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Synergistic cytotoxicity of busulfan, melphalan, gemcitabine, panobinostat, and bortezomib in lymphoma cells

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Abstract

DNA alkylators busulfan (B) and melphalan (M) act synergistically with gemcitabine (G) against lymphoma cells. To further improve the cytotoxicity, we combined them with the histone deacetylase inhibitor panobinostat (P) and proteasome inhibitor bortezomib (V). Lymphoma cell lines U937 and J45.01, and patient-derived cell samples were exposed to these drugs and the effects on cell proliferation and apoptosis were quantified. The combination BMGPV was found to exert strong synergistic cytotoxicity. Drug exposure to these cells activated the ATM pathway and modified histones at the epigenetic level. Cell death was triggered by the production of reactive oxygen species (ROS), permeabilization of the mitochondrial membrane, upregulation of proapoptotic factors, and activation of caspases. Downregulation of anti-apoptotic proteins c-MYC, MCL-1, and BCL-2 and inhibition of the prosurvival PI3K-AKT-mTOR pathway, culminated in apoptosis. The results of this study support a clinical trial using BMGPV as a possible pre-transplant conditioning regimen for relapsed/refractory lymphoma patients.

Keywords

Conditioning regimen; lymphoma; stem cell transplant

Introduction

The backbone of pretransplant conditioning regimens for hematopoietic stem cell transplantation of patients with refractory lymphomas has involved the use of DNA alkylators such as busulfan and melphalan since the 1970s.[1–3] They act as crosslinking agents, preventing the double-strand separation required for DNA replication and damage repair.[4] This ubiquitous mechanism of action targets all rapidly proliferating cells, with

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consequent toxicity to many other tissues like normal gastrointestinal and white blood cells. The BEAM protocol, (BCNU, etoposide, cytosine arabinoside, melphalan) is predominantly DNA alkylator-based and is the most common high-dose chemotherapy regimen for refractory lymphoma patients undergoing autologous transplantation.[3]

Regimens that combine DNA alkylators with other agents were found to be more efficacious and less toxic compared with single-agent regimens, as they concurrently target different mechanisms of cytotoxicity.[5] Synergism was demonstrated when the repair of alkylator-mediated damaged DNA was further compromised by nucleoside analogs, which inhibited ribonucleotide reductase and got preferentially incorporated into DNA strands.[6] In a previous study, Valdez et al [7] showed that busulfan (B), melphalan (M), and gemcitabine (G) in combination with the histone deacetylase inhibitor (HDAC_i) suberoylanilide hydroxamic acid (SAHA) worked synergistically; with SAHA sensitizing lymphoma cells to the BMG combination at an epigenetic level. The addition of SAHA resulted in alterations of the chromatin structure, facilitating the genomic insult from BMG. When two alkylators were combined, the antitumor effect was more effective than just busulfan alone.[7] Moreover, in reducing the dosage of the alkylators and combining with nucleoside analogs and later HDAC_i, excessive normal tissue toxicity was decreased and efficacy was enhanced.[8–11]

In the present study, we investigated whether a combination of agents with vastly different molecular mechanisms of action would act synergistically. In particular, we explored the efficacy of combining DNA alkylating agents with a nucleoside analog, histone deacetylase inhibitor, and proteasome inhibitor. Since the pan-HDAC inhibitor panobinostat (P) is more potent than SAHA,[12] we hypothesized that it would be more efficacious in combination with BMG. Moreover, addition of bortezomib (V) as a proteasome inhibitor (PI), which acts on a completely different pathway, would further sensitize malignant cells to chemotherapy. In the present report, we show that panobinostat and bortezomib enhance the synergistic cytotoxicity of BMG in lymphoma cells.

Materials and methods

Cell culture

U937 (histiocytic lymphoma) and J45.01 (T-ALL) cell lines were obtained from the American Type Culture Collection (Manassas, VA). Mononuclear cells were obtained from the peripheral blood of patients with T-cell malignancies after obtaining written informed consent according to a protocol approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center, in accordance with the Declaration of Helsinki. Cells were cultured in RPMI 1640 with L-glutamine and 25 mM HEPES (Mediatech, Manassas, VA) and supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St Louis, MO), 100 IU/mL penicillin and 100 µg/mL streptomycin (Mediatech). All cultures were incubated at 37 °C in a fully humidified atmosphere of 5% CO₂ in air.

