Bendamustine is a bifunctional alkylating agent with some efficacy in the treatment of newly diagnosed and relapsed/refractory multiple myeloma (MM). Everolimus, an mammalian target of rapamycin (mTOR) inhibitor, is a additional promising chemotherapeutic agent that has efficacy in a variety of cancers. We investigated the individual and combinational cytotoxic effects of these drugs in MM cell lines (RPMI8226 and MM1.S) and primary MM cells. Our results demonstrated a synergistic effect of these drugs, which was effective for both p53-wild-type and p-53-deleted MM cells, but was minimal in mononuclear cells from a healthy donor. Combination treatment with the two agents inhibited proliferation and promoted cytotoxicity and apoptosis as assessed by Annexin-V/PI staining, caspase-3 degradation, and PARP cleavage. Cell death was associated with the up-regulation of the pro-apoptotic protein Bax and the down-regulation of the anti-apoptotic proteins Mcl-1 and survivin. The combination drug treatment also promoted a decrease in the levels of the downstream target proteins of the mTOR pathway, p70s6k, and 4EBP-1, as well as an increase in the level of phosphorylation of the tumor suppressor protein p53 in MM1.S cells. p21 was also down-regulated upon treatment with the two drugs, suggesting a mechanism of sensitization through the release of cell cycle arrest. Our results demonstrate a network of regulatory factors that may contribute to the synergistic cytotoxicity of everolimus and bendamustine, and provide a rationale for application for the combinatorial treatment of MM with alkylating agents and mTOR inhibitors in future clinical practice.

Keywords everolimus; bendamustine; myeloma; synergy; apoptosis

Received: September 16, 2012    Accepted: February 19, 2013

Introduction

Multiple myeloma (MM) is a malignant tumor with clonal proliferation of plasma cells. MM comprises 10%–15% of all hematologic malignancies and ~1% of all forms of cancer. The incidence of MM follows that of leukemia and lymphoma among hematologic malignances [1]. In recent years, there has been a significant improvement in the survival of MM patients due to the introduction of autologous stem cell transplantation and novel therapies [2]. However, MM remains as an incurable disease, primarily due to drug resistance. Therefore, the development of new drugs and new treatment options is imperative for improving the prognosis of patients with MM.

The clinical application of bendamustine in the treatment of MM and related cancers has been an increasing area of focus over the past decade. This bifunctional agent combines both alkylating and anti-metabolic properties [3]. As a result of its dual characteristics, bendamustine can overcome resistance to general alkylating agents to provide an alternative method to enhance anti-tumor activity [4]. Bendamustine induces G2 arrest mediated by ataxia–telangiectasia mutated (ATM), checkpoint kinase 2 (Chk2), and cell division control 2 (Cdc2); and also induces p53-mediated apoptosis in myeloma cell lines [5]. Bendamustine was originally synthesized at Jena University in Germany in 1963. Multiple clinical trials confirm that this bifunctional alkylating agent has anti-tumor activity in MM, Hodgkin’s lymphoma, non-Hodgkin’s lymphoma, and breast cancer [6–9]. A Phase III clinical trial indicated that treatment of bendamustine plus prednisone in patients with newly diagnosed MM leads to superior complete response rate and has a more lasting effect as compared with treatment with melphalan plus prednisone [7]. However, for relapsed/refractory MM, the event-free survival and overall survival times are short with bendamustine monotherapy. A clinical trial of refractory/refractory MM patients showed that treatment with bendamustine monotherapy achieves a median survival time of 17.5 months [10]. Furthermore, a subsequent clinical trial reported that relapsed MM patients who were administered high-dose bendamustine monotherapy had a median progression-free survival of 26 weeks [11]. We suggest that the efficacy of bendamustine might be strengthened by...
Everolimus were dissolved in dimethylsulfoxide (DMSO; Sigma). Bendamustine was stored in aliquots at $-80^\circ$C, and everolimus was stored in aliquots at $-20^\circ$C, as recommended by the manufacturers. Antibodies against caspase-3 and poly (ADP-ribose) polymerase (PARP) were obtained from BD Biosciences (San Diego, USA). Antibodies against Bim, Bax, XIAP, Survivin, s6k, Phospho-p70s6k (Thr389), Phospho-4EBP-1 (Ser65), p53, p21, and Phospho-p53 (Ser15) were from Cell Signaling Technology (Beverly, USA). Antibodies against Bcl-XL and Mcl-1 were from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies against Bcl-2 were from Upstate Technology (Lake Placid, USA). Mouse monoclonal antibody against B-actin was from Sigma-Aldrich. Anti-mouse IgG and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibodies were from Pierce Biotechnology (Rockford, USA).

