Dyclonine enhances the cytotoxic effect of proteasome inhibitor bortezomib in multiple myeloma cells

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Abstract. The proteasome has become an important target for cancer therapy with the approval of bortezomib for the treatment of relapsed/refractory multiple myeloma (MM). However, numerous patients with MM do not respond to bortezomib and those responding initially often acquire resistance. Recent clinical studies have also demonstrated that bortezomib is also inefficacious in the treatment of other types of cancer. Therefore, it is imperative to develop novel approaches and agents for proteasome-targeting cancer therapy. In the present study, it was revealed that dyclonine, a major component of the cough droplets Sucrets, markedly enhances the cytotoxic effects of bortezomib and minimizes drug resistance in MM cells. It was demonstrated that a combination of bortezomib and dyclonine markedly induced apoptosis of MM cells. The present study suggests a novel therapeutic use of an over-the-counter medicine for the treatment of MM.

Introduction

Proteasomal degradation has a critical role in a wide range of cellular processes, including but not limited to cell cycle control, gene transcription, DNA repair, protein trafficking, antigen presentation and protein quality control (1). Abnormal proteasome activity is implicated in the pathogenesis of cancer and other human diseases (2-4). The observation that inhibition of proteasome activity induced apoptosis preferentially in transformed cells led to the introduction of proteasome inhibitors in clinical trials for cancer therapy (5 and refs. therein). Bortezomib (also known as Velcade® or PS-341), a peptide boronate proteasome inhibitor, was subsequently approved by the US Food and Drug Administration for the treatment of relapsed/refractory multiple myeloma (MM) (6-8). Although bortezomib has become a standard agent for MM and gained certain success, numerous patients with MM do not respond to bortezomib, whereas others often develop resistance (5,8). Therefore, the development of novel approaches are required to increase the efficacy of bortezomib for the treatment of MM.

The molecular basis underlying bortezomib resistance in MM cells is unclear and may be attributed to a number of different mechanisms. For example, mutation or aberrant expression of ABC membrane transporters, such as P-glycoprotein, induces fast excretion and/or reduced uptake of the drug. Induction of functionally redundant or compensatory pathways as a natural defense mechanism may diminish the effect of bortezomib. Other major factors that may contribute to bortezomib resistance include increased drug inactivation and metabolism, subcellular redistribution, and evasion of apoptosis, necrosis, mitotic catastrophe or senescence (9-11). Currently, there is no specific or efficient method to overcome bortezomib resistance in MM or other cancer types due to its complex nature. Current attempts to solve this limitation are mainly focused on combination therapy using a second anticancer drug along with bortezomib. The majority of combined therapies thus far lack a rigorous rationale and fail to highlight synergistic effects. The key to the success of combination therapy is to attack a specific target, which is essential for cell growth and survival when proteasome activity is inhibited. Although it is difficult to directly identify targets for combination therapy in mammalian cells, the yeast *Saccharomyces cerevisiae* appears to be an ideal model system that is used to obtain required information. A number of the cellular pathways, including the proteasome system are conserved from yeast to humans. All open reading frames of the *S. cerevisiae* genome have been annotated and whole-genome technologies are mature in yeast (12,13). In fact, by taking advantage of the outcome of global synthetic lethal analysis and profiling of chemical-genomic interactions in yeast (12-14), it was revealed that dyclonine, a major component of the cough droplets Sucrets, was able to increase the cytotoxic effects of the proteasome inhibitor MG132 in breast cancer cells (15). The present study aimed to examine whether dyclonine may augment the cytotoxic effects of bortezomib and overcome bortezomib resistance in MM cells.

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Materials and methods

Cell culture and cell viability assay. RPMI8226 and bortezomib-resistant RPMI8226.BR MM cell lines were provided by Dr R Orlowski (16). The cells were maintained in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in 5% CO_2 at 37˚C. Cell viability was measured by an 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described (17). Specifically, the cells were inoculated in 96-well plates at 4,000-6,000 cells/well 24 h prior to the treatment with bortezomib, dyclonine or a combination of the two. Dyclonine and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas bortezomib was obtained from LC Labs (Woburn, MA, USA) and dissolved in dimethylsulfoxide. Data were derived from at least three independent experiments each conducted in quadruplicate.