Patients

Patient J-01 is a 62-year-old Caucasian male with relapsed Sezary syndrome, an aggressive variant of cutaneous T-cell lymphoma. Prior to obtaining peripheral blood sample for this study, he received multiple lines of chemotherapy, radiation, and allogeneic stem cell transplantation. His circulating Sezary cells, which were used in the present study, were isolated during an aggressive stage and ultimately fatal relapse after his transplantation.

Patient L-02 is a 61-year-old African American male who presented with three weeks of enlarging neck mass and elevated white blood cells (75% lymphocytes). This patient was diagnosed with leukemic-phase peripheral T-cell lymphoma, an aggressive variant of T-cell lymphoma. Peripheral blood samples were taken from the patient upon diagnosis, prior to treatment.

Patient B-03 is a 37-year-old Caucasian male with a past medical history of Li–Fraumeni syndrome with associated rhabdomyosarcoma and vestibular schwannoma. He presented with an enlarged lymph node in his neck and elevated level of white blood cells. He was started on CHOP (cyclophosphamide, adriamycin, vincristine, prednisone) therapy. Staging bone marrow biopsy demonstrated T-cell lymphoma/leukemia, and he was switched to the anti-CD52 monoclonal antibody alemtuzumab for three cycles. However, his lymphadenopathy and leukocytosis progressed, and repeat bone marrow biopsy demonstrated T-cell prolymphocytic leukemia (T-PLL). He was salvaged with four cycles of EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, adriamycin). Samples were then taken from his peripheral blood during an aggressive relapse and used in the present study.

Reagents

Busulfan and melphalan (Sigma-Aldrich, St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO); gemcitabine, panobinostat and bortezomib (SelleckChem, Houston, TX) were dissolved in DMSO and diluted in RPMI 1640. The final concentrations of DMSO did not exceed 0.08% by volume in all experiments.

Cytotoxicity assay

Cell suspension (6 mL) at a concentration of 0.5×10^6 cells/mL were aliquoted into culture flasks and incubated for 48 h at 37 °C, with solvent alone as control or in the presence of the study drug(s). The concentration of the drugs that resulted in 80–90% cell survival relative to the control was then added to the cell cultures in various combinations. Following incubation, 100 µL of the cell suspension was aliquoted into 96-well plate and further incubated for 4 h with 50 µL of 5 µg/µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent.[13] The resulting precipitate was dissolved by adding 100 µL of the stop solution (10% Triton X and 0.1 N HCl in isopropanol) and further incubated overnight at 37 °C. The absorbance at 570 nm was measured with the Victor X3 plate reader (Perkin Elmer Life and Analytical Sciences, Shelton, CT). Graphical representation of cell survival was constructed using Microsoft Excel.

Apoptosis assay

Cells were exposed to the drug(s) for 48 h and 60 μ L cell suspension was added to equal volume of Annexin-V-FLUOS reagent (Roche Diagnostics, Indianapolis, IN) plus 7-aminoactinomycin D (BD Biosciences, San Jose, CA). The solution was kept in the dark for 20 min at room temperature and Annexin V-positivity was assessed by flow cytometric measurement of the extent of phosphatidylserine translocation to the outer membrane of cells undergoing apoptosis using the MUSE cell analyzer (EMD Milipore, Hayward, CA). To further evaluate the extent of apoptosis, the cleavage of poly(ADP-ribose) polymerase (PARP) 1 and caspases 3, 8, and 9 was analyzed by western blotting.

