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Targeting glutamine metabolism in multiple myeloma enhances BIM binding to BCL-2 eliciting synthetic lethality to venetoclax

R Bajpai, SM Matulis, C Wei, AK Nooka, HE Von Hollen, S Lonial, LH Boise, and M Shanmugam

Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA, USA.

Abstract

Multiple myeloma (MM) is a plasma cell malignancy that is largely incurable due to development of resistance to therapy-elicited cell death. Nutrients are intricately connected to maintenance of cellular viability in part by inhibition of apoptosis. We were interested to determine if examination of metabolic regulation of BCL-2 proteins may provide insight on alternative routes to engage apoptosis. MM cells are reliant on glucose and glutamine and withdrawal of either nutrient is associated with varying levels of apoptosis. We and others have demonstrated that glucose maintains levels of key resistance-promoting BCL-2 family member, myeloid cell leukemic factor 1 (MCL-1). Cells continuing to survive in the absence of glucose or glutamine were found to maintain expression of MCL-1 but importantly induce pro-apoptotic BIM expression. One potential mechanism for continued survival despite induction of BIM could be due to binding and sequestration of BIM to alternate pro-survival BCL-2 members. Our investigation revealed that cells surviving glutamine withdrawal in particular, enhance expression and binding of BIM to BCL-2, consequently sensitizing these cells to the BH3 mimetic venetoclax. Glutamine deprivation-driven sensitization to venetoclax can be reversed by metabolic supplementation with TCA cycle intermediate α -ketoglutarate. Inhibition of glucose metabolism with the GLUT4 inhibitor ritonavir elicits variable cytotoxicity in MM that is marginally enhanced with venetoclax treatment, however, targeting glutamine metabolism with 6-diazo-5-oxo-L-norleucine uniformly sensitized MM cell lines and relapse/refractory patient samples to venetoclax. Our studies reveal a potent therapeutic strategy of metabolically driven synthetic lethality involving targeting glutamine metabolism for sensitization to venetoclax in MM.

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Correspondence: Dr M Shanmugam, Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322, USA. mala.shan@emory.edu.

AUTHOR CONTRIBUTIONS

RB and MS conceived the research with conceptual advice from LHB; RB, SMM, CW and MS performed experimentation and data analysis with advice from LHB. HEVH, AKN and SL oversaw collection of myeloma patient samples and provided conceptual advice. RB and MS wrote the manuscript and MS supervised the project.

CONFLICT OF INTEREST

LHB is a consultant for Onyx Pharmaceuticals and Novartis; AKN is consultant for Onyx Pharmaceuticals and Spectrum pharmaceuticals; SL is a consultant for Millennium, Onyx Pharmaceuticals, Novartis, BMS, Janssen and Celgene. The remaining authors declare no conflict of interest.

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INTRODUCTION

Multiple myeloma (MM) is a plasma cell neoplasia accounting for 13% of all hematologic malignancies.¹ Despite use of next generation immunomodulatory drugs, proteasome inhibitors and newer targeted therapies, a major problem commonly observed in the treatment of MM is the development of resistance leading to relapse and often recurrence of more aggressive disease. Importantly, ~ 20% of patients succumb to aggressive treatment-refractory disease within a short time of diagnosis necessitating new therapeutic strategies to target resistance.²

Evasion of apoptosis is integral to tumor development and resistance to therapy. Induction of the intrinsic pathway of apoptosis is dictated by the release of pro-apoptotic BH3-only activator proteins (BIM, PUMA, BID) from anti-apoptotic BCL-2 family members (BCL-2, BCL-x_L, MCL-1, BCL-w and A1) that in turn activate BAX and BAK leading to mitochondrial membrane permeabilization and cytochrome *c* release.^{3,4} BH3 activator proteins are released either by reduction in expression of an anti-apoptotic BCL-2 protein to which they are bound or if a sensitizer (such as NOXA, BAD or a BH3 mimetic) releases the BH3 activator protein from binding the anti-apoptotic.⁵ It is not surprising that resistance to many commonly used therapeutics lies in altered regulation of BCL-2 proteins. For example, the inability to decrease MCL-1 expression correlates with resistance to bortezomib,⁶ rapamycin,⁷ cyclin-dependent kinase inhibitors,⁸ the BCL-2/BCL-x_L/BCL-w selective antagonist ABT-737⁹ and death receptor (Fas/TRAIL)¹⁰-induced apoptosis in various cell types. Overexpression of BCL-2 on the other hand is linked to resistance to bortezomib, dexamethasone and melphalan in CD138+ MM cells¹¹ and resistance in chronic lymphocytic leukemia.¹² MM, acute myelogenous and lymphocytic leukemia and various solid tumors are found to be more resistant to chemotherapy when they are less primed, that is, below the threshold of apoptosis induction that is importantly dictated by levels of pro-apoptotics sequestered by anti-apoptotic BCL-2 proteins.¹³ Thus finding alternative strategies to effectively engage and target BCL-2 proteins can potentially circumvent resistance.

Altered metabolism is now recognized as a hallmark of cancer and nutrients promoting survival and proliferation directly or indirectly also prevent apoptosis. MM is characterized by altered glucose metabolism evident from increased FDG-PET avidity that correlates with poor prognosis.¹⁴ In addition, MM cells are highly reliant on glutamine metabolism.^{15,16} Glucose and glutamine metabolism generate bioenergy and provide precursors for synthesis of amino acids, nucleotides and maintenance of redox homeostasis. Apart from these functions glucose and glutamine are critical drivers of signaling promoting proliferation¹⁷ and evasion of apoptosis through discrete regulation of BCL-2 proteins such as PUMA, BIM, NOXA,¹⁸ BAX,¹⁹⁻²¹ BAD²⁰ and MCL-1.¹⁹

MM cells are highly dependent on MCL-1 for survival^{22,23} and MCL-1 sequesters and neutralizes the key apoptotic activator BIM. However, a subset of MM exhibit co-dependencies on BCL-2/x_L in addition to MCL-1 for sequestering and binding BIM.²⁴ We previously demonstrated that glucose-deprived MM cells exhibit a reduction of MCL-1 expression that did not necessarily correlate with cell death.²⁵ These observations prompted

our hypothesis that MM cells surviving nutrient deprivation may re-configure BCL-2 protein expression and/or binding to maintain survival. Our studies indeed reveal altered regulation of BCL-2 proteins in cells surviving nutrient deprivation that importantly enables sensitization to the BH3 mimetic venetoclax (ABT-199) with efficacy in a broad range of MM cell lines and relapse/refractory MM patient samples.

