Targeting the Metabolic Plasticity of Multiple Myeloma with FDA Approved Ritonavir and Metformin

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Running Title: Targeting myeloma cell metabolism with ritonavir and metformin

Translational Relevance: Multiple myeloma (MM) is a largely incurable plasma cell malignancy characterized primarily by the development of resistance confounded by underlying clonal molecular heterogeneity. We have previously demonstrated the therapeutic utility of targeting abnormal glucose metabolism and MM growth and/or survival with the GLUT4 inhibitor ritonavir. Our recent studies have determined that a subset of myeloma cells survive glucose deprivation/ritonavir treatment likely by engagement of mitochondrial oxidative phosphorylation. We therefore tested combined use of ritonavir with the anti-diabetic metformin, which targets mitochondrial complex 1. Administration of FDA approved ritonavir with metformin effectively elicited apoptosis in vitro in MM cell lines and patient samples and reduced tumor burden in an in vivo xenograft model of MM. In addition, this combinatorial regimen was effective in breast, melanoma and ovarian cancer cell lines. Importantly, diabetic HIV patients have previously been administered this combinatorial regimen, supporting the investigation of re-purposing ritonavir and metformin for MM therapy.
Abstract:

Purpose: We have previously demonstrated that ritonavir targeting of glycolysis is growth inhibitory and cytotoxic in a subset of MM cells. In this study our objective was to investigate the metabolic basis of resistance to ritonavir and to determine the utility of co-treatment with the mitochondrial complex I inhibitor metformin to target compensatory metabolism.

Experimental Design: We determined combination indices for ritonavir and metformin, impact on myeloma cell lines, patient samples and myeloma xenograft growth. Additional evaluation in breast, melanoma, and ovarian cancer cell lines was also performed. Signaling connected to suppression of the pro-survival BCL2 family member MCL-1 was evaluated in MM cell lines and tumor lysates. Reliance on oxidative metabolism was determined by evaluation of oxygen consumption and dependence on glutamine was assessed by estimation of viability upon metabolite withdrawal in the context of specific metabolic perturbations.

Results: Ritonavir-treated MM cells exhibited increased reliance on glutamine metabolism. Ritonavir sensitized MM cells to metformin, effectively eliciting cytotoxicity both in vitro and in an in vivo xenograft model of MM and in breast, ovarian and melanoma cancer cell lines. Ritonavir and metformin effectively suppressed AKT and mTORC1 phosphorylation and pro-survival BCL-2 family member MCL-1 expression in MM cell lines in vitro and in vivo.

Conclusions: FDA-approved ritonavir and metformin effectively target MM cell metabolism to elicit cytotoxicity in MM. Our studies warrant further investigation into repurposing ritonavir and metformin to target the metabolic plasticity of myeloma to more broadly target myeloma heterogeneity and prevent the re-emergence of chemo-resistant aggressive MM.
Introduction:

Multiple myeloma (MM) is a largely incurable plasma cell malignancy accounting for 11,000 deaths annually in the US (1, 2). Median survival remains 5-7 years primarily due to the development of chemoresistance, necessitating the need for new therapeutic strategies (1, 2). Targeting abnormal cancer cell metabolism could potentially provide a broader means to targeting the cellular and molecular heterogeneity of myeloma that is in part responsible for the re-emergence of chemo-resistant aggressive MM. Tumor cells generate ATP, biosynthetic intermediates and reducing equivalents by abnormally engaging biochemical pathways such as glycolysis, glutaminolysis and the pentose phosphate pathway. To target cancer cell metabolism effectively for therapy, one must identify tumor specific primary metabolic pathways and alternative compensatory metabolic pathways.

MM is one of several cancers that exhibits abnormal glucose metabolism (3). Aggressive late stage myeloma exhibits elevated glucose uptake evident from increased PET positivity (4), which correlates with lower event-free survival (5, 6). Glucose regulates multiple biochemical, cellular and molecular pathways to maintain viability and proliferation, and induce chemoresistance in various cancers (7, 8) including MM (3), thus supporting the utility of targeting abnormal glucose metabolism for therapy. We have demonstrated that MM cells are glycolytic and rely on the insulin-responsive glucose transporter GLUT4, which is constitutively localized in the plasma membrane in MM (3). Targeting this rate-limiting step in glucose metabolism by knockdown or inhibition of GLUT4 leads to apoptosis in and/or cytostasis of a subset of MM cells. Our previous studies demonstrated the utility of targeting GLUT4 with the HIV protease inhibitor ritonavir (9-11). Ritonavir binds GLUT4 to reversibly inhibit glucose transport in a non-competitive manner (9-11). In the current study we sought to determine the metabolic basis for continued survival and resistance of MM cells to ritonavir. Inhibition of glucose metabolism can lead to compensatory engagement of mitochondrial metabolism and use of alternative carbon sources to counteract the loss of glucose derived metabolites to maintain survival. We have previously demonstrated that ritonavir in combination with complex I inhibitor metformin is cytotoxic in CLL (12). In this study, we investigate metabolic compensation in MM cells, exploring a role for glutamine and fatty acid oxidation in maintaining survival of ritonavir treated cells and the utility of combining ritonavir with metformin to target compensatory mitochondrial complex 1 activity in in vitro and in vivo myeloma xenograft studies with closer evaluation of dosing regimens easily translatable to humans.

