0026-895X/05/6706-1991–1998$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 6
7021/3031985

Mol Pharmacol 67:1991–1998, 2005
Printed in U.S.A.

Farnesyltransferase Inhibitor BMS-214662 Induces Apoptosis in Myeloma Cells through PUMA Up-Regulation, Bax and Bak Activation, and Mcl-1 Elimination

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Received September 17, 2004; accepted February 28, 2005

ABSTRACT

We have studied the mechanism of apoptosis elicited by the farnesyltransferase inhibitor (*R*)-7-cyano-2,3,4,5-tetrahydro-1-(1*H*-imidazol-4-ylmethyl)-3-(phenylmethyl)-4-(2-thienylsulfonyl)-1*H*-1,4-benzodiazepine (BMS-214662) in human myeloma cell lines. Low concentrations of BMS-214662 efficiently inhibited protein farnesylation but did not affect the activation of Akt. BMS-214662 treatment increased levels of the BH3-only protein PUMA; induced proapoptotic conformational changes of Bax and Bak; reduced Mcl-1 levels; caused mitochondrial transmembrane potential loss; induced cytochrome *c* release, caspase activation, apoptosis-inducing factor (AIF) nuclear translocation, and phosphatidylserine exposure; and allowed the development of apoptotic morphology. Western blot analysis of cell extracts revealed the activation of caspases 2, 3, 8, and 9 upon treatment with BMS-214662. The general caspase inhibitor Z-VAD-fmk significantly prevented BMS-214662–induced death in U266 and RPMI 8226 cells but not in NCI-H929 cells. A mixture of selective caspase inhibitors for caspases 9 [*N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethyl ketone (Z-LEHD-fmk)], 3 (Z-DEVD-fmk), and 6 (Z-VEID-fmk) approached the protective effect of Z-VAD upon cell death. However, Z-VAD-fmk did not prevent BMS-214662–induced Bax and Bak activation and decrease of Mcl-1 levels. According to its effect on cell death, Z-VAD-fmk inhibited nuclear translocation of AIF in RPMI 8226 and U266 but not in NCI-H929 cells. These results suggest that apoptosis triggered by BMS-214662 is initiated by a PUMA/Bax/Bak/Mcl-1–dependent mechanism. In some cell lines, Bax/Bak activation is not sufficient per se to induce mitochondrial failure and release of apoptogenic proteins, and so caspases need to be activated to facilitate apoptosis. After $\Delta\Psi_m$ loss, execution of apoptosis was performed in all cases by a cytochrome *c*-enabled, caspase-9–triggered, caspase cascade and the nuclear action of AIF.

Farnesyltransferase is a cytosolic enzyme that catalyzes the transfer of the 15-carbon isoprenoid chain from the farnesyl pyrophosphate to the cysteine residue of a conserved CAAX sequence at the carboxyl terminus of proteins. This allows protein association to cell membranes via the farnesyl group. Substrates of farnesyltransferase include Ras (Haluska et al., 2002), which critically control proliferative signals in normal and malignant cells; RhoB, a small G-protein involved in control of actin cytoskeleton and endocytosis (Du et al., 1999); the cytosolic chaperone HDJ-2 (Adjei et al., 2001); peroxysomal protein PxF; and lamins A and B (Haluska et al., 2002). Because Ras proteins require farnesylation to localize at the plasma membrane and become biologically active, a number of farnesyltransferase inhibitors (FTIs) have been developed as potential cancer therapeutics (Sebti and Hamilton, 2000). In addition to Ras, current evidence suggests that the anticancer activity of the FTIs may be caused by the blocking of function of other farnesylated proteins (Haluska et al., 2002). A suitable candidate for treatment with FTIs is multiple myeloma. Myeloma is the second most frequent hematological malignancy, with a median survival inferior to 4 years (San Miguel et al., 1999). Gain-of-function mutations of Ras are found in up to 50% of myeloma cases, and their frequency increases as

This work was supported by grants PIO20065 and Thematic Network G03/136 from Fondo de Investigaciones Sanitarias (Ministerio de Sanidad, Spain) and from Diputación General de Aragón.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.007021.

ABBREVIATIONS: FTI, farnesyltransferase inhibitor; AIF, apoptosis-inducing factor; $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; BMS-214662, (*R*)-7-cyano-2,3,4,5-tetrahydro-1-(1*H*-imidazol-4-ylmethyl)-3-(phenylmethyl)-4-(2-thienylsulfonyl)-1*H*-1,4-benzodiazepine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SCH66336, 4-(2-(4-(8-chloro-3,10-dibromo-6,11-dihydro-5*H*-benzo-(5,6)-cyclohepta(1,2-*b*)-pyridin-11(*R*)-yl)-1-piperidinyl)-2-oxoethyl)-1-piperidinecarboxamide; R115777, (*R*)-6-(amino(4-chlorophenyl)(1-methyl-1*H*-imidazol-5-yl)methyl)-4-(3-chlorophenyl)-1-methyl-2(1*H*)-quinolinone; Z-, *N*-benzyloxycarbonyl; fmk, fluoromethyl ketone.

disease progresses (Reuter et al., 2000). Ras is critically implicated in proliferation, through the activation of the Raf/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway, and survival, via phosphatidylinositol 3-kinase/Akt pathway (Ogata et al., 1997; Mitsiades et al., 2002; Hu et al., 2003) of myeloma cells. BMS-214662 is a nonthiol, nonpeptide inhibitor of farnesyl transferases (Singh and Lingham, 2002). Preclinical studies have shown that some types of human leukemia cell lines are sensitive to growth inhibition by BMS-214662 (Rose et al., 2001). In addition, results from a phase I trial indicate that BMS-214662 may be useful for the treatment of human acute leukemias and myelodysplasias (Kurzrock et al., 2002). Herein, we show that low concentrations of BMS-214662 efficiently inhibit protein farnesylation and induce apoptosis in human myeloma cell lines. Apoptosis is associated in most cases with an increase in PUMA levels, conformational changes of Bax and Bak, elimination of Mcl-1, caspase activation, and AIF translocation to cell nucleus. These results provide a molecular framework for the possible use of BMS-214662 in the treatment of multiple myeloma.