**Materials and Methods**

**Reagents and antibodies**

Bendamustine was purchased from Sigma-Aldrich (St Louis, USA). Everolimus was kindly provided by Novartis Pharma AG (Basel, Switzerland). Both bendamustine and everolimus were dissolved in dimethylsulfoxide (DMSO; Louis, USA). Everolimus was kindly provided by Novartis

**Cells and cell culture**

The dexamethasone-sensitive MM1.S human MM cell line was kindly provided by Professor Steven Rosen (Northwestern University, Chicago, USA). The RPMI8226 cell line was purchased from The American Type Culture Collection (Rockville, USA). Both cell lines were cultured in RPMI 1640 (Hyclone, Logan, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone), at 37°C in 5% CO$_2$. MM1.S cells have wild-type form of the tumor suppressor gene p53 [19], whereas RPMI8226 cells harbor a mutant form of p53 [20]. The bendamustine and everolimus aliquots were thawed and diluted in corresponding medium just before addition to cell cultures.

**Clinical samples**

Six MM patients who had met the clinical criteria of MM in the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) were recruited, subsequent to obtaining informed consent in accordance with the institutional guidelines and the Declaration of Helsinki principles. The patients included four males and two females with an average age of 54 years (range 40–61). The diagnosis and Durie–Salmon staging of the six patients were IgA-\lambda IIIA, IgG-\kappa II A, IgA-\lambda IIIB, \kappa light chain IIIB, \lambda light chain IIIA, and IgG-\kappa IIIA, respectively. The median MM cell ratio was 54.5% (range 36%–91%). Patient 1 had been exposed to traditional therapy (vincristine + doxorubicin + dexamethasone VAD protocol) and developed VAD protocol resistance. Patient 2 had been exposed to bortezomib and developed bortezomib resistance. Patient 4 is p53-deleted as analyzed by fluorescence in situ hybridization, whereas the others are not diagnosed with p53 deletion. One healthy volunteer was enrolled as control.

**Primary cells from MM patients**

Bone marrow samples were obtained from the MM patients. Mononuclear cells were isolated by Histopaque gradient
Cell proliferation assays
The CCK-8 assay (Cell counting kit-8; Dojindo Molecular Technologies, Inc. Shanghai, China) was used to evaluate the effects of bendamustine and everolimus on MM cell viability. The assay was performed according to the manufacturer’s recommendations. Briefly, cells were seeded in triplicate in 96-well plates (Corning, Steuben County, USA) and incubated in the presence of different concentrations of reagents for 48 h. Four hours before culture termination, 10 µl of CCK-8 solution was added to the culture. Absorbance or optical density was read on a 96-well plate reader at a wavelength of 450 nm. Data from cell proliferation experiments were expressed as percentage of cell proliferation in untreated cells. The bendamustine concentration resulting in 50% inhibition of cell growth (IC50) was determined by curve fitting to a dose–response curve.

Trypan blue staining
MM cells were cultured at 2 × 10^5 cells/ml in 12-well plates, and then cells were treated with increasing concentrations of bendamustine, everolimus, or a combination of the two drugs for 48 h at 37°C. Viable and dead cells were assessed by counting with a hemocytometer after trypan blue staining.

