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Panobinostat and venetoclax enhance the cytotoxicity of gemcitabine, busulfan, and melphalan in multiple myeloma cells

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Abstract

Gemcitabine (Gem), busulfan (Bu) and melphalan (Mel) are used for hematopoietic stem cell transplantation (HSCT). To further improve their efficacy, a preclinical study on their synergism with the HDAC inhibitor panobinostat (Pano) and the BCL2 inhibitor venetoclax/ABT199 was performed. Multiple myeloma (MM) cell lines MM.1R and MC/CAR were exposed to $\sim$ IC₂₀ levels of the drugs. Synergistic cytotoxicity was observed in cells exposed to the 5-drug combination as indicated by combination indexes < 1 , supported by $\sim$ 86% inhibition of proliferation and $\sim$ 84% annexin V-positivity in MM.1R and $\sim$ 58% inhibition of proliferation and $\sim$ 46% annexin V-positivity in MC/CAR cells. Activation of the DNA-damage response and apoptosis were suggested by a modest increase in the phosphorylation of ATM and its substrates, significant cleavage of PARP1, caspase 3 and HSP90, DNA fragmentation, mitochondrial membrane depolarization and reactive oxygen species production. The 5-drug combination significantly decreased the levels of PI3K, AKT, mTOR, RAPTOR, P-P70S6K, and eIF2 α with concomitant increase in the P-AMPK and its substrate Tuberin/TSC2, suggesting that the mTOR signaling pathway was compromised. Endoplasmic reticulum (ER) stress through activation of the unfolded protein response (UPR) was also observed as suggested by an increase in the level of calnexin, BiP/GRP78, ERO1-L α , and PDI, which may relate to venetoclax-mediated inhibition of BCL2 in the ER. This is the first report on the effects of venetoclax-containing regimen on UPR. These results provide a rationale to propose a clinical trial on using (Gem+Bu+Mel+Pano +Venetoclax) as part of a conditioning regimen for MM patients undergoing auto-HSCT.

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Conflict of interest disclosure

All authors declare no competing financial interests.

Keywords

Pre-transplant chemotherapy; myeloma; synergistic cytotoxicity; gemcitabine; busulfan; melphalan; panobinostat; venetoclax/ABT199

Introduction

Multiple myeloma (MM) is a malignancy of plasma cells that primarily reside in the bone marrow and in most cases involves the secretion of monoclonal paraproteins. Treatments for MM include corticosteroids, alkylating agents, anthracyclines, proteasome inhibitors, and immunomodulatory agents [1]. High-dose chemotherapy (HDC) in combination with autologous hematopoietic stem cell transplantation (auto-HSCT) has improved clinical responses [2–7] and the success of this treatment is partly due to the efficacy of pre-transplant HDC regimens.

The most commonly used HDC regimens for MM contain busulfan (Bu) and melphalan (Mel) [8]. The combination of Bu, Mel and gemcitabine (Gem) as part of conditioning therapy for auto-HSCT is safe and active for refractory myeloma [7]. In the search for a more efficacious pre-transplant regimen for MM, we hypothesized that addition of other drugs with different mechanisms of action to the (Gem+Bu+Mel) combination would provide better efficacy.

Panobinostat is a non-selective histone deacetylase (HDAC) inhibitor approved by the FDA for use in combination with bortezomib and dexamethasone for patients with MM. Overexpression of HDAC is associated with poor prognosis in MM [9], and panobinostat improves median progression-free survival of refractory MM patients [10]. The anti-myeloma activity of panobinostat involves alteration of gene expression through epigenetic modifications and inhibition of protein metabolism [11].

The efficacy of Gem, Bu, Mel and panobinostat may be limited by the ability of tumor cells to evade apoptosis. Venetoclax/ABT199 binds to and inhibits the anti-apoptotic B-cell lymphoma-2 (BCL2) protein, causing cells to undergo apoptosis [12]. Preclinical studies have demonstrated the cytotoxicity of venetoclax in leukemia and lymphoma cells [13–14]. It has an anti-myeloma activity as a monotherapy [15] or in combination with other drugs [16].