Materials and Methods

Materials. BMS-214662, an imidazole-containing tetrahydrobenzodiazepine (Rose et al., 2001) was kindly provided by Bristol-Myers Squibb Co. (Stamford, CT). Cycloheximide and MTT were from Sigma-Aldrich (Madrid, Spain). Peptide caspase inhibitor Z-VAD-fmk was from Bachem (Bubendorf, Switzerland); Z-DEVD-fmk, Z-LEHD-fmk, Z-IETD-fmk, and Z-VEID-fmk were from BD PharMingen (Madrid, Spain); and Z-VDVAD-fmk from Calbiochem (Madrid, Spain).

Cell Proliferation and Toxicity Assays. Human myeloma cell lines RPMI 8226, NCI-H929, U266 (clone B1), and the B-leukemia IM-9, obtained from the peripheral blood of a patient with myeloma, were from the American Type Culture Collection (Manassas, VA). All cell lines were routinely cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin (hereafter, complete medium), using standard cell-culture procedures. In proliferation assays, cells (3–5 $\times$ 10^5 cells/ml) were treated in flat-bottom, 24- (1 ml/well) or 96-well plates (100 μl/well) with different concentrations of BMS-214662 (0.075–1 μM) in complete medium for different times, as indicated. For apoptosis inhibition assays, cells were preincubated with either one or with mixtures of the following inhibitors: 100 μM Ac-DEVD-fmk, 100 μM Z-LEHD-fmk, 100 μM Z-IETD-fmk, 100 μM Z-VEID-fmk, 200 μM Z-VDVAD-fmk, or 100 μM Z-VAD-fmk for 1 h, before the addition of BMS-214662. The dependence of apoptosis on de novo protein synthesis was evaluated by adding cycloheximide (0.5 μg/ml) to cultures 1 h before BMS-214662 treatment. Cell proliferation was determined by a modification of the MTT reduction method (Alley et al., 1988), and viability was determined by microscopical inspection of Trypan blue-stained cells. Cells exhibiting a blebbing morphology, defined by the appearance of distinct protrusions of the plasma membrane and/or vacuolization, were also scored as nonviable. Nuclear alterations during apoptosis were analyzed by labeling with *p*-phenylenediamine in oxidized glycerol and visualized by fluorescence microscopy (Gamen et al., 1998).

Flow Cytometry Analysis. Phosphatidylserine exposure and mitochondrial membrane potential ($\Delta\Psi_m$) were simultaneously evaluated in the same cells. In brief, cells (2.5 $\times$ 10^5 in 200 μl) were incubated with 2 nM $DiOC_6(3)$ (Molecular Probes, Eugene, OR) at 37°C for 10 min in binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, and 10 mM HEPES/NaOH, pH 7.4). Then, 0.5 μg/ml annexin V-PE (Caltag Laboratories, Burlingame, CA) was added and incubated at room temperature for 15 min. Cell suspension was diluted to 1 ml

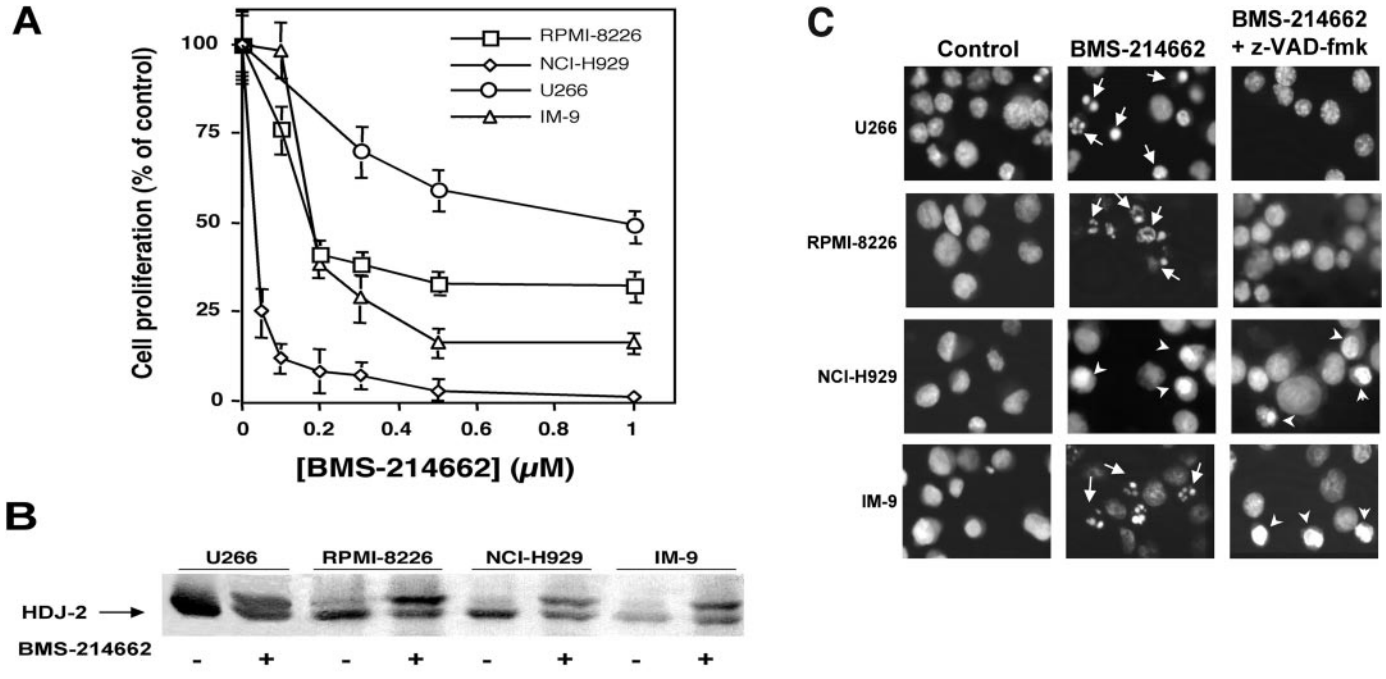

Fig. 1. Effect of BMS-214662 on viability of myeloma cells. A, cells (3 $\times$ 10^5 cells/ml) were treated in 96-well plates (100 μl/well) in complete medium with different concentrations of BMS-214662. Cell proliferation was estimated at 24 h by the MTT-assay. Results are means of three to four individual determinations on two different experiments and are expressed as a percentage of cell proliferation relative to controls (without drug). Bars indicate S.D. B, inhibition of protein farnesylation by BMS-214662. Cells were treated with 1 μM (U266), 0.3 μM (RPMI 8226, IM-9), or 75 nM (NCI-H929) BMS-214662 for 24 h, and the mobility of the farnesylated chaperon marker HDJ-2 was analyzed by Western blot. BMS-214662 treatment caused the appearance of a slower mobility band corresponding to the unprocessed protein. C, induction of nuclear apoptosis by BMS-214662. Cells were treated with the drug for 24 h, stained with *p*-phenylenediamine, and photographed under epifluorescence illumination. Arrows, cells showing chromatin condensation and nuclear fragmentation; arrowheads, cells with chromatin condensation. Original magnification, 400×.

