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Proteasome Inhibitor MG132 Induces Apoptosis and Inhibits Invasion of Human Malignant Pleural Mesothelioma Cells

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Abstract

Malignant pleural mesothelioma (MPM) is an aggressive malignancy tightly associated with asbestos exposure. The increasing incidence of MPM and its resistance to all therapeutic modalities necessitate an urgent development of new treatments for MPM. Proteasome inhibitors (PIs) have emerged as promising agents for treating human cancers that are refractory to current chemotherapies. In this study, we characterized MG132, a commonly used PI, for its proapoptotic and anti-invasion activities in NCI-H2452 and NCI-H2052 human thoracic MPM cell lines to determine the therapeutic effect of PIs on MPM. We found that as low as 0.5 μM MG132 caused a significant apoptosis in both cell lines as evidenced by DNA damage, cleavage of poly ADP-ribose polymerase and caspases 3, 7, and 9, and mitochondrial release of Smac/DIABLO and Cytochrome *c*. Mitochondrial caspase activation was found to be the underlying mechanism of the MG132-induced apoptosis. Mcl-1, among the Bcl-2 and IAP (inhibitor of apoptosis protein) antiapoptotic family proteins tested, was proved to be a major inhibitor of the MG132-induced apoptosis in MPM cells. Meanwhile, subapoptotic doses of MG132 inhibited the invasion of both MPM cell lines through reducing Rac1 activity. These observations demonstrate that MG132 possesses proapoptotic and anti-invasion activities in human MPM cells, therefore encouraging further investigations on the value of PIs for treating MPM.

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Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignancy that arises from the mesothelium-lined surfaces of the pleural cavities after exposure to asbestos [1]. There are approximately 3000 new MPM cases diagnosed each year in the United States, with an even higher number worldwide. There are three distinct common histologic subtypes of MPM based on the microscopic appearance of the major malignant elements: epithelial, sarcomatoid, and biphasic (mixed). Although measures have been put in place to limit further asbestos exposure, the long latency of disease development after exposure has resulted in a dramatically increasing current incidence of MPM, with a projected peak in 2010 in the United States [2].

General resistance to currently available therapeutic modalities is the major reason for the overall very poor clinical outcome of MPM, which, regardless of treatment, has a median survival rate of less than 1 year [3]. Malignant pleural mesothelioma is extremely resistant to most chemotherapy regimens examined, and radiation therapy is generally ineffective as a primary treatment as well [4,5]. Targeted therapies, such as antiangiogenic drugs and inhibitors of the epidermal growth factor receptor tyrosine kinase [6], have proved equally ineffective in prolonging MPM patient survival despite substantial overexpression of the relevant molecular targets in MPM cells. Pemetrexed (Alimta) and cisplatin combination chemotherapy was recently shown in a prospective randomized trial to be the best chemotherapy regimen for MPM examined so far. However, median survival with this therapy was still less than 1 year, and the response rate was lower than 50% [7].

Most chemotherapeutic molecules trigger tumor cell eradication and patient survival by inducing apoptotic cell death [7]. Caspases play the most important role in regulating the apoptotic program. These proteolytic enzymes are divided into initiators, such as caspases 8, 9, and 10, and effectors, such as caspases 3 and 7, depending on

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the timing of activation [8]. The mitochondrial or intrinsic caspase activation pathway represents a fundamental mechanism in the apoptotic response triggered by different cellular stresses [9]. Antiapototic Bcl-2 family protein members antagonize the mitochondrial pathway through controlling outer mitochondrial membrane permeability and the release of mitochondrial proapoptotic factors, including Cytochrome *c* (Cyto *c*) and Smac/DIABLO (Smac), into the cytosol [10]. After release into the cytosol, Cyto *c* stimulates formation of the apoptosome to activate the initiator caspase 9, whereas Smac relieves the inhibition imposed by inhibitor of apoptosis proteins (IAPs) on caspases 9, 3 and 7. The synergistic action of Cyto *c* and Smac leads to full activation of caspases and cell death [9].