Combination index determination
Combinations of the two drugs were prepared using serial fixed-ratio dilutions. Cell proliferation assays were performed using the CCK-8 assay described above. The effects of combination drug treatment were estimated using CalcuSyn software, as described previously [21]. The combination index (CI) was the ratio of the combinational concentration to the sum of the single-agent concentration at an isoeffective level. CI < 0.9 indicates synergy; CI > 1.1, antagonism; and 0.9 ≤ CI ≤ 1.1, additivity.

Cell apoptosis assays
Cells (4 × 10^5/ml) were incubated with vehicle control (0.1% DMSO), bendamustine, everolimus, or a combination of the two drugs in 6-well plates for 48 h. Apoptosis was measured by Annexin-V/PI staining using the Annexin-V-FITC kit according to the instruction of the manufacturer (Sigma-Aldrich), and analyzed using a FACSCalibur flow cytometer (BD Biosciences) [22].

Western blot analysis
The whole cells were lysed in radioimmunoprecipitation assay buffer [1 × phosphate-buffered saline (PBS), 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)], and supplemented with freshly added 10 mmol/l β-glycerophosphate, 1 mmol/l sodium orthovanadate, 10 mmol/l NaF, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 × Roche Complete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland) [23] on ice for 30 min. Equal volumes of lyses were electrophoresed with SDS–polyacrylamide gels (8%–12%) and electrotransferred to nitrocellulose filters. The filters were blocked in PBS containing 5% skimmed milk and incubated with the primary antibody at 4°C overnight, and then incubated with the secondary HRP-conjugated antibody. Protein expression was assessed by enhanced chemiluminescence system. An anti-β-actin monoclonal antibody was used as an internal control.

Statistical analysis
Data are expressed as mean ± standard deviation (SD) for three replicates. SPSS Version 16.0 software (SPSS Software, Munich, Germany) was used to perform the statistical analysis. Student’s t-test was used for combination drug treatment. P < 0.05 was considered to be statistically significant.

Results
Effects on proliferation following single drug treatment of MM cells lines with bendamustine or everolimus
To provide a basis for the use of bendamustine and everolimus as potential combinatorial chemotherapeutic agents against MM, we first investigated the inhibitory effect of these drugs individually on the proliferation of the MM cell lines MM1.S and RPMI8226. These cell lines were selected based on the difference in their pathways of the myeloma progression: MM1.S cells have wild-type form of the tumor suppressor gene p53 [18], whereas RPMI8226 cells harbor a mutant form of p53 [19]. Through serial dilution and measurement of cell viability with a CCK-8 kit, we showed that bendamustine inhibited the viability of MM1.S and RPMI8226 cells in a dose-dependent manner. The IC50 of bendamustine at 48 h was 56.2 ± 0.7 M for RPMI8226 [Fig. 1(A)], suggesting that wild-type p53 cells may be more sensitive to bendamustine. Following the same method, the inhibitory effects of everolimus were determined. The results showed that everolimus provided minimal levels of suppression of proliferation for both cell lines. After treatment with the maximal dose of everolimus (1000 nM) for 48 h, the proliferation of MM1.S and RPMI8226 cells was decreased by 58% and 32.1%, respectively [Fig. 1(C,D)]. This finding suggests that bendamustine can cause a dose-dependent inhibition of proliferation for both a p53-wild-type and a p53-mutant MM cell line with a variable level of sensitivity, but that everolimus
Everolimus enhances bendamustine cytotoxicity in multiple myeloma

To determine whether a low dose of bendamustine could exert an enhanced effect in providing tumor cell cytotoxicity in the presence of everolimus, doses less than the IC_{50} for bendamustine treatment were tested in combination with everolimus. To obtain a broader understanding of the effects of the drug combination, we measured cytotoxicity over a time-course of treatment. MM1.S cells were treated with bendamustine (0, 12.5, 25, 50 \text{mM}) with or without 10 nM everolimus for 24, 36, and 48 h, followed by trypan blue staining to count cell viability. The results showed that everolimus could enhance bendamustine-induced death of MM1.S cells at all times and doses tested. Treatment with 50 \text{\mu M} bendamustine in combination with 10 nM everolimus showed the greatest effect with \sim 80\% of cells dead at 48 h [Fig. 2(B)]. These results suggest that everolimus enhances cell death induced by bendamustine for both p53-wild-type and p53-mutant MM cell line.