with binding buffer and analyzed in a flow cytometer (Epics XL-MCL; Beckman Coulter, Fullerton, CA). Conformational changes of Bax and Bak proteins were assessed by intracellular immunostaining using specific antibodies recognizing only the proapoptotic conformation of these proteins (Griffiths et al., 1999; Panaretakis et al., 2002; Yamaguchi et al., 2002). Cells (1×10^6) were cultured in complete medium (controls) or medium containing BMS-214662 for 20 h in the absence or presence of Z-VAD-fmk. Then, cells were fixed with 0.5% paraformaldehyde in PBS (15 min, 4°C) and incubated for 25 min at room temperature with 0.5 μg of anti-Bax (6A7; BD PharMingen), anti-Bak (TC100; Calbiochem, San Diego, CA), or an irrelevant mouse IgG in 100 μl of PBS containing 0.1% saponin and 5% goat serum. Cells were washed with 0.03% saponin in PBS, incubated with a FITC-labeled anti-mouse IgG antibody (Caltag Laboratories), and analyzed by flow cytometry. Quantitative analysis of cytochrome *c* release from mitochondria during apoptosis was assayed by the method described by Waterhouse and Trapani (2003). In brief, cells (1×10^6) were cultured in complete medium alone (controls) or medium containing BMS-214662 in the absence or presence of Z-VAD-fmk. Cells were permeabilized with 100 μl of digitonin (50 μg/ml in PBS containing 100 mM KCl) for 5 min on ice, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and resuspended in blocking buffer (3% bovine serum albumin, 0.05% saponin in PBS). Cells were incubated overnight at 4°C with a 1:200 dilution of anti-cytochrome *c* antibody (6H2.B4; BD PharMingen) in blocking buffer, washed, and incubated with a FITC-labeled anti-mouse IgG antibody. Finally, cells were resuspended in PBS and analyzed by flow cytometry (Epics XL-MCL; Beckman Coulter). For cell-cycle analysis, cells (1×10^6) were washed with PBS, pH 7.4, containing 1 mg/ml glucose and fixed with 70% ethanol at −20°C for 24 h. Cells were next incubated for 1 h at room temperature in PBS containing 0.5 mg/ml RNase and 20 μg/ml propidium iodide and analyzed by flow cytometry.

Immunofluorescence Analysis. AIF translocation from mitochondria to nucleus was analyzed by confocal microscopy. Cells (1.5×10^6) were cultured in complete medium with or without BMS-214662, fixed in 4% paraformaldehyde for 15 min, and centrifuged onto poly-L-lysine–coated coverglasses. Coverglasses were washed in PBS, briefly immersed in 0.1% saponin in PBS, placed onto a drop of a 1:200 dilution of a rabbit anti-AIF antiserum (kindly provided by Drs. Santos Susin and Guido Kroemer, Centre National de la Recherche Scientifique, Villejuif, France), and incubated at room temperature in a humidified chamber for 30 min. Coverglasses were washed twice with 0.1% saponin and incubated in a FITC-labeled anti-rabbit IgG antibody. Finally, coverglasses were sequentially washed with 0.1% saponin, PBS, and distilled water and mounted onto glass slides over a drop of Mowiol (Calbiochem). Preparations were observed in a Zeiss 310 confocal microscope and analyzed using the LSM 3.95 software (Carl Zeiss GmbH, Jena, Germany).

Subcellular Fractionation and Western Blot Analysis. Akt activation, HDJ-2 farnesylation, caspase activation, and levels of Bcl-2 superfamily proteins were analyzed by Western blot essentially as described previously (Perez-Galan et al., 2002). For the detection of the active, phosphorylated form of Akt, myeloma cells (1×10^6 cells/ml) were incubated for 1 h with 200 IU/ml interleukin-6 in the presence or absence of BMS-214662. Cells were lysed in 50 mM Tris/HCl, pH 7.4, buffer containing 0.15 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Solubilized proteins from equal numbers of Trypan blue-negative cells (1×10^6/lane) were resolved by SDS-12% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with primary antibodies diluted in 10 mM Tris/HCl, pH 8.0, 0.12 M NaCl, 0.1% Tween 20, and 0.05% sodium azide, containing 5% skimmed milk, as described previously (Gamen et al., 1997). Primary antibodies anti-human proteins used were the following: anti-COX-1 (Molecular Probes), anti-HDJ-2 (Neomarkers, Fremont, CA); anti-phospho-Akt (Ser473; Cell Signaling Technology Inc., Beverly, MA); anti-caspase-8 and anti-Bak (Upstate Biotechnology, Lake Placid, NY); anti-caspase-3 (clone 19), anti-caspase-9, anti-active caspase-3, anti-Bax, anti-cytochrome *c*, and anti-p53 (all from BD PharMingen); anti-Bim (Calbiochem); anti-caspase-2, anti-Bcl-2, anti-Bcl-x_L, anti-Mcl-1, and anti-Bik (all from Santa Cruz Biotechnology, Santa Cruz, CA); and anti-PUMA (AbCam Inc., Cambridge, MA). Membranes were washed with 10 mM Tris/HCl, pH 8.0, 0.12 M NaCl, 0.1% Tween 20, and 0.05% sodium azide and incubated with 0.2 μg/ml of the corresponding phosphatase alkaline-labeled secondary antibody (Sigma-Aldrich). Western blots were revealed with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate, as described previously (Gamen et al., 1997). Control of protein loading was achieved by reprobing with anti-β-actin or anti-α-tubulin (Sigma-Aldrich). Western blots were sequentially analyzed for several proteins by a modification of the multiple-blotting assay method (Krajewski et al., 1996), as described previously (Perez-Galan et al., 2002). Release of cytochrome *c* from mitochondria was determined by subcellular fractionation of digitonin-treated cells as described by Piqué et al. (2000) and Western blot analysis. Purity of fractions was assessed by analyzing the presence of the inner mitochondrial membrane protein COX-1 and cytosolic tubulin in the same blots.

