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Dual regulation of glucocorticoid-induced leucine zipper (*GILZ*) by the glucocorticoid receptor and the PI3-kinase/AKT pathways in multiple myeloma

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Abstract

Glucocorticoids (GCs) are effective therapeutics commonly used in multiple myeloma (MM) treatment. Clarifying the pathway of GC-induced apoptosis is crucial to understanding the process of drug resistance and to the development of new targets for MM treatment. We have previously published results of a micro-array identifying glucocorticoid-induced leucine zipper (*GILZ*) as GC-regulated gene in MM.1S cells. Consistent with those results, GCs increased *GILZ* in MM cell lines and patient samples. Reducing the levels of *GILZ* with siRNA decreased GC-induced cell death suggesting *GILZ* may mediate GC-killing. We conducted a screen to identify other pathways that affect *GILZ* regulation and report that inhibitors of PI3-kinase/AKT enhanced *GILZ* expression in MM cell lines and clinical samples. The combination of dexamethasone (Dex) and LY294002, wortmannin, triciribine, or AKT inhibitor VIII dramatically up regulated *GILZ* levels and enhanced apoptosis. Addition of interleukin-6 (IL-6) or insulin-like growth factor (IGF1), both which activate the PI3-kinase/AKT pathway and inhibit GC killing, blocked up regulation of *GILZ* by GC and PI3-kinase/AKT inhibitors. In summary, these results identify *GILZ* as a mediator of GC killing, indicate a role of PI3-kinase/AKT in controlling *GILZ* regulation and suggest that the combination of PI3-kinase/AKT inhibitors and GCs may be a beneficial MM treatment.

Keywords

Multiple myeloma; Glucocorticoid; Glucocorticoid receptor; *GILZ*; PI3-kinase; AKT; Signal transduction

1. Introduction

Multiple myeloma (MM) is the second most commonly diagnosed hematologic malignancy in the US with an estimated 16,570 new cases and 11,310 deaths in 2006 [1,2]. MM is a malignant plasma-cell tumor located at multiple sites in the bone-marrow compartment [3]. Targeting aspects of the bone marrow microenvironment (BMME) that contribute to MM

pathogenesis has emerged as new area of therapeutic development. Interleukin(IL)-6 and insulin like growth factor 1 (IGF1) are two important paracrine growth factors secreted by the BMME that enhance the growth and survival of myeloma cells. Both have been reported to signal through the PI3-kinase/AKT pathway stimulating pro-survival and proliferative mechanisms via many downstream targets [2–4]. 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 [5].

The ability of GCs to induce growth arrest of lymphoid cells has resulted in their widespread use in the treatment of MM and various leukemias and lymphomas [6]. Despite this fact, the molecular details of GC-induced apoptosis remain largely undefined. Most of the effects of GCs are mediated through interaction with the glucocorticoid receptor (GR) as it has been shown that an intact functional receptor is required for cytotoxicity [7]. 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 [6]. The GR regulates gene expression through either trans-activation or trans-repression of gene targets. GCs are known to inhibit the transcription of cytokines and other mediators of immune and inflammatory responses necessary for cell proliferation and growth in part due to direct interactions between GR and the transcription factors NF- κ B, AP-1 and other transcription factors without GR-DNA binding [6,7]. However, evidence supporting the requirement for GR-induced gene activation also exists. Denovo RNA and protein synthesis are necessary as GC-induced thymocyte apoptosis is blocked upon inhibition of either transcription or translation [8]. The DNA binding potential of GR is required for GC-induced apoptosis of thymocytes as mice expressing a dimerization deficient GR mutant that cannot bind to DNA have defects in that process [9].

Since GC-induced apoptosis has been shown to be mediated by the regulation of gene expression, 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. Over 900 different genes have been reported to be GC-regulated, but only about 70 have appeared in more than one publication [7]. 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 contributing to cell death may be involved [7].

Glucocorticoid-induced leucine zipper (GILZ) has been identified in a number of cell types as a GC-induced gene [10]. In a cDNA micro-array screen, *GILZ* was rapidly up regulated by dexamethasone (5.9-fold) [11]. GC treatment up regulates *GILZ* expression in T cells (CD4+ and CD8+), B cells, and macrophages suggesting a possible role in the control of immune cell compartment growth and death [12–14]. 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- κ B and AP-1 and the kinases Raf-1 and ERK [10,15–17]. The data on the role of *GILZ* in B cells and MM cells is limited. Up regulation of *GILZ* is observed in resting and tolerant B cells compared to activated B cells where it was hypothesized to maintain quiescence while down regulation of *GILZ* facilitates B cell activation [13].