The synergistic effects of bendamustine and everolimus occur at the level of cell proliferation suppression and cell death induction

Effects on cell viability could result from inhibition of proliferation, induction of apoptosis, or a combination of the two. To determine whether the synergistic effect of bendamustine and everolimus are observed at the level of viability, we determined the CI following 48 h treatment of MM1.S cells with bendamustine (0–400 \text{mM}) and everolimus alone or in combination. The CI was < 0.9 at a range of doses, indicating a favorable synergistic suppressive effect of bendamustine and everolimus on the proliferation of MM1.S cells [Fig. 3(A)]. Using the same procedure, a synergistic suppressive effect of bendamustine (0–500 \text{mM}) and everolimus (0–250 nM) was shown on the proliferation of RPMI8226 cells [Fig. 3(B)].
Flow cytometry was used to determine the effect on apoptosis of bendamustine in combination with everolimus. MM1.S cells were treated for 48 h with everolimus (20 nM), bendamustine (20 μM), or the two drugs in combination, followed by double staining with Annexin-V/PI. Compared with control cells, treatment with everolimus alone and bendamustine alone induced about 30% and 20% apoptosis, respectively. However, combination treatment induced >90% apoptosis in MM1.S cells (Fig. 4). Collectively, these results show that bendamustine and everolimus function synergistically to inhibit cell viability both by inhibiting cell proliferation and by inducing apoptosis.

Changes in expression of cell death signaling proteins after combination treatment with bendamustine and everolimus

To explore the mechanisms underlying the combined effects of everolimus and bendamustine on MM cells, we treated MM1.S cells for 48 h with 20 nM everolimus and 20 μM bendamustine alone or in combination, and assessed the levels and activation of cell death signaling proteins. Caspase-3 degradation and downstream cleavage of PARP were assessed as markers of apoptosis. Our results showed that caspase-3 decreased and cleaved PARP significantly increased after combination treatment with both drugs as compared with either drug alone, suggesting that apoptosis following combination treatment is dependent on this pathway [Fig. 5(A)]. To further assess the effects of the drug combination on other apoptosis-related proteins, we assayed the expression of Bcl-2 and inhibitor of apoptosis proteins (IAPs) family proteins. Compared with treatment with bendamustine alone, Bax (a pro-apoptotic protein) was up-regulated, while Mcl-1 (an anti-apoptotic protein) and survivin (a member of the IAP family) were markedly down-regulated [Fig. 5(B)].

To further determine signaling pathways affected by the combination of bendamustine and everolimus, the activation of the mTOR and p53 pathways was also assessed. Levels of two activation markers of the mTOR pathway, phosphorylated p70s6k (P-p70s6k), and phosphorylated 4EBP-1 (P-4EBP-1), were dramatically decreased after combination treatment, though no decrease was observed in the levels of total s6k [Fig. 5(C)]. Furthermore, treatment with the drug combination did not up-regulate the expression of the tumor suppressor p53 but markedly increased its activation by phosphorylation (P-p53). We also assessed the expression of the cyclin-dependent kinase inhibitor p21, which classically functions as a downstream transcriptional target of p53, but is also known to be inhibited translationally as a potential mechanism of drug sensitization. Interestingly, the expression p21 was decreased, rather than increased in the presence of bendamustine plus everolimus [Fig. 5(D)].
Everolimus enhances the cytotoxicity of bendamustine in primary MM cells