Based on the observed anti-myeloma activities of Gem, Bu, Mel, panobinostat, and venetoclax, we hypothesized that their combination would be efficacious as part of a pre-transplant regimen for MM. A preclinical study was therefore performed using MM cell lines. The five drugs elicited synergistic cytotoxicity and the possible molecular mechanisms were determined. The results from this study provide a rationale for the development of a pre-transplantation conditioning regimen for MM patients undergoing auto-HSCT.

Methods

Cell lines and chemicals

MM.1R, MC/CAR and U266B1 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI 8226 vr10 was a kind gift from Dr. R. Orlowski (UT MD Anderson Cancer Center, Houston, TX, USA). Cells were cultured in RPMI 1640 (Mediatech, Manassas, VA, USA) supplemented with 15% heat-inactivated fetal bovine serum (Atlanta Biologicals, Inc., Flowery Branch, GA, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Mediatech) at 37°C in a fully humidified atmosphere of 5% CO₂ in air. Cells were validated by short tandem repeat DNA fingerprinting using the Amp-FISTR Identifier kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Mycoplasma contamination was determined using the EZ-PCR mycoplasma detection kit (Biological Industries, Cromwell, CT, USA). Busulfan and melphalan (Sigma-Aldrich, St Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO); gemcitabine, panobinostat and venetoclax (SelleckChem, Houston, TX, USA) were dissolved in DMSO and diluted in RPMI 1640 prior to use.

Cytotoxicity and apoptosis assays

Cells (6 ml of 0.5×10^6 cells/ml) in T25 flasks were exposed to drugs for 48 h, aliquoted (100 µl) into 96-well plates and analyzed by the 3 (4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [17]. Briefly, 50 µl of 1 mg/ml MTT reagent (Sigma-Aldrich) in phosphate-buffered saline (PBS) was added per well and incubated for 3–4 h at 37°C. The solid reaction product was dissolved by adding 100 µl of solubilization solution (0.1 N HCl in isopropanol containing 10% Triton X-100), mixing, and incubating at 37°C overnight. Absorbance at 570 nm was measured using a Victor X3 (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA) plate reader. The number of MTT-positive cells was determined relative to the solvent control cells.

Apoptosis was determined by flow-cytometric measurements of phosphatidylserine externalization with Annexin-V-FLUOS (Roche Diagnostics, Indianapolis, IN, USA) and a fluorescent DNA-binding marker 7-aminoactinomycin D (BD Biosciences, San Jose, CA, USA) using a Muse Cell Analyzer (EMD Millipore, Billerica, MA, USA). Drug combination effects were estimated based on the combination index (CI) values [18] calculated using the CalcuSyn software (Biosoft, Ferguson, MO, USA).

Protein analysis

Western blot analysis was performed to determine the mechanisms of synergism by analyzing changes in the level of key proteins and their modifications. Cells were incubated with the study drug(s) for 48 h, centrifuged, washed with ice-cold PBS and pelleted. Cells were lysed with lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and total protein concentration was determined using the BCA protein Assay kit (Thermo Scientific, Rockford, IL, USA). The protein extracts were combined with the loading buffer, boiled for 5 min, and aliquots of equal amount of proteins were loaded onto polyacrylamide-SDS gels for electrophoresis. The proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The required antibodies were added and detected using the

chemiluminescent substrate Immobilon (EMD Millipore). Autoradiograms were scanned and analyzed using the UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT, USA). The primary antibodies, catalog numbers, sources, and dilutions are listed in Table 1.