RESULTS

MM cells are variably dependent on glucose or glutamine for cell survival

We have previously demonstrated that MM cells are variably dependent on glucose or glutamine for cell survival with some cells remaining viable in the absence of either nutrient.¹⁶ Given the role of glucose in regulating expression of BCL-2 proteins such as PUMA, BIM, NOXA,¹⁸ BAX,^{19–21} BAD²⁰ and MCL-1,¹⁹ we were interested to investigate how these proteins were regulated in cells surviving in the absence of either glucose or glutamine. We first evaluated the impact of glucose or glutamine withdrawal or of both metabolites on the induction of apoptosis in a broader panel of MM cell lines. While all cells are growth inhibited on removal of either glucose or glutamine (data not shown), the extent of apoptosis elicited was highly variable (Figure 1a). RPMI-8226, L363 and KMS18 cells exhibit significant apoptosis (>50%, that we term ‘sensitive’) on glucose withdrawal while the rest of the cell lines tested were resistant. RPMI-8226, KMS11, JLN3 and AMO1 are sensitive to glutamine deprivation exhibiting >50% cell death. KMS11 and MM.1S cell lines are equally sensitive to removal of either nutrient. The U266 cell line is the most resistant while the RPMI-8226 cell line is most sensitive to removal of either glucose or glutamine. All MM cell lines tested exhibit reduced viability on withdrawal of both glucose and glutamine. The majority of patient samples tested are resistant (exhibiting <50% death) on glucose or glutamine withdrawal (Figure 1b). Characteristics of patient samples used in the study are given in Table 1. Cytogenetic and fluorescent *in situ* hybridization results from these patient samples indicate complex genetic abnormalities characteristic of relapse/refractory patients. In sum, MM cells are variably dependent on glucose or glutamine for maintenance of viability.

Increased expression of pro and anti-apoptotic BCL-2 proteins in MM resistant to glucose or glutamine deprivation

To investigate altered regulation of anti-apoptotic and pro-apoptotic proteins for continued survival of glucose or glutamine-deprived MM, we evaluated expression of key pro-survival and pro-apoptotic BCL-2 proteins, namely MCL-1, BCL-2, BCL-x_L, BIM, BID, PUMA and NOXA in seven MM lines excluding RPMI-8226 that exhibit significant cell death on removal of either nutrient. Our preliminary investigation of BIM and BCL-2 protein expression demonstrated that expression levels are upregulated as early as 5 h, peaking at 18–24 h when cells are not yet permeabilized based on DAPI uptake (data not shown). Based on this preliminary analysis, we evaluated nutrient deprivation-associated regulation of BCL-2 proteins at 24 h. Most lines exhibited an induction of anti-apoptotic BCL-2 on glucose or glutamine withdrawal and variable induction or suppression of BCL-x_L expression. Expression of MCL-1 decreased with removal of glucose in L363 and KMS18 and with removal of glutamine in U266 cells; correlating with a reduction in viability, while

expression of MCL-1 was increased or maintained irrespective of glucose or glutamine deprivation in all other lines tested (Figure 2). We detect an induction of BIM and NOXA in the majority of MM lines deprived of glutamine (except U266, which does not express NOXA). PUMA expression is decreased (Figure 2) and BID expression was also decreased (data not shown). In sum, removal of glucose or glutamine is associated with regulation of both pro-survival and pro-apoptotic BCL-2 proteins with a more consistent induction of BIM and BCL-2 across all lines. The patterns of expression of the BCL-2 proteins on nutrient deprivation, however, do not clearly suggest a basis for continued survival.

Glucose or glutamine deprivation increase BIM binding to BCL-2

MM cells are primarily MCL-1 dependent, however, can exhibit a co-dependency on MCL-1, BCL-2 and BCL-x_L.²⁴ Since both anti-apoptotic BCL-2 and pro-apoptotic BIM were induced in the majority of glutamine-deprived MM cell lines, we hypothesized that pro-apoptotic BIM may be sequestered by BCL-2 in cells remaining viable after glutamine deprivation. Consequently, if this were the case, addition of a BH3 mimetic like ABT-199²⁶ would release BIM bound to BCL-2 and induce apoptosis. To test this hypothesis, we evaluated the impact of either glucose or glutamine withdrawal on binding of BIM to BCL-2, MCL-1 and BCL-x_L. MCL-1, BCL-2 and BCL-x_L proteins were co-immunoprecipitated from cell lysates prepared from MM cell lines that had been nutrient deprived for 24 h treated with or without ABT-199. BIM bound to each of the immunoprecipitated anti-apoptotics was assessed by densitometric quantification and percent distribution among the anti-apoptotics calculated. Indeed, co-immunoprecipitates of MCL-1, BCL-2 or BCL-x_L from glucose or glutamine-deprived L363, JJN3 and KMS11 cell lines demonstrate increased association of BIM with BCL-2. Each cell line, however, exhibits differential distribution of BIM binding to the anti-apoptotics as described below.

In glucose- or glutamine-deprived L363, BIM remains bound to MCL-1 at levels comparable to that detected on MCL-1 in cells cultured in nutrient replete media (Figure 3a). Under glucose or glutamine deprivation, we detect a greater than twofold increase in BIM binding to BCL-2 (interestingly not BCL-x_L, in glutamine-deprived L363, Figure 3a) which is removed on treatment with ABT-199.

JJN3 cells on the other hand which are more resistant to glucose deprivation (Figure 1) exhibit minimal induction of BCL-2 and BIM and maintain MCL-1 and BCL-x_L expression on glucose deprivation (Figure 2) that is, however, associated with increased binding of BIM to MCL-1 and BCL-2 (Figure 3b). Glutamine withdrawal in JJN3 maintains MCL-1, BCL-2, BCL-x_L and BIM expression (Figure 2) that is again associated with a greater than twofold increase in binding of BIM to BCL-2 (Figure 3b).

As previously demonstrated, KMS11 cells cultured in the absence of glucose increase expression levels of BIM and MCL-1, maintain BCL-2 and decrease BCL-x_L expression (Figure 2). Correspondingly, in the absence of glucose the quantity of BIM bound to MCL-1 is increased by approximately twofold with marginal increases in BIM bound to BCL-2 and BCL-x_L (Figure 3c). Glutamine-deprived KMS11 cells increase expression of BIM and MCL-1 and decrease expression of BCL-2 and BCL-x_L (Figure 2). Glutamine deprivation increases BIM bound to BCL-x_L and MCL-1 and importantly increases the amount of BIM

bound to BCL-2 by greater than twofold (Figure 3c). Summary of fold change in BIM bound to BCL-2 in relation to percent death elicited on glutamine deprivation with or without ABT-199 treatment is now included as Supplementary Table S1.

Glutamine deprivation sensitizes MM to ABT-199

Based on our detection of increased BIM bound to BCL-2 after nutrient deprivation, we tested the impact of ABT-199 treatment on the viability of nutrient-deprived MM. As demonstrated in Figures 4a and b, glucose- or glutamine-deprived MM lines exhibit significant apoptosis when co-treated with ABT-199, with glutamine deprivation-insensitive cells uniformly demonstrating sensitization to ABT-199. JLN3 and the U266 cells are the only two lines among the eight cell lines tested where glucose deprivation sensitizes to ABT-199 while glutamine deprivation appears to sensitize six of the eight MM cell lines to ABT-199 with the exception of RPMI-8226 cells and AMO1, which exhibit significant apoptosis on culture in the absence of glutamine (Figures 4a and b). These results suggest that increased BIM binding to BCL-2 on glutamine deprivation facilitates sensitization to ABT-199. ABT-199 treatment in cells cultured in nutrient replete media did not elicit cell death, suggesting lack of release of sufficient pro-apoptotic proteins bound to BCL-2 to elicit death (Figures 4a and b).