Methods:
Cell culture

KMS11, L363 and JJN3 cell lines were obtained from Dr. Michael Kuehl (National Cancer Institute). We have not authenticated these cell lines in our laboratory. KMS11 cells were engineered to express green fluorescent protein – hereafter referred to as KMS11-GFP cells to aid in detection in in vivo studies. DLBCL and Mantle Cell lines were obtained from Dr. Leo Gordon Northwestern University and Dr. Varsha Gandhi, MD Anderson Cancer Center, respectively. All cell lines were cultured in complete RPMI 1640 (Invitrogen) with glutamine supplemented with 10% FBS, 2mM glutamine, 100U/ml penicillin, 100mg/ml streptomycin, 2.5μg/ml fungizone, 0.5μg/ml plasmocin (InvivoGen) and maintained at 37°C with 5% CO2. NCI-60 breast, ovarian and melanoma cell line experimentation was performed by Northwestern University Center for Developmental Therapeutics. For glucose and/or glutamine deprivation experiments, cells were cultured in glucose and glutamine free medium (Rainbow Scientific, Inc.) supplemented with dialyzed FBS (Invitrogen) and supplemented with indicated concentrations of glucose or glutamine.

Isolation of primary myeloma cells

Approval for collection of all primary samples was obtained from the Institutional Review Board of Northwestern University. Patients provided written informed consent in all cases at time of enrollment in accordance with the Declaration of Helsinki. An AutoMacs cell sorter (Miltenyi Biotec) was used to purify CD138+ cells from MM patient bone marrow aspirate as described previously (3). Normal peripheral blood mononuclear cells (PBMCs) were harvested by layering whole blood over a Ficoll histopaque (Sigma) gradient and subject to standard buffy coat purification.

Chemicals and reagents

Ritonavir was purchased from Euroasia Inc., Metformin, dimethyl a-ketoglutarate (DMK) and 6-Diazo-5-oxo-L-norleucine (DON) from Sigma-Aldrich. The following antibodies were purchased: MCL-1 (Santa Cruz) and GAPDH from Millipore; pAKT (S473), AKT, pAMPK (T172), AMPK, mTORC1 and pmTORC1 from Cell Signaling Technology. Antisera to human GLUT4 was generously provided by Dr. S. Cushman (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Oligomycin, carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone, antimycin and rotenone were purchased from Sigma.

Cell growth, proliferation and death assays

CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) was used to determine cell growth according to the manufacturer’s instructions. Cell proliferation and viability were quantified using a Beckman Coulter ViCell automated cell viability analyzer. Annexin V/4,6-
diamidino-2-phenylindole (DAPI) staining was used to assess cell death via flow cytometry with a BD Biosciences LSR Fortessa Analyzer. During treatments cells were maintained in RPMI-1640 media supplemented with 2mM glutamine, 5% dialyzed FBS and 5mM glucose. Gentamycin was added to all media at a concentration of 50ug/ml in keeping with the NCI60 protocol.

**Oxygen consumption**

Seahorse bioscience extracellular flux (XF24) analyzer was utilized to measure oxygen consumption rates (OCR). Myeloma cells subject to specific treatments were harvested, washed with PBS and then suspended in glucose and glutamine free DMEM (Sigma) supplemented with 1% dialyzed FBS + 5ml pen/strep (ph7.5) and 5 mM glucose or 2 mM glutamine as required. Cells were plated in 5 replicates in 24-well plates custom designed for XF24 analysis at a density of 0.4x 10⁶ cells per well after pre-coating plates with Cell-Tak (Becton Dickinson) following manufacturer’s recommendations. OCR was evaluated over time basally as well as after injection of oligomycin, (final concentration 5uM), carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP) (final concentration 5 uM) and antimycin + rotenone (final concentration of each 2 uM).