Analysis of reactive oxygen species (ROS)

Cells exposed to drug(s) for 48 h were analyzed for production of ROS using CM-H2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), an ROS indicator which diffuses into cells where it is oxidized to a fluorescent product (Life Technologies, Grand Island, NY, USA). Briefly, cells were aliquoted (0.5 ml) into 5 ml tubes and 1 μ l of 1.5 mM CM-H2DCFDA (dissolved in DMSO) was added. Cells were incubated at 37°C for 1 h and immediately analyzed with a Gallios™ Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA) using excitation/emission wavelengths of 492/520 nm. Geometric means of the fluorescence intensities were compared and the relative fold increase in ROS production was calculated.

Analysis of mitochondrial membrane potential (MMP)

An MMP detection kit (Cayman Chemical Co., Ann Arbor, MI, USA) was used to determine changes in MMP using the JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) reagent. Cells to be analyzed were aliquoted (0.5 ml) into 5 ml tubes. Diluted (1:10 with cell growth medium, 40 μ l) MMP-sensitive fluorescent dye JC-1 reagent was added to each tube, incubated at 37°C for 20 min, and immediately analyzed by flow cytometry as described by the manufacturer.

Statistical analysis

Results are presented as the mean $\pm$ standard deviation of at least three independent experiments and statistical significance of the difference between two groups was determined by non-parametric Mann-Whitney test using GraphPad Prism 7.03. P values 0.05 were considered statistically significant.

Results

The (Gem+Bu+Mel+panobinostat+venetoclax) combination exerts synergistic cytotoxicity towards multiple myeloma cell lines

We previously showed substantial activity of Gem, Bu and Mel for auto-HSCT in patients with refractory Hodgkin and non-Hodgkin lymphoma, and myeloma [7,19,20]. Addition of epigenetic modifiers or a PARP inhibitor to this combination enhanced its efficacy in preclinical [21–23] and clinical [24,25] studies. We sought to determine whether addition of HDAC and BCL2 inhibitors would further enhance the cytotoxicity of (Gem+Bu+Mel) combination. Continuous exposure of MM.1R and MC/CAR cells for 48 h to each drug inhibited cell proliferation by 10%–23% versus control cells as measured by MTT assay; cell apoptosis increased to a maximum of ~22% as indicated by annexin V-positive cells (Figure 1). Exposure to (Gem+Bu+Mel) resulted in ~48% and ~38% inhibition of proliferation of MM.1R and MC/CAR cells, respectively, versus control cells; annexin V-positive cells increased to ~51% and ~27%, respectively (Figure 1). When combined with the indicated concentration of panobinostat or venetoclax, the efficacy of the triple-drug

combination was increased as shown by a decrease in cell proliferation and increase in apoptosis. Exposure to (Gem+Bu+Mel+panobinostat+venetoclax) significantly inhibited MM.1R and MC/CAR cell proliferation by ~86% and ~58% compared to control, respectively; annexin V-positive cells increased to ~84% and ~46%, respectively (Figure 1). These results show that the combined action of panobinostat and venetoclax further synergistically sensitized MM cells to the (Gem+Bu+Mel) combination, suggesting strong synergism of the five drugs in the two MM cell lines.

To further show drug synergistic interactions, cells were exposed to different concentrations of individual drugs or to five-drug combination at a constant concentration ratio and the MTT assay was performed after 48 h. Combination index values at increasing drug effects were graphically analyzed according to the Chou-Talalay method [18] as shown in Figure 1 (below the bar graphs). At 50% cell proliferation, or 0.5 Fa, the calculated CI values were 0.6 and 0.4 in MM.1R and MC/CAR cell lines, respectively, suggesting strong synergism ($CI < 1$) of the five drugs.

Cellular sensitivity to venetoclax correlates with the level of BCL2 protein family members

Venetoclax/ABT199 is a selective BCL2 inhibitor [26]. We compared the level of endogenous BCL2 protein with the sensitivity of various human MM cell lines to venetoclax. Among the four cell lines tested, U266B1 was the most resistant to venetoclax ($IC_{50} = 24 \mu M$) and MM.1R was the most sensitive ($IC_{50} = 3 \mu M$, Figure 2A). Western blot analysis showed a direct correlation between the level of BCL2, BCL-xL or MCL-1 protein and the IC_{50} values of venetoclax (Figure 2B) with correlation coefficients (R^2) of 0.66, 0.95 and 0.89, respectively.