In L363, the shift in binding of BIM to BCL-2 on glucose deprivation that is released with ABT-199 treatment is sufficient to induce apoptosis. In glutamine-deprived L363, BIM remains bound to MCL-1 at levels comparable to that detected on MCL-1 in cells cultured in nutrient replete media. BIM, however, exhibits a greater than twofold increase in binding to BCL-2 (interestingly not BCL-x_L) (Figure 3a) that is released on treatment with ABT-199 correlating with the observed cytotoxicity (Figure 4b). In KMS11 cells, the marginal increase in BIM bound to BCL-2 and enhanced binding of BIM to MCL-1 on ABT-199 treatment may account for a lack of additional sensitization to ABT-199 on glucose deprivation (Figure 4a). Glutamine deprivation in KMS11 increases BIM bound to BCL-x_L and MCL-1 and importantly increases the amount of BIM bound to BCL-2 by greater than twofold (Figure 3c) accounting for increased sensitization to ABT-199 (Figure 4b).

NOXA by virtue of increased affinity for MCL-1^{27,28} can shift BIM from MCL-1 to BCL-2. We therefore wanted to test whether the induction of NOXA seen across all the nutrient-deprived cell lines contributes to increased binding of BIM to BCL-2. We knocked down NOXA in glucose- or glutamine-deprived L363, JLN3 and U266 (negative control, which does not express NOXA) cells and assessed sensitivity towards ABT-199. Suppression of NOXA improved basal viability and partially reversed the sensitivity to ABT-199 in glucose- or glutamine-deprived L363 cells, however, had no impact on the sensitization of the JLN3 and U266 cells to ABT-199. These results overall suggest that NOXA does not promote a shift in binding of BIM to BCL-2 to account for increased binding of BIM to BCL-2 and sensitization to ABT-199 (Supplementary Figure S1).

Nutrient deprivation-elicited sensitivity to ABT-199 is reversed by metabolic supplementation

We next wanted to examine the metabolic specificity of glutamine deprivation-induced sensitization to ABT-199. Glutamine-deprived cells were supplemented with various metabolites generated by metabolism of glutamine and evaluated for their ability to reverse sensitization to ABT-199. We supplemented glutamine-deprived MM lines with galactose, dimethyl fumarate, dimethyl succinate, oxaloacetic acid, 2 methyl pyruvate, a mixture of non-essential amino acids or cell permeant dimethyl alpha ketoglutarate (DMK). Among these metabolites only the addition of DMK reversed sensitization to ABT-199 in the L363, KMS11, MM.1S and AMO1 cell lines (Figures 5a–d and data not shown). We focused on the L363 cell line cultured in glutamine-free media as the L363 cells are resistant to glutamine withdrawal (however, sensitive to glucose withdrawal) allowing us to better parse out metabolism tied to glutamine withdrawal and sensitization to ABT-199. To investigate the mechanistic basis of the reversal of sensitization to ABT-199, we examined the effects of DMK on BIM, MCL-1, BCL-2 and BCL-x_L expression. DMK suppressed induction of BIM in glutamine-deprived L363 without impacting the induction of BCL-2 (Figure 5e). In addition, evaluation of BIM binding demonstrated that DMK treatment reduced binding of BIM to BCL-2 and increased binding to MCL-1 in glutamine-deprived cells (Figure 5f). Investigation of DON (6-diazo-5-oxo-L-norleucine)-treated L363 likewise demonstrated increased association of BIM with BCL-2, underscoring the ability of both glutamine deprivation and inhibition of glutamine metabolism to increase BIM binding to BCL-2 (Figure 5g).

Evaluation of ritonavir targeting GLUT4 and DON targeting glutamine metabolism for sensitization to MM to ABT-199

Ritonavir is an Food and Drug Administration (FDA)-approved HIV protease inhibitor and a non-competitive reversible inhibitor of the glucose transporter GLUT4.²⁹ We have previously demonstrated that MM cells exhibit increased expression of GLUT4 on the plasma membrane and are highly reliant on this particular transporter for cell survival and proliferation.²⁵ Consequently ritonavir exhibits growth inhibitory/cytotoxic effects in MM cells. To phenocopy glucose deprivation, we treated MM cells with ritonavir. Ritonavir elicits cell death, however, as seen previously with glucose-deprived MM we do not detect uniform sensitization to ABT-199 (Figures 6a and b). To target glutamine metabolism, we tested the glutamine antagonist DON that effectively blocks the glutaminases that facilitate glutaminolysis of glutamine to glutamate.^{30,31} DON increased sensitivity to ABT-199 in MM cell lines and patient samples (Figures 6c and d) similar to that seen in glutamine-deprived MM. The MM patient samples highly sensitized to the combination were relapse/refractory and high-risk patients exhibiting common resistance-associated genetic deletions, that is, del (17p) or del (1p) or translocations like t(4;14), as indicated in Table 1. Our data thus highlight a novel metabolically driven therapeutic strategy that can be further evaluated for MM therapy.

DISCUSSION

Tumor cells are constantly subject to genotoxic, metabolic and redox stress that is in part related to prior therapy leading to induction of pro-apoptotics counter-balanced by elevated expression of anti-apoptotics. This circuitous addiction to BCL-2 proteins, however, can effectively increase the primed state of the cell making them more susceptible to apoptosis.¹³ Our results demonstrate that targeting glutamine metabolism in particular as opposed to glucose metabolism increases the primed state and thereby lowering the apoptotic threshold by increasing binding of BIM to BCL-2. In the majority of glutamine-deprived cells BIM is induced and while exhibiting increased binding to MCL-1 and some cases BCL-x_L the additional BIM bound to BCL-2 when released is sufficient to induce apoptosis. The model presented outlines the basis for how glutamine deprivation regulates BCL-2 family members facilitating sensitization to ABT-199 (Figure 6e).

BH3 mimetics like ABT-737, ABT-263 and ABT-199 are potent small molecule mimetics that target anti-apoptotic BCL-2 family members.^{32,33} These compounds mimic BH3 domains, bind the BH3 binding groove of anti-apoptotic BCL-2 proteins releasing BIM or other bound pro-apoptotics to induce apoptosis.³⁴ ABT-199 targeting BCL-2 is particularly attractive as it has overcome drug delivery and resistance-related problems and lacks the deleterious side-effects of thrombocytopenia and T-cell lymphopenia seen with ABT-263.^{26,34-36} ABT-199 is, however, not successful as a single agent in MM except for a minority contingent of t(11;14) cells³⁷ likely because BIM is bound to MCL-1/BCL-x_L, not BCL-2. Our analysis of cell lines and patient samples likewise demonstrates minimal sensitivity to ABT-199 as a single agent. However, glutamine deprivation (and glucose deprivation in a subset of lines) significantly sensitized cells to ABT-199. Previous examination of neuroblastoma and Ewings sarcoma demonstrated increased sensitivity of DON-treated cells to ABT-263³⁸ that may mechanistically be explained by induction and altered associations of BCL-2 proteins similar to that identified in our study. New MCL-1 selective inhibitors have been developed recently;³⁹ however, studies demonstrating the requirement for MCL-1 in myocardial homeostasis may preclude targeting MCL-1 for therapy.⁴⁰ While MCL-1 inhibitors will likely be effective in displacing BIM from MCL-1 and eliciting apoptosis in MCL-1-dependent MM, excess BIM may be sequestered by BCL-2/X_L in MCL-1 co-dependent²⁴ cells reducing cell death on treatment with an MCL-1 inhibitor. We believe our strategy of increasing BIM binding to BCL-2 will provide to be a more potent strategy than targeting MCL-1, with efficacy in both MCL-1-dependent and co-dependent MM.

