

KSP inhibitor SB743921 induces death of multiple myeloma cells via inhibition of the NF- κ B signaling pathway

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SB743921 is a potent inhibitor of the spindle protein kinesin and is being investigated in ongoing clinical trials for the treatment of myeloma. However, little is known about the molecular events underlying the induction of cell death in multiple myeloma (MM) by SB743921, alone or in combination treatment. Here, we report that SB743921 induces mitochondria-mediated cell death via inhibition of the NF- κ B signaling pathway, but does not cause cell cycle arrest in KMS20 MM cells. SB743921-mediated inhibition of the NF- κ B pathway results in reduced expression of SOD2 and Mcl-1, leading to mitochondrial dysfunction. We also found that combination treatment with SB743921 and bortezomib induces death in bortezomib-resistant KMS20 cells. Altogether, these data suggest that treatment with SB743921 alone or in combination with bortezomib offers excellent translational potential and promises to be a novel MM therapy. [BMB Reports 2015; 48(10): 571-576]

INTRODUCTION

Multiple myeloma (MM) is a type of hematological malignancy characterized by abnormal plasma cells in the bone marrow (1). In order to treat MM, it is important to develop anticancer drugs targeting abnormal plasma cells. Since the introduction of combination therapy involving melphalan and prednisone, numerous multidrug chemotherapies employing agents such as dexamethasone, thalidomide, nitrosoureas, and bortezomib have been used to treat MM (2, 3). However, many compounds have been reported to have resistance to anticancer activities when

used as single treatment because of the multistep process of tumorigenesis (4, 5). Thus, it is required to develop novel compounds and strategies of optimized combinations of these drugs to improve the survival of MM patients.

The spindle protein kinesin (KSP, human Eg5) is one of several microtubule-associated motor proteins required for proper spindle dynamics (6). KSP is essential for the formation of the bipolar spindle and proper segregation of sister chromatids during mitosis (7, 8). Furthermore, it is required for other critical physiological functions, such as intracellular transport and organelle positioning (9). Consequently, KSP-targeted novel antimetabolic agents have been developed to cancer therapy, and several KSP inhibitors have entered clinical trials.

KSP inhibitors have been developed as a new generation of antimetabolic agent with a novel mechanism of action for cancer therapy, and several KSP inhibitors have entered clinical trials. These KSP inhibitors, such as ispinesib (SB715992), ARRY-520, and MK0731, provide new opportunities for the development of hematological malignancy therapeutics. In particular, SB743921 is a selective KSP inhibitor that is being investigated in ongoing clinical trials for the treatment of myeloma, leukemia, and solid tumors (10, 11). However, little is known about the molecular events underlying the induction of cell death in multiple myeloma (MM) by SB743921, alone or in combination with other agents.

In this study, we first investigated the antimyeloma activity and mechanism of action for SB743921 in human MM cells. We found that this compound induces mitochondria-mediated cell death via inhibition of the NF- κ B signaling pathway in these cells. To maximize the efficacy of SB743921 in treating MM, we investigated the antimyeloma activity by combined treatment with SB743921 and bortezomib in KMS20 bortezomib-resistant cells and found that the combination treatment induces cell death in KMS20 cells. Here, we present evidence that the mechanism of action of SB743921 is the inhibition of SOD2 and MCL-1 expression, which leads to bortezomib resistance.