The dose–effect relationships of the five-drug combination relative to the individual drugs were analyzed based on kinetic principles to assess the degree of synergism, antagonism or summation using the Chou and Talalay method.[14]

Protein analysis

Western blot analysis was performed to determine the mechanisms of synergism by analyzing changes in the level of proteins and their modification status. Briefly, cells were incubated with the study drug(s) for 24 h (to assess the early DNA-damage response) or 48 h. Thereafter, they were centrifuged, washed with ice-cold PBS and pelleted. Cells were lysed with lysis buffer (Cell Signaling Technology, Danvers, MA) and total protein concentration was determined using the BCA protein Assay kit (Thermo Scientific, Rockford, IL) relative to albumin standards. 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 then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The required antibodies were added and detected using the chemiluminescent substrates Immobilon (EMD Milipore), Hyglo (Denville Scientific), or Femto (Thermo Scientific). The primary antibodies, their sources, dilutions, and antigen molecular weights are listed in Table 1 (Supplementary Materials).

Analysis of mitochondrial membrane potential (MMP)

Determination of the integrity of the mitochondrial membrane was assessed using the dual-emission JC-1 fluorescent probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbo cyanine iodide) (Sigma-Aldrich) which detects changes in the MMP. Cells were exposed to drug(s) for 48 h and 0.5 mL of the resulting cell suspension was aliquoted into 5-mL tubes. Valinomycin (2 μ M) was added to untreated cells and used as a positive control and incubated for 1 h at 37 °C and 5% CO₂. JC-1 reagent (5 μ L at dilution of 1:10) was added per sample and incubated at 37 °C for 20 min. The samples were immediately analyzed using the Gallios Flow Cytometer (Beckman Coulter, Brea, CA).

Analysis of reactive oxygen species (ROS)

U937 and J45.01 cells were exposed to drug(s) for 24 h as detailed above and analyzed for production of ROS using the indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluoresceine diacetate acetyl ester (CM-H₂DCFDA) (Life Technologies, Grand Island, NY). Hydrogen peroxide (2 mM) was used as a positive control in an

untreated sample. The CM-H₂DCFDA assay measured hydroxyl, peroxy and other ROS activity, when the indicator diffused into the cell and oxidized into a fluorescent product. CM-H₂DCFDA (1.5 nM dissolved in DMSO) was added to 0.5 mL cell suspension and incubated for 1 h at 37 °C, and the fluorescent intensities were immediately analyzed by flow cytometry at excitation/emission wavelengths of 492/520 nm.

Statistical analysis

All results were presented as the mean $\pm$ standard deviation of at least three independent experiments and statistical analysis was performed using Student's paired t-test with a two-tailed distribution.

Results

Busulfan, melphalan, gemcitabine, in combination with panobinostat and bortezomib (BMGPV) exert synergistic cytotoxicity when administered concurrently to lymphoma cell lines.

Subtoxic concentrations of individual drugs were used to attain an IC₁₀₋₂₀ for the lymphoma cell lines. For the U937 histiocytic lymphoma cells, the drug concentrations used were as follows: 81 μ M busulfan, 0.9 μ M melphalan, 18 nM gemcitabine, 7 nM panobinostat, and 4.2 nM bortezomib. These individual drug concentrations decreased the relative proliferation by 0–10% and there was no statistically significant difference among them. When busulfan, melphalan, and gemcitabine (BMG) were combined, the proliferation dropped to ~73% (Figure 1a). With the addition of panobinostat, the proliferation further decreased to ~53% ($p < 0.001$), and with addition of bortezomib, it decreased to ~40% ($p < 0.001$). But upon exposure to the five-drug combination (BMGPV), cell proliferation decreased the most to ~22% ($p < 0.001$). This was confirmed with the Annexin V assay which demonstrated that the four- or five-drug combination (BMGP, BMGV, or BMGPV) significantly increased the percentage of cells undergoing early or late apoptosis compared with the three-drug combination (BMG) (47% vs. 55%; $p < 0.03$). Synergistic cytotoxicity was similarly demonstrated in J45.01 cells when the five-drug combination BMGPV decreased the cell proliferation to ~39% from ~71% in the BMGP sample ($p < 0.007$); apoptosis increased from ~59% to ~80% ($p < 0.04$) (Figure 1b). The kinetics of drug-induced cell death (from 8 to 48 h), as measured by Annexin V-positivity, shows significant effects of combined drugs after 24 h in U937 cells and after 32 h in J45.01 cells (Figure S1). Using the lymphoma patient cell sample, the BMGPV combination decreased cell proliferation from ~66% to ~34%, compared with BMGP ($p < 0.005$) and increased the Annexin V-positive apoptotic cells from ~28% to ~61% ($p < 0.007$) (Figure 1c, using 2 nM bortezomib).