The (Gem+Bu+Mel+panobinostat+venetoclax) combination activates the DNA-damage response (DDR)

To determine possible mechanism(s) of the observed drug synergism, cells were exposed to drug(s) for 48 h and changes in selected key DDR protein levels and their modifications were analyzed. Following treatment with either of the 5 drugs individually, there was minimal phosphorylation of ATM at S1981 over the control level. However, this effect was increased with all drug combinations, with the phosphorylation being most pronounced for the 5-drug combination in both cell lines (Figure 3). The apparently low level of P-ATM (S1981) seen in MM.1R cells exposed to the 5-drug combination may be due to the relatively low level of endogenous ATM in these cells. Since auto-phosphorylation of ATM is known to activate its kinase activity [27], we also examined the phosphorylation of its known substrates.

The phosphorylation of histone 2AX (γ -H2AX) is a widely used indicator of DDR [28]. Figure 3 shows the highest level of γ -H2AX in both cell lines exposed to (Gem+Bu+Mel+panobinostat+venetoclax). CHK1 is another ATM substrate and analysis by Western blotting shows the highest increase in the level of its phosphorylated form in cells exposed to the 5-drug combination (Figure 3). These results are consistent with strong activation of the DDR pathway in cells treated with the 5-drug combination.

Analysis of selected key proteins involved in DNA repair showed an increase in the phosphorylation of NBS1 and RAD50 in cells treated with (Gem+Bu+Mel) which was not significantly increased by the addition of panobinostat and/or venetoclax or both (Figure 3). The phosphorylated forms of these two proteins actually appeared to decrease in the presence of the 5-drug combination, which might be attributed to a decrease in the level of total protein. NBS1 and RAD50 are subunits of the MRN complex involved in the repair of double-strand DNA breaks and DNA interstrand crosslinks [29,30].

The (Gem+Bu+Mel+panobinostat+venetoclax) combination activates apoptosis

The observed DDR can reflect an underlying complex genomic injury which may cause the cells to undergo apoptosis. The increase in annexin V-positive cells in the presence of the 5-drug combination (Figure 1) indicates induction of apoptosis in both cell lines. This observation is further supported by the extensive cleavage of PARP1 and caspase 3 and downregulation of the pro-survival protein c-MYC – most notably with the 5-drug combination (Figure 4A). An increase in acetylation of histone 3 at Lys4 (AcH3K4) suggests that the panobinostat-mediated inhibition of HDACs and facilitation of chromatin relaxation may make the DNA more susceptible to alkylation (Figure 4A). *In vitro* assay also shows increased caspase 3 enzymatic activity in these cell lines following exposure to the 5-drug combination as well as in RPMI 8226 vr10 cells (Figure 4B). Moreover, exposure of cells to the 5-drug combination increased DNA fragmentation, a biochemical hallmark of apoptosis [31], as shown by agarose gel analysis (Figure 4C), and suggesting activation of caspase-dependent DNase.

To further determine the underlying mechanism of drug-induced apoptosis, we analyzed changes in the MMP, which plays a major role in programmed cell death. The relative monomeric (cytoplasm) and aggregated (mitochondria) forms of JC-1 were measured by flow cytometry; increased monomer would suggest depolarization of the mitochondrial membrane. Exposure of MM.1R cells to (Gem+Bu+Mel) increased the ratio of monomeric/aggregated JC-1 molecule from 0.2 (Control) to 1.7, suggesting a small decrease in MMP (Figure 4D). Addition of panobinostat or (panobinostat+venetoclax) to the 3-drug combination further increased the ratio to 10.0 and 15.1, respectively, suggesting significant damage to the mitochondrial membrane. This membrane disruption may result in leakage of electrons from the electron transport chain causing increased production of ROS. Exposure of MM.1R cells to individual drugs resulted in relative ROS levels of 1–1.8; exposure to (Gem+Bu+Mel) and (Gem+Bu+Mel+panobinostat+venetoclax) resulted in relative ROS levels of 3.0 and 4.9, respectively (Figure 4E). The observed decrease in MMP, increase in the relative level of ROS and activation of caspase 3 (Figure 4) collectively suggest upregulation of pro-apoptotic factors which may contribute to drug synergism.