BH3 sensitizers have critical roles releasing BIM/BID from the anti-apoptotics. Our evaluation revealed that NOXA induction did not have a role in sensitization of nutrient-deprived cells to ABT-199. In cell lines like L363 and KMS18 where MCL-1 is reduced in absence of glucose, the contribution of induced NOXA in degradation of MCL-1 and induction of apoptosis cannot be ruled out.²⁸ BID is the second important BH3 activator protein. We find that nutrient deprivation decreases total BID expression and therefore is unlikely to have a role in the sensitization of nutrient-deprived cells to ABT-199. Among the other BH3-only activators, PUMA is also regulated by glucose.¹⁸ We demonstrate that both glucose and glutamine deprivation lead to a suppression of PUMA, suggesting a lack of its

role, as well in the sensitization to ABT-199. Since PUMA is regulated by p53⁴¹ the ability to induce sensitivity to ABT-199 on nutrient deprivation is likely in a background where p53 activity is suppressed suggesting the utility of this approach in targeting tumor cells lacking p53. In the cell lines where BIM is induced on nutrient deprivation one can speculate that the induction of BIM above a threshold saturates BIM binding to MCL-1 forcing excess BIM to preferentially bind BCL-2. BIM is also sequestered to microtubules by binding dynein light chain 1 (DYNLL1/LC8)⁴² and released from LC8 by phosphorylation on T116 by JNK.⁴³ While we have not investigated BIM release from DYNLL1 this could potentially explain detection of increased binding of BIM to BCL-2 in glucose-deprived L363 and glutamine-deprived JJN3 cells that importantly do not induce BIM on nutrient deprivation. Treatment of all cell lines cultured in nutrient replete media with ABT-199 releases BIM bound to BCL-2 (Figure 3); however, as our results on evaluation of viability suggest is not sufficient to induce apoptosis (Figures 4a and b). In addition, our co-immunoprecipitation data suggest that BIM released on ABT-199 treatment is not completely sequestered back by MCL-1 or BCL-x_L and thus free to induce apoptosis.

Glutamine is metabolized to glutamate by glutaminase I and then to α -ketoglutarate to supplement the TCA cycle, transaminase reactions, GSH synthesis and a number of other metabolic pathways.⁴⁴ An important implication of our study is the need to elucidate whether glutamine deprivation or inhibition of glutamine metabolism impacts the TCA cycle, electron transport chain and oxidative phosphorylation to increase BIM–BCL-2 association. Glutamine can support the electron transport chain through supply of NADH or succinate as electron donors to mitochondrial complex I and II, respectively. We tested a dose range of the complex I inhibitor rotenone and find that low doses that are not cytotoxic sensitize only the L363 cell line to ABT-199 while the KMS11, MM.1S, KMS18 and AMO1 MM lines were not sensitized to ABT-199 on rotenone treatment (data not shown). These results underscore the need to further explore the contribution of TCA cycle activities to the regulation of BIM–BCL-2 binding. The ability of a key proximal metabolite of glutamine metabolism, that is, DMK to significantly but partially reverse glutamine deprivation-induced sensitization to ABT-199 and lack of the ability of other TCA cycle intermediates to do so suggests specificity to processes more closely tied to α -ketoglutarate metabolism. The mechanistic basis of DMK reversal also appears to be complex in that addition of DMK not only suppresses induction of BIM on glutamine deprivation but also appears to promote BIM binding to MCL-1 in glutamine-deprived L363. The inability of metabolites like fumarate (that like DMK supplements NADH generation) to reverse sensitization to ABT-199 in nutrient-deprived MM further suggests specificity among TCA cycle intermediates and warrants further investigation of the additional critical metabolites required for induction and increased binding of BIM to BCL-2. We also tested the GLS1 inhibitor CB-839 and while we detect some single agent cytotoxicity in MM cell lines we detect more of an additive as opposed to synergistic effect on sensitization to ABT-199 (data not shown). Myeloma cells are known to express the GLS2 glutaminase isoform⁴⁵ that is not targeted by CB-839.⁴⁶ Our conclusion was that inhibition of GLS1 alone was not sufficient to elicit sensitization to ABT-199 and that further investigation of the path of glutamine flux would be required to identify enzymes that were being targeted on glutamine deprivation/DON treatment to elicit robust sensitization to ABT-199.

The cell lines utilized in our study exhibit a range of translocations and mutations commonly detected in MM. For example, U266 exhibits a t(11; 14) translocation and upregulation of CCND1 expression; JJN3 has a t(14; 16) translocation identified in 5–10% of myeloma cases and exhibits overexpression of c-MAF; KMS18 exhibits a t(4; 14) translocation observed in 15% MM cases and also has increased overexpression of FGFR3 and MMSET,⁴⁷ while KMS11 has both t(14;16) and t(4; 14) translocations. The patient samples utilized in the study are relapsed and/or refractory myeloma patient samples with a range of underlying genetic complexity. Most of the patient samples tested are bortezomib refractory but sensitized on targeting glutamine metabolism in conjunction with ABT-199. Our data suggest that targeting glutamine metabolism in conjunction with ABT-199 uniformly elicits cytotoxicity across heterogenic MM cell lines and patient samples.

While we have not further examined the metabolic basis for survival of nutrient-deprived cells our past research has indicated that metabolic re-programming and/or autophagy may sustain critical metabolic processes required for survival.^{16,48} Our insights on how the BCL-2 family of proteins adapts to nutrient deprivation-elicited stress to maintain survival highlight metabolism-based approaches of synthetic lethality that may be more universally applicable. Given that normal cells are generally less primed,⁴⁹ identification of metabolic targets connected to regulation of BCL-2 proteins in tumor cells will bolster development of targeted strategies of inducing synthetic lethality to BH3 mimetics with likely less toxic effects in normal cells.

MATERIALS AND METHODS

MM cell lines

MM cell lines were obtained from the following sources: KMS18 (Dr L Bergsagel, Mayo Clinic, Phoenix, AZ, USA), RPMI-8226 (American Type Culture Collection, Manassas, VA, USA), AMO1 (Calithera Biosciences, San Francisco, CA, USA), U266, L363, KMS11, JJN3 (Dr M Kuehl, NCI, MD) and MM.1S (Dr S Rosen, City of Hope, CA).

Cell culture

Cells were routinely cultured in complete RPMI-1640 (Invitrogen, Life Technologies Corporation, Grand Island, NY, USA) with 10% FBS and 100 U/ml penicillin, 100 mg/ml streptomycin and maintained in a 37 °C incubator as previously described.¹⁶ Under conditions of nutrient deprivation, cells were cultured in glucose- or glutamine-free RPMI-1640 (Rainbow Scientific, Inc., Windsor, CT, USA) supplemented with 10% dialyzed FBS (Invitrogen, Life Technologies Corporation) and glucose (5 mM) or glutamine (2 mM) as indicated.