Mcl-1, or commonly known as the long isoform of Mcl-1 (Mcl-1_L), acts as an antiapoptotic member of the Bcl-2 family proteins and is expressed in multiple cell lineages [11,12]. It has emerged as a key member of this family of apoptosis control molecules. In contract to Mcl-1_L, the short isoform of Mcl-1 (Mcl-1_S), like other BH3-only proteins of the Bcl-2 family, serves as a proapoptotic protein [13]. Increased expression of Mcl-1 (or Mcl-1_L) is associated with the maintenance of cell viability and decreased expression is associated with cell death [14].

Due to the resistance to currently available chemotherapies and the increasing incidence of MPM, development of new treatments for MPM is urgently needed. Proteasome inhibitors (PIs) are becoming potential therapeutic agents for various types of human cancers that are refractory to currently available therapeutic modalities. The proteasome is a large catalytic complex that is responsible for most nonlysosomal intracellular protein degradation. This structure is a promising target for cancer therapy because it has been shown to impact the cell cycle, apoptosis, proliferation, and other physiological processes by regulating the levels of important signaling proteins such as CDC25A, CDC25C, MDM2, p21/WAF-1, p27/KIP1, and I-κB [15].

Limited attempts have been made to address the possibility of using PIs in the treatment of MPM [16,17]. Although the PIs have been claimed to induce apoptosis in MPM cells, determination of caspase involvement and other critical molecules in regulating this process and other anticancer activities of PIs in MPM cells warrant further investigations. In this study, we examined anticancer activities of MG132, a commonly used PI, in two human MPM cell lines. We demonstrate that MG132 induces a caspase-dependent apoptotic cell death in MPM cells and that subapoptotic doses of MG132 inhibit MPM cell invasion possibly through inhibiting Rac1 activation, suggesting that MG132 possesses antimesothelioma activities through either activating the apoptotic pathways or targeting regulatory molecules for MPM cell invasion.

Materials and Methods

Cells, Chemicals, and Antibodies

Cell lines. NCI-H2052 and NCI-H2452, two human MPM cell lines, and NCI-H358, a human non–small cell lung carcinoma cell (NSCLC) line, were purchased from American Type Culture Corporation (Rockville, MD) and cultured in RPMI-1640 medium with 10% fetal bovine serum.

Chemicals. MG-132, an S26 proteasome inhibitor, Src kinase inhibitor I, caspase inhibitors, Z-VAD-fmk for broad spectrum caspases, Z-IETD-fmk for caspase 8, Z-LEHD-fmk for caspase 9, and Z-FA-fmk used as negative control for all caspase inhibitors, were from EMD-CalBiochem (San Diego, CA). Caspase substrates, Ac-DEVD-Amc for caspase 3, Ac-IETD-Amc for caspase 8, and Ac-LEHD-Amc for caspase 9, were from BD Biosciences (San Jose, CA). Fibronectin and proteinase inhibitor cocktail were from Sigma. Matrigel was from Becton Dickinson Labware (Bedford, MA). Mcl-1 and XIAP siRNAs and the scrambled siRNA (control siRNA) were from Santa Cruz Biotechnology (Santa Cruz, CA). EZ-Detect Activation Kits for Rho, Cdc42, or Rac1 were from Pierce (Rockford, IL).

Antibodies. The antibodies used in against caspases 3, 7, and 9, poly ADP-ribose polymerase (PARP), XIAP, c-IAP1, Survivin, Cyto *c*, and Smac were from Cell Signaling Technologies (Danvers, MA). Antibodies against Bid, Bcl-2, β-actin, and HA tag were from Sigma. Antibodies against Mcl-1 and Bcl-X_L were from Santa Cruz Biotechnology.