Effects of (Gem+Bu+Mel+panobinostat+venetoclax) on the AMPK/mTOR signaling pathway

Drug-mediated increases in ROS production in MM cells were previously shown to inhibit protein translation through the PI3K/AKT/mTOR signaling pathway [32]. We, therefore, sought to determine if the synergistic cytotoxicity of (Gem+Bu+Mel+panobinostat+venetoclax) was associated with inhibition of the mTOR signaling pathway by examining changes in the level and modification of upstream proteins. The combination of (Gem+Bu

+Mel) decreased the level of PI3Kp85 (a regulatory subunit of PI3K) and of AKT; addition of panobinostat and venetoclax almost abolished their expression (Figure 5). Another key protein in this pathway is AMP-activated protein kinase (AMPK), a protein that monitors shifts in the cellular AMP/ADP to ATP ratio and becomes activated by decreased MMP and/or increased ROS [33,34]. Figure 5 shows a significant increase in the phosphorylation of AMPK at T172 in MM.1R cells exposed to the 5-drug combination. An increase in the phosphorylation of Tuberin/TSC2, a known substrate for AMPK [35], was also observed. Such activation by phosphorylation of AMPK and TSC2 may lead to inhibition of mTOR and protein translation [36].

Consistent with this observation is the downregulation of mTOR and its binding partner RAPTOR [37, Figure 5]. Downstream of mTOR is the activation of P70S6K via phosphorylation at T389 and subsequent phosphorylation of the S6 ribosomal protein of the 40S ribosomal subunit which is involved in protein translational control [38]. The level of both phosphorylated proteins decreased in the presence of (Gem+Bu+Mel+panobinostat+venetoclax) (Figure 5). The level of translation initiation factor eIF2 α [39] was also downregulated in cells exposed to 5 drugs (Figure 5). Overall, these results suggest that the mTOR signaling pathway was compromised in MM.1R cells exposed to (Gem+Bu+Mel+panobinostat+venetoclax), possibly resulting in inhibition of protein translation.

(Gem+Bu+Mel+panobinostat+venetoclax) combination affects the level of chaperone proteins

Besides its mitochondrial localization, BCL-2 can also localize to the endoplasmic reticulum (ER) where it interacts with transporters to control Ca²⁺ trafficking [40]. The effects of the drugs on ER proteins primarily involved in proper protein folding were determined. The level of calnexin, an ER membrane calcium-binding protein, increased in MM.1R cells exposed to (Gem+Bu+Mel+panobinostat+venetoclax), suggesting an ER stress (Figure 6) which may involve the unfolded protein response (UPR). This finding is consistent with an increase in the level of BiP/GRP78, which binds to misfolded proteins to prevent aggregation [41], and of factors involved in oxidative protein folding such as ERO1-L α and protein disulfide isomerase PDI [42, Figure 6]. The increase in the level of calnexin, BiP/GRP78, ERO1-L α and PDI suggests activation of the UPR in cells exposed to the 5-drug combination.

We also examined the effects of the 5-drug combination on additional chaperone proteins including HSP90 and HSP70, which mostly localized to the cytoplasm. (Gem+Bu+Mel) marginally decreased the level of HSP90 and a low level of its cleavage product was observed. A more significant cleavage was observed when venetoclax was added to the 3-drug combination which was further enhanced in the presence of panobinostat (Figure 6); cleavage of HSP90 is known to be mediated by ROS and to activate apoptosis [43]. The level of HSP70 decreased in the presence of (Gem+Bu+Mel+panobinostat+venetoclax), further supporting the activation of UPR in cells exposed to the 5-drug combination.

