Dex and LY294002 were analyzed by western blotting for PARP. Enhanced PARP cleavage was observed with the combination treatment of Dex and LY294002 compared to either agent alone at 6 and 24 hours (Figure 40). Similar to the IL-6 or IGF1 inhibition of *GILZ* expression by the combination treatment of Dex and LY294002, IL-6 or IGF1 inhibited or delayed Dex-induced and Dex + LY294002-induced PARP cleavage (Figure 41). Experiments utilizing PI staining to identify an increase in the percentage of cells with a sub-G1 phase DNA content which is indicative of cells undergoing apoptosis found that both LY294002 and wortmannin when added with Dex enhanced apoptosis over each agent alone (Figure 42). These results were further confirmed with Annexin-V/7-AAD staining which detects cells undergoing apoptosis where the phospholipid phosphatidylserine has inverted from inner to outer leaflet of the plasma membrane, an early event in apoptosis. The combination of Dex and LY294002 resulted in an apoptotic induction greater than the additive combination of either agent alone (Figure 43).
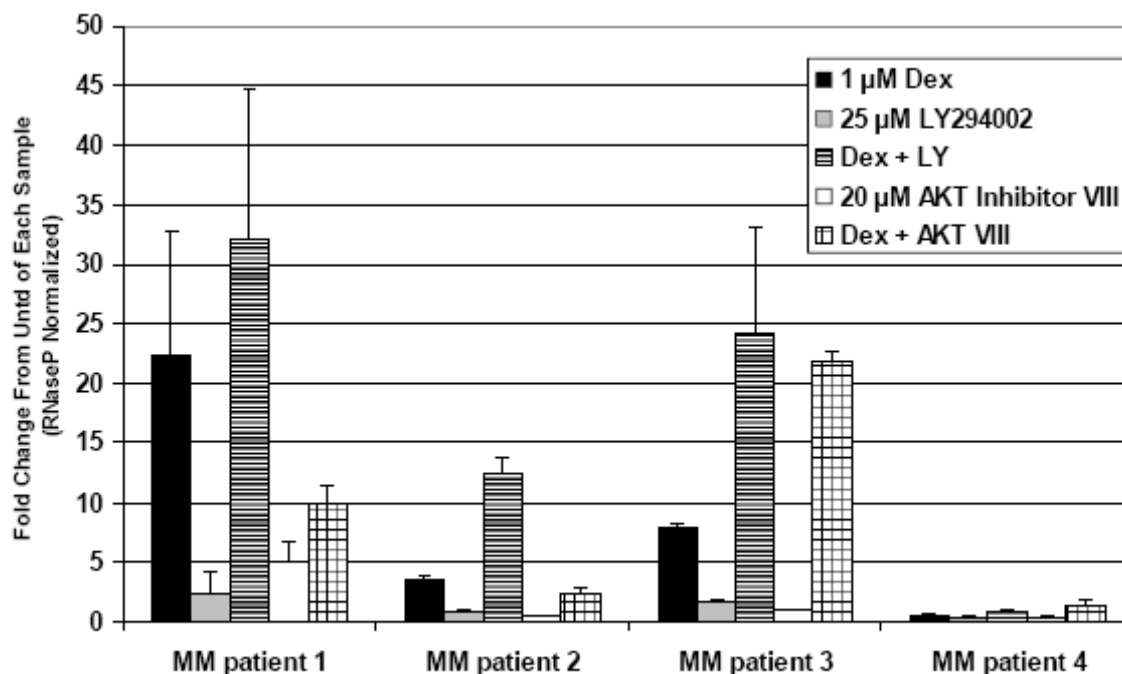
Figure 38. *GILZ* **regulation by PI3-kinase and AKT inhibitors in MM patient samples.**

MM cells isolated from bone marrow patient samples obtained from the clinic were treated for 6 hours with 1 μM Dex, 25 μM LY294002 and 20 μM AKT inhibitor VIII alone or in combination. Total RNA was isolated and the level of *GILZ* in each sample was measured using real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from the untreated level in each patient sample.
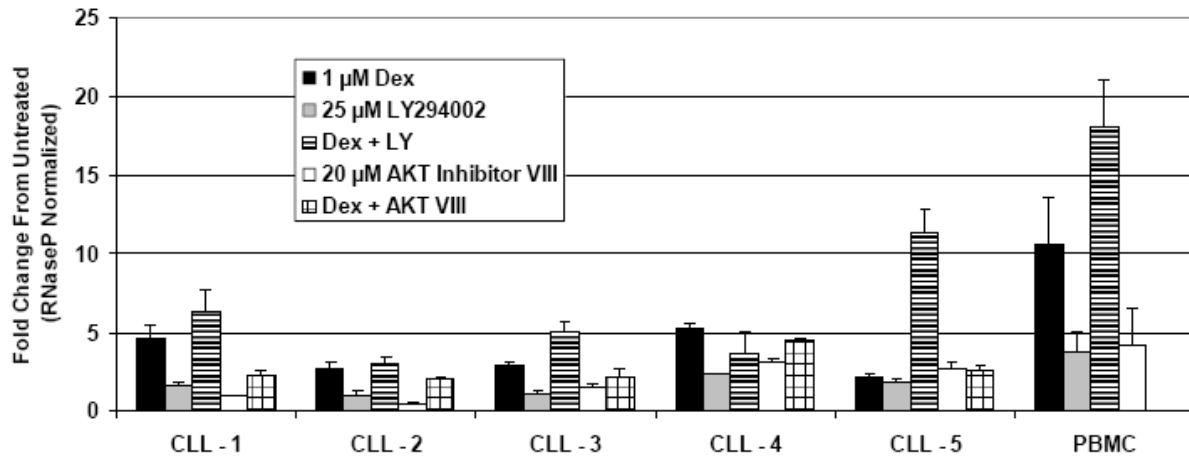
Figure 39. *GILZ* **regulation by PI3-kinase and AKT inhibitors in CLL patient and PBMC samples.** CLL cells and PBMCs isolated from peripheral blood of donors were obtained from the clinic were treated for 6 hours with 1 μM Dex, 25 μM LY294002 and 20 μM AKT inhibitor VIII alone or in combination. Total RNA was isolated and the level of *GILZ* in each sample was measured using real time PCR. *GILZ* levels were normalized to *RNaseP* and expressed as fold change from the untreated level in each patient sample.
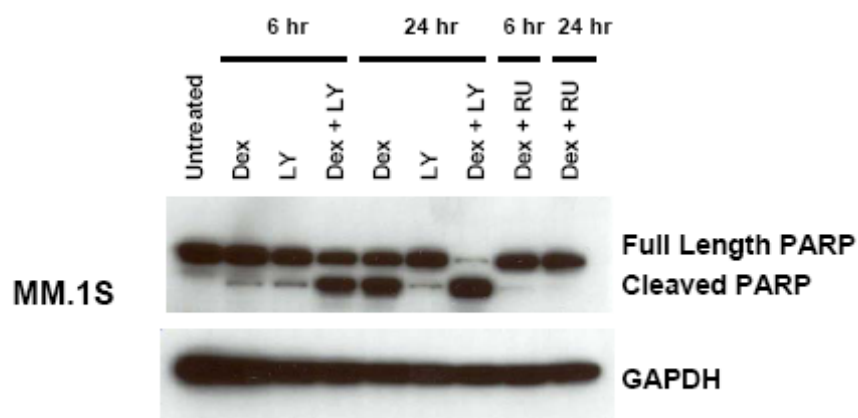
Figure 40. **LY294002 enhances Dex-induced PARP cleavage.** Whole cell lysates of MM.1S cells treated with 1 μM Dex, 25 μM LY294002, and/or 10 μM RU486 (added 1 hour prior to Dex) for 6 and 24 hours were analyzed by western blotting. Blots were probed with antibodies to PARP and GAPDH
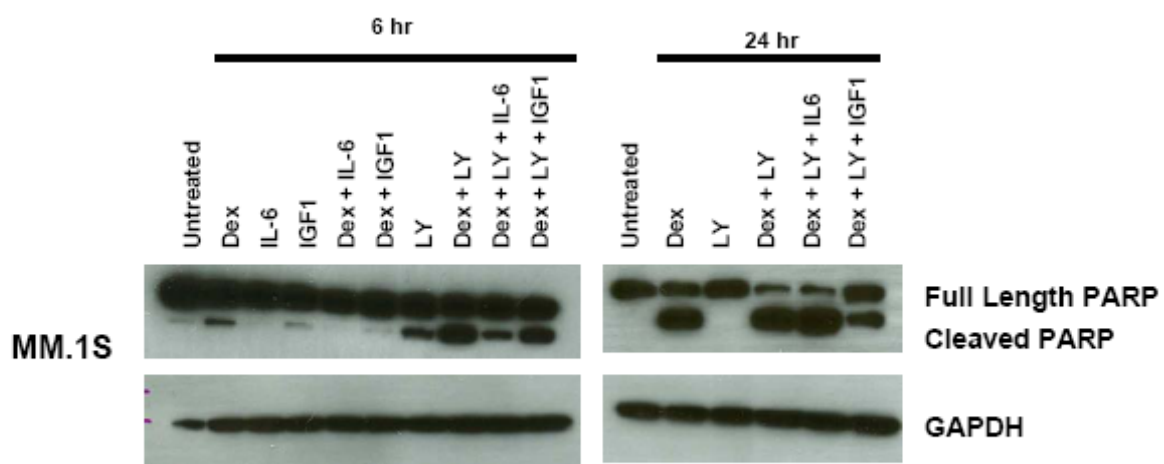
Figure 41. **IL-6 and IGF1 inhibit Dex and LY294002-induced PARP cleavage.** Whole cell lysates of MM.1S cells treated with 1 μM Dex, 25 μM LY294002, and/or 100 ng/mL IL-6 and 250 ng/mL IGF1 (added 30 min prior to Dex or LY294002) for 6 and 24 hours were analyzed by western blotting. Blots were probed with antibodies to PARP and GAPDH.
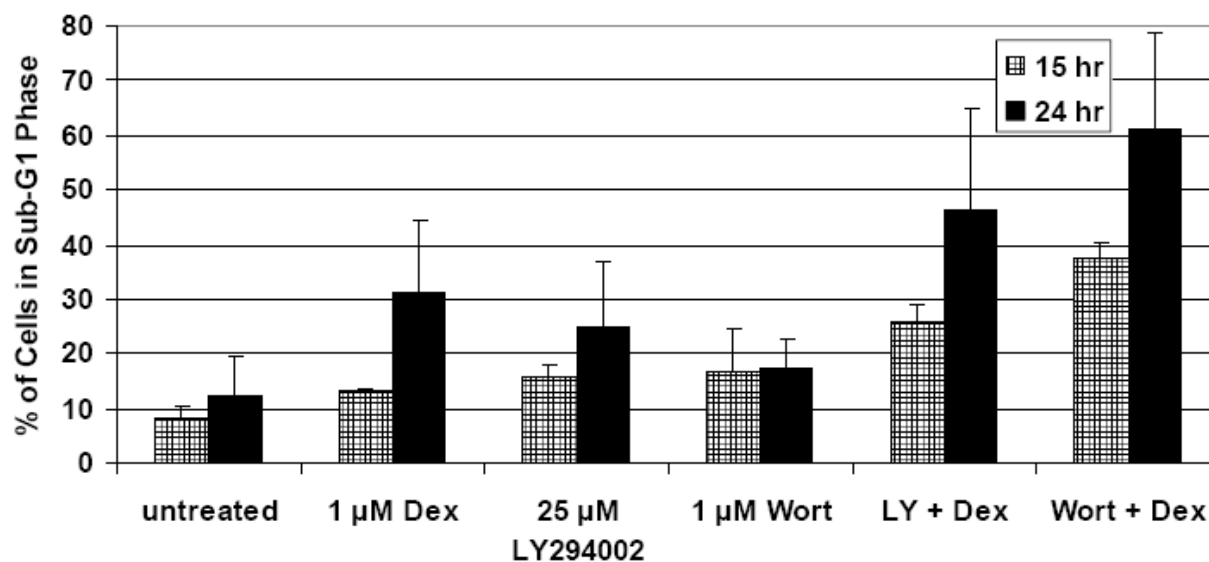
Figure 42.   LY294002 and wortmannin enhance GC-induced cell death of MM.1S cells.
MM.1S cells were treated with 1 µM Dex and/or 25 µM LY294002 or 1 µM wortmannin. Cells
were analyzed by propidium iodide staining after 15 and 24 hr treatments and the percentage of
cells with a sub-G1 DNA content are expressed.
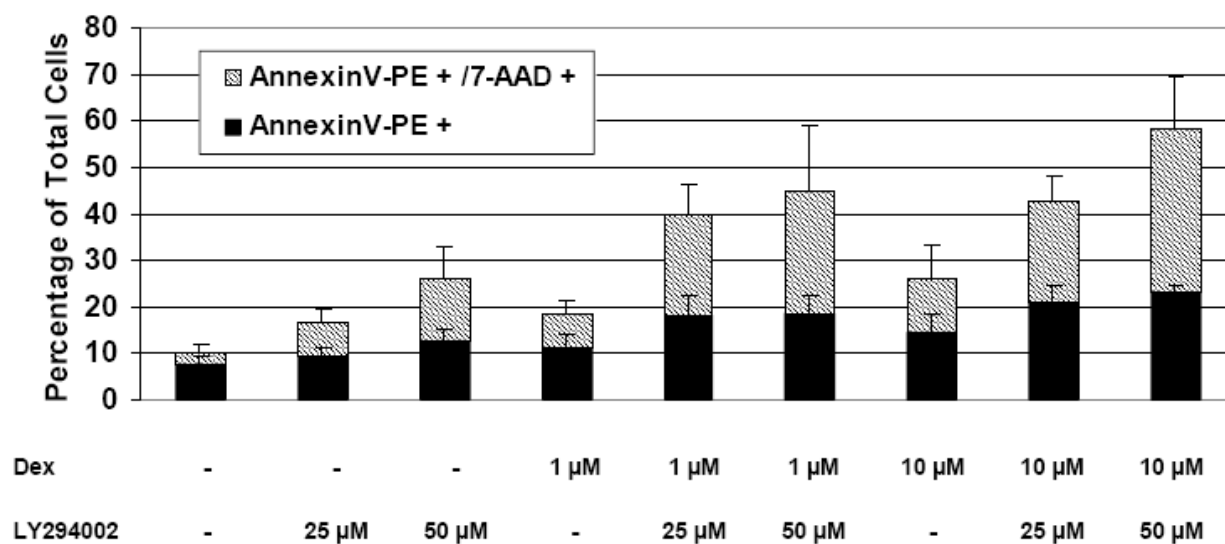
Figure 43. **LY294002 enhances GC-induced cell death of MM.1S cells.** MM.1S cells were treated with 1-10 µM Dex and/or 25-50 µM LY294002 for 24 hours. The percentage of cells undergoing apoptosis were stained with Annexin V-PE and 7-AAD. Cells staining Annexin-V-PE+/7-AAD- are early stage apoptotic and cells staining Annexin-V-PE+/7-AAD+ are late stage apoptotic or necrotic.