Immunoblotting analysis. The cells were washed three times with ice-cold phosphate-buffered saline and scraped for centrifugation at 1,500 x g for 5 min. The cell pellets were lysed in buffer (50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1X protease inhibitor cocktail). The cell extracts were separated by SDS-PAGE (8% gel), followed by immunoblotting with anti-poly (ADP-ribose) polymerase (PARP; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-β-actin (Sigma-Aldrich) antibodies, respectively. The signals were detected by horseradish peroxidase-conjugated secondary antibody and Visualizer Western Blot Detection kit (Upstate, Lake Placid, NY, USA).

Results

Cytotoxicity analysis. To assess the potential application of dyclonine in combination therapy with bortezomib for MM, RPMI8226 and RPMI8226.BR cell lines were used. First, the cytotoxicity of dyclonine in RPMI8226 and RPMI8226.BR cells was investigated using an MTT assay. Dyclonine appeared to have only a mild effect on the viability of the two cell lines up to the concentration of 15 μM (Fig. 1A and B). Then, it was examined whether dyclonine augmented the cytotoxic effects of bortezomib in RPMI8226 cells. Various doses of bortezomib were used to treat the cells in the absence or presence of 15 μM dyclonine. As demonstrated in Fig. 1A, dyclonine substantially enhanced the cytotoxic effects of bortezomib. Approximately 10 and 30% of RPMI8226 cells were killed by 2 and 5 nM of bortezomib, respectively, in the absence of dyclonine, but the numbers increased to 40 and 75% with the addition of dyclonine.

Overcoming drug resistance. It was then determined whether dyclonine may facilitate in overcoming drug resistance in bortezomib-resistant MM cells. As demonstrated in Fig. 1B, ~8 and 20% of RPMI8226.BR cells died following treated with 20 and 40 nM bortezomib in the absence of dyclonine. The percentage of inviable cells reached 35 and 80% with addition of dyclonine. Therefore, dyclonine was able to reduce bortezomib resistance in MM cells. Consistent with the MTT assay, examination of morphological changes revealed cell

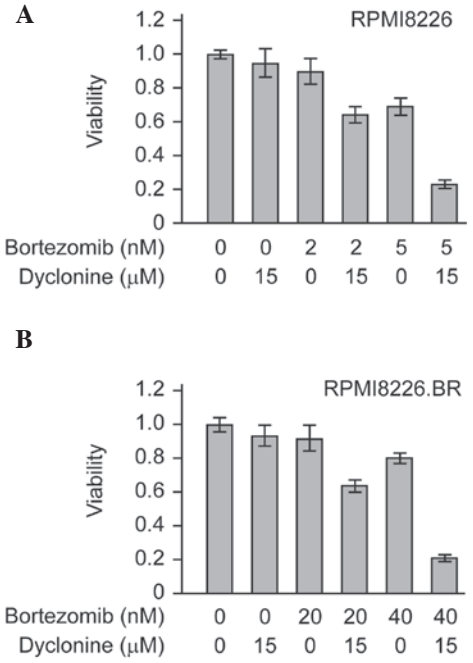

Figure 1. Dyclonine enhances the cytotoxic effects of bortezomib in multiple myeloma cells. (A) RPMI8226 and (B) RPMI8226.BR cells were treated with different concentrations of bortezomib in combination with dyclonine or vehicle for 48 h. The cell viability was measured by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Data are expressed as the means from at least three independent experiments.