**Mice and drug administration**

5-6 week old NOD/SCID CB17- strain 394 (white) mice were used in this study. 1x10⁷ KMS11-GFP cells suspended in 100uL of RPMI 1640 and 100uL of matrigel were injected in the back of mice. Following tumor engraftment (one week), mice were administered ritonavir by oral gavage and/or metformin by intraperitoneal injection (i.p). Ritonavir was dissolved in 5% ethanol: 95% propylene glycol with two molar equivalents of p-toluene sulfonic acid. p-toluene sulfonic acid was weighed and added to the solution and stirred overnight at room temperature. Ritonavir was administered daily by oral gavage. Metformin (125mg/Kg, equivalent to a human dose of 10mg/Kg by normalization to surface area) was delivered via i.p daily for the duration of the experiment. In combinatorial treatment regimens, the second drug was administered after one week.

Treatments were started after the average size tumor reached 125-250 mm³. Tumor volume was calculated using: Volume (mm³) = 0.5 x (W²xL). Percent inhibition of tumor growth was calculated as follows: (1 - (mean tumor volume of vehicle treated group ÷ mean tumor volume of ritonavir treated group)) x 100.

**Immunoblot analysis**

Mouse tumors were lysed in Complete Lysis-M buffer (Roche Applied Science) supplemented with phosphatase inhibitor cocktail tablets (Roche Applied Science), 1mM DTT (Sigma-Aldrich) and 0.5% NP-40 (CalBiochem) following homogenization. Cell line cytosolic lysates were
prepared using the Complete Lysis-M buffer (Roche Applied Science) supplemented with phosphatase inhibitor cocktail tablets (Roche Applied Science). Immunoblotting was carried out according to standard protocols with horseradish peroxidase–linked secondary antibodies (Cell Signaling Technology).

**Immunohistochemistry**

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded 4-μm sections of mouse xenograft tumors. The tissue sections were deparaffinized, and incubated in 1% hydrogen peroxidase for 10 minutes to quench endogenous tissue peroxidase. Antigen retrieval was carried out in citric buffer in microwave (high power) for 10 min. The tissue sections were then incubated with the anti-Glut1 or anti-Glut4 antibody overnight at +4°C. The slides were stained using a standard EnVision+ System-HRP kit (DAKO, Carpinteria, CA) according to the manufacturer’s protocol. Immunohistochemical reactions were developed with diamino-benzidine as the chromogenic peroxidase substrate, and slides were counterstained with hematoxylin. Images were taken using an Olympus BX40 microscope.

**Results:**

**MM cells resistant to ritonavir rely on glutamine metabolism**

To start to investigate basis for resistance to ritonavir in MM cells we first determined the impact of ritonavir alone on a panel of MM cell lines. MM cells treated with ritonavir for 72 hours exhibit varying levels of cell death as evaluated by AnnexinV/DAPI staining (Figure 1A). To examine the basis for resistance to ritonavir in MM, we explored the ability of cells to utilize alternative substrates and oxidative phosphorylation (OXPHOS). Since myeloma cells are known to be highly reliant on glutamine (13) we tested the impact of glutamine withdrawal in the context of glucose deprivation on MM cell viability. Indeed, removal of both glucose and glutamine elicited significant apoptosis in a panel of MM cell lines (Figure 1B). To further confirm reliance on glutamine in ritonavir treated cells, the KMS11 MM cell line was treated with ritonavir in absence of glutamine with or without the addition of cell permeant dimethyl α-ketoglutarate (DMK). DMK rescued ritonavir-treated glutamine-deprived KMS11 cells (Figure 1C). Evaluation of the contribution of fatty acid oxidation by treatment with etomoxir indicated minimal contribution to maintaining cellular viability of ritonavir treated KMS11 cells (data not shown).

**Oxygen consumption is maintained in ritonavir-treated KMS11-GFP cells and can be targeted with complex I inhibitor metformin**
While our results suggest a role for glutamine in sustaining MM cell viability, there are additional mitochondrial metabolites that can sustain OXPHOS. We therefore rationalized that treatment with a complex 1 inhibitor would block any resistance-promoting OXPHOS. To this end we first evaluated oxygen consumption in KMS11-GFP cells treated with ritonavir with or without metformin (that is known to target complex 1) (14-16)) using a sea horse bioenergetics analyzer. Cells treated for 17 hours with ritonavir, metformin or the combination were evaluated for oxygen consumption rate (OCR) at baseline and following the addition of oligomycin A, to determine the amount of oxygen consumption coupled to ATP synthesis; FCCP, to determine maximal respiratory capacity; and lastly antimycin and rotenone to determine the spare respiratory capacity (Figure 1D). Our results demonstrate that OCR is indeed maintained in KMS11-GFP cells upon treatment with ritonavir (Figure 1D). The combination of ritonavir with metformin however completely suppressed OCR in the KMS11-GFP cells. In addition, evaluation of viability demonstrated that ritonavir in combination with metformin elicits significant apoptosis in KSM11-GFP cells (Figure 1E).