Cell Proliferation Assay

A total of 2×10^5 MPM cells were seeded in each T25 culture dish and were treated continuously with MG132 for a total of 96 hours. Living cells stained negative by trypan blue were counted at 24-hour intervals.

Cell Viability Assay

A total of 1×10^4 cells growing in each well of a 96-well microplate were treated with MG132 for 36 to 48 hours and then incubated with 10 μl of WST-1 reagent (Roche, Indianapolis, IN) for 1 to 4 hours. The increase of absorbance at 420 to 480 nm relative to the blank well control was measured for each sample using a microplate spectrophotometer.

Flow Cytometry Assay

DNA fragmentation suggesting apoptotic DNA damage was indicated by a distinct sub–G_0/G_1 peak in flow cytometry assay [18]. After treatment, approximately 1×10^5 cells were collected, fixed in 70% ethanol, and stained with propidium iodide, and DNA content was determined on a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA).

Caspase Activity Assay

A total of 40 μg of protein isolated from the cells treated with MG132 was incubated in each reaction with 10 μg of corresponding caspase substrates for 1 hour, and the activities for caspases 3, 8, and 9 were measured in a CytoFluor Multiwell Plate Reader (PerSeptive Biosystems, Framingham, MA).

Western Blot Analysis

Conventional procedures of Western blot analysis were followed to monitor expression and/or cleavage of apoptosis-related proteins in MPM cells treated with MG132. RIPA buffer supplemented with proteinase inhibitor cocktail was used to collect cell lysates.

In Vitro *Cell Migration and Invasion Assays*

A transwell (8-μm pore size, 24-well format; BD Biosciences) coated with fibronectin on the bottom side of the transwell membrane was used in the migration assay. The same type of transwell coated also with Matrigel on the upper chamber was used in the invasion assay. A total of 0.5 to 1×10^5 cells were loaded in each transwell and incubated with MG132 for 24 to 48 hours in migration and

invasion assays. Nonmigrated cells were removed by cotton swab, and migrated cells were fixed and stained with Diff-Quick solutions. The transwell membrane was then mounted on a glass slide, and migratory cells of five fields under a microscope were counted for each chamber, and the average number of migrating cells was calculated from the total number of cells counted per chamber.

Pull-down Assay

EZ-Detect Rho Activation Kits for Rho, Cdc42, or Rac1 were used to measure GTP-bound Rho, Cdc42, or Rac1 proteins from MG132-treated NCI-H2452 cells. The protocol provided in the kits was followed to precipitate GTP-bound proteins. Both GTP-bound proteins and total proteins were subjected to Western blot analysis.

siRNA Transfection

Malignant pleural mesothelioma cells growing at approximately 60% confluence in each 60-mm culture dish were incubated with a transfection mixture containing 60 pmol of target-specific siRNA or scrambled siRNA and 3 μg of Lipofectamine 2000 for 5 hours in serum-free medium. A total of 3 ml of complete medium with 10% fetal bovine serum was then added to the cells for another 24 to 48 hours of incubation. After siRNA transfection, the cells were treated with MG132 and examined for apoptosis induction by both cell viability assay and Western blot analysis.

Construction of Rac1-G12V Expression Vector and Gene Transfection

cDNA of a constitutively active mutant of Rac1 (Rac1-G12V) tagged with HA antigen in pcDNA3 vector (UMR cDNA Resource Center, Rolla, MO) was transferred into pCEP4 vector (Invitrogen, Carlsbad, CA). A total of 5 μg of new construct DNA with 3 μg of Lipofectamine 2000 was mixed and applied to 1×10^6 NCI-H2052 cells. After the initial 48 hours of incubation, tumor cells were trypsinized and selected by 20 μg/ml hygromycin for 25 days. The pooled hygromycin-resistant cells were examined for expression of the Rac1-G12V protein in Western blot analysis and then used in the invasion assay. Vector alone–transfected cells were used as a control.