These results show that addition of panobinostat sensitized cells to the three-drug combination, as expected from similar results of BMG with SAHA.[6] Our results also show that the combined action of panobinostat and bortezomib further synergistically sensitized the cells to the nucleoside analog-alkylating agents combination.

Strong synergism was demonstrated when the effect (fraction of dead cells; Fa) of single drug was compared with the BMGPV combination at fixed concentration increments (Figure

1d). At 50% Annexin-V-positive cells, the calculated combination index (CI) was 0.04, suggesting strong synergism.

Bortezomib was shown to sensitize myeloma cells to conventional chemotherapy when given 24 h afterwards, via the inhibition of the NF κ B pathway.[15] We therefore investigated the optimal timing of exposure and found that the relative survival was impacted the most when bortezomib was given 4 h prior to or concurrently with BMGP ($p = 0.04$). The relative survival was the same as the BMGP control, if bortezomib was added more than 6 h beforehand or afterwards (Figure 1e), emphasizing the importance of the precise sequence and timing of drug exposure.

BMGPV combination activates an early DNA-damage response and results in extensive apoptosis

Protein analysis after 24-h incubation with the subtoxic concentrations of drugs, alone or in combination, revealed that the DNA-damage response (DDR) involving the ataxia-telangiectasia-mutated (ATM) pathway was activated. The DDR is a highly sensitive surveillance system attuned to recognizing any DNA strand breakage and initiating repair before these mutations are propagated. At single drug doses, there was minimal phosphorylation of ATM at Ser 198 but with the two-, three-, and four-drug combinations, there was increasing phosphorylation of ATM, with the phosphorylation being most pronounced in the five-drug combination BMGPV in both U937 and J45.01 cell lines (Figure 2). When busulfan and melphalan form DNA adducts, DDR initiates repair mechanisms via ATM kinase by activating other substrates downstream of this pathway, like SMC1 and CHK-2, which are involved in checkpoint regulation.

After 48 h of drug exposure, phosphorylation of other substrates downstream of this pathway like γ -H2AX- and KRAB-associated protein-1 (KAP1) was demonstrated in all cells analyzed (Figures 2 and 3). These protein modifications are involved in recruiting DDR proteins, modifying chromatin [16], and histones to anchoring the broken DNA strands together.[16–17] There was an increased level of phosphorylated p53 at Ser15 in p53-positive cell samples (J45.01 and three patient cell samples (Figure 3b and c), which may lead to cell cycle arrest.[18]

The cleavage of apoptotic proteins PARP1, and caspases 3, 8, and 9, was most notably significant in the BMGPV sample, compared with the single drugs or other combinations, suggesting robust activation of apoptosis (Figure 3). This coincided with a decrease in the inhibitor of apoptosis protein XIAP. The level of γ -H2AX was increased more in BMGP and BMGPV compared with BMGPV combination in U937 and J45.01 cells (Figure 3a and b), which suggests enhanced synergism in combinations containing a histone deacetylase inhibitor. This is especially more pronounced when combined with bortezomib.

Similar results were observed in three patient-derived cell samples. DNA-damage response is suggested by the phosphorylation of H2AX in cells exposed to BMGP and/or BMGPV in all three patient samples; although sample B-03 was more sensitive to bortezomib alone, probably due to exposure of the patient to excessive chemotherapy prior to sampling (Figure 3c). Cleavage of PARP1 was significant in patient cell samples exposed to BMGPV. There

was also cleavage of caspases in the BMGPV combination compared with the four-drug regimen suggesting activation of apoptosis. Partial cleavage of caspase 3 in sample J-01 and caspase 9 in samples L-02 and B-03 with subtoxic doses of bortezomib occurred, suggesting an activity of bortezomib alone in T-cell lymphoma samples, consistent with previous reports.[19] Drug-induced phosphorylation of P53 was also observed in J-01 and L-02 samples, another indication of DNA-damage response activation; patient sample B-03 was P53-negative, consistent with his Li-Fraumeni syndrome.