Results

BMS-214662 Inhibits Farnesylation and Induces Apoptosis in Myeloma Cells. Treatment with BMS-214662 caused cell death in a dose-dependent way (Fig. 1A) in all cell lines tested, although their relative sensitivities

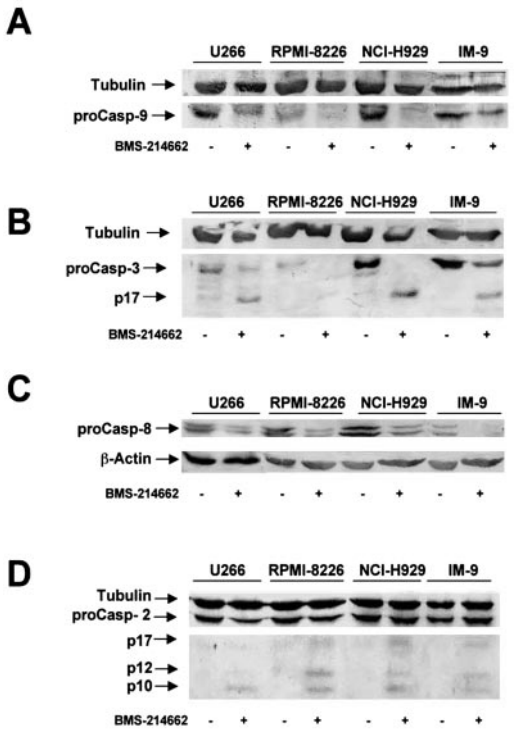

Fig. 2. Caspase activation induced by BMS-214662. Western blot analysis of the activation of caspase-9 (A), caspase-3 (B), caspase-8 (C), and caspase-2 (D). Cells were treated for 20 h with 1 μM BMS-214662 (U266), 0.3 μM (RPMI 8226, IM-9), or 75 nM (NCI-H929), and cell extracts were analyzed by Western blot with antibodies to caspases 9, 3, 8, and 2. Immunoblots were reprobed with anti-actin or anti-tubulin antibody as a control for equal loading. Activation was characterized by the disappearance of procaspase and, in the case of caspases 2 and 3, by the presence of active subunits.

varied. Analysis of cell proliferation by the MTT assay indicated that IC_{50} value increased in the following order: NCI-H929 (50 nM) < RPMI 8226 ≈ IM-9 (0.2 μM) < U266 (0.8 μM) after a 24-h incubation. To assess the inhibition of farnesyltransferase activity in cells, farnesylation of cytosolic chaperone HDJ-2 and lamin A, recognized as suitable markers (Adjei et al., 2001), was analyzed by Western blot. BMS-214662, at apoptosis-inducing doses, significantly inhibited farnesylation of HDJ-2 (Fig. 1B) in all cell lines and lamin A in cells expressing this protein (RPMI 8226, NCI-H929, and IM-9; data not shown). The appearance of a lower mobility band corresponding to the nonfarnesylated HDJ-2 is probably caused by a blockade of postfarnesylation proteolytic processing (Adjei et al., 2001; Karp, 2001). On the other hand, treatment with BMS-214662 had no effect on the levels of activated Akt (data not shown), ruling out the possibility that apoptosis was caused by Akt inhibition. Moreover, BMS-214662 did not modify the levels of activated mitogen-activated protein kinases c-Jun NH_2-terminal kinase, p38, and extracellular signal-related kinase (data not shown). Morphological evaluation revealed that cells treated with BMS-214662 exhibited typical features of apoptosis such as cell shrinking, chromatin condensation, and, except for NCI-H929 cells, nuclear fragmentation and formation of apoptotic bodies (Fig. 1C).

BMS-214662 Causes Caspase Activation, $\Delta\Psi_m$ Loss, and Phosphatidylserine Exposure. Caspase activation during BMS-214662 induced-apoptosis of RPMI 8226, U266, NCI-H929, and IM-9 cells was analyzed by Western blotting. In all cell lines, BMS-214662 induced a significant activation of caspase-9 (Fig. 2A) and caspase-3 (Fig. 2B), as shown by reduction in the intensity proenzymes and, in the case of caspase-3, by the appearance of the 17-kDa band of the active subunit (Fig. 2B). RPMI 8226 cells expressed low levels of caspase-3 (Fig. 2B). Caspase-8 and caspase-2 were also activated upon BMS-214662 treatment in all cell lines (Fig. 2, C and D).

Treatment of myeloma cells with BMS-214662 caused phosphatidylserine exposure and loss of $\Delta\Psi_m$ (Fig. 3A). Cotreatment with Z-VAD-fmk did not prevent $\Delta\Psi_m$ loss, although it attenuated phosphatidylserine translocation in NCI-H929 cells (Fig. 3). However, in RPMI 8226 and U266 cells, Z-VAD-fmk completely prevented $\Delta\Psi_m$ loss and phosphatidylserine exposure induced by BMS-214662 at 24 h (Fig. 3). Inhibition by Z-VAD-fmk of $\Delta\Psi_m$ loss caused by BMS-214662 in IM-9 cells was not complete (Fig. 3). A significant proportion of cells showed an intermediate $\Delta\Psi_m$ ($\Delta\Psi_m^{int}$) compared with untreated cells ($\Delta\Psi_m^{high}$) and cells treated with BMS-214662 alone ($\Delta\Psi_m^{low}$) (Fig. 3). Cotreatment of cells with Z-VAD-fmk also prevented the develop-