RESULTS

SB743921 potently induces cell death in multiple myeloma cells

We first examined the capability of SB743921 to induce cell

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death using a CCK-8 assay. As shown in Fig. 1A, SB743921 induced cell death in a dose-dependent manner. SB743921-treated cells also displayed an abrupt time-dependent increase in cell death, visualized by annexin V/propidium iodide (PI) staining (Fig. 1B). Interestingly, SB743921 treatment induced cell death of more than 30% even at an early time point (12 h) and low concentration (1 nM). Further, cell death was not significantly altered between 24 h and 48 h. We next measured the change in cell death based on dose and duration of treatment using annexin V/PI staining (Fig. 1C). Cell death increased from 30% to 50% between 12 h and 24 h. However, consistent with the data shown in Fig. 1A, cell death did not increase with an increase in dose from 1 nM to 2 nM. Thus, the optimal dose and duration of treatment for SB743921-induced MM cell death were 1 nM and 24 h, respectively, and these conditions were used in subsequent experiments. We next used western blot analysis to determine whether SB743921 treatment modified caspase-3, caspase-8, and poly-(ADP-ribose) polymerase (PARP) levels in a time-dependent manner (Fig. 1D). Western bands for cleaved caspase-8, caspase-3, and PARP increased in density with SB743921 treatment in a time-dependent manner. Conversely, levels of procaspase-3, -8, and -9 gradually decreased with SB743921 treatment. Next, we examined whether mitochondria were involved in SB743921-induced cell death. In the mitochondrial apoptotic pathway, the signal mediator between the cytosol and the mitochondrion is cytochrome c. Thus, cytochrome c release was measured in SB743921-treated KMS20 cells (Fig. 1E). Cytochrome c release into the cytosol was markedly increased in

cells treated with SB743921. As a result of this release, caspase-3 cleavage was subsequently enhanced in KMS20 cells. Finally, we investigated whether SB743921 induces cell death in human primary bone marrow mononuclear cells. As shown in Fig. 1F, SB743921 did not induce cell death in these cells. Collectively, these results indicate that treatment with SB743921 alone specifically induces cell death in MM cells.

SB743921 induces cell cycle arrest at early time points, but G2M arrest may not lead to cell death in MM cells

To determine the effect of SB743921 on the cell cycle, we performed cell cycle analysis in KMS20 cells treated with 0.5 nM and 1 nM SB743921. A time-course analysis showed that cell cycle arrest was detectable prior to cell death. In the case of cells treated with 0.5 nM SB743921, G2M arrest was detectable at 6 h and similarly decreased at 12 h and 24 h, whereas cell death (subG1 phase) was detectable at 12 h (Fig. 2). However, G2M arrest in 1 nM-treated cells was detectable at 6 h and gradually decreased at 12 h-24 h, whereas cell death (subG1 phase) was detectable at 12 h and more pronounced at 24 h (Fig. 2B and C). This means that dead cells were primarily derived from the G2M-arrested population at a low concentration of SB743921, whereas cell death is independent of cell cycle status at a lethal dose. Next, we examined the level of p53 in SB743921-induced cell death. It has been suggested that an increase in p53 level is required for maximal cell sensitivity to microtubule-targeting agents (12, 13). However, p53 level was not increased in KMS20 cells treated with SB743921 (Fig. 2D). Collectively, our results suggest that SB743921 induces