Statistics

Data from cell proliferation, cell viability, caspase activation, and cell invasion and migration assays were expressed as the means ± SD of at least two separate experiments. Comparison between group means was assessed using a one-way ANOVA with the Newman-Keuls posttest (GraphPad Prism 3.0 Software, Inc., San Diego, CA). $P < .05$ was considered significant.

Results

MG132 Inhibits Growth and Induces Apoptosis of Human MPM Cells

In our initial attempt to determine the therapeutic effect of PIs on MPM, we chose MG132 as a prototype PI to treat both NCI-H2052 and NCI-H2452 cells. Apoptosis induction by MG132 has been reported in different types of human cancer [19,20]. Our recent observations indicated that as low as 0.25 μM MG132 can induce a significant apoptotic response in human NSCLCs [21]. We therefore treated both MPM cell lines with 0.25 to 2 μM MG132 and then analyzed MG132's cytotoxicity. It was observed in cell proliferation assay that greater than 0.25 μM MG132 caused a clear inhibition of cell proliferation in a 4-day observation period in both cell lines, whereas greater than 0.5 μM MG132 induced a significant cell death with a higher potency demonstrated in NCI-H2452 cells (Figure 1*A*). A WST-1 cell viability assay indicated that most cells from both MPM cell lines died at 72 hours after exposure to greater than 1 μM MG132, with more significant cell death seen again in NCI-H2452 cells (Figure 1*B*). To determine the apoptosis induction by MG132, we tested DNA fragmentation in MPM cells by examining sub–G_0/G_1 cells in a flow cytometry assay [18]. A dose-dependent increase in sub–G_0/G_1 cells was observed in both cell lines at 36 to 48 hours after exposure to MG132, suggesting an apoptosis induction in tumor cells (Figure 1*C*). In addition to the increase of sub–G_0/G_1 population, a clear increase in G_2/M cells, or G_2/M arrest, was also observed in both cell lines (Figure 1*C*).