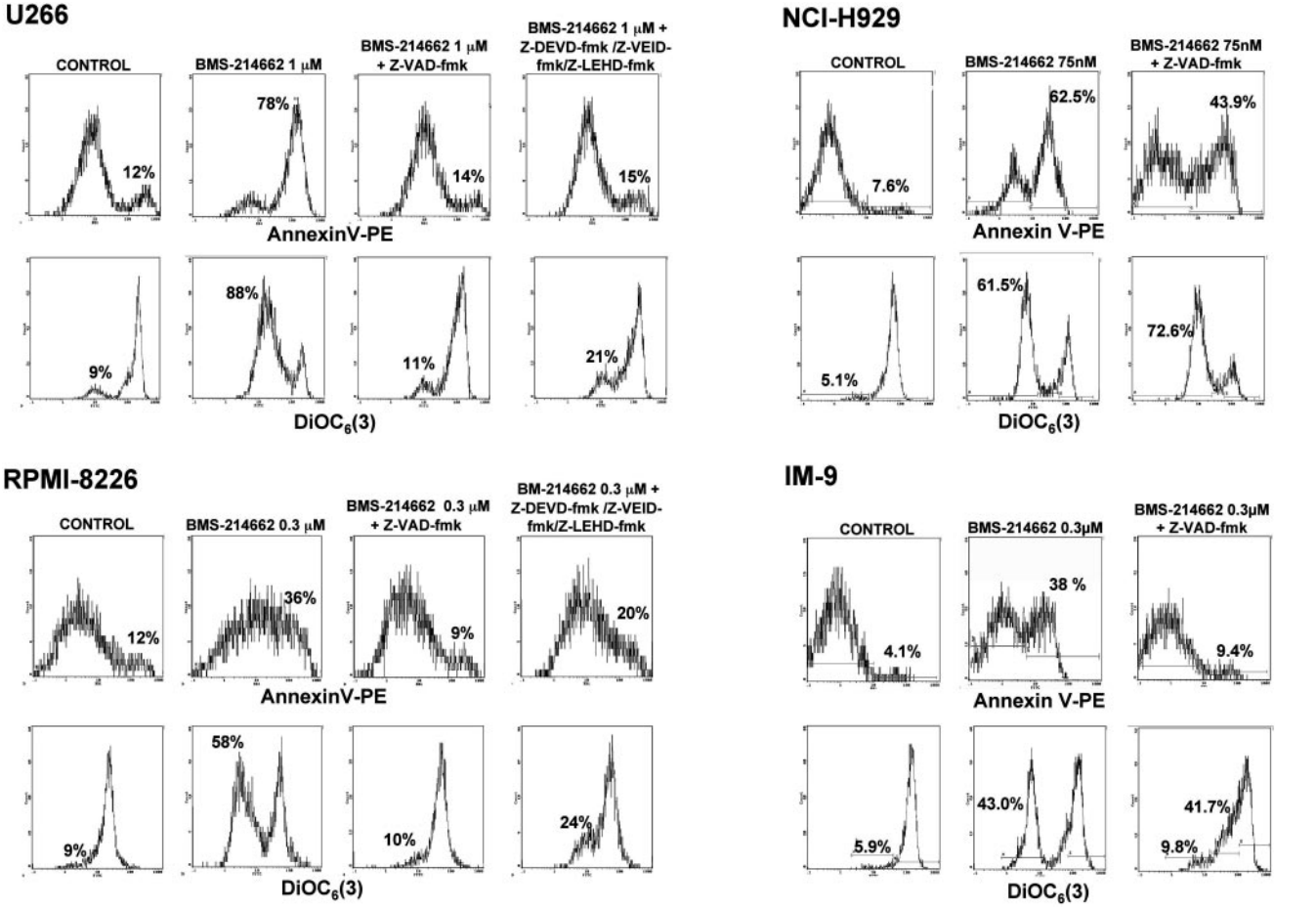

Fig. 3. Effect of peptide caspase inhibitors on BMS-214662–induced apoptosis. Cells (3×10^5 cells/ml) were incubated for 24 h with BMS-214662 at the indicated doses in the absence or presence of either 100 μM Z-VAD-fmk or Z-DEVD-fmk + Z-VEID-fmk + Z-LEHD-fmk (100 μM each). Phosphatidylserine exposure and $\Delta\Psi_m$ were analyzed by annexin V-PE and $DiOC_6(3)$ labeling, respectively. Experiments shown are representative of five different determinations for each cell line.

ment of apoptotic morphology, although peripheral chromatin condensation, characteristic of AIF action (see later), was still noted in NCI-H929 and IM-9 cells (Fig. 1C). We also analyzed the effect of selective caspase inhibitors on BMS-214662–induced apoptosis in cell lines in which the protective effect of Z-VAD was the greatest (RPMI 8226 and U266). Z-DEVD-fmk (inhibitor of caspases 3, 7, and 8), Z-VDVAD-fmk (minimal inhibitor of caspase-2), Z-IETD-fmk (caspases 8 and 10), and Z-LEHD-fmk (caspase-9) failed to prevent BMS-214662–induced $\Delta\Psi_m$ loss in U266, RPMI 8226, and NCI-H929 cells, although phosphatidylserine exposure was prevented in part (data not shown). Nuclear condensation and fragmentation was inhibited in U266, RPMI 8226, NCI-H929, and IM-9 cells treated with BMS-214662 in the presence of Z-DEVD-fmk, but a high percentage of cells exhibited peripheral chromatin condensation (data not shown), reminiscent of AIF action. Most (85–90%) of the protective effect of Z-VAD-fmk in RPMI 8226 and U266 cells could be mimicked by the addition of a mixture of inhibitors for caspases 9, 3, and 6 (Z-LEHD-fmk + Z-DEVD-fmk+ Z-VEID-fmk, 100 μM each) (Fig. 3). Any combination of two of these inhibitors, as well as the combination of Z-LEHD-fmk + Z-VDVAD-fmk, did not prevent cell death (data not shown). In other experiments, cells were incubated for longer periods with BMS-214662 in the continuous presence of Z-VAD-fmk, and cell death was evaluated by annexin V-labeling. Caspase inhibition by Z-VAD-fmk did not affect the toxicity in NCI-H929 cells at any time but preserved viability of RPMI 8226 and U266 cells for at least 48 and 36 h, respectively (data not shown). Protein synthesis inhibition with cycloheximide did not prevent BMS-214662 toxicity in myeloma cells (data not shown). In NCI-H929 cells, cycloheximide potentiated the toxicity of BMS-214662. Treatment of NCI-H929 cells with cycloheximide alone caused a reduction in Mcl-1 levels (data not shown) without significant toxicity, suggesting that this could be the mechanism of potentiation of BMS-214662 toxicity in these cells.