The promoter of *GILZ* contains 6 glucocorticoid responsive elements (GRE), along with binding sites for forkhead box class O (FOXO) family proteins, signal transducer and activator of transcription 6 (STAT6), nuclear factor of activated T cells (NFAT), Octamer, and c-myc [18–20]. The regulation of *GILZ* expression has been studied in a murine T lymphocyte line where FOXO3 was shown to activate *GILZ* expression independent of GCs [18,19].

Due to the compelling data in T cells, we hypothesize that GILZ is a component of the GR-signaling pathway in MM mediating GC-induced apoptosis. With these studies, we confirmed the micro-array findings that GILZ is a GC-induced gene in MM and identified a functional importance for GILZ in GC-induced apoptosis of MM cells. The regulation of *GILZ* expression in MM.1S and other myeloma cell lines was examined in order to gain insight into mechanisms of GR signaling in myeloma. We report the results of a large screen identifying additional regulators of *GILZ* and show that inhibition of the PI3-kinase/AKT pathway results in the up regulation of *GILZ* expression. We further demonstrate that inhibition of PI3-kinase/AKT can cooperate with the GR to dramatically enhance *GILZ* expression and cause synergistic cell killing of MM cells.

2. Materials and methods

2.1. Cell culture

All cell culture medium, serum and antibiotics were purchased from GIBCO/Invitrogen unless otherwise noted (Carlsbad, CA). The MM.1S, MM.1R_e, and MM.1R_L cell lines were developed previously in our laboratory [11,21]. The U266 cell line was purchased from ATCC. The RPMI-8226 and MDR10V lines were obtained from Dalton and coworkers [22]. The OPM-II cell line were obtained from Thompson and coworkers [23]. 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 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% CO₂. 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 with 15% Defined Premium Fetal Bovine Serum from Hyclone (Logan, UT).

2.2. Patient samples

Multiple myeloma patient cells were isolated from fresh bone marrow samples after informed consent. Mononuclear cells were isolated with Ficoll/Histopaque 1077 (Sigma, St. Louis, MI). The population of myeloma cells was enriched for with CD138+ microbeads and automated magnetic cell sorting using an AutoMacs cell sorter (Miltenyi Biotec, Auburn, CA).

2.3. Reagents

All glucocorticoids, wortmannin, RU486, thalidomide and ATRA were obtained from Sigma. LY294002, all AKT, p38, and MEK inhibitors 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), and the GILZ antibodies were obtained from Cao and coworkers [24] and Eddleston et al. [25]. All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

2.4. Reverse-transcriptase PCR

Total RNA was isolated from treated 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 (Forward: 5'-CAGCCCCGAGCCATGAACACC-3' and Reverse: 5'-CGCAGAACCACCAGGGGCCT-3') with 1 unit Taq DNA Polymerase (Fermentas), in 2.5 mM MgCl₂, 0.5 mM dNTPs (Roche) and 1× reaction buffer (Fermentas). Amplification

conditions were 94 °C 2 min, 23 cycles of 94 °C 30 s, 60 °C 30 s, 72 °C 60 s, followed by 72 °C for 12 min. In each reaction, GAPDH was also amplified to serve as a normalization control (Forward: 5'-AGGTGAAGGTCTGGAGTCAAC-3' and Reverse: 5'-CGCTCCTGGAAGATGGTGAT-3'). 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).

2.5. 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 h 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 (6-FAM-CGTTAAGCTGGACAACAG) and primers (Forward: 5'-CACAATTTCTCCATCTCCTTCTTCT-3' and Reverse: 5'-TCAGATGATTCTTCACCAGATCCAT-3'). 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 1 × 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 s 95 °C, 40 cycles of 3 s 95 °C, 30 s 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 [26]. 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 C_t 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 (6FAM-AACTCTTGGATTCTATGCATGAA) and primers (Forward: 5'-GCAGCGGTTTTATCAACTGACA-3' and Reverse: 5'-AATGTTTGGGAAGCAATAGTTAAGGAGAT-3') 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 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.