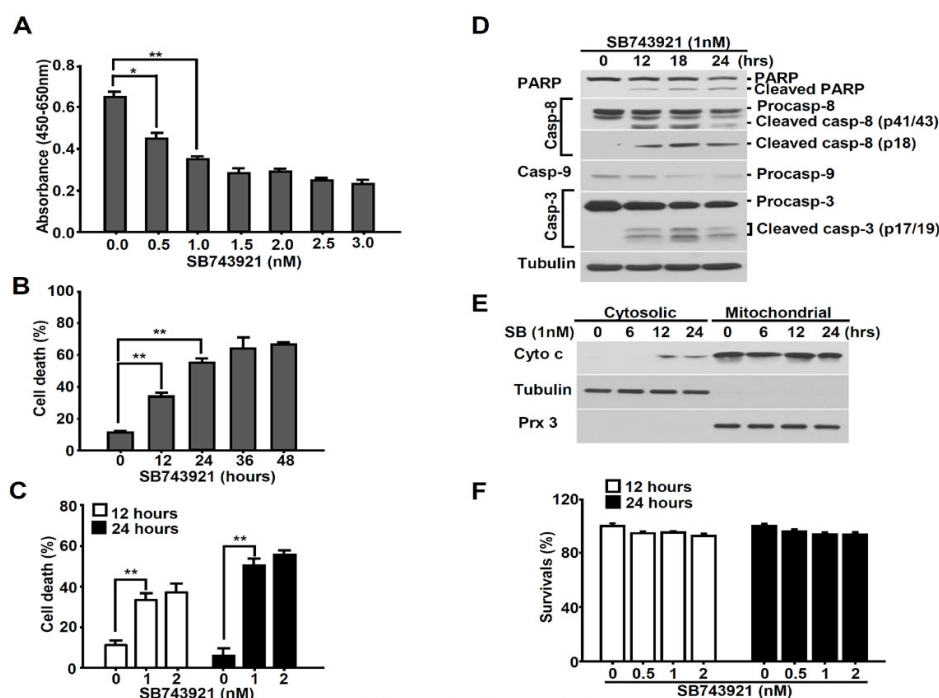


Fig. 1. SB743921 induces dose- and time-dependent cell death in MM cells. (A) Cells were treated with the indicated dose of SB743921, and cell death was determined at 24 h by CCK-8 assay. (B) KMS20 cells were treated with 1 nM SB743921 at the indicated time points, and cell death was determined by annexin V/PI staining. (C) KMS20 cells were treated with the indicated dose of SB743921, and cell death was determined at 12 h and 24 h by annexin V/PI staining. (D) KMS20 cells were treated with 1 nM SB743921 for the indicated times and subjected to western blotting using the indicated antibodies. (E) KMS20 cells were treated with 1 nM SB743921 for the indicated periods of times, and cell lysates were separated into cytosolic and mitochondrial fractions. Tubulin and peroxiredoxin 3 (Prx3) were used as cytosolic and mitochondrial markers, respectively. (F) To compare SB743921-induced cell death in a non-MM cells, human primary bone marrow mononuclear cells were treated with the indicated dose of SB743921 for the indicated times, and then subjected to CCK-8 assay to measurement of cell death.

cell death independent of p53-dependent cell cycle arrest.

SB743921 induces cell death via inhibition of the NF- κ B signaling pathway

NF- κ B is aberrantly activated in a wide range of human cancers, including MM, in which it promotes cell survival and malignancy by upregulating antiapoptotic genes (14, 15). Compelling evidence has established the paramount importance of aberrant NF- κ B sig-

naling in MM pathogenesis. To investigate the possible contribution of the NF- κ B signaling pathway to this process, we investigated whether I κ B α phosphorylation is decreased by SB743921 treatment. In the case of KMS20 cells, NF- κ B signaling was constitutively activated. As shown in Fig. 3A, I κ B α phosphorylation was clearly decreased in SB743921-treated KMS20 cells. Especially, the phosphorylation of I κ B α was dramatically decreased at 24 h after SB743921 treatment. Next, KMS20 cells were