Co-immunoprecipitation and immunoblotting

Whole cell lysates for co-immunoprecipitation or for total protein evaluation were prepared with RIPA buffer supplemented with phosphatase and protease inhibitors and 1% PMSF as previously described.^{16,24} Antibodies to BCL-x_L (#2764S), BIM (#2933BC), PUMA (#4976) and BCL-2 (#4223S) were purchased from Cell Signaling Technology (Danvers, MA, USA); MCL-1 (sc-819) and NOXA (sc-56169) from Santa Cruz Biotechnology

(Dallas, TX, USA) and GAPDH (GTX41027) from GeneTex Inc. (Irvine, CA, USA). Antibodies (used for co-immunoprecipitates) to MCL-1(559027) and BCL-2 were purchased from BD-Pharmingen (BD Biosciences, San Jose, CA, USA). The antibody used for the BCL-x_L IP was generated in the Boise Laboratory (Atlanta, GA, USA).⁵⁰

Chemicals and reagents

Standard chemicals, oxaloacetate and dimethyl alpha ketoglutarate, and DON (6-Diazo-5-oxo-L-norleucine) were purchased from Sigma-Aldrich (St Louis, MO, USA). Venetoclax from Biovision Inc. (Milpitas, CA, USA) and Ritonavir was purchased from Euroasia Inc. (Mumbai, India).

Cell viability assays

For cell viability assays 0.125×10^6 cells/ml were treated with the indicated concentration of drug and evaluated for viability by AnnexinV/DAPI flow cytometric analysis as previously described.⁵¹

Isolation of primary myeloma cells

Bone marrow aspirates or peripheral blood samples from consenting myeloma patients were diluted to 25 ml with $1 \times$ PBS and underlaid with lymphocyte separation media (Corning Life Sciences, Tewksbury, MA, USA). Following centrifugation, the buffy coat was collected and the cells were washed with PBS, and re-suspended in culture medium. Cells subject to various treatments were stained with anti-CD38-phyocerythrin and anti-CD45-allophycocyanin-Cy7 (BD Biosciences) to identify MM cells.⁵¹ All samples were collected following an Emory University Institutional Review Board-approved protocol.

Statistical analyses

Results are expressed as \pm s.e.m. of at least three independent experiments unless indicated otherwise. *P*-values were determined by an unpaired student *t*-test using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) five with *P*-values <0.05 considered statistically significant. Two way analysis of variance analyses was performed using Bonferroni post tests to compare replicate means where each column in a row was compared with all other columns. *P*-values were indicated as follows: *P* <0.1 as *, *P* <0.01 as **, *P* <0.001 as ***, *P* <0.0001 as **** and *P* >0.05 as not significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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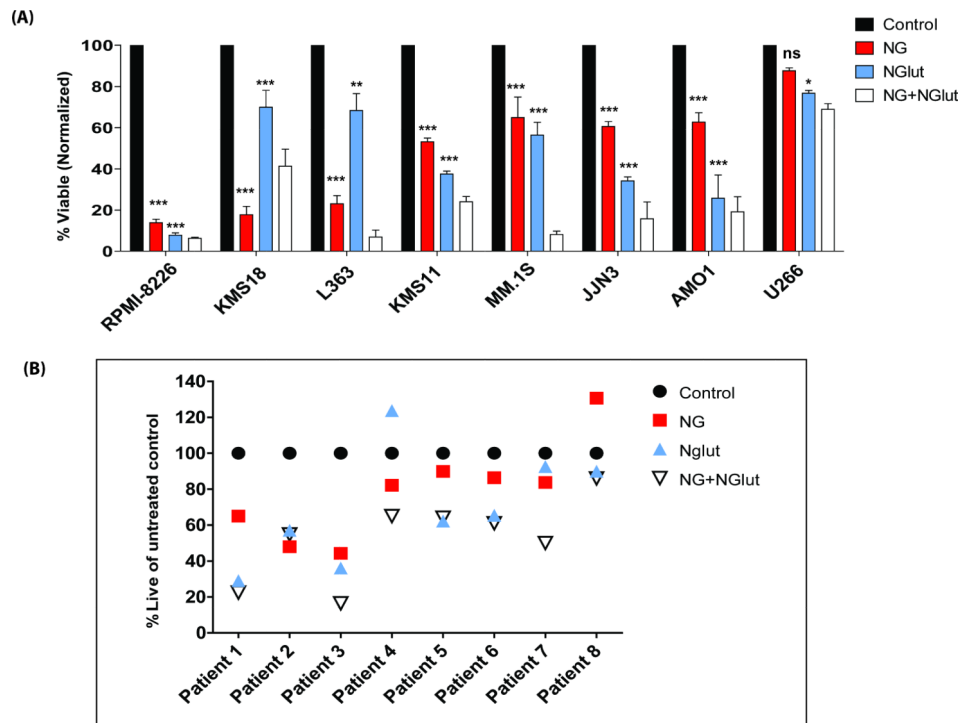


Figure 1. MM cell lines and CD138+ MM primary patient cells are variably sensitive to glucose or glutamine deprivation. (a) MM cell lines and (b) patient cells were cultured in the presence of glucose (5 mM) and glutamine (2 mM) (Control) or in the absence of glucose (NG), absence of glutamine (NGlut) or absence of both nutrients (NG+NGlut) for 72 h (cell lines) or 48 h (patient cells). Cell viability was assessed by AnnexinV/DAPI staining and Flow cytometry. Data (a) is mean \pm s.e.m. ($n = 3$). Control vs NG: P -value < 0.001 (***) in all cell lines except U266 which is > 0.05 . Control vs NGlut: P -value < 0.001 in all cell lines except U266 < 0.1 (*) and L363 < 0.01 (**).