Because of the greater than additive induction of apoptosis, formal analysis of synergism between Dex and LY294002 was done. Using the CalcuSyn software program, the median effect plot analysis and Annexin-V/7-AAD staining, it was determined that the cell killing resulting from the combination of Dex and LY294002 was indeed synergistic (combinatorial index (CI) = 0.211 for 1 μM Dex + 25 μM LY294002, CI = 0.105 for 10 μM Dex + 250 μM LY294002, where CI values less than 1.0 indicate synergy) (Table 5). PI3-kinase/AKT inhibition in concert with GC treatment synergistically enhances MM cell killing while up regulating GILZ levels dramatically. This suggests that GCs and inhibitors to the PI3-kinase/AKT pathway may be beneficial as a combinatorial therapy to treat MM patients in the clinic.

Deciphering the mechanism behind GC & PI3-kinase/AKT inhibitor synergism

The mechanism underlying PI3-kinase/AKT regulation of *GILZ* and synergistic induction of MM cell killing was explored. The effect of GC treatment on AKT phosphorylation at serine 473, a marker of AKT activation, was tested. The kinase inhibitors used in these studies are known to decrease the phosphorylation of AKT and the possibility that GC could also effect the level of AKT phosphorylation and activation was considered. As expected, a 30 minute treatment with all of the PI3-kinase/AKT inhibitors used in these studies dramatically reduced phosphorylated AKT levels, whereas treatment with IL-6 or IGF1 enhanced AKT phosphorylation at serine 473. However, the addition of Dex alone or in combination with LY294002 did not alter AKT phopshorylation levels any further (Figure 44).

As was shown in Figure 27, *GILZ* regulation by PI3-kinase/AKT inhibition appears to be independent of GR levels as up regulation is similar in MM.1S and MM.1R$_L$ cell lines despite very different GR levels. Even with this observation, the synergistic cell killing observed upon

Table 5: Dex and LY294002 cause synergistic cell killing of MM.1S cells. MM.1S cells were exposed to increasing concentrations of Dex and LY294002 at a fixed ratio (1:25) for 24 hours. Apoptosis was quantified by AnnexinV-PE/7-AAD staining. The combination treatment of Dex and LY294002 was analyzed for synergism using the commercially available software CalcuSyn and the median effect plot equation. Combinatorial indexes (CI) of less than 1.0 indicate synergism, greater than 1.0 antagonism, and equal to 1.0 additive.

	CI
1 µM Dex + 25 µM LY	**0.211**
µM Dex + 250 µM LY	**0.105**
CI values <1.0 = SYNERGY	
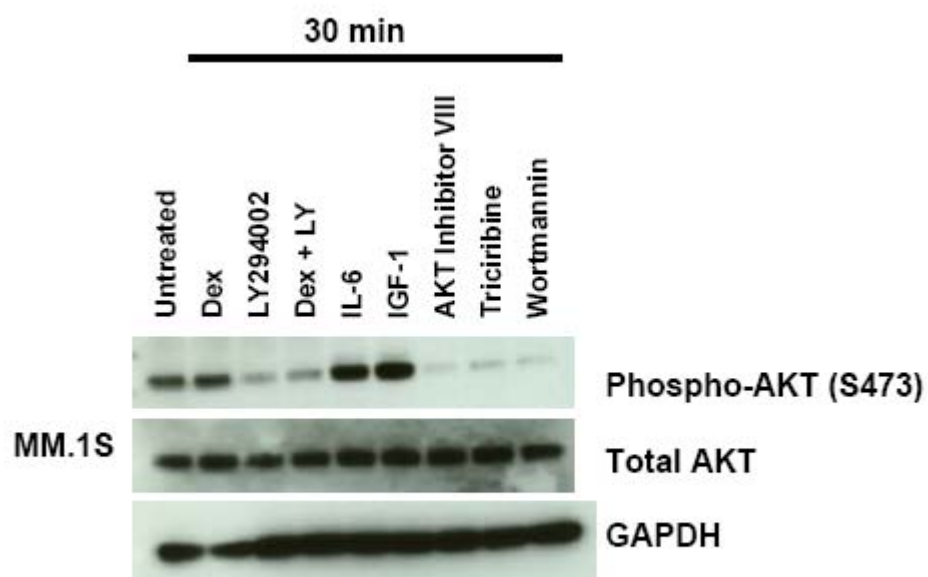
Figure 44. **Dex does not effect AKT phosphorylation.** Whole cell lysates of MM.1S cells treated with 1 μM Dex, 25 μM LY294002, 100 ng/mL IL-6, 250 ng/mL IGF1, 20 μM AKT inhibitor VIII, 5 μM triciribine, and/or 1 μM wortmannin for 30 minutes were analyzed by western blotting. Blots were probed with antibodies to phospho-AKT (Ser473), total AKT and GAPDH.

the combination treatment of GC and PI3-kinase/AKT inhibition, along with the dramatic enhancement of *GILZ* levels with the combination treatment is quite compelling and is suggestive of potential cross-talk between the two pathways. Therefore in MM.1S cells, the effect of the addition of the GR antagonist RU486 on the ability of LY294002 and wortmannin to regulate *GILZ* was tested. If the GR is not involved in LY294002/wortmannin up regulation of *GILZ*, no effect would be observed upon the addition of RU486. As shown with RT-PCR, the addition of RU486 did not block up regulation of *GILZ* by LY294002 or wortmannin in a manner similar to how it could when added with Dex (Figure 45). Surprisingly, upon quantitative real time PCR analysis, the combination treatment of RU486 with LY294002 or wortmannin enhanced *GILZ* levels 40 – 60 fold over control levels. This is quite significant as RU486, LY294002, and wortmannin alone each up regulate *GILZ* less than 10 fold (Figure 46). The combination of RU486 and LY294002 also enhanced MM.1S cell apoptosis as shown with increased PARP cleavage compared to either agent alone (Figure 47). This unexpected observation that the addition of RU486 when added in conjunction with PI3-kinase inhibitors enhanced *GILZ* levels and MM cell apoptosis may be in part explained by the partial agonist functions of RU486 mentioned above or due to cellular effects of RU486 independent of GR (130).