2.6. Western blots

Western blotting was done as previously described with the following changes [27]. Myeloma cells (5×10^6) were harvested post-treatment, washed with $1 \times$ PBS and lysed with 35 μ L 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 ($\text{Na}_4\text{P}_2\text{O}_7$), 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 phenyl-methylsulfonyl fluoride

(PMSF), 60 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ pepstatin, 10 $\mu\text{g/mL}$ leupeptin) or PLB (0.5% Triton X-100, 150 mM NaCl, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 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 min or subjected to 3 freeze-thaw cycles and centrifuged at 10,000 rpm for 10 min. 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 h 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 3 $\times$ sample buffer, a specialized PVDF for low molecular proteins was used (Immobilion-P^{SQ}, Millipore), the proteins were transferred from gel to membrane overnight at 4 °C at 10 V, and the blots developed using SuperSignal West Femto ECL (Pierce Biotechnology, Rockford, IL).

2.7. siRNA knockdown

ON-TARGET plus SMARTpool siRNA to GILZ (Human TSC22D3) 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 in the 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 h 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 and western blotting. Using a FITC-labeled dsRNA oligomer (BLOCK-iTTM Fluorescent Oligo, Invitrogen), transfection of siRNA oligomers by MM.1S was consistently measured to be greater than 90%.

2.8. Propidium iodide (PI) staining

MM.1S cells (2×10^6) were treated for 15 or 24 h with Dex (1 μM). After harvesting, cells were washed with PBS and fixed overnight with 40% ethanol in PBS. On the day of staining, the cells were washed with PBS, incubated for 30 min at 37 °C with 50 $\mu\text{g/mL}$ RNaseA in PBS, and then stained with 43 μg propidium iodide in 38 mM sodium citrate. The cells were analyzed using an Epics Profile II flow cytometer (Coulter Electronics, Inc., Hialeah, FL) and the percentage of cells with a sub-G1 DNA content identified.

2.9. Annexin-V staining

Myeloma cells were harvested after drug treatment and washed twice with PBS. Cells were resuspended at 1×10^6 cells/mL in 1 $\times$ Annexin V Binding Buffer. 1×10^5 cells were stained with 5 μL of Annexin V-PE and 5 μL of 7-amino-actinomycin D (7-AAD; Annexin-FITC and PI was also used where noted) for 15 min at room temperature in the dark. 400 μL of 1 $\times$ Annexin V Binding Buffer was added and the cells were analyzed using an Epics Profile II flow cytometer (Coulter Electronics, Inc., Hialeah, FL). 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).

2.10. 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) [28]. 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 h. Apoptosis was quantified (24 h time point) by Annexin V-PE/7-AAD staining as described above.

2.11. Statistical analyses

All data presented here 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 available to be done one time per patient. The real time PCR analysis was performed multiple times on the isolated RNA to provide the error bars. The two tailed *p* values for Fig. 2 were calculated using the paired *t*-test and the software GraphPad InSTAT3 (San Diego, CA).

3. Results

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

Through DNA micro-array analysis, we have previously identified *GILZ* as a GC-responsive gene in MM.1S cells [11]. We have confirmed those results with quantification of the dexamethasone-induced mRNA up regulation measured by real time PCR (Fig. 1A). There is a concentration dependent increase in *GILZ* expression that is consistent with the concentration of GCs required to induce apoptosis in these cells. A time course of *GILZ* protein expression using 10 μ M Dex (Fig. 1B) indicates that *GILZ* is maximally expressed by 6 h of incubation and although it starts to decrease at 18 h, *GILZ* remains elevated for up to 48 h. To test whether the GR is required for *GILZ* up regulation, we utilized the GR antagonist RU486. If the receptor is required, we would expect that RU486 would inhibit the GC-induced expression of *GILZ*. At both the mRNA and protein level, 10 μ M of RU486 was able to block Dex-induced up regulation of *GILZ* (25-fold with Dex compared to 10-fold with Dex + RU486 or 7-fold with RU486 alone) (Fig. 1C and D). RU486 has been reported to act as a partial agonist in other cell types and 7-fold increase in *GILZ* in MM.1S cells treated with 10 μ M RU486 alone was observed [29,30].