Effect of BMS-214662 on Bcl-2 Superfamily Proteins. Western blot analysis revealed that BMS-214662 treatment did not significantly affect to the levels of Bax, Bak, and Bcl-2 (Fig. 4A) in any of the cell lines. However, treatment with BMS-214662 induced proapoptotic conformational changes in Bax and Bak, as determined by flow cytometry (Fig. 5). These conformational changes have been observed previously in other apoptotic models (Griffiths et al., 1999, 2001). However, Bak protein did not undergo the conformational change in RPMI 8226 cells treated with BMS-214662 (Fig. 5). Other drugs that induce apoptosis in RPMI 8226, such as doxorubicin and daunorubicin, were also unable to induce the conformational change of Bak (data not shown). Inhibition of caspases with Z-VAD-fmk did not prevent conformational changes of Bax and Bak in U266, NCI-H929, and RPMI 8226 cells (Fig. 5). A significant reduction in Mcl-1 levels was observed in RPMI 8226 and U266 cells after treatment with BMS-214662 (Fig. 4A) that was not prevented by Z-VAD-fmk (Fig. 4C). Levels of Bcl-x_L and of Bcl-x_s also diminished to a lesser extent in U266, RPMI 8226, and IM9 cells (Fig. 4A). The BH3-only proteins Bim, Bik, and Bad were expressed by U266 and NCI-H929 cells but not by RPMI 8226 and IM-9 cells. Levels of these proteins did not significantly change after treatment with BMS-214662 (Fig. 4A) in U266 cells, and only Bad levels were reduced in NCI-H929 cells. The BH3-only protein PUMA was expressed by all cell lines, and their α and β isoforms were detected by Western Blot (Fig. 4B). PUMA expression significantly increased in RPMI 8226 and NCI-H929 cells but not in U266 cells after 6 h of treatment with BMS-214662 (Fig. 4B). This increase of PUMA levels was concomitant in RPMI 8226 and NCI-H929 cells, with an increase in levels of p53 protein (Fig. 4B). BMS-214662 induced cell-cycle arrest at G_1 in NCI-H929 cells but not in U266 cells (data not shown). At longer incubation times, BMS-214662 produced a marked decrease of PUMA levels in all cell lines, independently of caspase activation (Fig. 4).

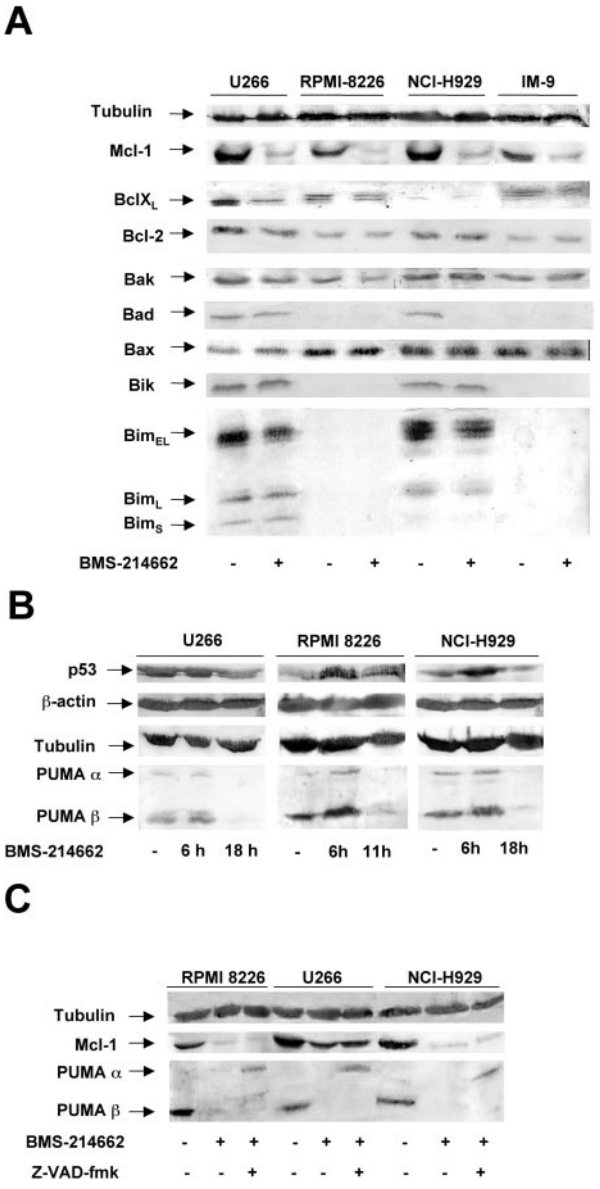

Fig. 4. Effect of BMS-214662 on the levels of Bcl-2 family proteins. A, cells were treated for 20 h with 1 μM BMS-214662 (U266), 0.3 μM (RPMI 8226, IM-9), or 75 nM (NCI-H929), and cell extracts were analyzed by Western blot with antibodies to Mcl-1, Bcl-X_L, Bcl-2, Bak, Bad, Bax, Bik, and Bim. Immunoblots were reprobed with anti-tubulin as a control for equal loading. B, cells were treated for 6 or 18 h with BMS-214662 1 μM (U266), 0.3 μM (RPMI 8226, IM-9), or 75 nM (NCI-H929), and levels of p53 and PUMA were analyzed by Western blot. C, cells were treated for 20 h with 1 μM BMS-214662 (U266), 0.3 μM (RPMI 8226, IM-9), or 75 nM (NCI-H929) in the absence or presence of Z-VAD-fmk, and levels of Mcl-1 and PUMA were analyzed by Western blot.

BMS-214662 Causes Release of Cytochrome *c* and AIF from Mitochondria. Mitochondrial release of cytochrome *c* from myeloma cells was determined by flow cytometry (Fig. 6) and by subcellular fractionation and Western blot analysis (data not shown), with equivalent results. BMS-214662 treatment induced the release to cytosol of cytochrome *c* in U266, RPMI 8226, and NCI-H929 cells (Fig. 6), although to a different extent. Cotreatment with the general caspase inhibitor Z-VAD-fmk prevented most of the cytochrome *c* release in U266 and RPMI 8226 cells but not in NCI-H929 cells (Fig. 6), according to the observed inhibitory effect of Z-VAD-fmk on $\Delta\Psi_m$ loss, phosphatidylserine exposure, and cell death in U266 and RPMI 8226 but not in NCI-H929 cells (Fig. 3). BMS-214662 treatment also induced AIF translocation from mitochondria to nucleus in RPMI 8226, U266, and NCI-H929 cells, as observed by immunofluorescence microscopy (Fig. 7). In untreated cells, AIF was only localized in cytoplasm, with a punctate pattern congruent with its mitochondrial location. After treatment with BMS-214662, AIF staining became diffuse and localized in cytoplasm and nucleus. Nuclear translocation of AIF in U266 and RPMI 8226 cells, but not in NCI-H929 cells, was blocked by caspase inhibition with Z-VAD-fmk (Fig. 7). Treatment with the caspase-3 inhibitor Z-DEVD-fmk (100 μM) did not prevent AIF nuclear translocation in any cell line (data not shown).