The binding of GCs to GR and subsequent induction of gene transcription activates a feedback mechanism that down regulates GR levels. This effect has been widely reported in the literature and has been shown to be dependent on GR hyperphosphorylation which occurs after ligand binding (131-134). The ligand-dependent phosphorylation of GR triggers ubiquitin-mediated degradation via the proteasome (41). The effect of the PI3-kinase and AKT inhibitors on GR turnover was investigated to see if the inhibition of PI3-kinase/AKT pathway effected the
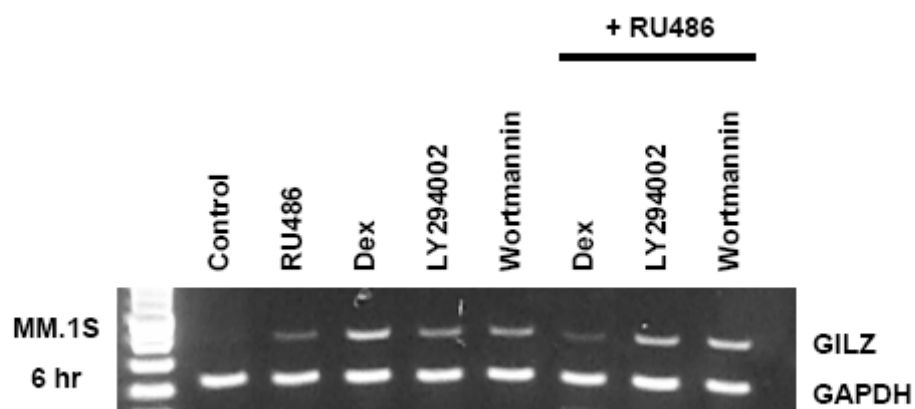
Figure 45. **RU486 treatment does not inhibit LY294002 or wortmannin-induced *GILZ* up regulation.** MM.1S cells were treated with 1 µM Dex, 25 µM LY294002, and 1 µM wortmannin for 6 hours. 10 µM RU486 was added 1 hour before the other drugs where indicated. *GILZ* and *GAPDH* levels in total RNA were measured with RT-PCR.

Dex (1 µM)	-	+	-	-	-	+	-	-
RU486 (10 µM)	-	-	+	-	-	+	+	+
LY294002 (25 µM)	-	-	-	+	-	-	+	-
Wortmannin (1 µM)	-	-	-	-	+	-	-	+
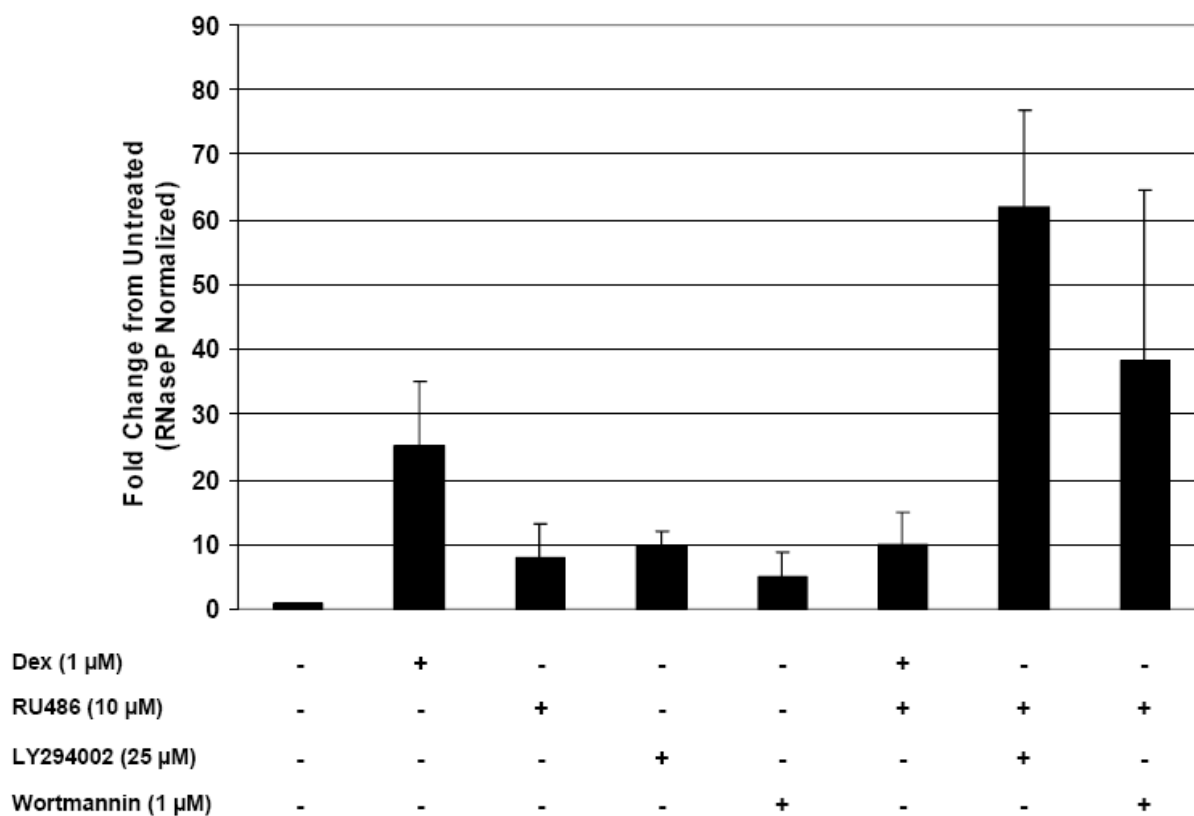
Figure 46. **RU486 enhances LY294002 and wortmannin induction of *GILZ*.** MM.1S cells were treated with 1 µM Dex, 25 µM LY294002, and 1 µM wortmannin for 6 hours. 10 µM RU486 was added 1 hour before the other drugs where indicated. *GILZ* was measured with real time PCR, normalized to *RNaseP*, and expressed as fold change from untreated sample.
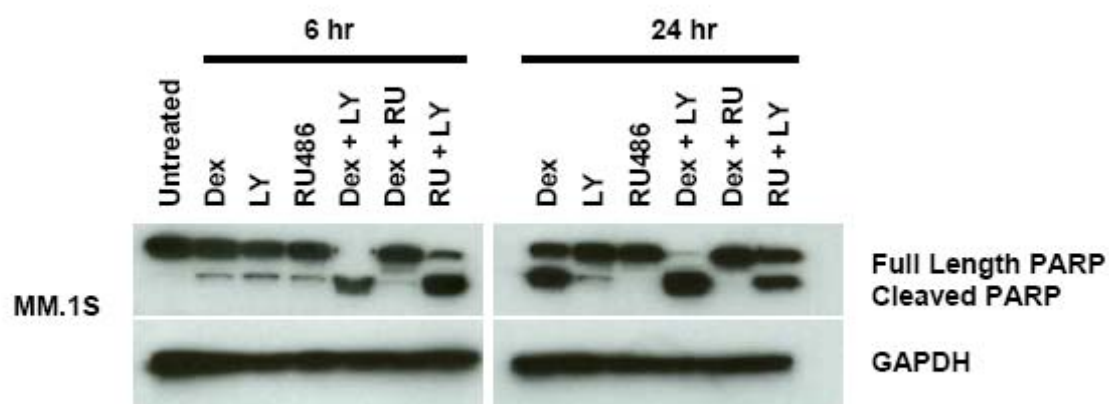
Figure 47. RU486 enhances LY294002-induced PARP cleavage of MM.1S cells. Whole cell

lysates of MM.1S cells treated with 1 μM Dex, 25 μM LY294002, and/or 10 μM RU486 (added

1 hour prior to Dex or LY294002) for 6 and 24 hours were analyzed by western blotting. Blots

were probed with antibodies to PARP and GAPDH.

kinetics of GR turnover. Because PI3-kinase and AKT are both kinases that affect phosphorylation of many cellular targets, this hypothesis was considered as the extent of GR phosphorylation contributes to GR degradation and it is possible that these kinase inhibitors could affect GR phosphorylation levels either indirectly or directly. This could explain the enhanced GILZ expression observed upon Dex treatment in combination with the kinase inhibitors used in these studies. The extent of GR down regulation at 24 hours was measured in Dex-treated and Dex and LY294002-treated samples by western blotting and was shown to be identical in the two samples. The down regulation of GR was independent of the extent of apoptosis as GR degradation was identical in both samples despite drastic differences in PARP cleavage (Figure 48) and the extent of *GILZ* up regulation (Figure 33).