To assess the glucocorticoid specificity of the *GILZ* up regulation, we examined the ability of additional glucocorticoids to induce *GILZ* expression in MM.1S cells. We found that *GILZ* expression was also up regulated by beclomethasone, beclomethasone DP, hydrocortisone, prednisolone, triamcinolone acetonide, and triamcinolone. All of these GC can kill MM.1S cells. The only GC tested that did not up regulate *GILZ* was prednisone which requires conversion to its active form by the liver and is not toxic to MM.1S cells *in vitro* (data not shown). We also measured Dex-induced up regulation of *GILZ* in a panel of other multiple myeloma cell lines including OPM-II, MDR10V, and RPMI-8226 (Fig. 1E). Up regulation of *GILZ* by Dex was not observed to the same extent in U266, MM.1R_e and MM.1R_L cell lines which correlates with the reduced level of GR expression in these lines (Fig. 1F). Together these data confirm that *GILZ* levels are increased by various GCs in a number of different MM cell lines and that this up regulation requires the GR.

3.2. Reduction in GILZ decreases GC-induced apoptosis

In order to determine if GILZ participates in GC-induced apoptosis of MM cells, siRNA was used to reduce the levels of GILZ in MM.1S cells and the effect on GC-induced apoptosis was quantified. Baseline *GILZ* mRNA levels were reduced by GILZ siRNA to approximately 50% the level observed in control siRNA transfected cells. Treatment with Dex increased *GILZ* levels in the GILZ siRNA transfected cells, however the extent of up regulation was reduced (9-fold increase in GILZ siRNA transfected cells compared to 30-fold increase in control siRNA transfected cells) (Fig. 2A). Similarly, GILZ protein levels were reduced by GILZ siRNA compared to the control siRNA transfected cells where a faint GILZ band can be seen in the untreated control siRNA sample. Up regulation of GILZ at the protein level occurred in both control and GILZ siRNA transfected samples, however the extent of GILZ protein up regulation was also reduced by GILZ siRNA compared to control cells (Fig. 2B).

GILZ siRNA transfected cells were treated with 1 μ M Dex for 48 h after which 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% that was shown to be statistically significant (Fig. 2C). Based on these results, it can be concluded that GILZ contributes to GC-induced apoptosis in MM.1S cells. It is remarkable that even with a 50% decrease in *GILZ* levels, a statistically significant decrease in the apoptotic potential is observed. The fact that the difference is a modest 10% can be explained by the fact that GILZ protein is still detectable in GILZ siRNA transfected cells and can contribute to GC-induced cell death. In addition, the modest decrease in GC-mediated cell death due to GILZ knockdown perhaps reflects that multiple factors are involved in GC regulation of cell death and GILZ is one factor in that process. These results provide an encouraging beginning to elucidating the functional importance of GILZ in the process of GC-induced apoptosis as a link between GILZ and GC-induced apoptosis in myeloma has not been identified prior to this report.

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

It has been previously reported that 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 treatment [2,5,31,32]. To determine if these growth factors can affect the GC regulation of *GILZ*, we tested the effect of IL-6 and IGF1 on *GILZ* expression levels in MM.1S cells. Pre-treatment of IL-6 or IGF1 partially inhibited *GILZ* up regulation induced by Dex. Shown with real time PCR, MM.1S cells treated with 1 μ M Dex for 6 h had a 25-fold increase in *GILZ* while increasing concentrations of either IL-6 or IGF1 limited Dex-induced up regulation of *GILZ* to only 5–10-fold (Fig. 3). We confirmed that these concentrations of IL-6 and IGF1 were sufficient to block Dex-induced apoptosis in MM.1S cells (data not shown). These results support our hypothesis that *GILZ* up regulation is involved in the process of GC-induced apoptosis in myeloma cells as the concentrations of IL-6 and IGF1 which block *GILZ* up regulation also inhibit GC killing.

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

In order to gain a better understanding into the regulation of *GILZ* and insight into glucocorticoid receptor signaling pathways in MM.1S cells, we screened a panel of cytokines and drugs for effect on *GILZ* expression levels using RT-PCR. The results of the screen are summarized in Table 1. We selected this panel of cytokines, growth factors, and growth conditions based on previous reports indicating up regulation of GILZ or its related family members in other cell lines and systems. These included IL-2, IL-7, IL-10, TGF- β , β -estradiol, sonic hedgehog, progesterone, EGF and serum starvation [14,19,33–36]. None of

these cytokines or growth conditions was found to up regulate *GILZ* in MM.1S cells. Because glucocorticoids are potent inducers of apoptosis in myeloma cells, we screened additional MM chemotherapeutic agents 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 [37–41]. Despite inducing apoptosis in MM.1S cells, none of these drugs up regulated *GILZ* in our screen.