BMGPV combination mediates chromatin remodeling

We hypothesized that exposure of lymphoma cells to a combination of HDAC_i and PI would modify chromatin structure and enhance the DNA damage mediated by the BMG combination. Therefore, the extent of chromatin modification after 48 h of drug exposure was examined.

There was increased acetylation of histone 3 at lysine 9 (Figure 4a) in U937 cells exposed to BMGPV, and an increasing trend of histone 3 acetylation in J45.01 cells (Figure 4b) and patient cell samples (data not shown) exposed to BMGP and BMGPV combinations. Such increase in histone 3 acetylation may be partly attributable to BMGPV-mediated decrease in the level of histone deacetylase HDAC 4 (Figure 4), which may promote the relaxation of the chromatin structure.[12] On an epigenetic level, this process may expose the DNA, making it more susceptible to damaging effects of even sub-toxic concentrations of busulfan, melphalan, and gemcitabine. There was also increased trimethylation of histone 3 at lysine 27 in the four-drug combination BMGP and BMGV but most prominently in cells exposed to BMGPV, further supporting the occurrence of chromatin remodeling.

BMGPV combination increases the ROS production and decreases the mitochondrial membrane potential, altering the interplay between pro- and anti-apoptotic proteins

Disruption of the mitochondrial membrane plays a major role in the orchestrated cell death process. The chemotherapy agents could have exerted an initial insult to the mitochondrial membrane and disrupt the electron transport process, causing electrons to leak onto oxygen to produce reactive oxygen species.[20] We therefore sought to evaluate the initiation and propagation of this process. When U937 cells were exposed to the BMGPV combination, there was a fourfold increase in ROS production relative to control within 24 h (Figure 5a).

Since ROS itself has been shown to increase the permeability of the membrane and aids in the release of proapoptotic proteins,[21,22] we were prompted to determine changes in the MMP. Exposure of cells to BMGPV resulted in a ~70% increase in JC-1 monomer accumulation in the cytoplasm. With the four-drug or single-drug combination, the extent of leakage was at a maximum of ~ 45% (Figure 5b). An increase in the level of proapoptotic NOXA and a decrease in the level of anti-apoptotic proteins c-MYC, MCL-1, and BCL-2 were more dramatic in cells exposed to the BMGPV combination (Figure 5c), suggesting that the status of mitochondria was compromised. These results, together with activation of caspases (Figure 4), suggest upregulation of proapoptotic factors and down-regulation of prosurvival proteins in cells exposed to BMGPV combination.

BMGPV combination inhibits the prosurvival PI3K/AKT/mTOR pathway

Owing to its proximal position, phosphatidylinositol 3-kinase (PI3K) plays a central role in several signaling pathways. Downstream of the pathway is AKT, a kinase that phosphorylates the effector mammalian target of rapamycin (mTOR) and is involved in the activation of prosurvival pathways.[23] The PI3K/AKT/mTOR pathway is found to be dysregulated in lymphoma, and therefore, it is a good therapeutic target; knocking it out would promote death mechanisms at multiple levels. [24] Upon exposure of U937 and J45.01 cells to BMGPV combination, there was a marked decrease in the levels of phosphorylated PI3K, AKT and mTOR (Figure 6a and b). The decrease was not as obvious in the BMGV combination, which suggests enhanced synergism when the cells were sensitized with panobinostat. These results also suggest that the cytotoxicity of BMGPV combination is partly due to inhibition of the PI3K/AKT/mTOR pathway.

Discussion

We previously demonstrated increased synergism upon combining BMG with SAHA in lymphoma cells.[7] In the present study, we demonstrate that the BMG combination's antineoplastic activity is enhanced if SAHA is replaced by a more potent HDAC_i panobinostat, and further sensitized with addition of the PI bortezomib.