**Ritonavir in combination with metformin synergistically elicits apoptosis in MM cell lines and patient samples**

We next investigated the efficacy of ritonavir and metformin in a panel of MM cell lines, myeloma patient samples (CD138 positive plasma cells isolated from patient bone marrow aspirates) and normal peripheral blood mononuclear cells (PBMC). Following treatment with the compounds alone or in combination for 72 hrs in cell lines and PBMC and for the indicated time periods in patient samples, we assessed viability by the MTS assay or AnnexinV/DAPI staining. The combination of ritonavir with metformin elicited significant reduction in MM cell lines and patient sample viability in contrast to normal PBMC (Figure 2). Estimation of combination indices (CI values) by the method of Chou (17) by evaluation of AnnexinV/DAPI staining in three MM cell lines treated with ritonavir or metformin or the combination for 72 hrs demonstrated that ritonavir synergizes with metformin to elicit cytotoxicity in MM with CI values in the range of 0.3 – 0.6 at ED50 (Figure 2, Table1).

**Ritonavir and metformin combination therapy is effective in an in vivo xenograft model of multiple myeloma**

To investigate the efficacy of combinatorial treatment in vivo, we generated KMS11-GFP expressing xenograft tumors. KMS11-GFP cells were inoculated on the backs of mice to generate myeloma xenografts. Following detection of palpable tumors, animals were administered one of the following treatments: 1) metformin vehicle; 2) ritonavir vehicle; 3) ritonavir (50mg/kg); 4) metformin (125 mg/kg); 5) ritonavir (50mg/kg) for one week followed by co-treatment with metformin (125 mg/kg); or 6) metformin (125 mg/kg) for one week.
followed by co-treatment with ritonavir (50mg/kg). These doses were chosen to approximate physiologically achievable dosing. A ritonavir dose of 100 mg/kg leads to a \( C_{\text{max}} \) of 33\( \mu \text{M} \) in mice, however humans administered ritonavir exhibit a \( C_{\text{max}} \) of 15 \( \mu \text{M} \) (18). We therefore chose a dose of 50 mg/kg. Average tumor volumes over time and fold change in tumor growth for the KMS11-GFP tumors are plotted in Figure 3A-B, respectively. Vehicle treated KMS11-GFP tumors continued to grow and reached 2500mm\(^3\) average volume at day 14. Consistent with the previous \textit{in vitro} findings, KMS11-GFP tumors regressed from 2500mm\(^3\) to 1500mm\(^3\) post ritonavir treatment (Figures 3A-C). It has been shown that metformin decreases tumor burden by targeting complex 1 activity (14). Surprisingly, metformin treated KMS11-GFP tumors remained smaller than vehicle treated animals until day 14 but grew exponentially after day 14 (Figure 3A). Consistent with our \textit{in vitro} findings, tumors regressed significantly in animals that received the combination therapy. Interestingly, tumor regression in mice treated with the combination therapy was greater in mice given metformin first prior to the addition of ritonavir (Figure 3A).

We also analyzed the effects of the combinatorial therapy on tumor weight (Figures 3C). Tumor weights were reduced post ritonavir and the combination treatments (Figure 3C), with the pretreated metformin + ritonavir group demonstrating the largest reduction in tumor weight. Body weights of the animals were not significantly decreased after combination therapy (Figure 3E) in comparison to the vehicle treated animals, suggesting that this dosing regimen is well tolerated and does not impact whole body glucose homeostasis significantly. Next, we sought to compare the survival of mice following treatments (Figure 3D). Consistent with the efficacy of the treatments, none of the vehicle treated mice survived after day 14 whereas 50% or more ritonavir or metformin treated animals survived longer (day 21). Consistent with the finding that tumors regressed after combination therapy, greater than 50% of mice that received this combination were surviving through the end of the study (35 days post treatment, Figure 3E), providing evidence that combination therapy improves survival of MM xenograft bearing mice.

**GLUT4 exhibits increased expression at the leading invasion front**

Immunohistochemical evaluation of GLUT1 and GLUT4 in xenograft tumors demonstrates for the first time GLUT4 at the leading invasion front of tumors while GLUT1 was more homogenously distributed (Figure 3F) further supporting the utility of targeting GLUT4 in MM.