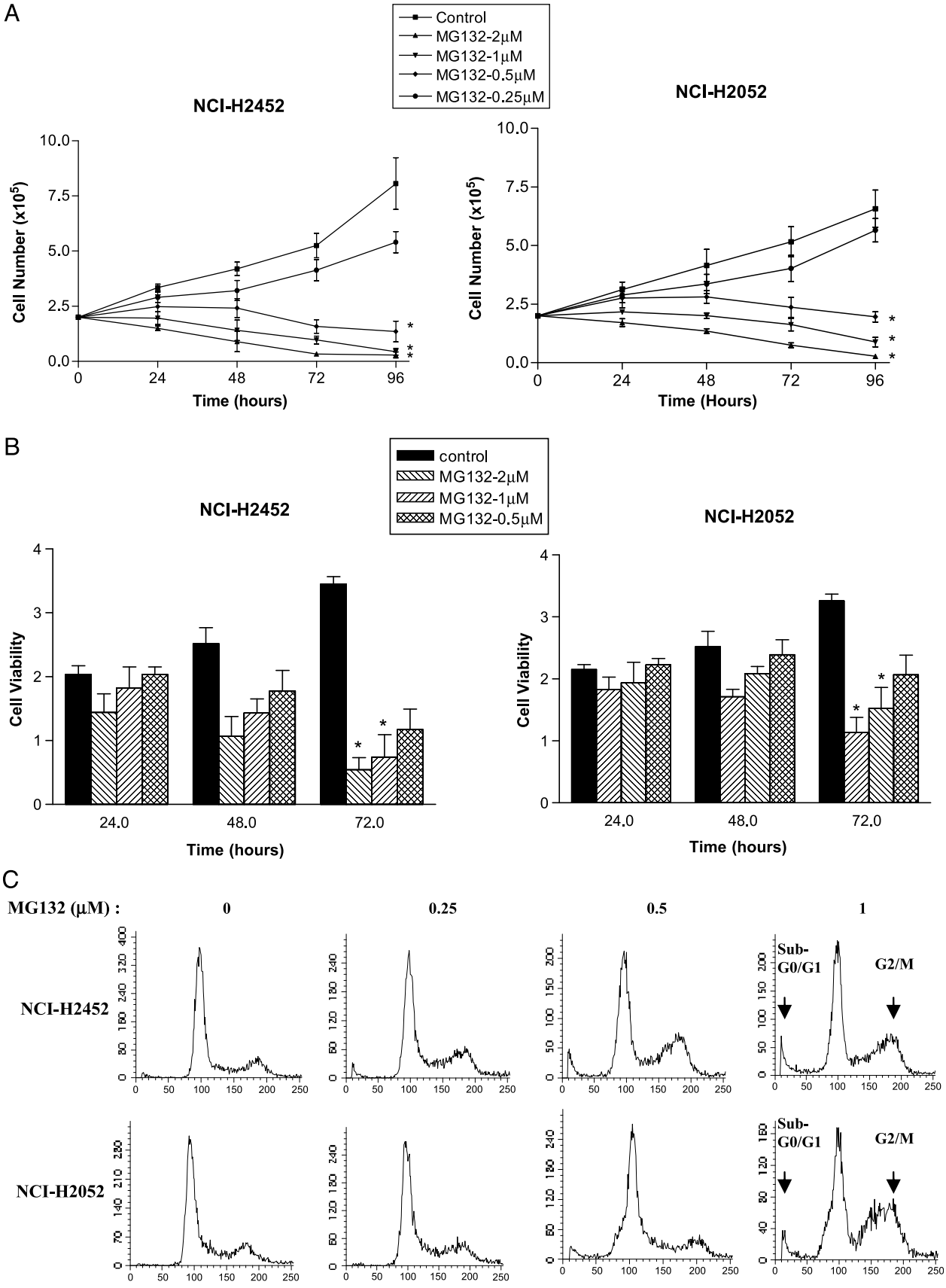
Caspase Activation Is Involved in MG132-Induced Apoptosis

Previous work demonstrated that various caspases are involved in PI-induced apoptosis in other types of tumor cells [19]. To analyze the MG132-induced apoptosis in MPM cells, we examined cleavage activities of caspases 3, 7, 8, and 9 as well as cleavage of PARP, a prominent substrate of executioner caspases, in both MPM cell lines after treatment with MG132. A significant increase in cleavage activities of caspases 3, 9, and 8 detected in caspase cleavage assay was observed in both cell lines in response to greater than 0.5 μM MG132 (Figure 2*A*). A dose-dependent cleavage for caspases 3 and 7 and PARP was further demonstrated by Western blot analysis in both cell lines (Figure 2*B*). Consistent with other parameters of apoptotic cell death, NCI-H2452 cells showed a more significant protein cleavage than NCI-H2052 cells. In addition, Bid protein cleavage was also observed in MG132-treated NCI-H2452 cells (Figure 2*B-1*). To determine whether the MG132-induced apoptosis is dependent on caspase activation, we cotreated NCI-H2452 cells with 1 μM MG132 and 10 μM of each caspase specific inhibitor. Z-FA-fmk was used as a negative control for all caspase inhibitors. It was observed that treatment with Z-VAD-fmk, Z-IETD-fmk, or Z-LEHD-fmk significantly reduced MG132-induced cleavage of PARP and caspase 7 as observed in Western blot analysis (Figure 2*C*) and significantly suppressed MG132-induced cell death as seen in the WST-1 assay (Figure 2*D*), demonstrating that MG132-induced apoptosis is caspase-dependent.