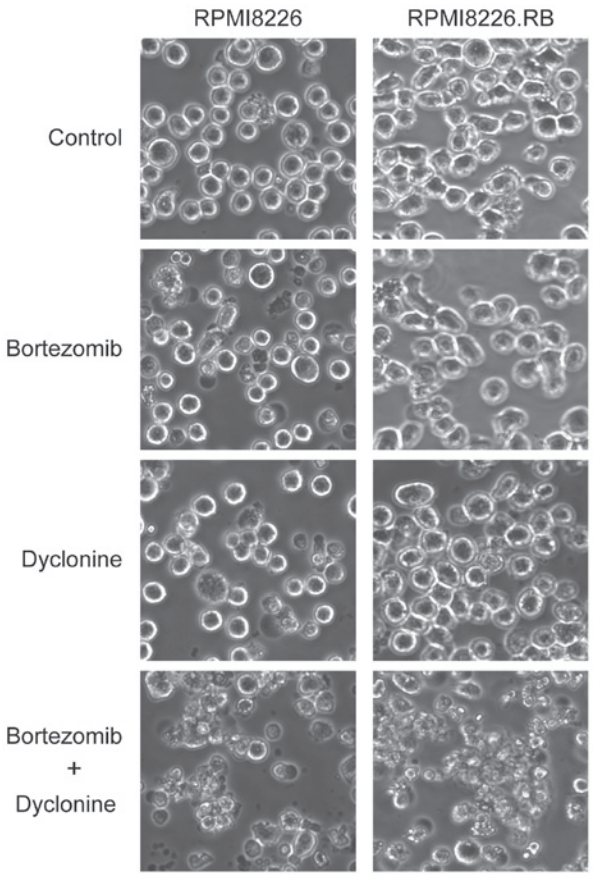

Figure 2. Combined treatment with bortezomib and dyclonine causes cell death. RPMI8226 and RPMI8226.BR cells were treated with bortezomib (2 nM for RPMI8226 and 20 nM for RPMI8226.BR), dyclonine (15 μM) or a combination of the two. The cell morphological changes were examined by microscopy (x100).

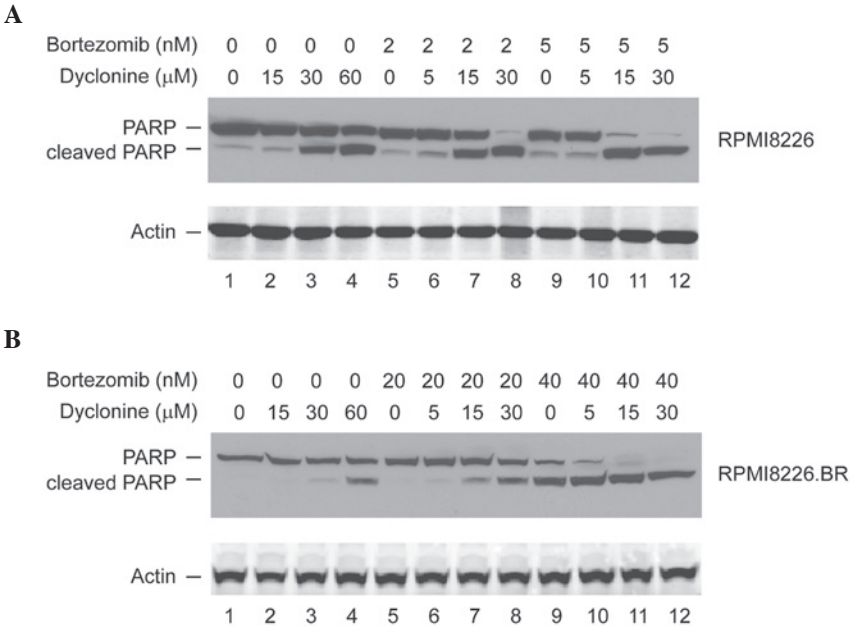

Figure 3. Combination of bortezomib with dyclonine induces cleavage of PARP. (A) RPMI8226 and (B) RPMI8226.BR cells were treated with various doses of bortezomib or dyclonine or a combination of the two as indicated. The cell extracts were subjected to immunoblotting analysis with anti-PARP and anti-actin antibodies, respectively. PARP, poly (ADP-ribose) polymerase.

death following simultaneous treatment with bortezomib and dyclonine (Fig. 2). By contrast, the treatment with individual agents did not result in cell death. Taken together, these results suggest that dyclonine enhanced the cytotoxic effects of bortezomib and minimized the drug resistance in MM cells.