**Combination therapy reduces pAKT, pAMPK, pmTORC1 and MCL-1 expression \textit{in vitro} in cell lines and in a mouse xenograft model of MM**

To begin to evaluate the mechanistic basis for efficacy of ritonavir and metformin we interrogated MM cell lines and cytosolic tumor lysates for protein expression of specific effectors known to regulate metabolism and or cell survival. Pro-survival BCL-2 family member
MCL-1 is regulated by glucose (3, 19) via the AKT/mTORC1 axis (19). Examination of AKT and mTORC1 phosphorylation both in vitro in cell lines and in tumor lysates exhibited suppression upon co-treatment with ritonavir and metformin correlating with suppression of MCL-1 expression (Figure 4A-C). Ritonavir and metformin did not impact MCL-1 mRNA levels (data not shown; estimation performed at the 72 hr time point). We also interrogated MCL-1 stability as a mode of regulation by the ritonavir metformin combination. Co-treatment with proteosome inhibitor bortezomib or MG-132 and inhibition of GSK-3β (that is involved in regulating MCL-1 stability) did not reverse the suppression of MCL-1 detected upon co-treatment with ritonavir and metformin (data not shown). Given the central role of AKT in promoting glycolysis (20) and of AMPK in promoting oxidative metabolism (21) we hypothesized that AMPK may be maintained in ritonavir treated cells consistent with their ability to maintain OXPHOS upon ritonavir treatment. Indeed, treatment with ritonavir maintained pAMPK expression levels in tumors (4/5 animals, Figure 4C). We importantly note that co-treatment led to significant suppression of pAMPK and pAKT both in vitro in cell lines and in 5/5 animals (Figure 4A and C), correlating with significant reduction in tumor burden that we detected with this treatment regimen. Treatment with metformin alone led to slight increases in total AMPK and reduction in pAKT levels across the animals tested however it is the combination of metformin and ritonavir that effectively reduce both pAMPK and pAKT levels, further supporting the growth inhibitory and apoptosis inducing effects of this combinatorial regimen.

**Ritonavir and metformin combination therapy is growth inhibitory in other hematological malignancies and solid tumors**

We have previously demonstrated the efficacy of ritonavir and metformin co-treatment in CLL (12) and hypothesized that this combination would be efficacious in other cancers. To understand if the combinatorial treatment is effective in other hematological malignancies, we investigated the effects of the combinatorial therapy in cell lines derived from diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphomas (Figure 5A and 5B). Similar to our findings in MM cell lines, the growth of these cell lines was reduced in a dose-dependent manner upon co-administration of ritonavir and metformin. We additionally, evaluated the effects of the combination therapy in solid tumors using cell lines derived from melanomas (Figure 6) as well as breast and ovarian cancers (Figure S1). The combinatorial regimen was growth inhibitory in all melanoma cell lines and a majority of the breast and ovarian cancer lines.
Discussion:

In the early 1900s Otto Warburg made the seminal discovery that tumor cells engage aerobic glycolysis even under normoxia (22). More recent studies have demonstrated the utilization of aerobic glycolysis by tumor cells despite the presence of functional mitochondria, suggesting inherent survival and/or proliferation benefits for tumor cells engaging this mode of glucose metabolism (23). While numerous studies provide significant rationale to target glycolysis for cancer therapy, the caveat remains in the identification of tumor specific targets and a need for increased understanding of the compensatory metabolic circuitry that tumor cells engage to circumvent perturbations in any given metabolic pathway.

We have previously determined that treatment of myeloma cell lines and patient samples with physiologically achievable doses of the GLUT4 inhibitor ritonavir exerts glucose transport-specific anti-myeloma effects (3). The selectivity of ritonavir for GLUT4 has been proven in vitro (9-11) and in vivo by the evaluation of ritonavir’s impact on glucose tolerance in GLUT4 knockout mice (10). In fact, it is the selective inhibition of GLUT4 that is responsible for the ensuing insulin resistance and dyslipidemia observed upon chronic administration of ritonavir (24). While the specificity for GLUT4 (in contrast to GLUTs1, 2, 3, 4 and 8) exhibited by this class of drugs (9) and greater potency of ritonavir versus indinavir (another GLUT4 inhibitor) (9) has been established in other cell types we cannot rule out minimal effects on other transporters within MM cells. While ritonavir has also been described to inhibit STAT3 and ERK1/2 in MM (25) our previous study established that inhibition of glucose uptake and glycolysis are required to elicit the growth inhibitory effects of ritonavir (3). In the current study we investigated the metabolic basis for resistance to ritonavir and found ritonavir-treated or glucose deprived cells to maintain survival associated with increased reliance on mitochondrial OXPHOS.