To determine whether everolimus could enhance the cytotoxicity of bendamustine in primary MM cells or not, six patients with clinically proven MM were recruited. Mononuclear cells were isolated from 10 ml of bone marrow from each patient and the healthy control donor using lymphocyte separation medium, resulting in a population primarily composed of CD3⁺ lymphocytes for the healthy donor and CD138⁺ cells for the MM patients. The cell density was adjusted to 4 x 10⁵ cells/ml, and cells were divided into the following six groups: the control group (DMSO treatment), 10 nM everolimus, 10 μM bendamustine, 10 nM everolimus + 10 μM bendamustine, 50 μM bendamustine, and 10 nM everolimus + 50 μM bendamustine. The cells were treated for 48 h, after which they were collected, stained with Annexin-V/PI, and analyzed by flow cytometry. The results clearly demonstrate that everolimus enhances the cytotoxicity of bendamustine in primary MM cells. Everolimus enhanced cell death when combined with bendamustine, both at the 10 μM and 50 μM doses and for the MM cells from both the p53-wild-type patients and the p53-deleted patient (Table 1). Significantly less effect was observed for the control mononuclear cells from the healthy donor.

Table 1 Percentage of cells that are non-viable following treatment of primary MM cells with DMSO (control), everolimus (10 nM), bendamustine (Ben, 10 μM or 50 μM), or a combination of the two drugs

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>Control (%)</th>
<th>Everolimus 10 nM (%)</th>
<th>Ben 10 μM (%)</th>
<th>Everolimus 10 nM + Ben 10 μM (%)</th>
<th>Ben 50 μM (%)</th>
<th>Everolimus 10 nM + Ben 50 μM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.68</td>
<td>1.31</td>
<td>0.21</td>
<td>2.35*</td>
<td>2.77</td>
<td>7.35*</td>
</tr>
<tr>
<td>1</td>
<td>0.40</td>
<td>40.96</td>
<td>60.53</td>
<td>78.78*</td>
<td>77.32</td>
<td>87.42*</td>
</tr>
<tr>
<td>2</td>
<td>0.82</td>
<td>12.56</td>
<td>35.44</td>
<td>52.04*</td>
<td>67.34</td>
<td>93.19*</td>
</tr>
<tr>
<td>3</td>
<td>0.36</td>
<td>29.07</td>
<td>55.84</td>
<td>83.92*</td>
<td>79.90</td>
<td>86.14*</td>
</tr>
<tr>
<td>4</td>
<td>1.66</td>
<td>35.44</td>
<td>28.69</td>
<td>59.82*</td>
<td>36.52</td>
<td>69.89*</td>
</tr>
<tr>
<td>5</td>
<td>3.29</td>
<td>15.69</td>
<td>24.54</td>
<td>37.41*</td>
<td>37.34</td>
<td>67.50*</td>
</tr>
<tr>
<td>6</td>
<td>2.73</td>
<td>26.64</td>
<td>30.62</td>
<td>62.70*</td>
<td>43.13</td>
<td>79.09*</td>
</tr>
</tbody>
</table>

Cells were assessed following 48 h treatment by flow cytometry using Annexin-V-FITC/PI staining.

*P < 0.05, χ² test for combination drug treatment vs. bendamustine alone.
donor, suggesting a potential specificity for inhibiting MM-activated pathways.

Discussion

In this study, we assessed the combinatorial effects of bendamustine, a bifunctional alkylating agent, and everolimus, an mTOR inhibitor, for activating cell death pathways in MM cells. Our results showed that these two drugs synergistically suppressed the growth of MM cells, both for the p53-wild-type MM1.S cells and the p53 mutant RPMI8226 cells. For RPMI8226 cells, the IC50 of bendamustine was higher than for MM1.S cells, suggesting that wild-type p53 MM cells may be more sensitive to bendamustine. The synergy between the two drugs was most obvious at low concentrations (Fig. 3), suggesting a potential ability of the drug combination to allow the use of reduced therapeutic levels of drug treatment. Everolimus also enhanced the cytotoxicity of bendamustine in primary MM cells, including both p53-wild-type and p53-deleted primary MM cells. These findings provide a basis for the clinical application of bendamustine and everolimus for both p53-wild-type and p53-deleted MM.