The complexity of the observed synergism is attributable to the inhibition and activation of several pathways which culminate in the disruption of the cell's innate DNA damage repair mechanisms and alterations of the integrity of the mitochondrial membrane. These processes ultimately lead to lymphoma cell apoptosis. As an HDAC inhibitor, panobinostat modifies histone, opens up the chromatin and makes it more accessible to cytotoxic agents. Busulfan and melphalan effect their insult through DNA adduct formation (Figure 7). The incorporation of the nucleoside analog gemcitabine inhibits DNA repair processes, causes accumulation of DNA breaks, exacerbates the DNA-damage response and marks the cells for apoptosis. At another level, bortezomib inhibits the prosurvival pathway PI3K/AkKT/mTOR axis and enhances apoptosis (Figure 7).

Moreover, the BMGPV combination damages the mitochondrial membrane, which is initiated and further perpetuated by the production of ROS. Such a process activates proapoptotic factors like NOXA, and downregulates anti-apoptotic factors like BCL-2, MCL-1, and cMYC, finally leading to the cleavage and activation of caspases which commits the cells to apoptosis.

These results are consistent with other published data combining busulfan with other nucleoside analogs and epigenetic modifiers,[25] showing their synergistic cytotoxicity in lymphoma and AML cell lines. Our study is the first to investigate the addition of a proteasome inhibitor into a purported pretransplant conditioning regimen. As BMGP exerts the effects on a genomic level, it was thought that the overall cytotoxicity could be augmented by targeting other signaling pathways, since PI had already been shown to sensitize myeloma cells to chemotherapy via the inhibition of NF κ B and the degradation of multiubiquitinated proteins.[26]

This study demonstrates the importance of incorporating novel agents into our armamentarium, as part of our continuing effort to overcome the evolution of drug resistance and progression of cancer. Our results underscore the relevance of preclinical studies to continuously search for more efficacious drug combinations for pretransplant conditioning regimens. For example, significantly improved patient survival was observed with the BMG combination when compared with the standard of care BEAM as a pre-transplant regimen for refractory lymphomas.[10] In a similar setting, the efficacy of BMG was further enhanced when SAHA was included in this pretransplant regimen.[11] Both clinical trials were based on our preclinical studies.[7,8] The present preclinical study strongly supports the design of a clinical trial using the BMGPV combination as a conditioning regimen prior to hematopoietic stem cell transplantation in patients with advanced refractory lymphoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Busulfan (B), melphalan (M), gemcitabine (G), panobinostat (P) and bortezomib (V) caused synergistic cytotoxicity in U937 cell line (a), J45.01 lymphoma cell line (b), and lymphoma patient-derived cell sample (c). Cells were continuously exposed to drug(s) for 48 h and analyzed using the MTT assay for cell proliferation and Annexin V assay (Ann V) for apoptosis. The drug concentrations for the lymphoma patient sample were 44 μ M B, 1.2 μ M M, 22.5 nM G, 6 nM P and either 1 nM or 2 nM V. (d) Dose effect graph of the fraction of dead cells (Fa) for individual drugs compared with the five-drug combination. The calculated combination index (CI) for the U937 cell line is 0.04 at Fa of 0.5, suggesting synergism. (e) Sequential drug exposure. U937 cells were exposed to bortezomib (V) 6 h or 4h prior to (minus) or after (plus) exposure to BMGP combination. The p values were calculated based on the averages of at least three independent experiments (a, b, c, and e). Panel d is a representative of two independent experiments.

Figure 2. Western blot analysis shows the activation of the early DNA-damage response pathway in U937 (a) and J45.01 (b) cells after exposure to the drug(s) at various combinations for 24 h. The abbreviations and drug concentrations are the same as those used in Figure 1. The results are representatives of at least two independent experiments.

Figure 3.

The apoptotic pathway was activated in established cell lines and patient-derived cell samples after 48 h exposure to drugs, alone or in combination. Western blot analysis was performed using cell extracts from U937 cell line (a), J45.01 cell line (b), and patient cell samples (c). The abbreviations and drug concentrations are the same as those used in Figure 1. The drug concentrations used for the other two patient samples were Patient L-02: 49 μ M B, 0.6 μ M M, 20 nM G, 4 nM P and 3 nM V; Patient B-03: 41 μ M B, 0.6 μ M M, 20 nM G, 4 nM P and 5 nM V.