Our results demonstrating increased apoptosis in ritonavir-treated KMS11 cells that are glutamine deprived suggest a role for compensatory glutamine metabolism. More importantly the rescue of ritonavir-treated and glutamine-deprived KMS11 cells with cell permeant α-ketoglutarate suggests a role for glutaminolysis in maintaining survival of ritonavir treated MM. Increased engagement of OXPHOS in KMS11 is further supported by the observation that ritonavir treated cells maintain OCR and exhibit increased sensitivity to the mitochondrial complex I inhibitor metformin. While one cannot rule out the contribution of fatty acid oxidation in maintaining MM survival upon ritonavir treatment, our results demonstrate efficacy of the combination of ritonavir and metformin in four MM cell lines exhibiting disparate genetic backgrounds. These results collectively underscore the utility of this strategy to target both glycolysis and resistance promoting-OXPHOS to elicit cytotoxicity in the broader spectrum of MM cell types.
Metformin is an FDA-approved anti-diabetic drug that has anti-cancer chemo-sensitizing properties now proven in various in vitro and pre-clinical in vivo studies (14, 26). Several epidemiologic studies have also correlated metformin treatment with reduced risk of cancer in diabetics (27, 28) and in cancer patients (29). Metformin is a mitochondrial complex 1 inhibitor that can be used to target OXPHOS (14-16). In addition, metformin impacts whole body glucose homeostasis by regulation of glucose production and insulin secretion (30) that can prove to be beneficial in regulating elevated levels of circulating glucose that may result from ritonavir treatment.

Evaluation of cell lines and tumor lysates importantly demonstrated that co-treatment of MM with ritonavir and metformin led to suppression of the AKT and mTORC1 pathways known to regulate synthesis of MCL-1 (19). MCL-1 is particularly important in the development of resistance, as the inability to decrease MCL-1 expression correlates to resistance to bortezomib (31), rapamycin (32), cyclin-dependent kinase inhibitors (33), and the BCL-2/BCL-XL/BCL-w selective antagonist ABT 737 (34) in MM and various cancers. Importantly, higher MCL-1 expression levels correlate with lower event free survival in MM (35), underscoring the need for effective strategies to target MCL-1. We and others have demonstrated a link between glucose and maintenance of MCL-1 (3, 19, 36). Therefore, there is reason to believe that suppression of MCL-1 seen in 4 of the 5 tumors in ritonavir treated mice may be linked to suppression of glucose uptake and responsible for the ensuing reduction in tumor burden. In targeting metabolism one would anticipate changes in the activity of AMPK, which like AKT has a pro-survival role in MM (37, 38). Regulation of AMPK was inconsistent between cell lines and tumor lysates that could be due to effects of the drugs in the context of the in vivo microenvironment. What is particularly interesting is the maintenance of pAMPK in ritonavir treated KMS11 tumor xenografts. In normal cells, AMPK is known to be activated under conditions of cellular stress such as upon glucose withdrawal to promote catabolic ATP synthesis, and mitochondrial biogenesis associated with activation of SIRT1 and PGC1α promoting increased oxidative metabolism (39). The maintenance of pAMPK in ritonavir only-treated mice may contribute to the resistance of the MM xenograft tumors in vivo to ritonavir therapy alone, which is targeted upon co-treatment with metformin.

There are several models of MM but none quite exactly recapitulate the in vivo bone marrow engagement and progression of MM. The subcutaneous xenograft model of MM has previously been used to test drugs such as bortezomib (40) and thus this model is able to provide an idea of the efficacy of this regimen in vivo. Our examination of GLUT4 localization in the tumors demonstrates for the first time expression of GLUT4 at the invasion front. GLUT4 could thus potentially be involved in promoting a highly active metabolic phenotype at the invasion front to promote dissemination promoting development of multiple myeloma skeletal lesions. Therefore, it will be important to test the impact of ritonavir and metformin on dissemination
and bone engagement in a tail vein disseminated model of myeloma (41) or in the spontaneous Vk*MYC genetically engineered mouse model of myeloma (42).

Among the MM cell lines tested in our study JJN3 cells exhibit the t(14;16) translocation associated with deregulation of cMAF, U266 cells express mutant BRAF and the t(11;14) translocation associated with deregulation of cyclin D1; L363 cells express mutant NKRAS and the t(14;16) translocation while KMS11 cells express the t(4;14) and t(14;16) translocations associated with deregulation of FGFR3 and cMAF (43, 44). MM is thus characterized by variety of genetic abnormalities in addition to exhibiting clonal heterogeneity within a given tumor. Any given patient thus requires therapy targeting diverse translocations and mutations to circumvent selection of aggressive clonal subpopulations (45). For e.g. in MM patients expressing BRAF mutations we have learnt that while BRAF inhibitors effectively inhibit MAPK in the MM cells expressing activating BRAF mutations, cells within the same patient expressing WT BRAF or KRAS increased activation of MAPK, essentially selecting for these subpopulations (45). Our data suggests that targeting abnormal cellular metabolism can circumvent the selective pressures associated with targeting individual upstream drivers of pathways that promote proliferation and survival by targeting the metabolism sustaining diverse genetic backgrounds. The reliance of MM cells on GLUT4 that is targeted with ritonavir that then elicits sensitivity to metformin allows for the selectivity of this strategy towards tumor cells.

