Establishment and characterization of bortezomib-resistant U266 cell line: Constitutive activation of NF-κB-mediated cell signals and/or alterations of ubiquitylation-related genes reduce bortezomib-induced apoptosis

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Abstract
Bortezomib has been known as the most promising anti-cancer drug for multiple myeloma (MM). However, recent studies reported that not all MM patients respond to bortezomib. To overcome such a stumbling-block, studies are needed to clarify the mechanisms of bortezomib resistance. In this study, we established a bortezomib-resistant cell line (U266/velR), and explored its biological characteristics. The U266/velR showed reduced sensitivity to bortezomib, and also showed cross-resistance to the chemically unrelated drug thalidomide. U266/velR cells had a higher proportion of CD138 negative subpopulation, known as stem-like feature, compared to parental U266 cells. U266/velR showed relatively less inhibitory effect of prosurvival NF-κB signaling by bortezomib. Further analysis of RNA microarray identified genes related to ubiquitination that were differentially regulated in U266/velR. Moreover, the expression level of CD52 in U266 cells was associated with NF-κB-mediated cell signals and/or alterations of ubiquitylation-related genes reduce bortezomib-induced apoptosis.

INTRODUCTION
The acquisition of anti-cancer drug resistance is a major issue with therapies in multiple myeloma (MM) (1). Studies focusing on the mechanisms of chemoresistance (2, 3) have helped us to understand the molecular pathogenesis of MM. Also, such efforts have led to bortezomib (PS-341, Velcade®) one of the most successful anti-cancer drugs, improving the clinical outcome of MM (4, 5). Although it has exhibited clinical success, some patients failed to respond to bortezomib, due to primary refractoriness, and acquisition of resistance (7).

The study of resistance to bortezomib has involved the elucidation of intrinsic mechanisms in cancer cells adapted to bortezomib in vitro. The mutation in the proteasome β5 subunit (PSMB5), and the increased expression of proteasome, have been shown in cancer cells with acquired resistance to bortezomib (8). Activation of NF-κB with inactivating abnormality of TNF receptor-associated factor 3 (TRAF3), in MM cells harboring genetic mutation of NF-κB pathways, correlated with bortezomib sensitivity (9). Extrinsic factors, bone marrow (BM) microenvironments can confer resistance to bortezomib, mediated by bone marrow stromal cells (BMSCs)-enhanced NF-κB activity (10, 11). However, to date, little is known about the mechanisms of bortezomib resistance. Therefore, it is necessary to identify the functional characteristics of resistant cells, to better understand the mechanisms.

In this study, we used soft agar assay, to isolate bortezomib-resistant U266 (U266/velR). The U266/velR had increased p-ERK and p-p65 following exposure to bortezomib, and less inhibitory effect of NF-κB, that resulted in the cells having less apoptotic effect by bortezomib. Moreover, the U266/velR cells showed an increased CD138 negative subpopulation, known as cancer-initiating cells with stem cell properties, characterized by quiescent cells and chemoresistance. We further analyzed the patterns of gene expressions, to identify molecular targets associated with bortezomib resistance. The expressions of proteasome subunit genes, including PSMB5, as known for the primary target of bortezomib, were not significantly changed in U266/velR; but genes involved in ubiquitination, such as transcription elongation factor B1 (TCEB1) and 2...
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Results

Establishment of bortezomib-resistant cell line (U266/velR)
To establish bortezomib-resistant cell lines, three different MM cells (U266, RPMI-8226, and IM9) were grown in soft agar plates, in the presence of 10 nM bortezomib. Only U266 colonies were visible on the soft agar plate, after 3-4 weeks of incubation. Pooled U266 colonies were subsequently plated on new soft agar plate, with 10 nM bortezomib (Fig. 1A). After 2 weeks, the colonies promptly grew again in agar plate (Fig. 1B). The bortezomib-resistant cell line (U266/velR) was grown and maintained in culture medium (RPMI 1640 containing 10% FBS), with 2 nM bortezomib. First, to confirm resistance to bortezomib in U266/velR, we tested the parental U266 and U266/velR, for sensitivity to bortezomib. U266/velR had less sensitivity to bortezomib-induced cell cytotoxicity, compared to the parental cells. In addition, U266/velR showed cross-resistance to thalidomide. U266/velR was 1.5 fold more resistant to both bortezomib and thalidomide, than their parental cells were (Fig. 1C). We further examined the effect of bortezomib-induced apoptotic signal in U266/velR. Treatment of bortezomib led to PARP cleavage and reduction of procaspase-3 expression, as well as induction levels of caspase-3 activities in the parental cells. The effects were substantially reduced in U266/velR (Fig. 1D and E).

NF-κB-mediated acquired bortezomib resistance in U266/velR
To assess changes in activation of cell signaling in U266, during acquisition of resistance to bortezomib, U266 cells that were cultured for 2 weeks with low dose of bortezomib (2 nM) were monitored for activation of ERK and NF-κB, by Western blotting. U266 cells that survived bortezomib pressure gradually increased pERK and p-p65 levels (Fig. 2A). Next, we further examined if the constitutive activation of NF-κB serves to blunt the efficacy of bortezomib. U266 cells dramatically reduced p65 and IκBα expression with exposure to bortezomib; whereas, their expression persisted in U266/velR that were treated with 2 nM bortezomib (Fig. 2B).

U266/velR cells contained higher proportion of CD138+
It has been postulated that a tumor-initiating population,