In an attempt to explain the RU486 enhancement of LY294002 or wortmannin up regulation of *GILZ*, the possibility that the combination of RU486 and PI3-kinase inhibitors activated a similar GR feedback degradation was tested next. A very slight down regulation of GR was observed in the RU486 treatment lane compared to the untreated, LY294002 treated, or wortmannin treated lanes, but not as much as was observed in the Dex treated lane. The addition of RU486 inhibited GR down regulation resulting from Dex treatment when added together. The addition of LY294002 or wortmannin in combination with RU486 did not enhance GR down regulation compared to RU486 alone. While RU486 when added in conjunction with Dex could block GR turnover, the addition of LY294002 or wortmannin alone and in combination with RU486 did not activate or inhibit GR turnover (Figure 49). This suggests that neither the addition of LY294002 with GCs nor the combination of RU486 and LY294002 or wortmannin affects the down regulation of GR upon GC-binding.
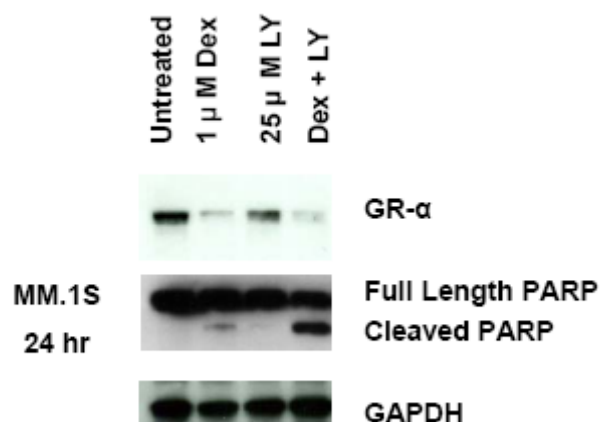
Figure 48.   GR degradation is not enhanced by LY294002 or enhanced apoptosis. Whole

cell lysates of MM.1S cells treated with 1 µM Dex and/ or 25 µM LY294002 for 24 hours were

analyzed by western blotting. Blots were probed with antibodies to GR-α, PARP and GAPDH.
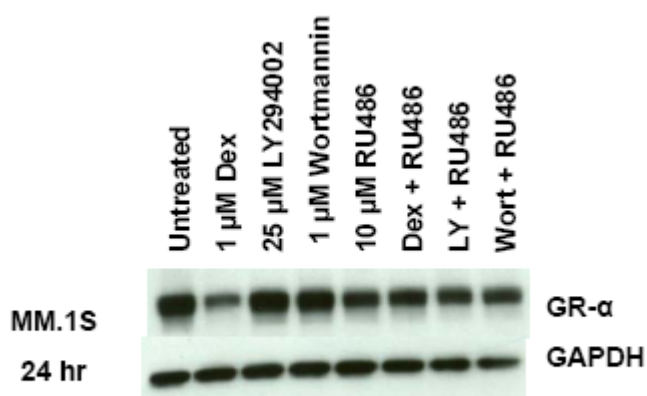
Figure 49. **GR degradation is not enhanced by RU486 and PI3-kinase inhibitor combination treatment.** MM.1S cells were treated with 1 µM Dex, 25 µM LY294002, 1 µM wortmannin, and/or 10 µM RU486 (added 1 hr before other drugs) for 24 hours. Whole cell lysates of samples were analyzed by western blotting. Blots were probed with antibodies to GR-α and GAPDH.

To further confirm that the amount of GR turnover is independent of the extent of cell death, the effect of the addition of the PAN caspase inhibitor ZVAD was measured. While ZVAD pretreatment could block caspase cleavage of PARP observed after Dex or Dex and LY294002 treatment, it had no effect on the extent of GR turnover observed at 24 hours (Figure 50). Using the same samples, the effect of ZVAD inhibition of caspase activation on *GILZ* up regulation was measured. The addition of ZVAD had no effect on *GILZ* up regulation by GC and/or PI3-kinase inhibition suggesting that *GILZ* up regulation and subsequent GR down regulation occurs upstream of cell death induction and is independent of caspase activation (Figure 51).

As reported by Asselin-Labat and colleagues, along with 6 glucocorticoid response elements (GRE), the promoter of *GILZ* contains 3 forkhead responsive elements (FHRE) (Figure 3). It was reported that the forkhead box O protein FOXO3 could activate the *GILZ* promoter independent of GR via the FHREs and that overexpression of FOXO3 increased *GILZ* levels (99, 100). FOXO3 and related family members are downstream targets of AKT where activated AKT phosphorylates and inhibits FOXO family members (22, 128). When phosphorylated, FOXO family members are sequestered in the cytoplasm and cannot activate gene transcription of many pro-apoptotic target genes. Because of these observations, the hypothesis that the FOXO family members mediate PI3-kinase/AKT inhibitor up regulation of *GILZ* by activating the *GILZ* promoter was considered next. RNAi constructs to FOXO3, the predominant FOXO family member in MM.1S, were used to decrease the levels of these proteins (Figure 52). FOXO3 protein levels were reduced by siRNA transfection, but the knockdown was not complete and some protein was still detectable in these samples. The ability of LY294002 with and without Dex was tested to see if a reduction in FOXO3 had any effect on the extent of GILZ
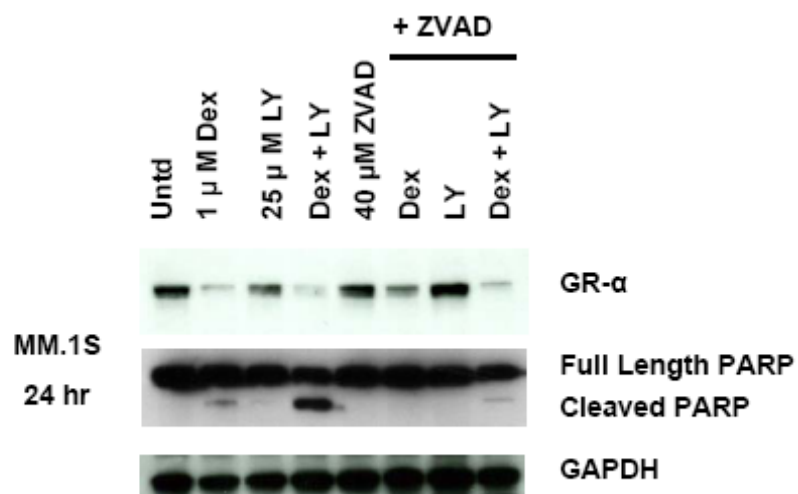
Figure 50. **GR degradation is not blocked by caspase inhibitor ZVAD.** Whole cell lysates of MM.1S cells treated with 1 μM Dex, 25 μM LY294002, and/or 40 μM ZVAD (added 1 hr before Dex or LY294002) for 24 hours were analyzed by western blotting. Blots were probed with antibodies to GR-α, PARP and GAPDH.
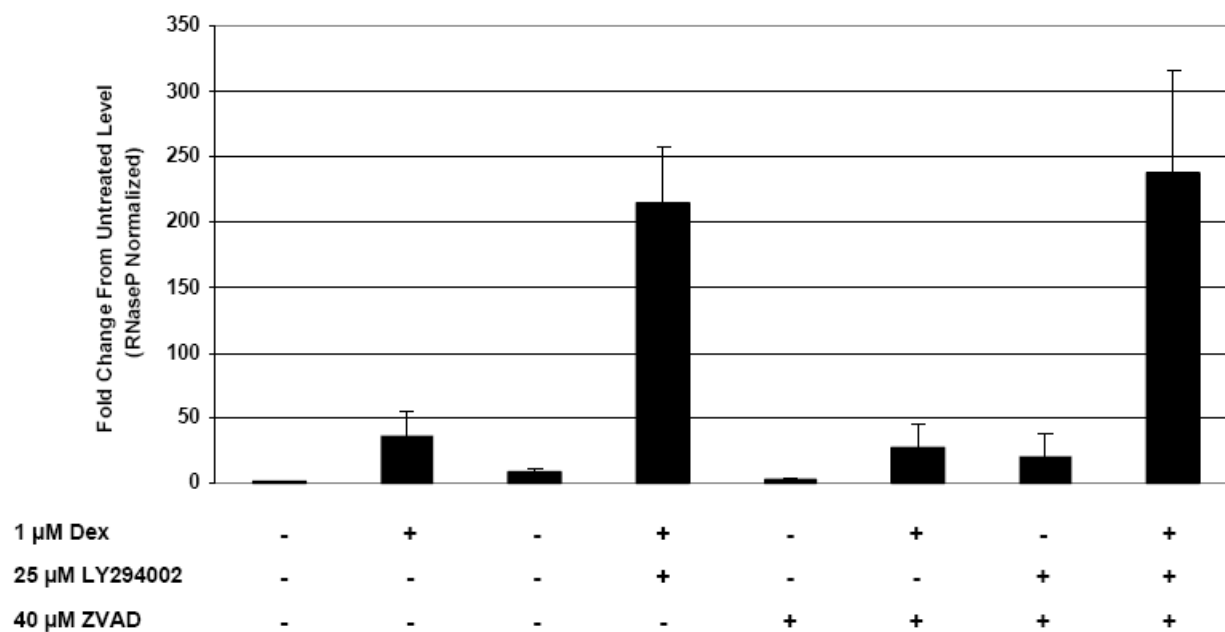
Figure 51. *GILZ* **induction is not blocked by caspase inhibitor ZVAD.** MM.1S cells were treated for 6 hours with 1 µM Dex, 25 µM LY294002 and/or 40 µM ZVAD (added 1 hr before Dex or LY294002). *GILZ* was measured with real time PCR, normalized to *RNaseP*, and expressed as fold change from untreated sample.

Figure 52. **Knock down of FOXO3 by siRNA in MM.1S cells.** MM.1S cells were

transfected with FOXO3 or control siRNA. 30 hours after transfection, the cells were harvested

and whole cell lysates analyzed by western blotting for FOXO3 and GAPDH protein expression.

Representative blots from one experiment is shown to give an example of the extent of FOXO3

knockdown.

up regulation. A decrease in GILZ baseline levels of 56% was observed upon FOXO3 siRNA transfection in MM.1S cells compared to control siRNA-transfected cells. However, no effect on *GILZ* induction by Dex and/or LY294002 was observed upon reduction FOXO3 protein levels (Figure 53).