Discussion

We recently demonstrated the safety and efficacy of (Gem+Bu+Mel) as part of a pre-transplant conditioning regimen for patients with multiple myeloma [7]. We now report an enhanced anti-myeloma activity of this drug combination in the presence of panobinostat and venetoclax in MM cell lines. The observed synergism might be attributed to activation of intrinsic apoptosis, inhibition of the mTOR signaling pathway, and activation of UPR.

This drug-induced apoptosis might have been initiated by the effects of (Gem+Bu+Mel +panobinostat) in the nucleus where the drugs inflicted damage to DNA (Figure 3A), which is then communicated to the mitochondria through complex signaling pathways that decrease the levels of NAD⁺ and acetyl-CoA [44]. Nucleoside analogs such as Gem, when phosphorylated, become incorporated into DNA during synthesis, cause DNA damage and histone modifications, and induce chromatin remodeling. The relaxed chromatin becomes more susceptible to alkylation by Bu and Mel and perpetuates the DNA damage initiated by Gem resulting in induction of apoptosis [45]. Increased acetylation of histones (Figure 4A) by panobinostat-mediated inhibition of HDAC maintains chromatin in its relaxed conformation, making it more susceptible to DNA alkylation [21]. These combined drug-mediated genomic insults send a stress signal to mitochondria, resulting in depolarization of the mitochondrial membrane and increased production of ROS (Figure 4D, E) with concomitant leakage of pro-apoptotic proteins into the cytoplasm. The BCL2 protein on the outer membrane of the mitochondria binds to pro-apoptotic proteins such as BAK and BAX, and prevents the release of pro-apoptotic factors. This interaction is inhibited by venetoclax which binds to the hydrophobic groove of BCL2 and interferes with BCL2 interaction with pro-apoptotic proteins [26], resulting in the activation of the apoptotic cascade followed by PARP1 cleavage and DNA fragmentation (Figure 4A, C). This model is consistent with increased Annexin V-positivity (Figure 1) and activation of caspase 3 (by cleavage) in myeloma cells exposed to (Gem+Bu+Mel+panobinostat+venetoclax) (Figure 4A). It is also consistent with the correlation between the IC₅₀ values of venetoclax and endogenous levels of BCL2 protein in various MM cell lines (Figure 2). Furthermore, the observed increase in ROS levels might have caused cleavage of HSP90 and activated apoptosis (Figure 6).

In addition to induction of apoptosis, the activation of AMPK by phosphorylation in the presence of (Gem+Bu+Mel+panobinostat+venetoclax) (Figure 5) may contribute to the observed drug synergism. AMPK is a central regulator of energy homeostasis [35] which is activated at low ATP/AMP ratio, resulting in caloric restriction by inhibition of anabolic pathways. One of these pathways is the regulation of protein synthesis through mTOR signaling. AMPK phosphorylates TSC2 which eventually inhibits the mTOR signaling pathway [46]. Consistent with this earlier report is the observed increase in the phosphorylation of AMPK and TSC2 in MM.1R cells exposed to the 5-drug combination (Figure 5). The drug-mediated decrease in the level of PI3Kp85 and AKT (Figure 5) might have compromised the PI3K/AKT/mTOR pathway, resulting in decreased levels of RAPTOR, P70S6K, P-S6 ribosomal protein and eIF2 α (Figure 5). All of these findings suggest inhibition of protein translation.