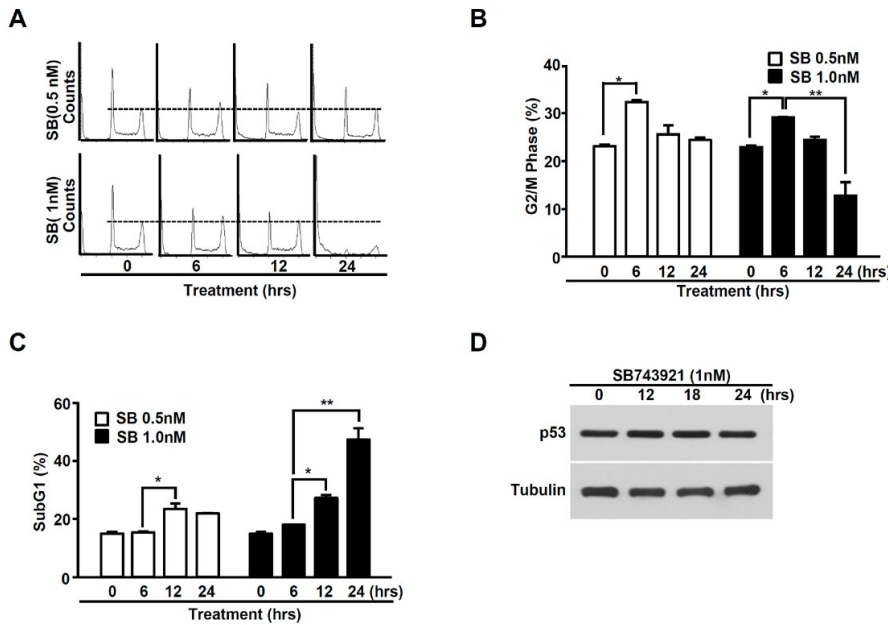


Fig. 2. SB743921 induces G2M arrest at an early time point prior to cell death. (A-C) KMS20 cells were incubated with the indicated doses of SB743921 for the indicated length of time, followed by FACS analysis of cell cycle distribution (A). Quantification of G2M arrest in (A) (B). Quantification of subG1 content in (A, C). (D) KMS20 cells were treated with 1 nM SB743921 for the indicated length of time, and cells were analyzed by western blotting using anti-p53 and anti-tubulin antibodies.

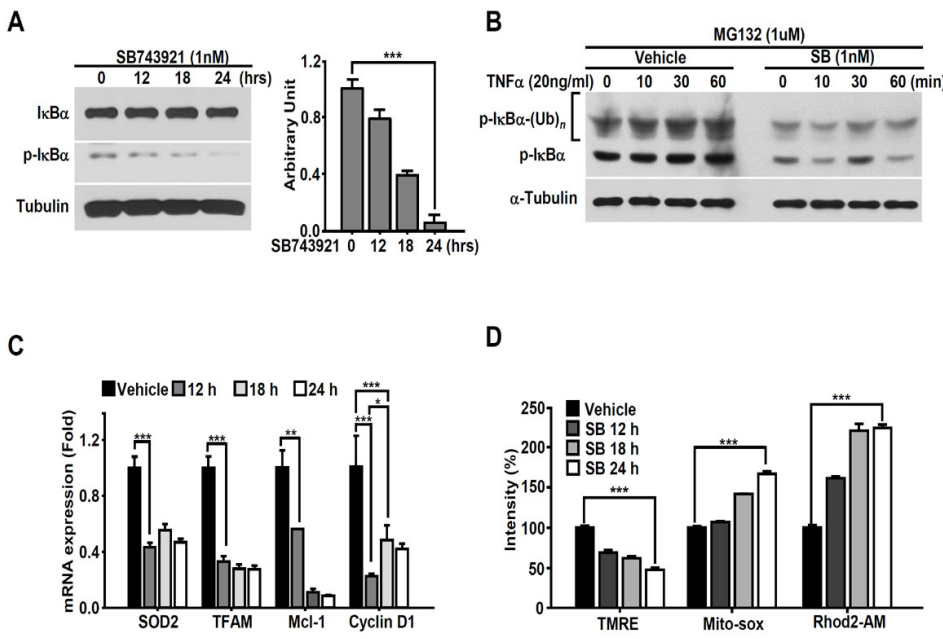


Fig. 3. SB743921 inhibits the NF- κ B pathway, resulting in suppression of transcriptional activation of mitochondria-related genes, such as Mcl-1 and SOD2. (A) KMS20 cells were treated with SB743921 for the indicated times and immunoblotted using anti-I κ B α and anti-phospho-I κ B α antibodies. (B) SB743921-pretreated KMS20 cells for 12hrs were stimulated with TNF- α (20 ng/ml) for the indicated times in the absence or presence of the proteasome inhibitor MG132 (1 hrs) and immunoblotted using an anti-phospho-I κ B α antibodies. The polyubiquitinated species of phospho-I κ B α are shown. (C) Relative expression levels of SOD2, TFAM, Mcl-1, and cyclin D1 in KMS20 cells pretreated with SB743921 for the indicated times were measured using qPCR. B2M served as the internal control. (D) Comparisons of mitochondrial calcium, ROS, and membrane potential in KMS20 cells treated with SB743921 for the indicated times generated using a FACSCanto II.