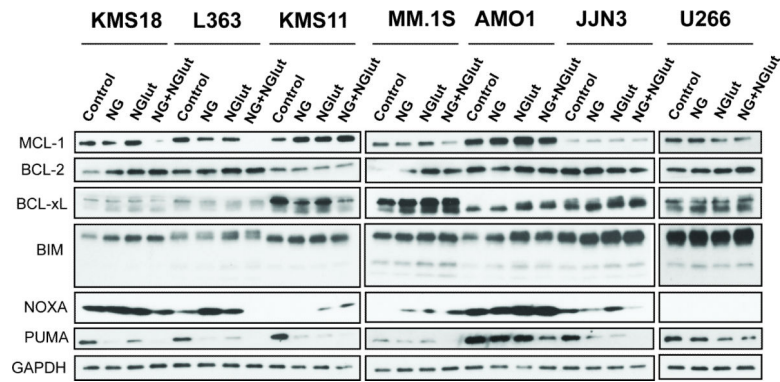
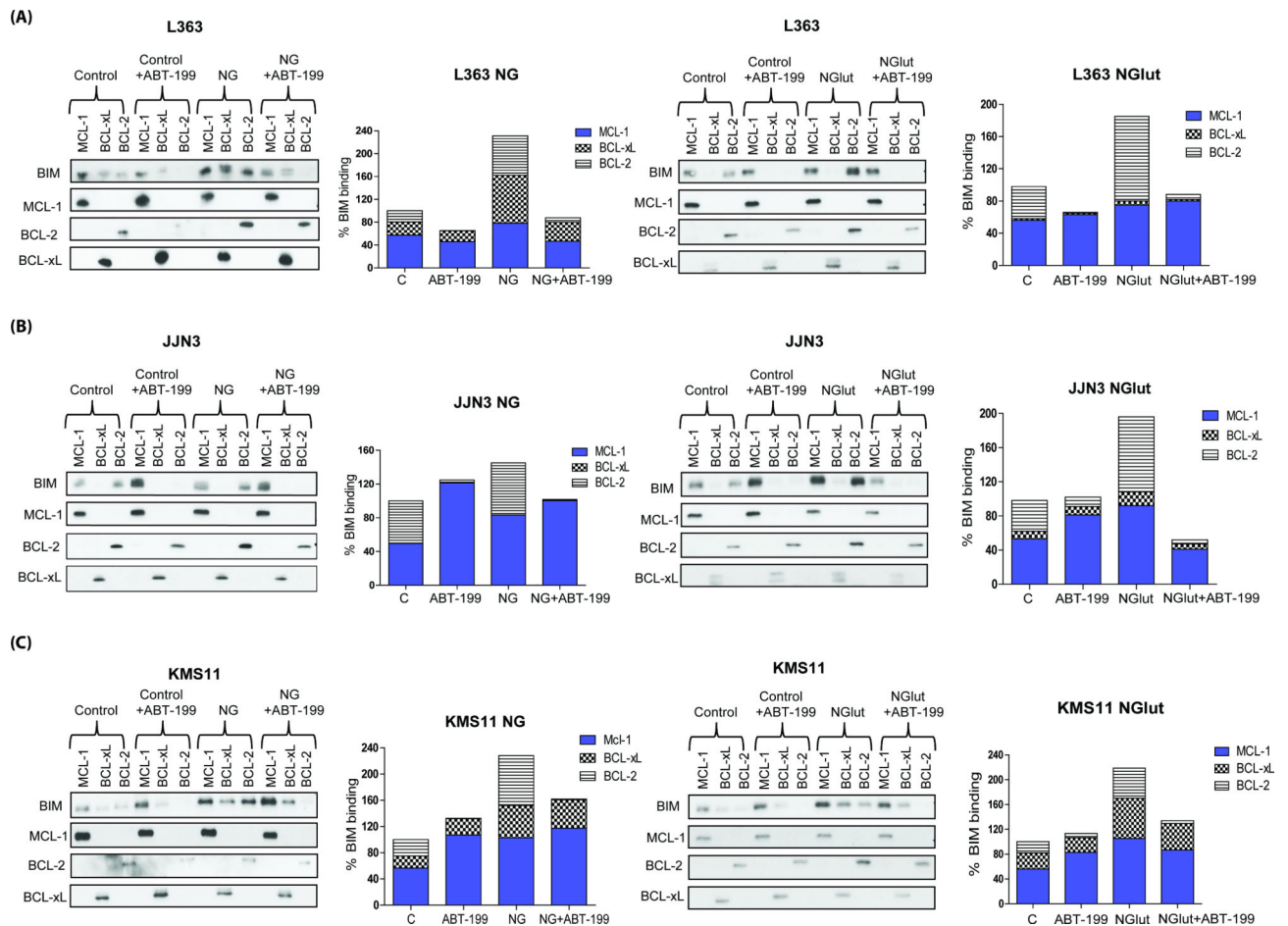


Figure 2.

Glucose or glutamine deprivation regulates expression of pro and anti-apoptotic BCL-2 proteins. MM cell lines cultured in the absence or presence of 5 mM glucose or 2 mM glutamine or both nutrients for 24 h were evaluated for expression of BCL-2 proteins. Cellular lysates were analyzed for expression of indicated proteins or GAPDH (loading control) by immunoblot analyses. One of two representative experiments is shown. NG, media without glucose; NGlut, media without glutamine.

**Figure 3.**

Glucose or glutamine deprivation increase binding of pro-apoptotic BIM to BCL-2. L363 (a), KMS11 (b) and JJJ3 cells (c) were cultured in the absence or presence of 5 mM glucose or 2 mM glutamine with or without (0.5 μ M) ABT-199 for 24 h. Immunoprecipitates of MCL-1, BCL-2 and BCL-x_L prepared from cellular lysates obtained from treated cells were evaluated for bound BIM, MCL-1, BCL-2 and BCL-x_L by immunoblotting. Densitometric analysis of the BIM immunoblot was performed using IMAGE J software (imagej.nih.gov) and percent BIM bound to MCL-1, BCL-2 and BCL-x_L presented in the corresponding stacked bar graph. NG, media without glucose; NGlut, media without glutamine. Representative blots from a minimum of three independent experiments are presented.

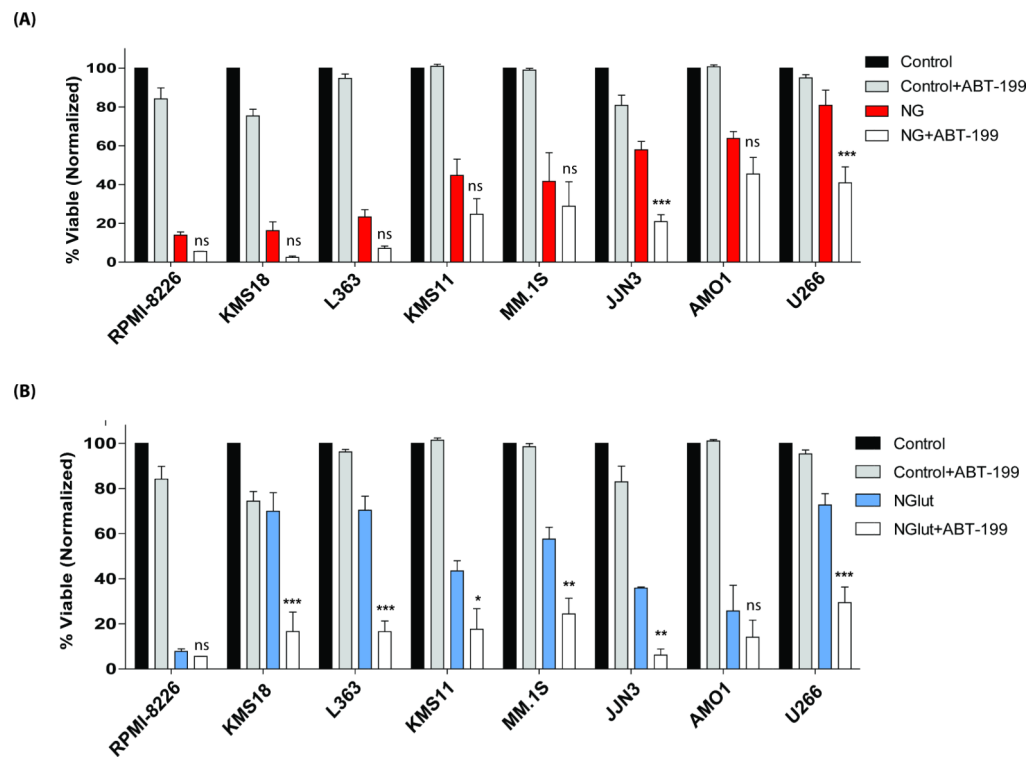


Figure 4.