The observed drug synergism may also be explained in part by the activation of the UPR in the ER as suggested by increased levels of calnexin, BiP/GRP78, ERO1- α and PDI which were most pronounced in cells exposed to the 5-drug combination (Figure 6). BCL2 is known to interact with the inositol trisphosphate receptor (IP3R) protein to control ER Ca^{2+} release [40], and its inhibition by venetoclax might have perturbed Ca^{2+} homeostasis in the ER leading to UPR activation. The unregulated Ca^{2+} flux through the ER-mitochondria contact sites might have resulted in excessive accumulation of mitochondrial Ca^{2+} , resulting in mitochondrial swelling and apoptosis (40). A similar activation of UPR together with increased phosphorylation of AMPK was previously observed in acute lymphoblastic leukemia cell lines exposed to combined 5-aminoimidazole-4-carboxamide ribonucleotide and methotrexate, leading to sustained ER stress and apoptosis [47].

In summary, (Gem+Bu+Mel+panobinostat+venetoclax) exerts synergistic cytotoxicity in MM cell lines by enhanced activation of the intrinsic apoptosis pathway, inhibition of the mTOR signaling pathway through the activation of AMPK and inhibition of PI3K, and activation of the UPR in the ER. These preclinical results provide a rationale to propose a clinical trial on using this 5-drug combination as part of a pre-transplant regimen for multiple myeloma patients undergoing auto-HSCT.

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B. Valdez contributed to the conception and design of the study, analysis and interpretation of data, and drafted the manuscript. Y. Li and Y. Liu provided technical support and helped in data acquisition. D. Murray helped in data interpretation and writing the manuscript. Y. Nieto, Q. Bashir, and M. Qazilbash provided expertise on the interpretation and analysis of results and writing the manuscript. B.S. Andersson was responsible for the research approach, funding, analysis of data and critical revision of the article. All authors contributed to the final version of the manuscript.

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Highlights

- GemBuMel is used as a pre-transplant conditioning therapy.
- Epigenetic modifiers enhance the efficacy of DNA alkylators+nucleoside analogs.
- Venetoclax cytotoxic is due to inhibition of pro-survival BCL2.
- GemBuMel +panobinostat+venetoclax provide synergistic cytotoxicity to MM cells.
- The 5-drug combination inhibits the mTOR signaling pathway.
- The 5-drug combination activates unfolded protein response.
- GemBuMel+Pano+Venetoclax may be used as pre-transplant conditioning regimen.

Figure 1.

Synergistic cytotoxicity of drugs in multiple myeloma cell lines. MM.1R (A) and MC/CAR (B) cells were continuously exposed to drugs alone, or in combination, for 48 h and analyzed by the MTT assay for cell proliferation and Annexin V assay (Ann V) for early cell death. Statistically significant differences are indicated by the P-values. Below the bar graphs is the analysis of synergism by graphing the combination index (CI) and fraction affected (Fa) using the Chou and Talalay method [18]. CI values less than 1 suggest synergism, more than 1 suggest antagonism, and equal to 1 suggest additive effects. The results are average $\pm$ standard deviation of at least three independent experiments. Gem, gemcitabine; Bu, busulfan; Mel, melphalan; Pano, panobinostat; ABT199 or ABT, venetoclax

Figure 2.

Correlation between IC₅₀ of ABT199 and the level of BCL2 family members in four myeloma cell lines. (A) Cells were exposed to various concentrations of ABT199 for 48 h and analyzed by the MTT assay. The dashed line indicates 50% cell proliferation and the IC₅₀ values of ABT199 are indicated next to the names of the cell lines. (B) Western blotting was performed to determine the relative endogenous level of BCL2, BCL-xL, MCL1 and GAPDH in untreated cells. Autoradiogram signals were scanned, analyzed using the UN-SCAN-IT software, and normalized relative to GAPDH. Correlation coefficients (R²) between the IC₅₀ of ABT199 and protein levels were determined using the Excel program.

Figure 3.

Activation of the DNA-damage response. Cells were either left untreated (control) or were exposed to drugs alone, or in combination, for 48 h and protein extracts were analyzed by Western blotting. Abbreviations are the same as in Figure 1.

Figure 4.