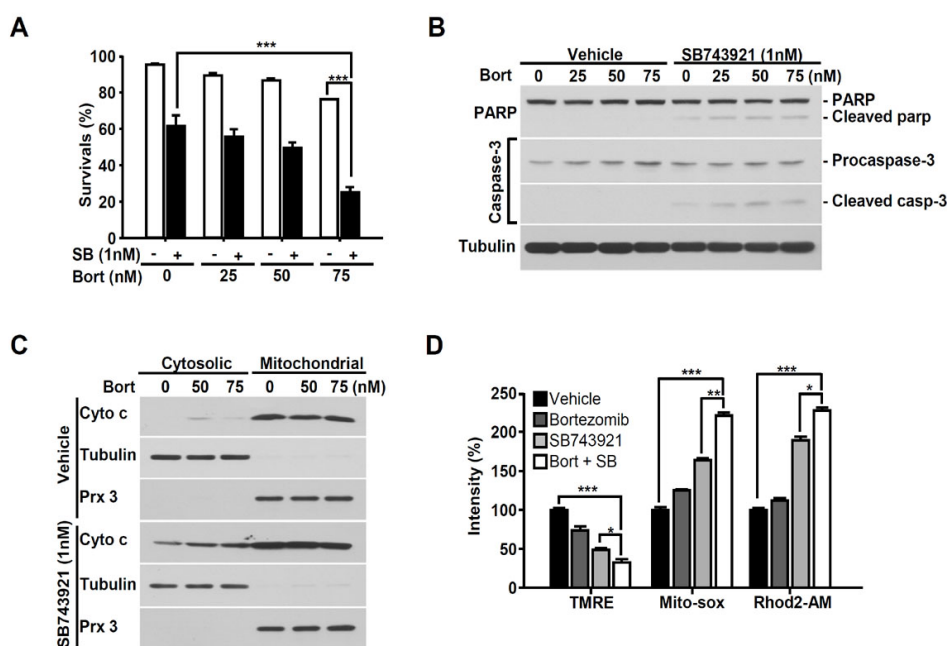


Fig. 4. Effects of combination treatment with SB743921 and bortezomib on apoptosis of KMS20 cells. (A) KMS20 cells were treated with SB743921 combined with bortezomib for 24 h at the indicated doses, and cell death was determined by annexin V/PI staining. (B) KMS20 cells were treated with SB743921 combined with bortezomib for 24 h at the indicated doses and analyzed by western blotting using the indicated antibodies. (C) KMS20 cells were treated with SB743921 combined with bortezomib for 24 h, and the cells were separated into cytosolic and mitochondrial fractions. Tubulin and Prx3 served as cytosolic and mitochondrial markers, respectively. (D) Mitochondrial calcium, ROS, and membrane potential in KMS20 cells treated with SB743921 and bortezomib for the indicated times were measured using a FACSCanto II.

co-treated with a proteasome inhibitor (MG-132) to determine whether the effect was due to a change in NF- κ B activation (Fig. 3B). In the presence of MG132, SB743921-pretreated cells showed a dramatic reduction in I κ B α phosphorylation. In contrast, the control cells showed a robust increase in the level of phosphorylated I κ B α , including the polyubiquitinated species, consistent with the activation of endogenous NF- κ B signaling. Additionally, induction of the endogenous NF- κ B target genes was examined in SB743921-treated cells. Levels of superoxide dismutase (SOD) 2; cyclin D1; TFAM; and an antiapoptotic Bcl-2 homologue, Mcl-1; were significantly reduced in SB743921-treated cells compared with control cells (Fig. 3C). Lastly, we examined whether SB743921-induced cell death was occurred through the mitochondrial dysfunction by the inhibition of the NF- κ B signal pathway. As shown in Fig. 3D, mitochondrial ROS and calcium levels were increased by treatment with SB743921, and resulted in depolarization of the mitochondrial membrane potential. Collectively, these results show that SB743921 is an inhibitor of NF- κ B signaling that mediates NF- κ B transcriptional activation.