Mitochondrial Caspase Activation Pathway Is Responsible for MG132-Induced Apoptosis

To determine whether the mitochondrial pathway is involved in MG132-induced apoptosis, we then tested mitochondrial release of Cyto *c* and Smac into the cytosol after treatment with MG132 in both MPM cell lines. A more abundant release of Smac with a less abundant release of Cyto *c* was observed in Western blot analysis supporting that the mitochondrial pathway was activated by MG132 (Figure 3*A*). Subsequently, we examined protein levels of Bcl-2, Bcl-X_L, and Mcl-1, the Bcl-2 antiapoptotic family proteins, in MG132-treated cells. MG132 treatment caused no changes in Bcl-2 and Bcl-X_L, but induced a significant, dose-dependent elevation of the 40-kDa-long isoform Mcl-1 protein (or Mcl-1_L; Figure 3*B*). No short Mcl-1 isoform (30 kDa or Mcl-1_S) was observed. Therefore, these data supported that the mitochondrial apoptotic pathway was activated by MG132 and the Mcl-1 protein (or Mcl-1_L) is likely a negative regulator of activation of this pathway.

IAPs are another group of apoptosis regulatory proteins, which include c-IAP1, XIAP, and Survivin and inhibit apoptosis by binding to different caspases [22]. In this study, we also examined protein levels for c-IAP1, XIAP, and Survivin. It was revealed by Western blot analysis that the c-IAP1 level was slightly reduced by MG132 in both MPM cell lines, whereas the Survivin level was lowered in NCI-H2052 cells but elevated in NCI-H2452 cells by MG132. However, the XIAP protein level was clearly increased in both cell



lines, suggesting that XIAP may serve as another negative regulator for MG132-induced apoptosis (Figure 3*B*).

Silencing of Mcl-1 Protein Expression by siRNA Sensitizes MPM Cells to MG132-Induced Apoptosis

To determine the regulatory effect of Mcl-1 and XIAP on MG132-induced apoptosis, we used siRNA to silence expression of each protein in MPM cells before MG132 treatment. The scrambled siRNA was used as control siRNA. NCI-H2052 cells were chosen in this experiment because NCI-H2052 cells displayed a lower response to MG132-induced apoptosis than NCI-H2452 cells, probably through a more elevated Mcl-1 protein level (Figure 3*B*). It was observed that only Mcl-1 siRNA enhanced MG132-induced cell death (Figure 4*A*), which was accompanied by the increased protein cleavage for PARP and caspase 3 (Figure 4, *A* and *B-1*) although both Mcl-1 and XIAP siRNAs significantly reduced expression of each protein (Figure 4, *B-1* and *B-2*). Although Mcl-1 siRNA alone induced a slightly visible caspase 3 cleavage, the most significant apoptosis was only observed after Mcl-1 siRNA transfection and MG132 treatment (Figure 4*B-1*), suggesting that Mcl-1 is a major regulator of the MG132-induced apoptosis. Further tests revealed that Mcl-1 siRNA also assisted MG132 in inducing Bid protein cleavage with a subsequent increase in mitochondrial release of Smac and caspase 9 activation (Figure 4, *C* and *D*). These observations strongly suggest that Mcl-1 acts as a major survival molecule in restricting MG132-induced apoptosis in MPM cells.

Low-Dose MG132 Inhibits In Vitro *MPM Cell Invasion*

Because invasion is involved in both mortality and morbidity of MPM, thoracic MPM in particular [1], we also analyzed whether MG132 possesses an anti-invasion activity in MPM cells. To distinguish such possible effect from a proapoptotic effect, MPM cells were treated with subapoptotic doses of MG132 (<0.25 μM), which elicited no visible apoptotic response in both MPM cell lines. It was observed that subapoptotic doses of MG132, from 0.125 to 0.0625 μM, exhibited no effects on the viability or mobility of both MPM cell lines as revealed by a migration assay and a cell proliferation assay (data not shown). However, they significantly inhibited Matrigel invasion of both MPM cell lines in a dose-dependent manner (Figure 5), demonstrating that whereas higher concentrations of MG132 induce apoptosis in MPM cells, lower concentrations of MG132 inhibit invasion of MPM cells.