The chronic use of ritonavir as part of a combinatorial anti-retroviral treatment regimen for HIV despite its GLUT4-inhibitory effects is proof of principle that humans can tolerate drugs that target GLUT4 (46). In the aforementioned HIV study, patients on 600 mg ritonavir twice a day were additionally dosed at 1500 mg/day with metformin to treat diabetic symptoms (46). This treatment combination was thus found to be well tolerated in humans (46). Metformin is normally dosed at 500 mg twice daily (47) and ritonavir is administered at 600 mg twice daily (18). The dosing of metformin and ritonavir that we utilized in our in vivo mouse study is comparable to clinically achievable doses in humans, thus can potentially be evaluated in humans for cancer therapy. Our rationale for treating with metformin prior to ritonavir was to approximate how the drugs would be administered in humans. This in fact turned out to be fortuitous as it appears like this dosing pattern may have better efficacy. We are unclear as to the mechanism but this order of administration may lead to better control of glucose homeostasis and circulating insulin levels in vivo.

Our studies also demonstrate the utility of combining metformin with ritonavir in a wide range of malignancies i.e. mantle cell lymphoma, diffuse large B cell lymphoma melanoma, ovarian, breast cancer and just recently our group reported efficacy in CLL (12). These observations, in addition to our findings, warrant further pre-clinical investigation into repurposing ritonavir and
metformin for short-term combinatorial and/or chemo-sensitization regimens for the treatment of GLUT4-dependent tumors such as MM. Our study also conceptually bolsters closer investigation into the development of agents that target GLUT4 in combination with agents that target compensatory mitochondrial activity for cancer therapy.

Author contributions: S.D-A. and M.S. conceived the research; S. D-A, R.B, M.M, K.U.A.A, C.W and M.S. performed experimentation and analyzed data with conceptual advice from S.T.R. IK performed and aided in interpretation of animal studies. S.S. oversaw collection of myeloma patient samples. NR and JK assisted with the design and implementation of the animal study. M.S. wrote the manuscript and supervised the project.

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Figure 1: (A-B) Ritonavir treated MM cells increase reliance on glutamine metabolism that is targeted with metformin. Impact of ritonavir on MM cell viability: (A) L363, JJN3 and KMS11 cell lines were cultured in the presence or absence of Ritonavir (40 μM) for 72hr or (B) in the absence of glucose (5mM) / glutamine (2mM) or both as indicated for 48hrs and viability assessed by AnnexinV-DAPI staining and flow cytometry. (C) Glutamine deprived ritonavir-treated KMS11 cells are rescued by cell permeant DMK: KMS11 cells were cultured in presence or absence of glucose (5mM), ritonavir (40 μM), glutamine (2mM) or DMK-1.25mM as indicated for 48hr. Cell viability was assessed by AnnexinV/DAPI staining. Data are mean ± SEM (n=3). (D) KMS11-GFP cells maintain oxygen consumption upon ritonavir treatment that can be targeted by co-treatment with metformin. KMS11-GFP cells were treated with 20μM ritonavir and/or 5 mM metformin for 17 hrs following which oxygen consumption rate (OCR) was evaluated in equal numbers of viable cells using a seahorse bioenergetics bioanalzyer. Specific
perturbations i.e. oligomycin, FCCP and antimycin/rotenone were injected and OCR measured over time. One of three representative experiments is shown that was derived from an average of 5 wells per treatment condition ± SEM. (E) KMS11-GFP cells exhibit significant apoptosis upon co-treatment with ritonavir and metformin. KMS11-GFP cells were treated with 20μM ritonavir and/or 5mM metformin for 72 hrs and viability assessed by AnnexinV/DAPI staining. Data are mean ± SEM (n=3).

Figure 2: Myeloma cell lines and patient samples exhibit significant apoptosis upon co-treatment with ritonavir and metformin. Indicated multiple myeloma (MM) cell lines were treated for 72 hrs with 20μM ritonavir and/or 1 mM metformin and the impact on cell proliferation determined by MTS assay. Data are mean ± SEM (n=3) PBMCs were treated for 72 hrs with 20μM ritonavir and 1 and/or 5 mM metformin and the impact on cell viability was evaluated by AnnexinV/DAPI staining. Data are mean ± SEM (n=5) Myeloma patient samples were treated with 20μM ritonavir and 5mM metformin for the indicated periods and impact on cell viability determined by AnnexinV/DAPI staining. Table 1: The CI values at ED50, ED75, ED90 and ED95 indicate a synergistic interaction between ritonavir and metformin in L363, JNJ3 and KMS11 cell lines. CI calculation of drug combination was based on the IC50s for inducing apoptosis evaluated by AnnexinV/DAPI staining i.e. 45 μM for ritonavir and 10 mM for metformin. CompuSyn Version 1 was used to calculate the CI value that measures the degree of interaction between two or more drugs, where a CI <1 indicates synergism.