Combined treatment with SB743921 and bortezomib overcomes bortezomib resistance

In Fig. 1, we showed that cell death was induced by treatment of SB743921. However, SB743921-induced cell death did not increase by more than 60%, even at a concentration of 3 nM or after 48 h of treatment in KMS20 cells. In a previous study, we reported that mitochondria play critical role in resisting cell death by bortezomib, and the potentiation of mitochondrial function resulted in the resistance of KMS20 cells to bortezomib (16). Moreover, we have showed that alteration of mitochondria-related genes ex-

pression led to changes in mitochondrial function and resistance or susceptibility to bortezomib; these alteration contributed to discriminative susceptibility or resistance to bortezomib in MM cells. Thus, the powerful activity of SB743921 in inducing cell death in MM (in even bortezomib-resistant cells) suggests the combination of SB743921 and bortezomib would increase therapeutic potency (Fig. 4). KMS20 cells were treated with 1 nM SB743921 and different concentrations of bortezomib for 24 h followed by labeling with annexin V-FITC/PI and flow cytometric analysis. As shown in Fig. 4, the combination of bortezomib with SB743921 showed strong additional cytotoxicity in KMS20 bortezomib-resistant cells, whereas the respective monotherapies had no or a moderately cytotoxic effect. To verify whether the cell death by combination treatment were induced by apoptosis, we performed immunoblot analysis with bortezomib or combination treatment in KMS20 cells. Immunoreactive bands for cleaved caspase-3 and PARP were more intense in lysates of combination-treated cells than in those of cells treated with bortezomib alone (Fig. 4B). Finally, we examined whether mitochondria were involved in combination treatment-induced cell death. Cytochrome c release into the cytosol was markedly increased in cells treated with both compounds compared with those treated with bortezomib alone (Fig. 4C). Finally, we examined whether combination treatment with SB743921 and bortezomib induced mitochondrial dysfunction in KMS20 cells. As shown in Fig. 4D, mitochondrial ROS and calcium levels were significantly increased by combination treatment compared to single treatment, and resulted in the depolarization of mitochondrial membrane potential. Collectively, these results indicate that combination treatment with SB743921 and bortezomib induces a cell death mechanism in bortezomib-resistant KMS20 cells.

DISCUSSION

SB743921 is being investigated in clinical trials for the treatment of myeloma, leukemia, and solid tumors, but its mechanism of action is unknown. Here, we show that SB743921 has significant cytotoxic activity in MM cells, supporting its potential use for therapeutic intervention in MM. Additionally, we demonstrated the induction of cell death specifically in MM cells, and not in human primary bone marrow cells (Fig. 1F). This study demonstrated that SB743921 inhibits the NF- κ B signaling pathway without the impairing of cell cycle progression at an early time point, leading to MM cell death via activation of the mitochondria-mediated pathway. This effect is independent of both p53 level and cell cycle arrest. SB743921 strongly inhibited transcriptional activation of NF- κ B target genes Mcl-1 and SOD2 (15, 17, 18). Inhibition of the NF- κ B survival pathway is possible and constitutes a promising candidate therapy that has no preclusive toxicity and could be of profound benefit for patients with MM (19).