From these studies, it can be concluded that GC treatment does not alter AKT activation. It can also be concluded that the addition of PI3-kinase/AKT inhibitors does not affect GR degradation after ligand binding and that GR turnover is independent of the extent of GILZ up regulation and cell death induction. FOXO3 was confirmed to play a role in regulating *GILZ* baseline levels, but reducing FOXO3 levels did not alter LY294002 or GC/LY294002 up regulation of *GILZ*. It is possible that other FOXO family members are compensating for the decrease in FOXO3 or that PI3-kinase/AKT regulation of *GILZ* is independent of FOXO family members all together. The details into the exact mechanism of PI3-kinase/AKT inhibitor up regulation of GILZ require more investigations.
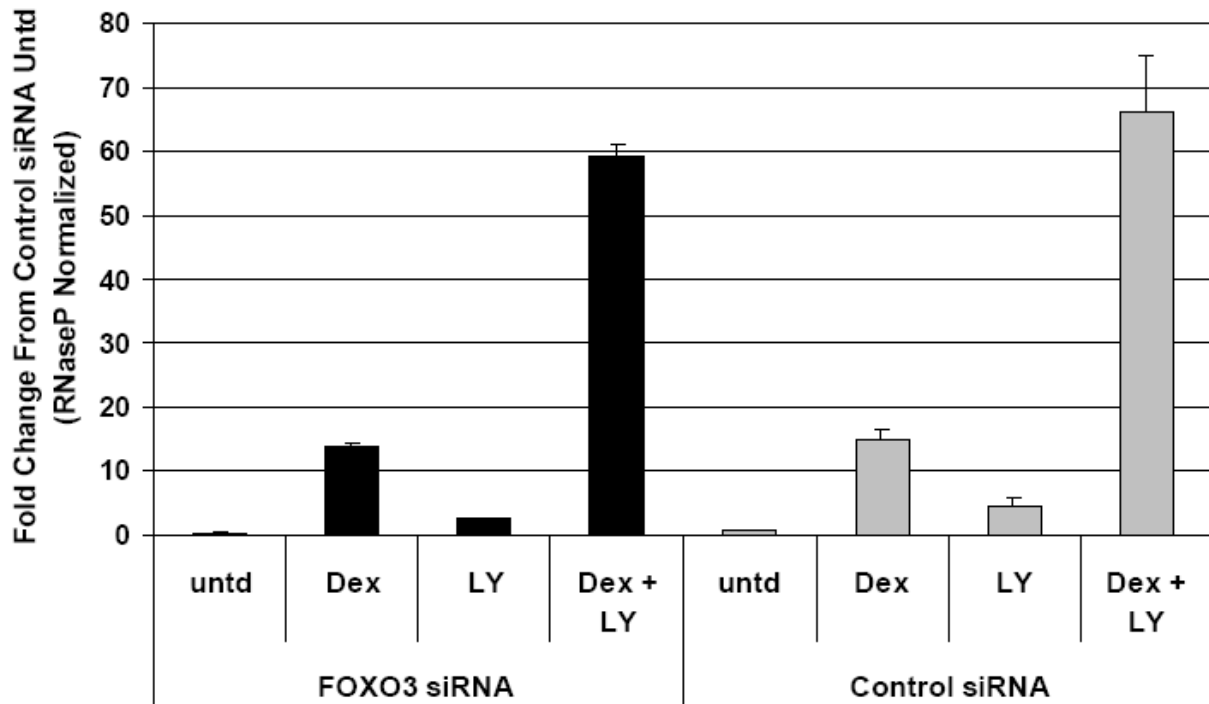
Figure 53. **Reduction in FOXO3 protein does not effect Dex or LY294002 up regulation of GILZ.** MM.1S cells were transfected with FOXO3 siRNA or control siRNA. 24 hours after transfection, the cells were treated with 1 µM Dex and/or 25 µM LY294002 for 6 hours. Total RNA was isolated and *GILZ* levels were measured with real time PCR, normalized to RNaseP, and expressed as a fold change from the control siRNA transfected untreated sample.

CHAPTER 6

EFFECT OF BONE MARROW MICROENVIRONMENT ON GILZ REGULATION AND GLUCOCORTICOID-INDUCED APOPTOSIS

The importance of the bone marrow microenvironment for promoting MM cell growth and resistance to chemotherapy has been reported in the literature (2, 6). In order to address the effects of the surrounding microenvironment on MM cells, a co-culture system of MM.1S and immortalized human bone marrow stromal cells was established for tissue culture experiments in the laboratory. Hs5 and Hs27 cells immortalized with HPV16 E6 and E7 viral oncogenes have been reported to support the growth of hematopoietic progenitor cells (108). The two cells lines represent components of the bone marrow microenvironment which include a variety of extracellular matrix proteins, bone remodeling osteoclasts and osteoblasts, vascular endothelial cells, and stromal cells. Hs5 are small fibroblastic-like cells and have been reported to secrete IL-6, IGF1, and MIP1α. Hs27 are larger epitheloid-like cells that express VCAM and secrete VEGF (108).

Stromal cell co-culture inhibits *GILZ* induction in MM.1S cells.

MM.1S cells were plated above cultures of adherent Hs5 or Hs27 cells set up 24 hours previous. After 24 hours of co-culture, treatments of Dex and/or LY294002 were added for 6 hours. After treatment, the upregulation of *GILZ* by Dex and/or LY294002 in MM.1S cells was determined with real time PCR and the effect of the co-culture was compared to MM cells cultured alone under normal conditions. As shown in Figure 54 and 55, co-culturing MM.1S cells with either Hs5 or Hs27 cells reduced *GILZ* up regulation by either GC or LY294002 treatment. It has been previously reported that Hs5 cells secrete IL-6 and IGF1 and in Chapter 5, these cytokines were shown to partially inhibit *GILZ* up regulation by Dex and LY294002 (Figure 24 and 34). However, Hs27 cells do not secrete measurable levels of either cytokine so the inhibition of *GILZ* up regulation observed upon co-culture with Hs27 cells
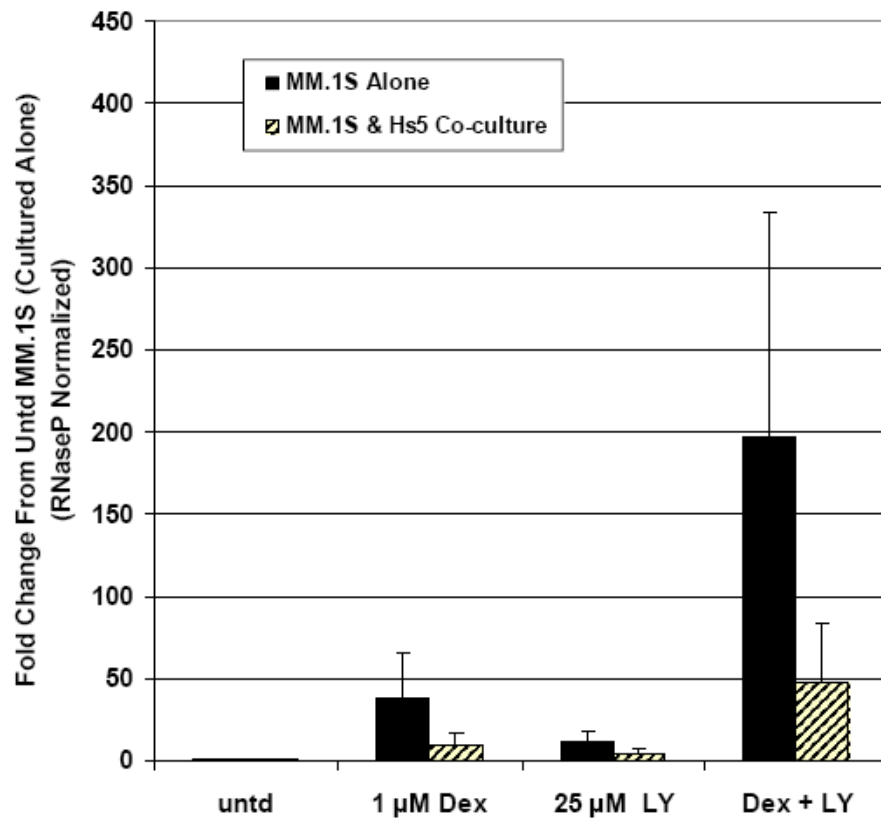
Figure 54. *GILZ* **expression is reduced in MM.1S cells when co-cultured with bone marrow stromal cell line Hs5.** MM.1S cells co-cultured with Hs5 cells or alone were treated with 1 µM Dex and/or 25 µM LY294002. *GILZ* levels were measured in total RNA isolated from MM cells after 6 hour drug treatment, normalized to *RNaseP*, and expressed as fold change from untreated sample.
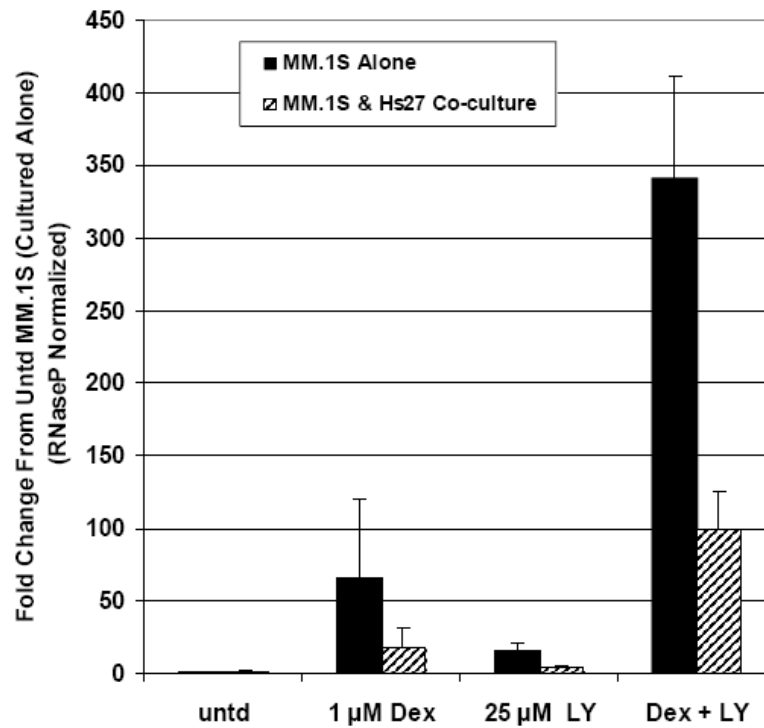
Figure 55. *GILZ* **expression is reduced in MM.1S cells when co-cultured with bone marrow stromal cell line Hs27.** MM.1S cells co-cultured with Hs27 cells or alone were treated with 1 µM Dex and/or 25 µM LY294002. *GILZ* levels were measured in total RNA isolated from MM cells after 6 hour drug treatment, normalized to *RNaseP*, and expressed as fold change from untreated sample.

cannot be explain by this mechanism. The secretion of IL-6 and IGF1 by Hs5 cells, but not Hs27 cells was confirmed in our laboratory (M. Shanmugam unpublished observation). It is possible that other undetermined cytokines secreted by Hs27 can activate PI3-kinase/AKT similar to IL-6 and IGF1. It is also possible that the physical interaction between the two cell types may play a role in activating signaling pathways within MM.1S resulting in *GILZ* down regulation.