Previous studies have investigated the effects of bendamustine or everolimus alone on MM cells; however, we provide the first mechanistic analysis of the two drugs in combination. Annexin-V/PI staining suggests that cytotoxic effects of the drugs may be mediated mainly through apoptosis. To elucidate the pathways underlying the synergistic effect of bendamustine, we assessed the activation of a variety of proteins known to be involved in cell death pathways. Following combination treatment, caspase-3 and PARP were significantly cleaved, which provides a hallmark event in the activation of apoptosis. Survivin is the most potent anti-apoptotic factor identified thus far and its anti-apoptotic activity is stronger than the members of the Bcl-2 family. At the cellular level, survivin may block caspase-3 and -7 to inhibit apoptosis [29]. Consistent with a role for survivin in the suppression of apoptosis during MM, we showed that the expression of survivin in MM cells was markedly decreased after combination treatment. The above findings indicated that Bax, Mcl-1, and survivin each are likely to play critical roles in the apoptosis induced by combination treatment of MM cells with bendamustine and everolimus.

mTOR is an effector of the PI3K/AKT signaling pathway and plays a key role in the regulation of multiple cellular functions, including proliferation, survival, apoptosis, migration, and angiogenesis [3]. p70s6k and 4EBP-1 are two downstream target proteins of the mTOR pathway. Activated p70s6k and 4EBP-1 can promote cell growth and proliferation by interfering with the initiation and extension of translation [3]. It was reported that everolimus enhances the cytotoxicity of cisplatin, an alternate alkylating agent, in liver cancer cells, through regulation of the expression of p-p70s6k and p-4EBP-1 [30]. Similarly, our study confirmed that everolimus can significantly down-regulate the expression of p-p70s6k and p-4EBP-1 when combined with bendamustine.

p53 is a tumor suppressor gene whose function is closely associated with the occurrence and development of cancers. p53 phosphorylation at serine 15 blocks its interaction with its inhibitor, murine double minute 2 (MDM2) [31], leading to the p53-mediated expression of apoptosis-regulated proteins and suppression of cell cycle-related proteins [32,33]. In the present study, monotherapy and combination treatment with bendamustine and everolimus did not markedly increase the expression of total p53, but the level of phosphorylated p53 was significantly increased after the combination treatment, suggesting a role for p53 pathway suppression in the synergy between the two drugs. p53 activation typically leads to the transcriptional up-regulation of the cell cycle kinase inhibitor p21, resulting in cell cycle arrest as a cytoprotective response. However, in our study, the activation of p53 upon combination treatment was associated with a decrease in p21, suggesting that p21 is inhibited by everolimus significantly in this model. Consistently, there is a basis for the inhibition of p21 translation following treatment with mTOR inhibitors as a mechanism for sensitizing tumor cells to DNA-damaged-induced apoptosis [34]. These results suggest a model whereby combination treatment can down-regulate the expression of p21, which promotes cell cycle progression in the absence of DNA repair. Once damaged DNA cannot be repaired, p53 may act as a checkpoint and directly induce apoptosis.

In summary, our results indicate that everolimus enhances the cytotoxicity of bendamustine both in wild-type p53 and...
mutant-p53 MM cells for cell lines and primary cells derived from MM patients. The synergistic effect of these two drugs involves a pathway of apoptosis activated by a variety of proteins associated with the regulation of pro-survival and pro-proliferation molecules. These findings provide a rationale for the use of mTOR inhibitors in combination with bendamustine as a promising strategy for the treatment of MM.

**Funding**

The study was supported by the grants from the Natural Science Foundation of Guangdong Province (8151008901000064) and the Science and Technology Planning Project of Guangdong Province (2010B060900022).

**References**