Due to previous reports highlighting the importance of the fork-head responsive elements (FHRE) in the *GILZ* promoter [18,19] and the regulation shown with IL-6 and IGF1 (Fig. 3), the effects of inhibitors of the PI3-kinase/AKT pathway were tested in our screen. These proteins were targeted because both PI3-kinase and AKT are known to be upstream of the forkhead box family O (FOXO) members and can be activated by IL-6 and IGF1 in MM cells [31,32]. Interestingly, the PI3-kinase inhibitors LY294002 and wortmannin up regulated *GILZ* levels in MM.1S cells as shown with both RT-PCR after 6 and 24 h and real time PCR after 6 h (Fig. 4A and B). AKT inhibitors, triciribine and AKT inhibitor VIII, also up regulated *GILZ* in MM.1S after 6 h as shown with real time PCR (Fig. 4B). 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 is 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 μ M LY294002 or 20 μ M 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 (Fig. 4C). 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 cell line and appears independent of the level of the GR. We also measured *GILZ* up regulation by PI3-kinase and AKT inhibitors in human multiple myeloma patient samples where 1.7–5-fold increase in *GILZ* expression with LY294002 and AKT inhibitor VIII treatment was observed in 2 of the 3 samples tested (Fig. 4D). Due to the limited amount of patient material available, we were not able to perform biological replicates with the myeloma patient samples and these data are presented as an indication of *GILZ* regulation in MM patients. Taken together, we have identified *GILZ* regulation by components of the PI3-kinase/AKT pathway in a number of MM cell lines and clinical samples and this regulation appears to be independent of the GR.

3.5. When combined, GCs and inhibitors to PI3-kinase/AKT dramatically enhance *GILZ* levels

To further investigate the ability of PI3-kinase and AKT inhibitors to up regulate *GILZ*, we explored the effect of simultaneous addition of GCs and inhibitors to PI3-kinase and AKT on *GILZ* expression levels. With these combinations, *GILZ* levels were dramatically enhanced 200-fold from untreated levels in MM.1S (Fig. 5A). Using a *GILZ* specific antibody, the effect of the PI3-kinase/AKT inhibitors on *GILZ* protein levels alone and in conjunction with GCs was tested. Treatment with LY294002 and AKT inhibitor VIII alone resulted in an increase in detectable *GILZ* protein. The combination of Dex with any of the four inhibitors tested (LY294002, wortmannin, triciribine, and AKT inhibitor VIII) resulted in increased protein expression to a level greater than the level observed with Dex alone (Fig. 5B). A similar result was observed in RPMI-8226 and OPM-II cell lines, but not in MM.1R_e, MM.1R_L, U266, or MDR10V myeloma lines. As shown in Figs. 1E and 4C, *GILZ* was not up regulated by GCs in MM.1R_e, MM.1R_L, and U266 or by LY294002 in MDR10V cells and therefore a similar enhancement in *GILZ* levels with the combination treatment would not be expected in these cells. The effect of the combination treatment on *GILZ* levels was also investigated in the MM patient samples where dramatic enhancement was observed in patients 2 and 3 compared to the induction of *GILZ* observed with either

agent alone (Fig. 5D). The addition of IL-6 or IGF1 (activators of PI3-kinase/AKT) with the combination of Dex and LY294002 blunted the enhanced up regulation of *GILZ* (Fig. 5E). These results reveal a dramatic enhancement of *GILZ* induction when GCs and PI3-kinase/AKT inhibitors are combined and suggest that the PI3-kinase/AKT and the GR signaling pathways converge to regulate *GILZ* expression.

Pharmacologic inhibitors to two other important signaling molecules, p38 (SB203580) and MEK (U0261, PD98059), were tested to determine if this observed effect on *GILZ* 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* and when combined with Dex, none of these inhibitors dramatically up regulated *GILZ* expression (data not shown). This suggests that enhanced up regulation of *GILZ* observed when combined with GCs is unique to inhibitors of the PI3-kinase/AKT pathway.