Glucose or glutamine deprivation sensitizes MM cell lines to the BH3 mimetic ABT-199. MM cell lines were cultured in the absence or presence of 5 mM glucose (**a**) or 2 mM glutamine (**b**) with or without the BH3 mimetic ABT-199 (0.5 μ M) for 72 h. Cell viability was assessed by AnnexinV/DAPI staining and flow cytometry. NG, media without glucose; NGlut, media without glutamine. Data (**a** and **b**) are mean \pm s.e.m. ($n = 3$). P -value of NG vs NG+ABT data is >0.05 (ns) in all cell lines except JJN3 and AMO1 where P -value is <0.001 (***)). P -value of NGlut vs NGlut+ABT is <0.001 (***) in KMS18, L363 and U266, <0.01 (**) in MM.1S and JJN3, <0.1 (*) in KMS11 and >0.05 in RPMI-8226 and AMO1.

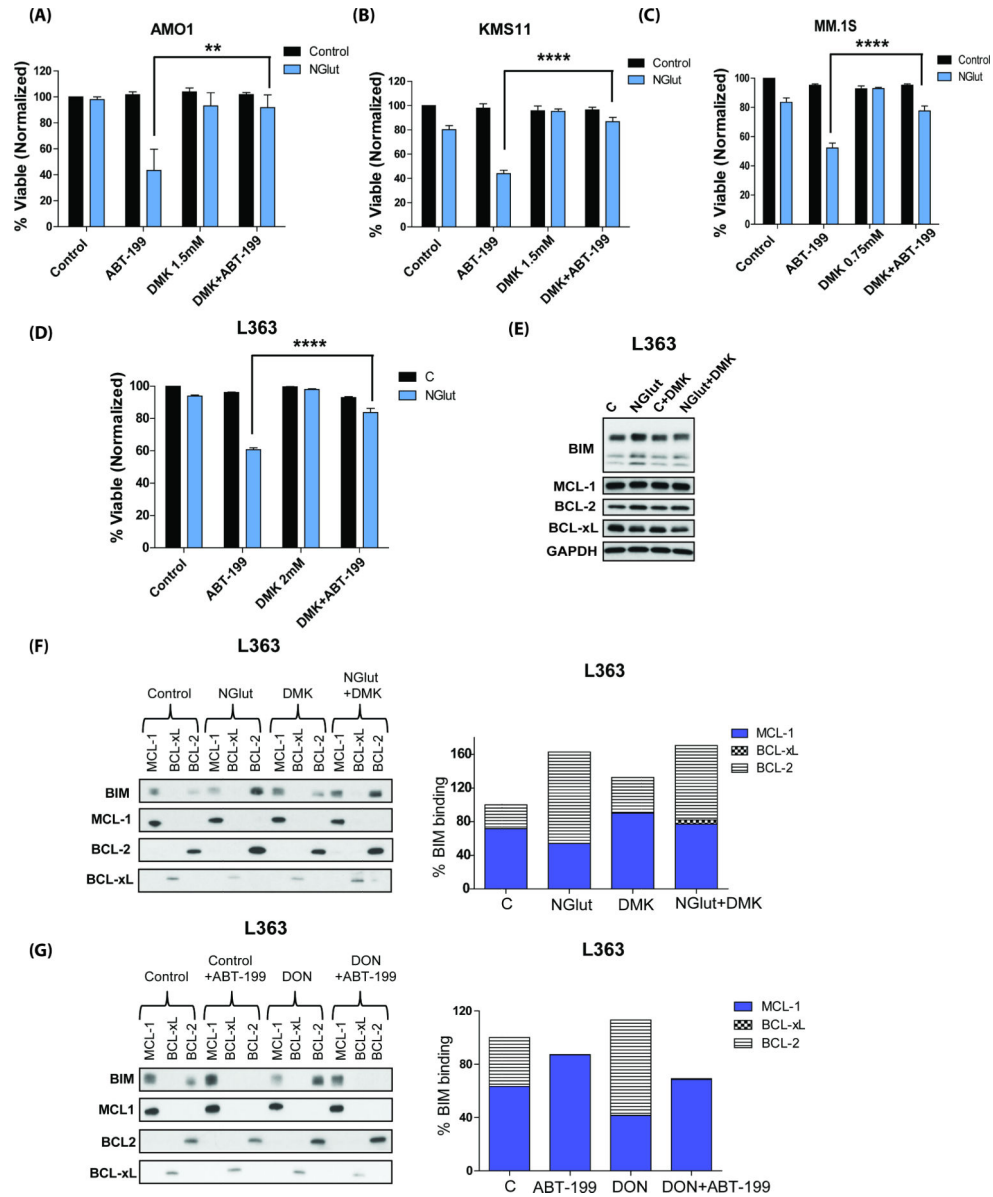
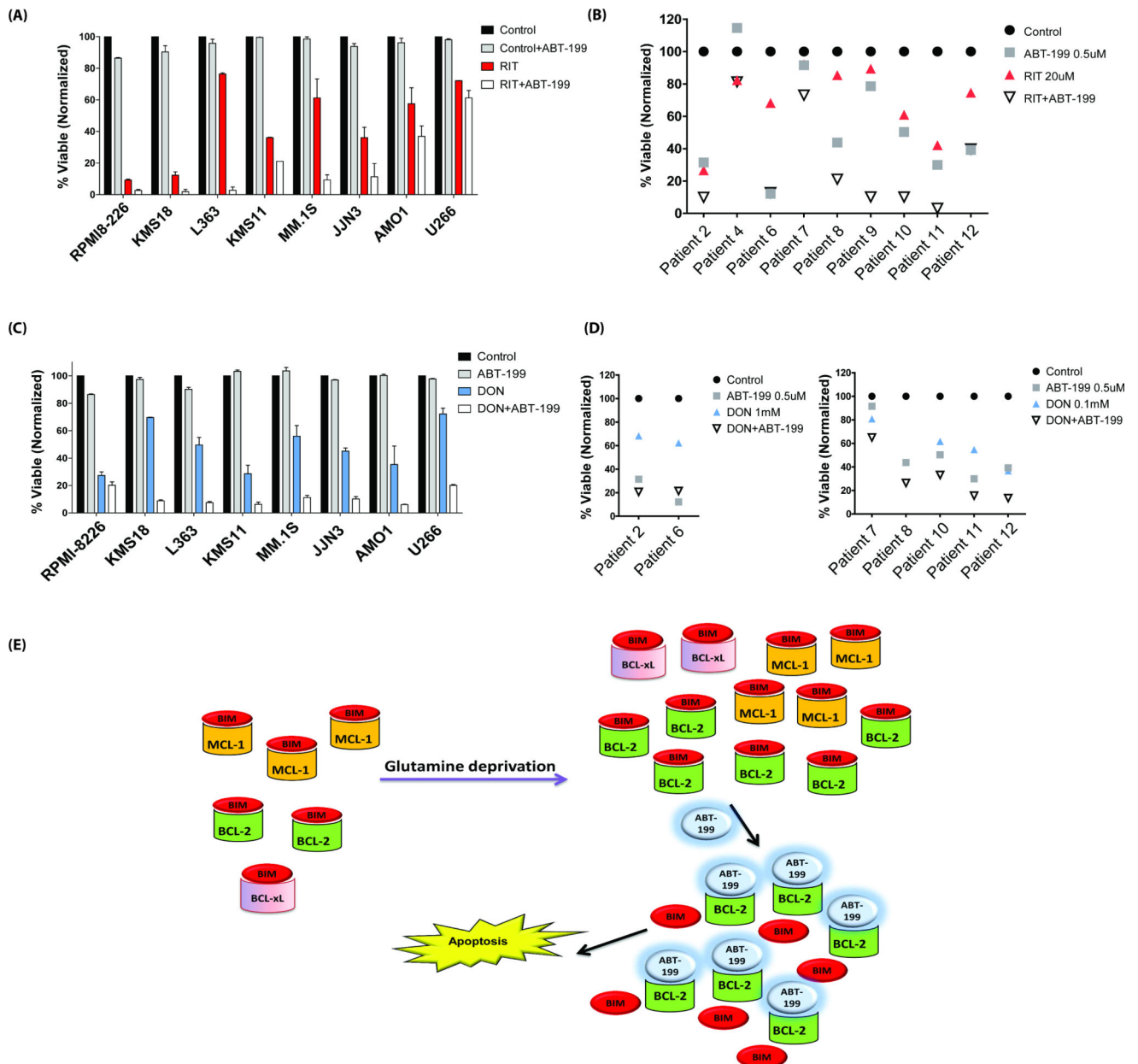