Figure 3: KMS11-GFP tumors regress post combinatorial therapy with ritonavir and metformin in vivo. KMS11-GFP were injected into the backs of BNX-SCID mice. Once palpable tumors (125-250 mm³) were detected, mice were administered respective vehicles, ritonavir (50mg/kg), metformin (125mgs/kg) or the combination. Combination treatments were started by administering one drug first for one week followed by the addition of the other. Injection of tumor cells and initiation of treatment were on day “-7” and day “0”, respectively. (A) Mean of KMS11-GFP tumor volumes at indicated days post treatment. (B) Fold change in tumor volume was calculated by normalizing mean tumor volume at day 14 post-treatment to mean tumor volume of vehicle treated mice on day 14. (C) Mean tumor weights at the time of sacrifice. (D) Percent survival of mice post each treatment. (E) Fold change in body weight of mice post respective treatments. Asterisks indicate that the difference is significant and *, **, *** indicate that p value is ≤ than 0.05, 0.01 and 0.001, respectively. NS depicts that the difference between groups shown is not significant. (F) GLUT4 is evident along the invasion front of the tumor is removed upon co-treatment with metformin and ritonavir. Immuno-detection of GLUT1 and GLUT4 in a representative vehicle treated KMS11-GFP tumor. Staining performed as described in materials and methods and representative images are shown. GLUT1 expression throughout tumor or GLUT4 expression at the leading edge of the tumor (indicated by arrows) is evident.
**Figure 4:** Combination of ritonavir and metformin effectively reduce MCL-1 expression and AMPK and AKT phosphorylation in MM. (A) L363, JJN3 and KMS11 cell lines were treated with ritonavir (40μM) or metformin (5mM) or both for 72hr. Protein lysates were analyzed for the expression of indicated proteins or GAPDH (loading control) by immunoblot analyses. (B & C) Combined administration of ritonavir and metformin effectively reduce MCL-1 expression and AMPK and AKT phosphorylation in MM xenograft *in vivo*. Cytosolic lysates of KMS11-GFP tumors were evaluated for the indicated proteins by immunoblot analysis. Data from an n=4 or 5 tumors from representative mice are shown.

**Figure 5:** Combination of ritonavir and metformin is growth inhibitory in other hematological malignancies. Cells derived from DLBCL (A) and mantle cell lymphomas (B) were cultured in the presence of 20μM ritonavir, increasing concentrations of metformin, or the combination for 72 hours. Impact of treatments on cell proliferation was evaluated by MTS assay. Doxorubicin (10 μM) was used as a positive control.

**Figure 6:** Combination of ritonavir and metformin is growth inhibitory in melanoma. Melanoma cancer cell lines were cultured in the presence of 20μM ritonavir, and/or increasing concentrations of metformin for 72 hours. Impact of treatments on cell proliferation was evaluated by an MTS assay. Doxorubicin (10μM) was used as a positive control.
References

44. https://myelomagenomics.tgen.org/.
Figure 2

Table 1: CI values of the combination of Ritonavir with Metformin in MM cell lines

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Figure 3

A

Tumor volume (mm$^3$)

Days post treatment

- metformin vehicle
- ritonavir vehicle
- ritonavir
- ritonavir and metformin
- metformin
- metformin and ritonavir

B

KMS11-GFP Tumors (Day 14)

Fold change in tumor volume

control          ritonavir          metformin          metformin + ritonavir

C

Tumor weight (g)

metformin vehicle          ritonavir vehicle          ritonavir          ritonavir + metformin          metformin          metformin + ritonavir

D

Survival

Percent survival

DAYS

E

Body weight

Fold change in body weight

ritonavir vehicle          ritonavir          ritonavir and metformin          metformin          metformin and ritonavir

F

GLUT1          GLUT4

Vehicle
**Figure 4**

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Figure 5

A

SUDHL-6

SUDHL-4

OLI-Ly93

B

SP-53

MINO

Granta
Figure 6

UACC-2677

M14

SKMEL-28

MALME

LOX-IMVI

SKMEL-2

UACC-62

SKMEL-5
Clinical Cancer Research

Targeting the Metabolic Plasticity of Multiple Myeloma with FDA Approved Ritonavir and Metformin

Sevim Dalva-Aydemir, Richa Bajpai, Maylyn Martinez, et al.

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