Discussion

FTIs were developed as potential cancer therapeutics, initially designed to target oncogenic Ras (Adjei et al., 2001). In sensitive cells, most FTIs tested cause cell-growth inhibition favoring death by neglect but not directly inducing apoptosis (Du et al., 1999; Le Gouill et al., 2002; Bolick et al., 2003). In some myeloma cell lines, FTIs such as R115777 and perillic acid induce apoptosis (Beaupre et al., 2003, 2004). Here, we show that BMS-214662 efficiently induces apoptosis in myeloma cells at much lower doses than those reported for other FTIs (Le Gouill et al., 2002; Bolick et al., 2003). As shown, treatment with BMS-214662 blocked protein farnesylation, inhibited cell growth, and induced apoptosis in cell lines that express either mutated (RPMI 8226, NCI-H929) or wild-type Ras (U266) (Bolick et al., 2003). These results agree with previous reports showing that the presence of mutated Ras is not essential for the action of FTIs (Du et al., 1999). In fact, at least some FTIs also act on alternative molecular targets. For instance, SCH66336, which is under clinical testing, efficiently inhibits nuclear factor-κB (Takada et al., 2004) and Akt activation (Chun et al., 2003) in tumor cells. Our present results indicate that BMS-214662 did not alter the levels of activated Akt under basal conditions or upon stimulation with interleukin-6. Nevertheless, our results indicate that at farnesylation inhibitory doses, BMS-214662 causes apoptosis in myeloma cells through the engagement of the mitochondrial pathway. The relative role of caspases and Bcl-2 superfamily proteins in the induction phase of apoptosis depends on the myeloma cell line. BMS-214662–induced apoptosis is caspase-dependent in U266 and RPMI 8266 cells but not in NCI-H929 cells. Moreover, Z-VAD-fmk blocks most, but not all, of the release of cytochrome *c* and AIF induced by BMS-214662 in RPMI 8226 and U266 cells. These results agree with a previous report showing that cytochrome *c* release may occur in two steps, with the first being caspase-independent and the second promoted by a caspase-amplification loop (Chen et al., 2000). Therefore, it is reasonable to speculate that the differences observed in myeloma cell lines are caused by the relative efficiency of proapoptotic multido-

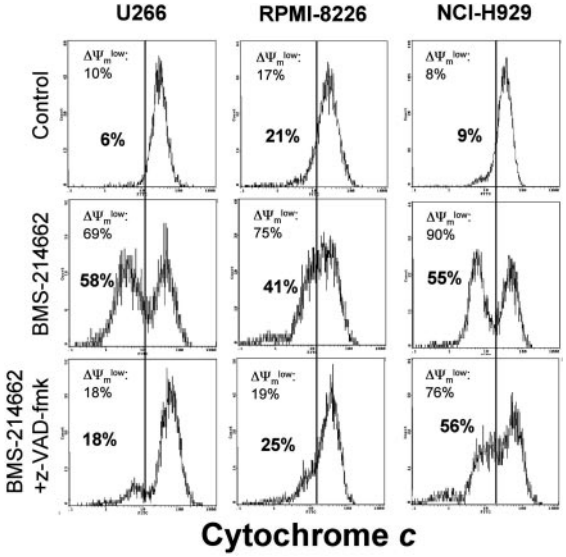

Fig. 6. Cytochrome *c* release induced by BMS-214662. Cells were left untreated or incubated for 20 h with BMS-216442 at 1 μM (U266), 0.3 μM (RPMI 8226, IM-9), or 75 nM (NCI-H929), in the absence or presence of 100 μM Z-VAD-fmk, as indicated. Cells were permeabilized with digitonin, fixed, immunostained with a monoclonal anti-native cytochrome *c* (6H2.B4) antibody, and analyzed by flow cytometry.

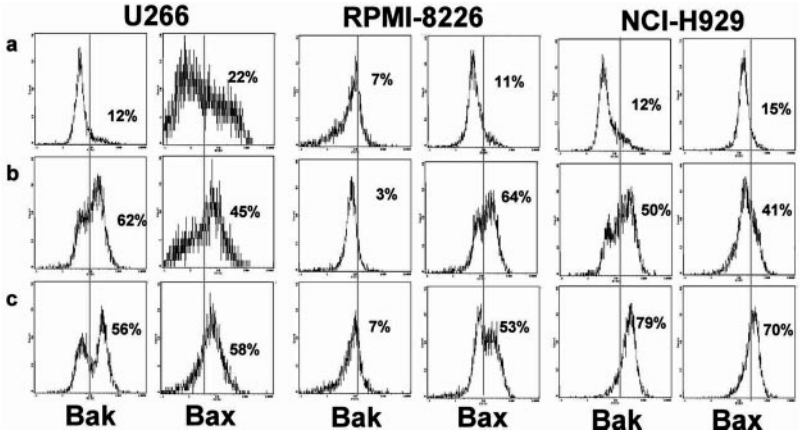

Fig. 5. BMS-214662 induces proapoptotic conformational changes of Bak and Bax. U266, RPMI 8226, and NCI-H929 cells were left untreated (a) or were incubated for 20 h with 1, 0.3, or 0.075 μM BMS-214662, respectively, in the absence (b) or presence (c) of 100 μM Z-VAD-fmk. Then, cells were fixed and immunostained with conformation-specific monoclonal anti-Bax (6A7) or anti-Bak (Ab-1) antibodies and analyzed by flow cytometry.

main proteins to cause the release of cytochrome *c* and AIF from mitochondria.