Figure 4.

Histones were modified in drugs exposed to drugs for 48 h, alone or in combination. Western blot analysis was performed using cell extracts from U937 (a) and J45.01 (b) cell lines. The abbreviations and drug concentrations are the same as those used in Figure 1 except for the patient samples. The results are representatives of two independent experiments.

Figure 5. Drug exposure increased reactive oxygen species (ROS), decreased mitochondrial membrane potential (MMP), up-regulated an apoptotic factor and down-regulated pro-survival proteins. U937 cells were exposed to drugs for 24 h (a) or 48 h (b and c) and analyzed by flow cytometry for the production of ROS (a) and changes in the MMP (b), and by Western blotting (c). The abbreviations and drug concentrations are the same as those used in Figure 1. Panels a and b show the averages of at least three independent experiments; panel c is a representative of two experiments.

Figure 6. BMGPV combination inhibited the PI3/AKT/mTOR prosurvival pathway in U937 (a) and J45.01 (b) cell lines. Western blot analysis was performed using protein extracts from cells exposed to drug(s) for 48 hrs. The abbreviations and drug concentrations are the same as those used in Figure 1. The results are representatives of two independent experiments.

Figure 7.
Suggested mechanism of the synergistic cytotoxicity of busulfan (B), melphalan (M),
gemcitabine (G), panobinostat (P) and bortezomib (V) in lymphoma cells.

Table 1.

List of primary antibodies, their sources, dilutions and molecular weights.

Antibodies	Source (Catalogue #)	Clone type	Dilutions	Molecular weight (kDa)
AcH3K9	Active Motif/39137	pAb	3500	17
AKT	Cell Signaling/4691	pAb	2000	60
ATM	Santa Cruz/23921	mAb	800	250
BCL-2	DAKO/124	mAb	1500	26
c-MYC	Cell Signaling/9402	pAb	2500	57–70
Cleaved Caspase 3	Cell Signaling/9661	pAb	2000	17, 19
Caspase 8	Cell Signaling/9746	mAb	2000	43
Caspase 9	Cell Signaling/9502	pAb	2000	35–47
CHK2	Cell Signaling/2662	pAb	1000	62
HDAC4	Cell Signaling/5392	mAb	3000	140
KAP1	Bethyl Lab A300-275A	pAb	2500	100–117
MCL-1	Santa Cruz/819	pAb	700	40
mTOR	Cell Signaling/2983	pAb	2500	289
NOXA	Calbiochem/0180	mAb	1500	15
p-ATM (Ser1981)	Rockland/200–301–400	mAb	2000	250
p-AKT (Ser473)	Cell Signaling/4060	pAb	2500	60
p-CHK2 (Thr68)	Cell Signaling/2661	pAb	2000	62
p-KAP1 (Ser824)	Cell Signaling/4127	pAb	2500	100
p-mTOR (Ser2448)	Cell Signaling/5536	pAb	1500	289
p-P53 (Ser15)	Cell Signaling/9284	pAb	2000	53
p-PI3K (Tyr199/Tyr458)	Cell Signaling/4228	pAb	2500	60, 85
p-SMCI (Ser957)	Novus Biologicals/100–205	pAb	1500	140
P53	Santa Cruz/126	pAb	2000	53
PARP1	Santa Cruz/8007	mAb	1000	116
PI3K	Santa Cruz/423	pAb	500	85
SMC1	Cell Signaling/4802	pAb	2500	140
XIAP	Cell Signaling/2045	pAb	2000	53
α-Tubulin	Cell Signaling/2144	pAb	1000	57

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Antibodies	Source (Catalogue #)	Clone type	Dilutions	Molecular weight (kDa)
β-ACTIN	Sigma/A5316	mAb	10000	42
γ-H2AX	Millipore/2554898	mAb	3000	15
3MeH3K27	Active Motif/39155	pAb	3500	17