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

Because we have shown that modulators of the PI3-kinase/AKT pathway can affect *GILZ* expression and Dex-induced apoptosis, we explored the possibility that the enhanced up regulation of *GILZ* observed with the combination treatment of GCs and inhibitors of PI3-kinase/AKT correlated with an increase in apoptosis. We tested the combination of Dex and LY294002 and showed that PARP cleavage, a marker of caspase activation and apoptotic induction, was enhanced with the combination treatment compared to either agent alone (Fig. 6A). Similar enhanced apoptosis was observed with Annexin V/7-AAD staining wherein the combination of Dex and LY294002 produced a greater response than the additive combination of either agent alone (Fig. 6B). Because of this observation, formal analysis of synergism was undertaken. Using the median effect plot and Annexin V/7-AAD staining, we determined that the cell killing caused by combination of Dex and LY294002 was synergistic (Table 2). We also measured enhanced cell killing with the combination of Dex and LY294002 in both RPMI-8226 and OPM-II cell lines (data not shown).

4. Discussion

Despite the fact that GCs have long been a mainstay of treatment for MM patients, the pathway of GC-induced apoptosis has not been fully elucidated. In addition, therapeutic resistance to GC-based therapies remains a critical issue in the treatment of MM patients. Insights into the molecular basis of GC signaling and induction of apoptosis are needed to aid in the development of novel therapeutics and strategies to combat GC resistance. In these studies, by screening for additional regulators of *GILZ*, a GC-induced gene in MM, we have identified dual regulation of *GILZ* by two key pathways necessary for MM proliferation and death: PI3-kinase/AKT and GR. Here we report the novel observation that in MM cells modulators of the PI3-kinase/AKT pathway affect the GC-induced gene *GILZ*. We also identify that *GILZ* plays a functional role in GC-induced killing of MM cells.

The importance of the PI3-kinase/AKT pathway in myeloma progression has been well characterized. IL-6 and IGF1 have been identified as crucial growth factors which signal through PI3-kinase/AKT and enhance MM cell growth [32]. Both factors have been identified as paracrine factors secreted from the bone marrow microenvironment supporting MM growth and drug resistance [3]. In addition, both IL-6 and IGF1 have been reported to inhibit GC-induced apoptosis in MM [5]. Here we show for the first time that the addition of these same growth factors inhibited GC-induced up regulation of *GILZ*. We also report that the inhibition of PI3-kinase or AKT up regulates *GILZ* expression and further that pharmacologic inhibition of this pathway in combination with GCs dramatically up regulates *GILZ* expression and synergistically enhances MM apoptosis. These observations suggest a

mechanism for the protective effects of IL-6 and IGF1 on GC-induced cell death through regulation of *GILZ* expression.

In this report, we have shown that PI3-kinase/AKT inhibitor up regulation of *GILZ* occurs independent of the GR status of the different MM cell lines (Figs. 1F and 4C). However, dramatic enhancement of *GILZ* up regulation and synergistic cell killing is observed in MM cells upon the combination of GCs and PI3-kinase/AKT inhibitors. The mechanism to explain this potential cross-talk or interaction between these two pathways needs to be investigated further.

Cooperation between the PI3-kinase/AKT pathway and the GR or other nuclear hormone receptors has been reported in other biologic models. In a genomics screen of acute lymphoblastic leukemia (ALL) cells, genes associated with the PI3-kinase/AKT pathway were highly enriched in a gene signature of GC-resistance [42]. 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 [43–45]. Interaction of nuclear hormone receptors with members of the FOXO protein family downstream of PI3-kinase/AKT has also been reported. 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*) [46,47]. Additionally, binding sites for both GR and FOXO1 exist in the glucocorticoid response element of the glucose-6-phosphate transporter gene promoter where both proteins cooperate to activate its transcription [48].

Further work needs to be conducted in order to determine whether the effect on *GILZ* expression reported here is the result of a direct protein interaction between GR and PI3-kinase, the result of activation of FOXO family members upon inhibition of PI3-kinase/AKT and mediated at the promoter level, or another unexpected mechanism. As reported previously, the *GILZ* promoter contains three forkhead responsive elements and mutation of those elements reduced Dex-induced up regulation of *GILZ* [18,19]. IL-6 and IGF1 have been shown to inhibit FOXO family members through the activation of PI3-kinase/AKT and it has also been reported that treatment with inhibitors to PI3-kinase and AKT activate FOXO family members in MM [5]. Experiments to clarify the molecular details regulating the *GILZ* promoter and the importance of FOXO family members are currently in progress.