To determine whether the physical contact between MM and stromal cells or the secreted cytokines from the stromal cells mediates inhibition of *GILZ* up regulation, experiments using transwell cell culture inserts were conducted. In these experiments, the stromal cells were plated as before and allowed to attach for 24 hours. The MM cells were either placed directly on top of the stromal cell layer or were placed on a cell culture insert membrane with pores large enough for cytokines and other media components to flow through, but too small to allow for cell passage. After 24 hours of co-culture, the cells were treated with Dex and LY294002 alone and in combination and the extent of *GILZ* up regulation was compared among three groups: MM and stromal cells separated by a transwell cell culture insert, MM and stromal cells cultured together without an insert where cell to cell contact could occur, and MM cells cultured alone on tissue culture plastic. As shown in Figures 56 and 57, co-culture with the stromal cells with or without a transwell insert resulted in inhibition of *GILZ* up regulation. The inhibition was greater in the sample set where cell to cell contact between the two cell types could occur than was in the sample set where the cells were separated by a cell culture insert. This suggests that both stromal cell types are secreting factors which are able to modulate *GILZ* levels and that a portion of the inhibition of *GILZ* up regulation is the result of the physical cell to cell interaction

between the MM and stromal cell lines. Further experiments need to be done to identify the components secreted by each stromal cell line which can activate PI3-kinase/AKT and inhibit *GILZ* levels and also identify the cell surface proteins mediating cell-cell contact. As reported previously, the BMME supports MM growth and drug resistance. The data in this chapter collectively supports the role of the BMME on regulating gene expression of GILZ which has been shown to play a functional role in GC-induced apoptosis (Chapter 4). These results provide one possible mechanism for how BMME supports MM growth and resistance to GCs.
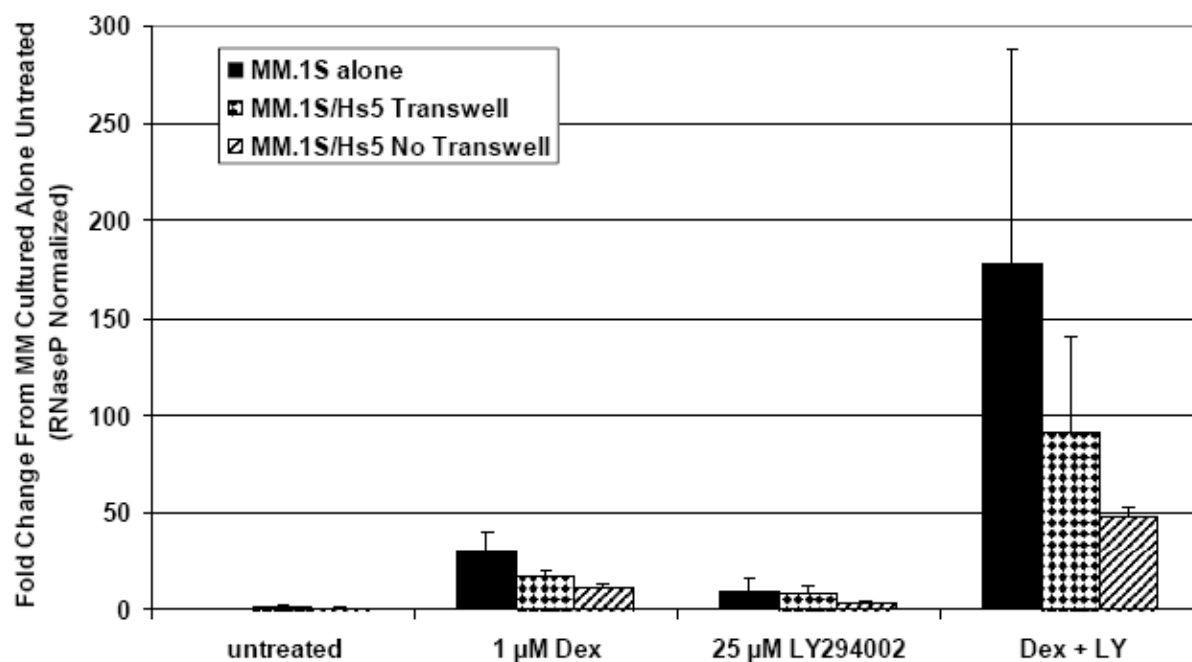
Figure 56.   Separation of MM cells from Hs5 cells with transwell filter insert reduced inhibition of *GILZ* up regulation. MM.1S cells co-cultured with attached Hs5 cells, separated from Hs5 cells by a transwell filter, or cultured alone were treated with 1 µM Dex and/or 25 µM LY294002 for 6 hours. *GILZ* levels were measured in total RNA isolated from MM cells after 6 hour drug treatment, normalized to *RNaseP*, and expressed as fold change from untreated sample.
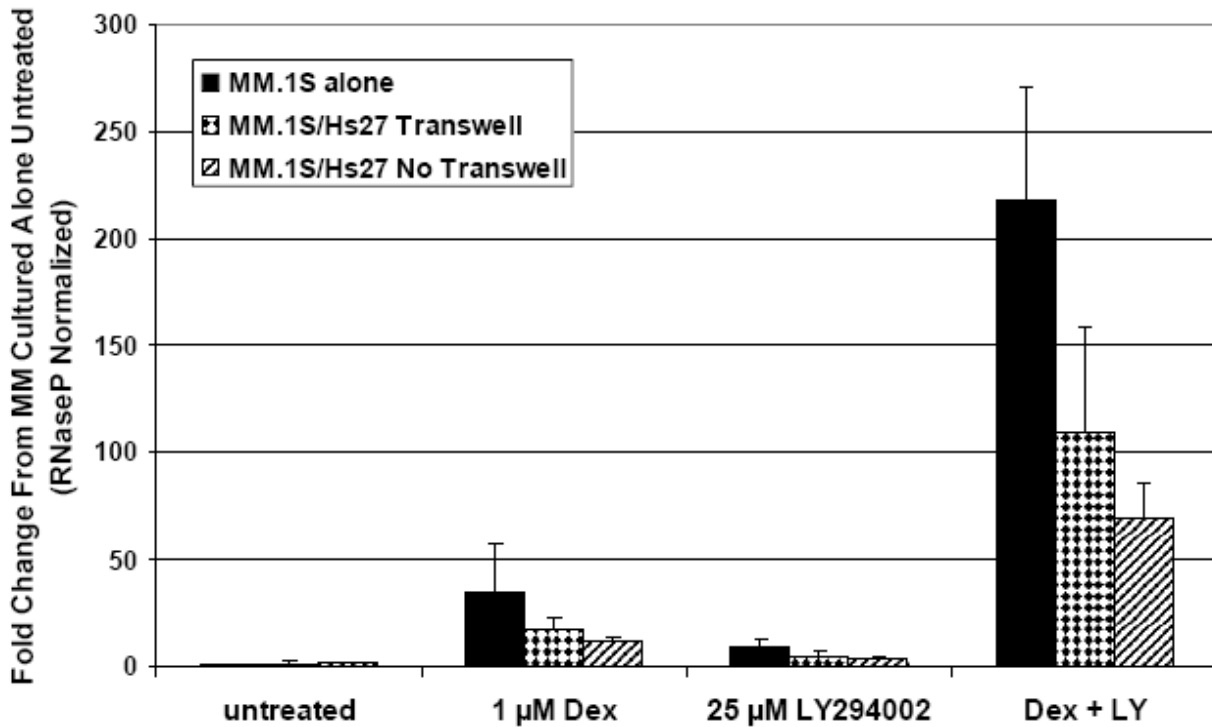
Figure 57.   Separation of MM cells from Hs27 cells with transwell filter insert reduced inhibition of *GILZ* up regulation. MM.1S cells co-cultured with attached Hs27 cells, separated from Hs27 cells by a transwell filter, or cultured alone were treated with 1 µM Dex and/or 25 µM LY294002 for 6 hours. *GILZ* levels were measured in total RNA isolated from MM cells after 6 hour drug treatment, normalized to *RNaseP*, and expressed as fold change from untreated sample.

CHAPTER 7

DISCUSSION

It has been estimated that there are over 50,000 people living with MM in the US today. Despite promising new therapeutic options and extended survival times, the disease currently remains incurable (150). Research into the biology behind this disease will aid in the development of additional therapeutics and ways to combat drug resistance. GCs are widely used in the treatment MM patients, however most patients relapse due to the development of resistance. The molecular details of GC-induced apoptosis in MM remain largely undefined, though GC activation of downstream gene targets is believed to play a role (29). Through micro-array analysis, *GILZ* was identified as a highly up regulated gene target of GCs in MM.1S cells (79, 80). This project began with the hypothesis that GILZ is a key component of GC-signaling in MM cells and acts as a mediator of GC-induced apoptosis. The goals of this research were to gain insight into the details of GC-induced apoptosis in order to benefit MM therapeutic development, to determine if GILZ was an important gene target of GCs playing a role in GC-induced killing, and to understand how GILZ was regulated in myeloma cells to provide insight into GC signaling and the involvement of other signaling pathways within the cell in GC-induced apoptosis.