Figure 5. Metabolic supplementation with DMK reverses glutamine deprivation-elicited sensitivity to ABT-199. (a–d) MM cell lines AMO1 (a), KMS11 (b), MM.1S (c) and L363 (d) grown in absence or presence of glutamine treated with or without 0.5 μ M ABT-199 were supplemented with indicated concentrations of dimethyl alpha ketoglutarate (DMK) for 18–24 h. Cells were harvested and evaluated for viability by AnnexinV/DAPI staining and flow cytometry. (e) Cellular lysates prepared from treated cells from d were evaluated for BIM, MCL-1, BCL-2 and BCL-x_L protein expression with GAPDH evaluated as a loading control. (f) Cells from d were utilized for co-IP of MCL-1, BCL-x_L and BCL-2 to evaluate BIM binding. MCL-1, BCL-x_L and BCL-2 protein levels in IPs are also evaluated as loading controls. (g) L363 cells grown in presence or absence of DON treated with or without ABT-199 for 18 h were subjected to co-IP of MCL-1, BCL-x_L and BCL-2 to evaluate BIM

binding. Distribution of BIM binding is presented in bar graph to the right of **f** and **g**. One of two representative co-IP results is presented in **f** and **g**. NG, media without glucose; NGlut, media without glutamine. Data (**a–d**) is mean \pm s.e.m. of $n = 3$; (**e–g**) co-IPs and immunoblots are representative of two independent experiments. *P*-value of NGlut+ABT vs NGlut+ABT+DMK in **a–d** is <0.0001 in KMS11 (**b**), MM.1S (**c**) and L363 (**d**) indicated as ‘****’ and $P<0.01$ in AMO1 (**a**) indicated as ‘**’.

**Figure 6.**

Evaluation of Ritonavir (targeting glucose transport) or DON targeting glutamine to ABT-199 sensitivity in MM. MM cell lines were cultured with 40 µM ritonavir (a) or 0.1 mM DON (c) with or without ABT-199 (0.5 µM) or in combination for 72 h. MM patient samples were cultured with ritonavir (20 µM) (b) or DON (1 mM or 0.1 mM), as indicated. (d) for 48 h with viability assessed by AnnexinV/DAPI staining. (e) Model showing the impact of glutamine deprivation on BCL-2 family members and the basis for synthetic lethality to ABT-199. This model is based on the L363 co-IP under glutamine deprivation. Data in a are mean ± s.e.m. ($n = 3$) and b are mean ± s.e.m. ($n = 2$). P -values of RIT vs RIT+ABT: >0.05 in RPMI-8226, KMS18, KMS11 and U266, <0.1 in AMO1, <0.01 in JJN3, <0.001 in L363

and MM.1S. *P*-value of DON vs DON+ABT is <0.001 in all cell lines except RPMI-8226. co-IP, co-immunoprecipitation.

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Patient characteristics: characteristics of myeloma patient samples used in this study

Table 1

No.	Diagnostic sample	Date of diagnosis	Date of sample	Age	Sex	Isotype	ISS stage	CTG	FISH	Prior lines ^a	LEN ref	BTZ ref	CFZ ref	POM ref
1	Myeloma	07/01/10	04/17/14	61	M	IgA kappa	3	Hypodiploid, complex karyotype	t(11;14), del 17p, monosomy 13, gain of 1q	8	Yes	Yes	Yes	Yes
2	Myeloma	01/21/10	01/17/15	72	M	Free lambda	2	46,XY	Gain of IgH; monosomy 13	6	Yes	Yes	Yes	No
3	Secondary PCL	08/01/09	06/06/14	80	M	IgG lambda	Unk	Complex karyotype	del 1p, gain of 1q, gain of 14q, trisomy 9	5	Yes	Yes	No	Yes
4	Extramedullary myeloma with plasmacytomas	11/29/06	08/19/14	74	M	IgG kappa	Unk	Complex karyotype	Gain of IgH; gain of 1q, trisomy 3 and 7, monosomy 13	5	Yes	Yes	Yes	No
5	Myeloma	11/01/08	09/03/14	50	F	IgG kappa	3	Complex karyotype	Gain of IgH; gain of 1q, trisomy 3, 7 and 11, monosomy 13, del 17p	3	Yes	Yes	No	No
6	Extramedullary myeloma with CNS involvement	07/10/14	11/07/14	69	M	IgG lambda	3	Complex karyotype	Gain of IgH, tetrasomy 1, trisomy 3 and 9, del 17p	2	Yes	Yes	No	No
7	Extramedullary myeloma with CNS involvement	06/30/14	02/13/15	55	F	IgG kappa	3	Complex karyotype	del 17p, monosomy 13	4	Yes	Yes	Yes	Yes
8	Myeloma	01/12/15	02/05/15	50	M	IgA kappa	1	46,XY	Gain of IgH, del 13q, trisomy 13, t(4;14)	0	No	No	No	No
9	Myeloma	11/12/09	07/23/14	60	M	IgA kappa	Unk	Complex karyotype	del 1p; gain of IgH, tetrasomy 1, trisomy 3, 7 and 9, del 13	4	Yes	Yes	No	Yes
10	Myeloma	03/14/15	03/14/15	66	F	IgG kappa	2	46,XX	Gain of 1q; gain of IgH; monosomy 13	0	No	No	No	No
11	Myeloma	09/02/09	03/17/15	61	F	IgG kappa	3	Hypodiploid, complex karyotype	del 17p, variant of t(11;14)	1	No	Yes	No	No
12	Secondary PCL	11/01/10	03/24/15	63	M	IgA lambda	3	Hypodiploid, complex karyotype	del 1p; gain of 1q; gain of IgH; monosomy 13; trisomy 3; del 17p	5	Yes	Yes	Yes	No

Abbreviations: BTZ, bortezomib; CFZ, carfilzomib; CNS, central neCTG; cytogenetics; F, female; FISH, fluorescent *in situ* hybridization; ISS, International Staging System; LEN, lenalidomide; M, male; PCL, plasma cell leukemia; POM, pomalidomide; ref, refractory; Unk, unknown.

^aNumber of prior lines of therapy.