Because the induction of apoptosis by GCs is so important in 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. Our data indicating *GILZ* knockdown results in reduced GC-induced apoptosis and compelling data in the literature implicate *GILZ* as a key component of GC-signaling. Eddleston et al. [25] report a similar siRNA induced decrease in GC-induced *GILZ* results in reduced anti-inflammatory actions of GC in HEK293 cells. The function of *GILZ* has been studied extensively in T cells where both pro- and anti-apoptotic functions have been reported [19,49]. However, the function of *GILZ* had yet to be characterized in B cells or myeloma before this study. We report that *GILZ* has a pro-apoptotic function in MM cells as reduction of GC-induced *GILZ* levels with siRNA results in a decrease in the potency of GCs to induce apoptosis. We also demonstrate that *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. We also find that *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* (data not shown). Collectively, these findings suggest that *GILZ* is an important mediator of GC-induced killing in MM cells.

Here we report that the combination treatment of PI3-kinase/AKT inhibitors with glucocorticoids 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 therapeutic benefit from the combination therapy of GC and PI3-kinase/AKT blockade is warranted as a beneficial alternative treatment regimen for MM patients. A clinically relevant AKT inhibitor Perifosine was recently reported to augment Dex killing of MM.1S cells [50] and our studies revealing dual regulation of GILZ and synergistic killing by PI3-kinase/AKT inhibitors and GCs add to this previous observation providing a strong rationale for clinical trials with this combination treatment. In addition, these studies reaffirm *GILZ* as an important gene product in the GC signaling pathway whose regulation may be a marker for successful therapy with GCs. 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. 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 [51]. The use of these agents in combination should be further investigated to establish their therapeutic potential.

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Fig. 1.

GILZ is up regulated by glucocorticoids in multiple myeloma cells and requires the glucocorticoid receptor. (A) Real time PCR of total RNA isolated from MM.1S cells (1 × 10⁶ cells) after 6 h of Dex treatment. *GILZ* expression levels are normalized to *RNaseP* levels in each sample and expressed as fold change compared to untreated sample. (B) Western blot for GILZ in whole cell lysates of MM.1S cells treated with 10 μM Dex for indicated time. GAPDH was probed as loading control. (C and D) MM.1S cells were treated with 10 μM RU486 and/or 1 μM Dex (RU486 was added 1 h before Dex). Total RNA or whole cell lysates was isolated after 6 h treatment and *GILZ* mRNA up regulation was measured by real time PCR (D) and GILZ protein up regulation by western blotting (D). *RNaseP* was used as a normalization control for real time PCR and GAPDH as a loading control for western blot. (E) Total RNA was isolated from MM.1S, MM.1Re, MM.1RL, OPM-II, U266, RPMI-8226, and MDR10V cells (0.25 × 10⁶ cells) treated with 1 μM Dex for 6 h and *GILZ* up regulation was measured by real time PCR. *GILZ* levels in each sample were normalized to *RNaseP* and expressed as fold change from the untreated control of each cell line. (F) The number of GR molecules in each total RNA sample from (E) was measured using absolute quantification real time PCR. The number of molecules was calculated following normalization to a standard curve generated using GR plasmid cRNA.

Fig. 2. GC-induced apoptosis in MM.1S is reduced by GILZ knock-down. (A–C) MM.1S cells were transfected with GILZ siRNA or control siRNA. After 24 h recovery, cells were treated with 1 μ M Dex for 6 h (A and B) or 48 h (C). GILZ knockdown and GC-induction was monitored at the mRNA level with real time PCR (A) and protein levels with western blotting (B). *RNaseP* was used as a normalization control for real time PCR and GAPDH as a loading control for western blotting. (C) 48 h after treatment, cells were stained with Annexin-V-FITC and propidium iodide and the percentage of cells staining single or double positive presented. 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).