MG132-Inhibited Invasion of MPM Cells Is Independent of Src Activity

Increased Src activity is generally associated with the enhanced motility and invasion of cancer cells [23]. To examine the change of Src activity in MG132-treated MPM cells, we tested Src phosphorylation at tyrosine 527 (Tyr527) and 416 (Tyr416) residues, which represent inactive and active forms of the Src kinase, respectively [24]. Apoptotic doses of MG132 (>0.25 μM) clearly reduced Src phosphorylation at Tyr416 in both MPM cell lines (Figure 6*A*). However, subapoptotic doses of MG132 (<0.25 μM) exhibited no such effect (data not shown). In addition, MG132 did not induce a visible change in Src phosphorylation at the Tyr527 residue, which was much more robust than the phosphorylation at Tyr416 in both cell lines regardless of MG132 treatment (Figure 6*A*). We next treated both MPM cell lines with an Src kinase–specific inhibitor (type I) to determine whether inhibition of the Src signaling can reduce MPM cell invasion. NCI-H358, a human NSCLC cell line that exhibits a high sensitivity to the Src inhibitor, was used as a positive control in this test. It was observed that 10 μM Src kinase inhibitor dramatically reduced migration of NCI-H358 cells (Figure 6*B*) but did not inhibit invasion of either MPM cell line (Figure 6*B*), suggesting that invasion by MPM cells tested in this study is independent of Src activity.