Drug-mediated activation of apoptosis. Cells were exposed to drugs alone, or in combination, for 48 h and cell extracts were analyzed for levels of key proteins by Western blotting (A) and for CASPASE 3 (CASP) enzymatic activity (B). DNA was isolated from 48-h treated cells and analyzed by agarose gel electrophoresis (C). MM.1R cells exposed to drugs for 48 h were stained and analyzed for changes in the mitochondrial membrane potential (D) and reactive oxygen species (E) as discussed under Materials and Methods. The results in B, D and E are average $\pm$ standard deviation of at least three independent experiments. G, gemcitabine; B, busulfan; M, melphalan; P, panobinostat; A, ABT199/Venetoclax

Figure 5.

Analysis of components of the mTOR signaling pathway. MM.1R cells were exposed to drugs alone, or in combination, for 48 h and cell extracts were analyzed by Western blotting. Abbreviations for drugs are the same as in Figure 4. Rib prot = ribosomal protein.

Figure 6. Drug-mediated effects on the level of molecular chaperones. MM.1R cells were exposed to drugs alone, or in combination, for 48 h and cell extracts were analyzed by Western blotting. Abbreviations are the same as in Figure 4.

Table 1.

List of primary antibodies, their sources and dilutions

Antigen	Company/Cat. #	Source	Dilution *
AcH3 (K4)	Active Motif/39381	Rabbit	2500
β-ACTIN	Sigma/A5316	Mouse	6000
AKT	Cell Signaling/4691	Rabbit	3500
AMPK	Cell Signaling/5831	Rabbit	1500
P-AMPK (T172)	Cell Signaling/50081	Rabbit	1500
ATM	Santa Cruz Biotech/25921	Mouse	750
P-ATM (S1981)	Rockland/200-301-400	Mouse	2000
BiP/GRP78	Cell Signaling/3177	Rabbit	1500
BCL2	Santa Cruz Biotech/7382	Mouse	1500
BCL-xL	Cell Signaling/2764	Mouse	3500
Calnexin	Cell Signaling/2679	Rabbit	2000
Cleaved Caspase 3	Cell Signaling/9661	Rabbit	2500
CHK1	Cell Signaling/2345	Rabbit	2000
P-CHK1 (S317)	Cell Signaling/2344	Rabbit	2000
eIF2α	Cell Signaling/9722	Rabbit	2500
ERO1- α	Cell Signaling/3264	Rabbit	2000
GAPDH	GeneTex/627408	Mouse	4000
γ -H2AX	EMD Millipore/05-636	Mouse	3000
HSP90	Cell Signaling/4877	Rabbit	3500
HSP70	Cell Signaling/4872	Rabbit	3500
MCL1	Santa Cruz Biotech/819	Rabbit	1500
mTOR	Cell Signaling/2983	Rabbit	2000
P-mTOR	Cell Signaling/2971	Rabbit	1500
c-MYC	Cell Signaling/9402	Rabbit	3500
NBS1	Cell Signaling/3002	Rabbit	2500
P-NBS1	Cell Signaling/3001	Rabbit	2000
PARP1	Santa Cruz Biotech/8007	Mouse	1000
PI3Kp85	Cell Signaling/4257	Rabbit	2500
P70S6K	Cell Signaling/2708	Rabbit	3500
P-P70S6K (T389)	Cell Signaling/9234	Rabbit	2000
PDI	Cell Signaling/3501	Rabbit	2000
RAD50	Cell Signaling/3427	Rabbit	2500
P-RAD50 (S635)	Cell Signaling/14223	Rabbit	2000
RAPTOR	Cell Signaling/2280	Rabbit	1500
TSC2/Tuberin	Cell Signaling/4308	Rabbit	4000
P-TSC2 (Thr1462)	Cell Signaling/3617	Rabbit	2500
S6 Ribosomal protein	Cell Signaling/2217	Rabbit	3500
P-S6 Rib protein	Cell Signaling/4858	Rabbit	2000

* Fold dilution in PBS with 0.05% Tween 20