Induction of apoptosis following combination treatment. To examine the induction of apoptosis by the combined treatment with bortezomib and dyclonine, the cleavage of PARP was examined using immunoblotting analysis. A small fraction of PARP was constantly cleaved in RPMI8226 cells in the absence of bortezomib and dyclonine (Fig. 3A, lane 1). The addition of dyclonine to the medium up to a concentration of 15 μM did not induce PARP cleavage (Fig. 3A, comparing lanes 1 and 2). Exposure of the cells to bortezomib at 2 and 5 nM only marginally increased the cleavage of PARP (Fig. 3A, comparing lanes 1, 5 and 9). Notably, a marked increase in cleaved PARP product was detected when the cells were simultaneously treated with bortezomib (2 or 5 nM) and 15 μM dyclonine (Fig. 3A, comparing lanes 2, 5, 7, 9 and 11). In addition, dyclonine at high concentrations (30 or 60 μM) also induced cleavage of PARP, but to a notably weaker extent compared with that caused by the combined treatment (Fig. 3A, comparing lanes 3, 4, 8 and 12). Similarly, the cleavage of PARP in RPMI8226.BR cells was markedly increased by the combined treatment (Fig. 3B). These results indicate that a combination of bortezomib and dyclonine has a greater potential to induce apoptosis in MM cells than each individual agent alone.

Discussion

While the proteasome has emerged as an important target for cancer therapy, it remains a challenging task to improve the efficacy of proteasome inhibitors, such as bortezomib, and to overcome the occurrence of drug resistance (18). Combination therapy is in principle a promising approach to increase the therapeutic effects of bortezomib. The current strategy in preclinical studies and in clinical trials is an application of a second anticancer agent along with bortezomib, and rarely produces a satisfactory synergistic effect (5,8). Taking advantage of the research conducted in budding yeast (12-14), we hypothesized that dyclonine may have a synergistic effect with proteasome inhibitors in mammalian cells, and in fact, we previously demonstrated that dyclonine enhanced the cytotoxic effects of MG132 in breast cancer cells (15). The present study further demonstrated that dyclonine augments the cytotoxic effects of bortezomib and reduces drug resistance in MM cell lines.

It is currently unclear how dyclonine enhances the cytotoxic effects of bortezomib. Two possible mechanisms have been considered. First, dyclonine may facilitate bortezomib-induced apoptosis. It is well established that the inhibition of the proteasome induces apoptosis in cancer cells (5). Previous studies have suggested that dyclonine is an inhibitor of the C-14 sterol reductase Erg24, which is essential for ergosterol biosynthesis in yeast (19). There are two Erg24 homologs in human cells with C-14 sterol reductase activity, including 3β-hydroxysterol C-14 reductase and the lamin B receptor (20,21). Dyclonine likely inhibits the lipid biosynthesis pathway and therefore, may impair the fluidity of cellular membranes, i.e. plasma and mitochondrial membranes, in cancer cells. This may augment the induction of apoptosis by inhibiting proteasome activity. Second, dyclonine may have a synergistic effect with bortezomib in deregulating lipid metabolism. Recent studies have demonstrated that endoplasmic reticulum-associated degradation (ERAD) has a crucial role in feedback regulation of cholesterol synthesis (22,23). When sterols are depleted, the membrane-bound transcription factors (SREBPs) are activated

by ERAD in which SREBPs are cleaved by the proteasome and the N-terminal fragments are released from the membrane and enter the nucleus to induce the genes required for cholesterol synthesis. By contrast, when sterols build up in the cell, activation of SREBPs by ERAD is inhibited. It is therefore hypothesized that bortezomib inhibits the ERAD process and, as a consequence, prevents the activation of SREBPs. Therefore, the inhibition of cholesterol synthesis by dyclonine is not compensated by the ERAD-mediated feedback mechanism. While further studies are required to decipher the molecular basis of the interaction between bortezomib and dyclonine, the present study unveils a potential novel use of dyclonine in combination therapy with bortezomib for patients with MM.

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