Summary of Findings

The initial studies verified the GC-induced up regulation of GILZ and work was continued with further characterization of the parameters of this observation. The up regulation of GILZ by GC treatment has been confirmed at the mRNA and protein level in a panel of myeloma cell lines and patient samples as well as in CLL patients and normal PBMCs. Up regulation of GILZ was observed with therapeutic doses of Dex capable of inducing apoptosis of MM.1S cells and only by GCs that are cytotoxic to MM.1S cells. GILZ up regulation occurs via

the GR as the GR antagonist RU486 blocked GC-induction of GILZ and GC-induced GILZ expression did not occur in cell lines with low to no measurable GR levels.

The importance of GILZ was investigated further in myeloma cell lines. The function of GILZ had been studied mostly in T cells and its role in B cells or myeloma had not been identified previously. With these studies, GILZ has been identified to be play a functional role in GC-induced apoptosis of MM.1S cells in experiments utilizing siRNA knock-down of GILZ. Reducing the level of GILZ at the mRNA and protein level decreased the killing potential of GCs in MM.1S cells. This connection between GILZ and MM cell GC-induced apoptosis had not been confirmed prior to these studies.

In vivo, myeloma cells receive important growth and survival signals from the surrounding tumor microenvironment. Therefore, an important component of therapeutic drug development is understanding the contribution of the surrounding tissue. In MM, the tumor microenvironment activates the PI3-kinase/AKT pathway which plays an important role in supporting MM cell growth and survival. With this work, it was observed that GILZ is regulated by members of the PI3-kinase/AKT signaling pathway. IL-6 and IGF1, both activators of PI3-kinase/AKT, reduce the baseline levels of *GILZ* and inhibit GC-induced up regulation of *GILZ* and GC-induced cell killing. Inhibitors of PI3-kinase and AKT (LY294002, wortmannin, triciribine, and AKT inhibitor VIII) up regulate GILZ levels at both the mRNA and protein level in MM cell lines and patient samples.

The regulation of GILZ by PI3-kinase/AKT pathway became even more compelling when treatments were given in conjunction with GC. When used together, GCs and PI3-kinase/AKT inhibitors dramatically enhance GILZ levels within the cell at both the mRNA and protein level. This enhanced up regulation occurs at both the mRNA and protein level and is

observed in multiple MM cell lines and in MM patient cells from the clinic. The addition of IL-6 or IGF1 could inhibit this up regulation in a similar manner to the effects on GC-induced up regulation of *GILZ*.

Interestingly, the combination of GCs and PI3-kianse/AKT inhibitors also enhanced cell killing of MM cells compared to either agent alone as reported with PARP cleavage western blot, PI staining, and Annexin-V staining. Formal synergism analysis demonstrated that the combination of Dex and LY294002 resulted in synergistic cell killing of MM.1S cells suggesting the potential of cross-talk between these two pathways.

Finally, experiments conducted when MM cells were co-cultured with bone marrow stromal cells lines have established that the tumor microenvironment can regulate gene expression within MM cells. Upon co-culture with either Hs5 or Hs27 immortalized human bone marrow stromal cell lines, MM.1S cells treated with GC and/or LY294002 had reduced *GILZ* up regulation compared to the up regulation observed upon treatment of MM.1S cells cultured alone. Two hypothesis could explain this observation. The reduced *GILZ* expression could be the result of the physical interaction between the two cell types or the result of the effect of paracrine factors released from the stromal cells . Experiments to distinguish between these two hypotheses were conducted using a cell culture insert with a membrane that physically separates the two cell types, yet allows for passage of cytokines and growth factors. Results from these experiments indicate that both physical cell-to-cell contact and released paracrine factors contribute to the reduced *GILZ* induction in myeloma cells observed upon co-culture with bone marrow stromal cells

Importance of GILZ to GC-induced apoptosis

Because the induction of apoptosis by GCs is so critical for the treatment of hematologic malignancies, a number of laboratories have searched for GC-regulated death-inducing genes which may be important mediators of GC-induced apoptosis. The results presented here based on GILZ knockdown experiments and compelling data in the literature implicate GILZ as a key component of GC signaling in MM cells and a candidate death-inducing gene. The function of GILZ has been studied extensively in T cells where both pro- and anti-apoptotic functions have been reported (97, 100). However, before this study the function of GILZ had yet to be characterized in B cells or in myeloma. Here it is reported that in MM cells, GILZ has a pro-apoptotic function where a reduction in GILZ levels with siRNA results in a decrease in the potency of GCs to induce apoptosis. *GILZ* up regulation by GCs occurs in the MM.1S myeloma cell line which is sensitive to GC killing, but not in the MM.1R$_e$ or MM.1R$_L$ myeloma cell lines which are resistant to GCs. *GILZ* is up regulated by all GCs tested that induce apoptosis, while those GCs that cannot induce apoptosis of MM.1S cells fail to up regulate *GILZ*. Collectively, these findings suggest that GILZ is an important mediator of GC-induced killing in MM cells.

These studies have identified a functional importance of GILZ in GC-induced killing of MM cells. Reduction of GILZ levels by 50% in MM cells decreased GC killing potential. The knockdown of GILZ was not complete and GC treatment could trigger GILZ induction albeit not to same level as in cells with control siRNA. A 10% reduction in apoptosis was observed in GILZ siRNA cells. It is possible that with more durable knockdown of GILZ perhaps with stable transfection of lentiviral shRNA contructs would result in a more dramatic effect on GC-induced apoptosis.

GILZ has been reported to interact directly with NF-κB, AP-1, and members of Ras/Raf MAPK pathway in T cells (51, 86, 92). The interacting targets proteins of GILZ in MM cells have not been investigated, nor have targets of downstream gene repression been identified. Experiments over-expressing GILZ independent of GC treatment may help identify GILZ interacting partners and micro-array of GILZ over-expressing cells should be done to identify changes in gene expression. Because GCs treatment alone results in alternations in gene expression including the induction of *GILZ*, a micro-array study of GILZ over-expressing cells would separate the GILZ target genes from those regulated by GCs and other targets.

One of the goals of these studies was to establish a connection between the ability of GC to kill MM cells and the level of GILZ up regulation. As observed in Figures 12 & 19, GILZ up regulation was higher in MDR10V and RPMI-8226 cells that were less sensitive to GC killing than MM.1S cells. As a link between *GILZ* levels and GC-induced killing in MM.1S cells was established in Figure 18, other mechanisms contributing to GC resistance may exist in the MDR10V and RPMI-8226 cells and likely play a role in their diminished response to GCs. Experiments to investigate the importance of GILZ with siRNA and potential mechanisms of GC resistance in these cells could help to explain this contradictory findings. In a different hematologic malignancy, childhood acute lymphoblastic leukemia (ALL), GC-induction of GILZ still occurred in Dex-resistant human ALL biopsies whereas resistance was reported to be associated with a failure to induce the pro-apoptotic BH3-only gene *bim* (135). Studies looking into Bim in myeloma cells may also be beneficial.

As described in Chapter 1, GILZ function has mirrored the function of GC in all cell types and systems studied (85, 93, 96, 98) with the exception of the reports by Asselin-Labat and colleagues where GILZ counteracted the function of GC-induced cell death (99, 100) and a

report in mesenchymal cells where GILZ inhibited GC-induced adipocyte differentiation (95). From the preliminary functional data presented in this report, it appears that GILZ mirrors the functions of GC in MM cells. Further work deciphering the details of systems where GILZ counteracts GCs and systems where GILZ mirrors GC function need to be done to understand these conflicting reports. As described in Chapter 1, GC affect a myriad of physiological processes and GC functions varies from cell type to cell type (38, 40-43). The molecular details of GC induced apoptosis are believed to be cell type specific and multiple GC-gene targets are important mediators of this effect (38). Because of this overall complexity, it is not surprising that GILZ function also varies in different cell types and may work in conjunction with GCs at times and against GCs at other instances.

Potential cross-talk between GR and PI3-kinase/AKT in MM cell signaling.

In this report, the PI3-kinase/AKT inhibitor up regulation of *GILZ* occurs independent of the GR status of the MM cell lines tested (Figure 30). However, a dramatic enhancement of GILZ up regulation and synergistic cell killing is observed in GR expressing MM cell lines upon the combination of GCs and PI3-kinase/AKT inhibitors (Figure 37). This suggests that potential cross-talk between these two pathways may exist. The mechanism underlying this cross-talk may provide important information for the development of combined therapeutic strategies and deserves further exploration.

Clues to the mechanism may be found in previous studies. Cooperation between the PI3-kinase/AKT and nuclear receptor pathways has previously been reported in other biologic models. A physical interaction between the GR and the p85 regulatory sub-unit of PI3-kinase has been reported in a number of different cell systems and this interaction was shown to counteract the tumorigenicity of activated AKT in a mouse skin cancer transgenic model (136-

138). GR protein-protein interactions with Raf-1 and 14-3-3 have been detected in the cytoplasm of rat liver cells and the GR was suggested to regulate the MAPK pathway in this manner (139). In MM cells, GC effects on AKT activation as measured by phosphorylation were not observed (Figure 41), but further experiments to investigate GC effects on PI3-kinase/AKT and potential interactions in MM cells may be informative.

Interestingly, in a genomics screen of ALL cells, genes associated with the PI3-kinase/AKT pathway were highly enriched in a gene signature of GC-resistance (140). In that study, rapamycin had a drug associated gene expression profile which matched the gene signature of GC sensitivity. Rapamycin inhibits mTOR, a downstream target of AKT, and has been reported to sensitize resistant MM cells to Dex-induced apoptosis (141). Downstream of AKT, the immediate substrates of mTOR are p70S6 kinase and 4E-BP1 translation repressor both which play roles in cap-dependent translation of cell cycle proteins. Thus inhibition of mTOR by rapamycin is hypothesized to inhibit cell cycle progression due to an inhibition of protein translation. In the results of the RT-PCR screen presented in Chapter 5, rapamycin did not up regulate *GILZ* in MM.1S (Table 4 and Figure 27). The effect of GC added in combination with rapamycin warrants testing using quantitative real time PCR to see if there is a combinatorial effect that was not picked up in the original RT-PCR screen. This will test the hypothesis that inhibition of a downstream target of AKT in conjunction with GC treatment may also effect *GILZ*. Complicating the signaling however in MM cells, rapamycin was recently reported to enhance PI3-kinase/AKT activation due to lack of negative feedback regulation on insulin receptor substrate (IRS) that did not occur upon inhibition of mTOR (142). This could explain why up regulation of GILZ does not occur upon rapamycin treatment as the drug activates AKT while at the same time inhibiting downstream mTOR.