Dipeptidyl-boronic acid derivative, bortezomib inhibits the proteasomes activity and is used in patients with relapsed/refractory MM. Unfortunately, such MM patients have a low susceptibility to agents, and resistance have frequently occurred in early stage of treatment (20, 21). Here, we showed that the combination treatment of bortezomib and SB743921 induces cell death via inhibition of the NF- κ B pathway in bortezomib-resistant MM cells. Human KMS20 MM cells were quite resistant at a high concentration bortezomib (22). In previous study, we reported that bortezomib resistance occurs because of changes in the expression of important mitochondrial genes, such as cyclophilin D, SOD2, and the mitochondrial calcium uniporter (16). Thus, SB743921-induced cell death may be the result of mitochondrial dysfunction via suppression of SOD2 and Mcl-1, induced by inhibition of the NF- κ B pathway. However, the mechanism to NF- κ B inhibition by SB743921 is not yet clear. Activation of the PI3K/AKT pathway, which is mostly related to signaling from major growth factors in MM (23), is also not yet verified in response to SB743921. Therefore, future research topics include examining potential candidates linking SB743921 to the IKK/NF- κ B signaling cascades and PI3K/AKT activation.

In summary, our study reports that SB743921 alone and in combination with bortezomib induce cell death of myeloma cells as a novel therapeutic tool for relapsed/refractory MM. SB743921 alone and combined treatment with bortezomib facilitates reduction in protein levels of Mcl-1 and SOD2 due to the inhibition of the NF- κ B pathway, and induces cell death in MM. Collectively, our results offer that treatment with SB743921, alone or in combination with bortezomib, offers excellent translational potential and novel strategies for MM therapy.

MATERIALS AND METHODS

Reagents, Cell culture, Measurement of cell death, Measurement of mitochondrial ROS, membrane potential ($\Delta\psi_m$), and calcium concentration are described in the online

data supplement, available at <http://www.bmbreports.org/>.

Western analysis and subcellular fractionation

Cells were lysed in lysis buffer A [20 mM HEPES (pH 7.5), 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and protease cocktail I/II (Sigma-Aldrich)]. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked in 5% skim milk in 0.01 M Tris-buffered saline (pH 7.5) containing 0.5% Tween 20 and then incubated with the appropriate primary antibodies. For cytochrome c release after treatment with SB743921 alone or in combination with bortezomib, subcellular fractions were prepared using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, La Jolla, CA, USA). The purity of each fraction was verified using α -tubulin and peroxiredoxin 3 (Prx3) as specific markers of cytosol and mitochondria, respectively (16).

Measurement of cell cycle distribution

Cell cycle distribution was determined by DNA staining with PI (Sigma-Aldrich). Cells (1×10^6) were collected and fixed in 70% ethanol. The cells were suspended in PI and simultaneously treated with RNase at 37°C for 30 min (24). The proportion of cells in each phase of the cell cycle was measured using a FACSCanto II flow cytometer (BD Biosciences).

Quantitative PCR (qPCR)

qPCR was performed using the first strand complementary DNA as template and specific primers for SOD2 (forward, 5'-ggaagc-catcaaacgtgact-3'; reverse, 5'-acacatcaatccccagcagt-3'), Mcl-1 (forward, 5'-agaaagctgcatcgaacat-3'; reverse, 5'-ccagctc ctactcagcaac-3'), TFAM (forward, 5'-ccgaggtggtttcatctgt-3'; reverse, 5'-tccgcctataagcatcttg-3'), B2M (forward, 5'-ctcgctccgtggccttag-3'; reverse, 5'-caatgaggcgcattcctca-3'), cyclin D1 (forward, 5'-caatgaccccgacgattc-3'; reverse, 5'-catggaggcggattggaa-3'). Amplification was carried out using the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in 15- μ l reaction mixtures containing 2 μ l diluted DNA template, 2 pmol each primer, and 7.5 μ l 2x SYBR®Premix Ex Taq™ (TaKaRa, Shiga, Japan). B2M was used as a control, and all reactions were performed in triplicate. Quantification of relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method (25).

Statistical analysis

Data were analyzed using Student's t-test and SigmaPlot 8.0 software; P values were derived to assess statistical significance, as follows: *P < 0.05; **P < 0.01; and ***P < 0.001. Data in all figures are expressed as the mean \pm standard deviation (SD) of three independent experiments.

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