NCI-H929 cells express the BH3-only proteins Bad, Bik, and, at higher levels, Bim and PUMA. Treatment with BMS-214662 increases the levels of p53 and its regulated protein PUMA. PUMA, Bim, Bad, and Bik can bind to and neutralize Mcl-1, Bcl-2, and Bcl-x_L and thus facilitate the conformational change of Bax and Bak. This change is necessary and sufficient to provoke the release of cytochrome *c*, which seems to be primarily responsible for $\Delta\Psi_m$ loss, and of AIF. AIF causes caspase-independent cell death, and so caspase inhibition by Z-VAD-fmk only affects the morphology of dying cells but not to the extent of cell death. U266 cells express only mutant p53, and RPMI 8226 cells express both wild-type and mutant p53 proteins (Teoh et al., 2000; Liu et al., 2003). Therefore, after BMS-214662 treatment, levels of p53 and PUMA remained constant in U266 cells and increased in RPMI 8226 cells. However, RPMI 8226 cells do not express the BH3-only proteins Bim, Bad, and Bik, and Bak does not acquire the proapoptotic conformation upon treatment with BMS-214662 or other drugs (data not shown). In RPMI 8226 cells, the action of PUMA would only enable the release of limited amounts of cytochrome *c*, being dependent on active caspases to cause $\Delta\Psi_m$ loss and the release of AIF and further amounts of cytochrome *c*. These results could explain why Z-VAD-fmk completely blocks cell death for nearly 2 days in U266 and RPMI 8226 cells but not in NCI-H929 cells. On the other hand, only the combination of inhibitors of caspases 9, 3, and 6, but not others, offered a degree of protection approaching that of Z-VAD-fmk in RPMI 8226 and U266 cells. This agrees with the canonical model of apoptosis execution with caspase-9 as the apical caspase, and the improved protection offered by Z-VAD-fmk versus Z-LEHD-fmk is probably caused by its better accessibility to cellular caspase-9 (Scoltock and Cidlowski, 2004). In addition, a possible role of Bid can be ruled out because Bid requires its previous proteolytic activation by caspase-8 (Gross et al., 1999), and inhibition of this caspase with Z-IETD-fmk did not prevent cell death. Protection by Z-VAD in these cell lines is not permanent, and 48 h after the initiation of treatment, cells began to die in the absence of caspase activation. BMS-214662 also induced a marked decrease in the levels of Mcl-1, a short-lived protein critical for myeloma survival (Derenne et al., 2002; Zhang et al., 2002; Jourdan et al., 2003), and down-regulation of Mcl-1 occurs during apoptosis of myeloma cells (Pei et al., 2003; Van de Donk et al., 2003; Gomez-Bougie et al., 2004). Apoptosis triggered by BMS-214662 was associated with reduction of Mcl-1 protein expression in all cell lines analyzed. Moreover, treatment of H929 cells with cycloheximide decreased the levels of Mcl-1 and increased their sensitivity to BMS-214662. A similar phenomenon of acceleration of apoptosis after cycloheximide-induced Mcl-1 down-regulation has been described in endothelial (Bannerman et al., 2001) and HeLa cells (Nijhawan et al., 2003). Down-regulation of Mcl-1 could result from both protein synthesis blockade and degradation of existing Mcl-1 by the proteasome (Nijhawan et al., 2003). Moreover, preliminary ongoing experiments from our laboratory indicate that overexpression of Mcl-1 in myeloma cell lines protects them from BMS-214662–induced apoptosis (data not shown). All of these data support a key role for Mcl-1 in the susceptibility to apoptosis of myeloma cell lines.

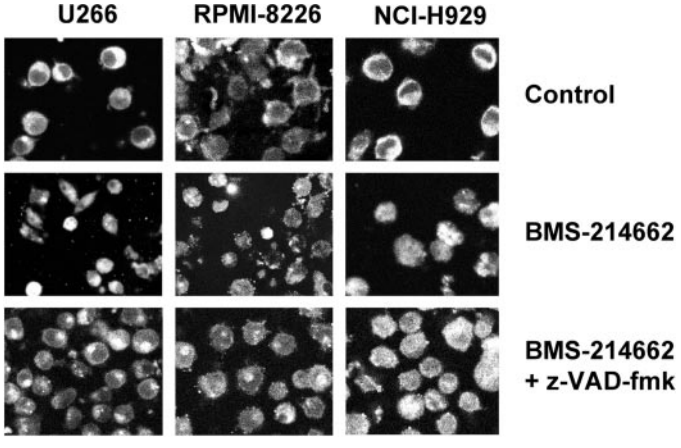

Fig. 7. Nuclear translocation of AIF. U266, RPMI 8226, and NCI-H929 cells were left untreated or treated with BMS-214662 in the absence or presence of Z-VAD-fmk, as described. After 20 h, cells were immunostained with an anti-AIF antibody and analyzed by confocal microscopy. BMS-214662–treated cells show the nuclear localization of AIF, which, except in the case of NCI-H929 cells, is blocked by Z-VAD-fmk.

In summary, BMS-214662 inhibits farnesylation and induces an increase in PUMA levels, conformational changes of Bax/Bak, and Mcl-1 elimination in myeloma cells. This facilitates the release of cytochrome *c* and AIF from mitochondria and cause loss of $\Delta\Psi_m$, caspase activation, and nuclear translocation of AIF. The combined action of AIF and caspases leads to the apoptotic phenotype. In some cell lines like RPMI 8226 and U266, activation of proapoptotic Bcl-2 family proteins is not sufficient per se to cause mitochondrial destabilization. In these cases, after the induction of Bax/Bak conformational change, caspases activated downstream of mitochondria are needed to cause rapid loss of $\Delta\Psi_m$ and the complete release of cytochrome *c* and AIF. Whatever their necessity, caspases accelerate the onset of cell death and are responsible for the morphology and correct dismantling of apoptotic cells. Differences observed among myeloma cell lines could be reminiscent of differences in apoptotic sensitivity found in myeloma cells from patients. Further work is obviously needed to precisely characterize the molecular targets of BMS-214662 and how apoptotic signals are generated, leading to the activation of BH3-only proteins and Mcl-1 degradation. Nevertheless, the FTI BMS-214662 has proved to efficiently induce apoptosis in myeloma cell lines and merits to be evaluated in cells of patients with multiple myeloma.

Acknowledgments

We thank Dr. Veeraswamy Manne (Bristol-Myers Squibb) for providing us with BMS-214662 and Dr. Antonio Campos-Caro (Hospital Puerta del Mar, Cádiz) for human myeloma cell lines.

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