Role of FOXO3 in PI3-kinase/AKT regulation of *GILZ*

Interaction of nuclear hormone receptors with members of the FOXO protein family downstream of PI3-kinase/AKT has also been reported in the literature. The progesterone receptor, which shares a consensus DNA binding sequence with the GR, has been reported to cooperate with FOXO1 and bind adjacent on the promoter of the insulin-like growth factor-binding protein-1 (*IGFBP1)* (143, 144). Additionally, binding sites for both GR and FOXO1 exist in the GRE of the glucose-6-phosphate transporter gene promoter where both proteins cooperate to activate its transcription (145). Based on the sequence of the *GILZ* promoter and location of the GRE and FHRE binding sites (Figure 3), adjacent binding of GR and FOXO proteins next to each other would not be predicted, however an interaction may still occur (50).

As reported previously, mutation of the three FHRE within the *GILZ* promoter reduced Dex-induced up regulation of *GILZ* in IL-2 dependent cytotoxic T cells (99, 100). IL-6 and IGF1 have been shown to inhibit FOXO family members through the activation of PI3-kinase/AKT and in MM, treatment with inhibitors to PI3-kinase and AKT results in the activation of FOXO family members (20). These results together support an interaction between the GC and PI3-kinase pathways at the level of the FOXO family. Preliminary experiments reducing the levels of FOXO3 in MM.1S cells suggest that FOXO3 regulates GILZ baseline levels, but is not the mediator of LY294002-induced upregulation of GILZ alone or in cooperation with Dex, as there was no change in the extent of GILZ upregulation upon FOXO3 reduction by siRNA (Figure 53). The down regulation of FOXO3 with siRNA was not complete as FOXO3 protein was still detectable with western blotting (Figure 52). It is possible that a complete loss of FOXO3 is required in order to see an effect on the LY294002 induction of GILZ or that other FOXO family members can compensate for the loss of FOXO3 preventing

any observable effect. Future work with a more complete knockdown of FOXO3 and studies clarifying the importance of other FOXO family members may provide more information into the importance of FOXO3 in GILZ induction by PI3-kinase/AKT inhibition.

Post-translational modifications on GR by PI3-kinase/AKT pathway

Another potential mechanism to explain the effects of the PI3-kinase/AKT inhibitors on GILZ regulation and potential cross-talk with the GR pathway could involve post-translational modifications of the GR. GR protein can undergo a variety of complex post-translational modifications including phosphorylation, sumoylation, and ubiquitination (146). The human GR can be phosphorylated at specific sites, most located within the N terminal domain. A number of kinases including p38 MAPK, cyclin dependent kinases (CDK), glycogen synthase kinase-3 (GSK-3), and c-Jun N terminal kinases (JNK) have been reported to phosphorylate some of these sites (146, 147). In some cases, these phosphorylation events are dependent on ligand binding, but other sites are phosphorylated in a ligand independent manner. GR phosphorylation has been shown to affect its cellular localization, transactivation potential and protein turnover (146). This N terminal region known as the AF1 region of the GR where the majority of known phosphorylation sites are located has been shown to not be required for the GC-induction of GILZ (147, 148). It is possible that other phosphorylation sites exist on the GR protein and that the PI3-kinase/AKT inhibitors used in these studies can affect these sites. As stated above, GSK-3 was previously identified to phosphorylate rat GR and is a downstream target of AKT (148). Research into the effect of the PI3-kinase and AKT inhibitors on GR phosphorylation in MM cells should be investigated to determine if GR phosphorylation at known and novel sites is altered by the PI3-kinase/AKT inhibitors with and without GC and if changes in phosphorylation by these drug combinations result in enhancing GR transactivation and *GILZ* induction.

Previously it was shown that the mutation of phosphorylation sites in GR increased its half-life and abolished GR down regulation which occurs after ligand binding suggesting that the phosphorylation of GR is involved in mediating protein turnover. Experiments presented in this dissertation tested the hypothesis that the PI3-kinase/AKT inhibitors could affect GR turnover. These experiments showed that there was no effect on GR turnover by PI3-kinase/AKT inhibition in MM.1S cells (Figures 47 - 49) and that the amount of GR turnover was not dependent on the extent of apoptosis induced in these cells. While no effect on GR degradation was observed by combining PI3-kinase/AKT inhibitors with GCs, it is possible that the PI3-kinase/AKT inhibitors may effect other aspects of GR function still to be determined.

RU486 as an agonist and enhancer of GILZ expression

While investigating the importance of the GR to PI3-kinase/AKT inhibitor up regulation of GILZ, it was observed that the addition of RU486 with PI3-kinase inhibitors enhanced GILZ up regulation and MM apoptosis. As described in Chapter 5, partial agonist properties of RU486 have been identified when RU486 is added without GC resulting in a slight induction of *GILZ*. Based on the findings of combining RU486 with LY294002 or wortmannin, it is possible that the presence of PI3-kinase/AKT inhibitors enhances the agonist properties of RU486 in MM cells. Investigations using chromatin immunoprecipitation to identify binding proteins on the GILZ promoter would test the hypothesis if GR bound to RU486 can bind to the GILZ promoter and if the addition of PI3-kinase/AKT inhibitors enhances that binding. Effects of RU486 and the kinase inhibitors on GR phosphorylation and *GILZ* message stabilization should also be considered as possible mechanisms to explain the observed RU486 agonist-like properties.

Effect of the tumor microenvironment on MM gene expression

Experiments involving co-culture of MM.1S cells with two human bone marrow stromal cell lines (Hs5 and Hs27) have revealed that the tumor microenvironment can regulate *GILZ* expression in MM.1S cells. Using cell culture transwell inserts, it was shown that both paracrine factors released from the stromal cells and the physical cell-to-cell contact between the two cell types in part regulates *GILZ* expression. Hs5 cells have been shown to secrete IL-6 and IGF1, two cytokines that were shown in this report to regulate GILZ. However Hs27 cells do not secrete these growth factors. Further experiments to identify the other factors secreted by Hs27 cells that can affect GILZ need to be done, as well as studies to characterize the potential cell surface receptors that mediate the cell-to-cell contact between the MM and stromal cells which results in inhibition of *GILZ* up regulation.

Previous knowledge from the literature suggests that the co-culture of MM cells with bone marrow stromal cells should protect MM cells from GC-induced killing. Based on these results identifying GILZ as an important mediator of GC-induced killing and that the stromal cells can block *GILZ* up regulation in MM cells, the co-culture of MM cells with stromal cells should decrease GC killing potential. This apoptotic protection from the microenvironment needs to be investigated with this co-culture system and also using bone marrow stroma isolated from MM patient samples from the clinic to see if similar effects can be observed with non-immortalized stromal cells.

Therapeutic effect of combination of GC and PI3-kinase/AKT inhibitors

The data presented in this dissertation has shown that the combination treatment of PI3-kinase/AKT inhibitors with GCs dramatically up regulates a GC-induced gene product and synergizes to enhance MM cell killing. This observation provides the biologic basis for rational

drug design wherein studies deciphering the therapeutic benefit of the combination therapy of GC and PI3-kinase/AKT blockade are warranted as a beneficial alternative treatment regimen for MM patients. Many PI3-kinase and AKT inhibitors are currently being developed as this pathway has been shown to be mutated in a variety of cancer types (149). The use of these agents in combination should be further investigated to establish their therapeutic potential. A clinically relevant AKT inhibitor perifosine was recently reported to augment Dex induced killing of MM.1S cells though not synergistically (27). The effects of perifosine on GILZ induction may provide data to determine if GILZ upregulation is necessary for the observed synergism between GC and PI3-kinase/AKT inhibitors. These studies revealing dual regulation of GILZ and synergistic killing by PI3-kinase/AKT inhibitors and GCs add to the observation of the therapeutic benefit of perifosine together providing a strong rationale for clinical trials with GCs and inhibitors of PI3-kinase/AKT combination treatment. Most intriguingly, these studies indicate that inhibition of the PI3-kinase/AKT pathway may be an effective therapeutic approach in the face of GC resistance.

In conclusion, the work presented in this dissertation identifies GILZ as an important mediator of GC-induced apoptosis in myeloma cells. It also reveals regulation of GILZ by the PI3-kinase/AKT pathway where inhibition of this pathway by pharmacologic inhibitors up regulated GILZ levels and synergizes with GCs to induce MM cell death. This strengthens the importance of the PI3-kinase/AKT pathway in MM and reveals potential cross-talk with the GR signaling. The combination of GC and PI3-kinase/AKT inhibitors have potential use as dual treatment that may prove beneficial to patients with multiple myeloma.

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Publications

Journals

- **Davies, KM**, Ma, C, Kurosawa Y, Singhal, S, Krett NL, and Rosen ST. Dual Regulation of Glucocorticoid-Induced Leucine Zipper (*GILZ)* By the Glucocorticoid Receptor and the PI3-kinase/AKT Pathways in Multiple Myeloma. J Steroid Biochem Mol Bio, *submitted*
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Seminars

- "Dual Regulation of Glucocortioid-Induced Leucine Zipper (GILZ) by the Glucocorticoid Receptor and Phosphatidylinositol 3-Kinase/AKT Pathways in Multiple Myeloma." Final Thesis Seminar, Northwestern University Graduate School, Robert H. Lurie Comprehensive Cancer Center, Chicago, IL. June 8, 2007.
- "Regulation of GILZ in Multiple Myeloma Reveals Potential Cross-talk between PI3-kinase/Akt and the Glucocorticoid Receptor Pathway." Tumor Cell Biology Seminar Series, Robert H. Lurie Comprehensive Cancer Center. Northwestern University, Chicago, IL. October 19, 2006.
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