Fig. 3. IL-6 and IGF1 block Dex-induced up regulation of *GILZ*. Real time PCR of MM.1S total RNA isolated from MM.1S cells (1×10^6 cells) treated with 1 μ M Dex for 6 h with and without IL-6 or IGF1 (added 30 min prior to Dex).

Fig. 4.

GILZ is up regulated by inhibitors of PI3-kinase and AKT. (A) MM.1S cells were treated with Dex (1 μ M), LY294002 (25, 50, and 75 μ M), and wortmannin (0.1 and 1 μ M) for 6 or 24 h. *GILZ* and *GAPDH* levels were analyzed using RT-PCR. (B) MM.1S cells (1×10^6 cells) were treated with indicated concentrations of PI3-kinase and AKT inhibitors for 6 h. *GILZ* expression levels were measured using real time PCR, normalized to *RNaseP*, and expressed as fold change from untreated cells. (C) Total RNA was isolated from MM.1S, MM.1Re, MM.1RL, OPM-II, U266, RPMI-8226, and MDR10V cells (0.25×10^6 cells) treated with 25 μ M LY294002 or 20 μ M AKT inhibitor VIII for 6 h and *GILZ* up regulation was measured by real time PCR. *GILZ* levels in each sample were normalized to *RNaseP* and expressed as fold change from the untreated control of each cell line. (D) Freshly isolated myeloma patient cells were exposed to 25 μ M LY294002 and 20 μ M AKT inhibitor VIII for 6 h. *GILZ* expression levels were measured using real time PCR, normalized to *RNaseP*, and expressed as fold change from the untreated of each sample. Due to the limited amount of patient material available, this data represents a single biologic experiment where the measurement of *GILZ* expression was performed twice.

Fig. 5.

PI3-kinase and AKT inhibitors combined with Dex dramatically up regulate *GILZ* levels. (A) MM.1S cells (1×10^6 cells) were treated for 6 h with 1 μ M Dex, 25 μ M LY294002, 1 μ M wortmannin, 20 μ M AKT inhibitor VIII, or 5 μ M 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. (B) MM.1S cells were treated for 6 h with 1 μ M Dex, 25 μ M LY294002, 1 μ M wortmannin, 20 μ M AKT inhibitor VIII, or 5 μ M triciribine alone or in combination as indicated. Western blotting was done on whole cell lysates and probed with antibodies to *GILZ* and GAPDH. (C) MM.1S, MM.1Re, MM.1RL, OPM-II, U266, RPMI-8226, and MDR10V cells (0.25×10^6 cells) were treated for 6 h with 1 μ M Dex, 25 μ M LY294002 or 20 μ M AKT inhibitor VIII alone or in combination as indicated. *GILZ* levels were measured by real time PCR, normalized to *RNaseP* and expressed as fold change from the untreated control level of each cell line. (D) Freshly isolated myeloma patient cells were exposed to 1 μ M Dex, 25 μ M LY294002 or 20 μ M AKT inhibitor VIII alone or in combination as indicated for 6 h. *GILZ* expression levels was

measured using real time PCR, normalized to *RNaseP*, and expressed as fold change from untreated of each sample. (E) MM.1S cells (1×10^6 cells) were treated for 6 h with Dex and LY294002 with and without IL-6 and IGF1 (added 30 min prior to Dex and LY294002). *GILZ* levels were measured by real time PCR as above.

Fig. 6.

PI3-kinase inhibitor LY294002 enhanced Dex-induced cell death in MM.1S. (A) Whole cell lysates of MM.1S cells treated with 1 μ M Dex, 25 μ M LY294002, and/or 10 μ M RU486 (added 1 h prior to Dex) for 6 and 24 h were analyzed by western blotting. Blots were probed with antibodies to PARP and GAPDH. (B) Cells were stained for Annexin V-PE and 7-AAD after 24 h treatment with LY294002 and/or Dex.

Table 1

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

Cytokines/growth factors	Up regulate GILZ?	Cytotoxic drugs/kinase inhibitors	Up regulate 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

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.

Table 2

Dex and LY294002 cause synergistic cell killing of MM.1S cells

	CI
1 μ M Dex + 25 μ M LY	0.211
10 μ M Dex + 250 μ M LY	0.105

MM.1S cells were exposed to increasing concentrations of Dex and LY294002 at a fixed ratio (1:25) for 24 h. Apoptosis was quantified by Annexin V-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 values <1.0 = synergy