Inhibition of Rac1 Activation Contributes to MG132-Inhibited MPM Cell Invasion

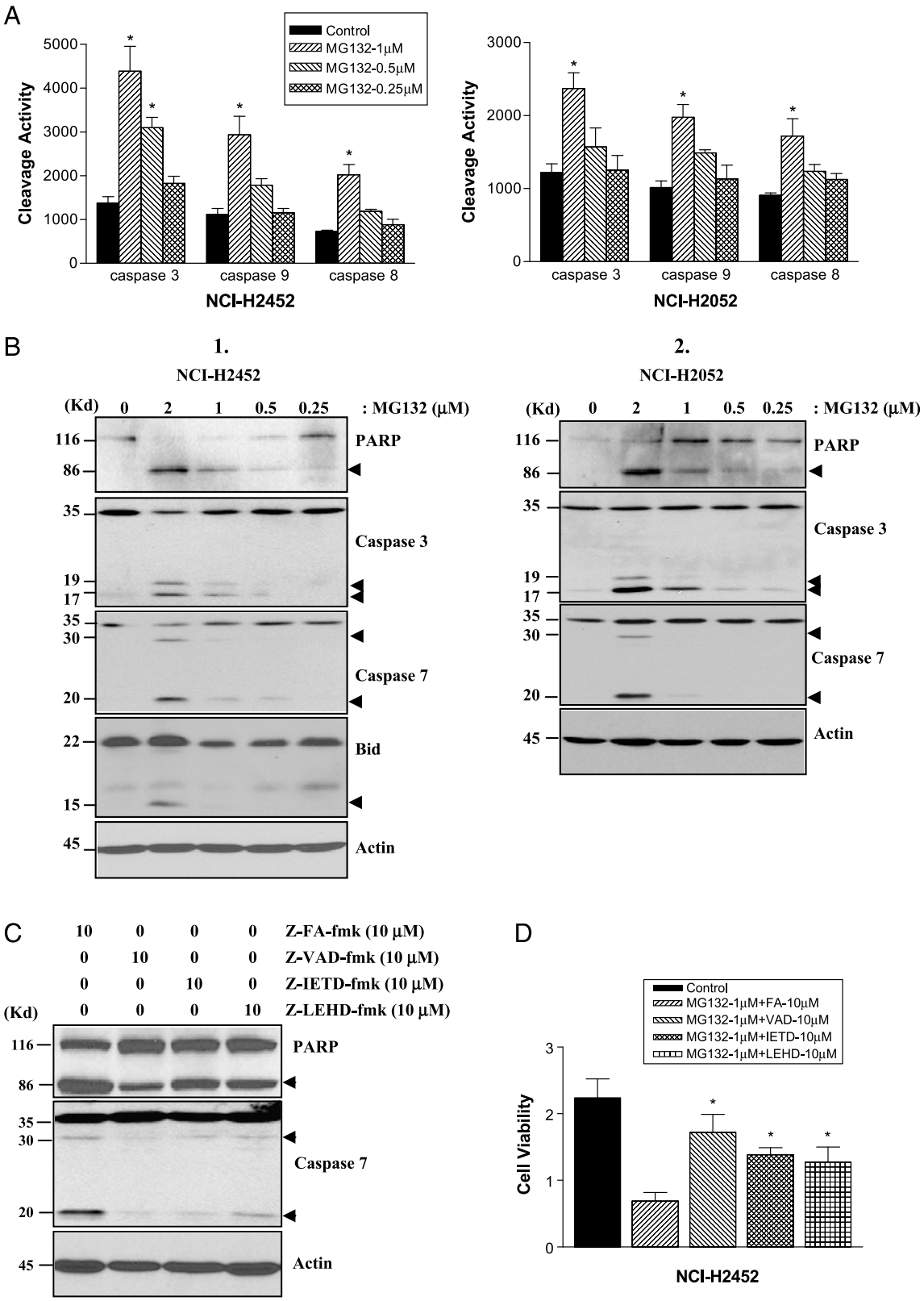
Rho subfamily members of the small GTPase Ras superfamily proteins, such as RhoA, Cdc42, and Rac1, represent another category of proteins that mediate tumor cell metastasis mainly through regulating actin cytoskeleton reorganization [25]. The activity of these proteins is switched on and off through a GTP- to GDP-binding cycling [25]. To further analyze MG132-inhibited MPM cell invasion, we performed a pull-down assay to determine the change of activity of the RhoA, Cdc42, and Rac1 proteins in response to MG132 treatment in NCI-H2452 cells, which displayed a higher invasion potential than NCI-H2052 cells. The GTP-bound Rac1 protein, but not the RhoA and Cdc42 proteins, was significantly reduced by subapoptotic doses of MG132 in a dose-dependent manner (Figure 7*A*), suggesting that reduction of Rac1 activity may be responsible for MG132-inhibited MPM cell invasion. To support this observation, we stably transfected a cDNA of a constitutively active Rac1 mutant, Rac1-G12V, into NCI-H2452 cells to first determine the effect of the mutant Rac1 on MPM cell invasion. Expression of Rac1-G12V, confirmed by Western blot analysis (Figure 7*B-1*), conferred a more invasive potential to MPM cells, especially during the earlier time of cell invasion (Figure 7*B-2*). It was next observed that the Rac1-G12V mutant significantly reduced MG132-inhibited NCI-H2452 cell invasion (Figure 7*B-3*), further supporting that Rac1 serves as a target of inhibition of tumor cell invasion by MG132.

Discussion

The lethality of MPM is due to both the resistance to currently available chemotherapies through its evasion of apoptosis induction

Figure 1. MG132 induces apoptotic cell death in human MPM cells. (A) Cell proliferation assay indicates that greater than 0.5 μM MG132 induces significant cell death in both NCI-H2452 and NCI-H2052 cells. Values are expressed as the means ± SD of two experiments. **P* < .05 *versus* control. (B) Cell viability assay using WST-1 reagent shows that most cells in both MPM cell lines died at 72 hours after treatment with greater than 1 μM MG132. Values are expressed as the means ± SD of three experiments. **P* < .05 *versus* control. (C) Flow cytometry assay demonstrates that MG132 induces the appearance of sub–G_0/G_1 cells with a distinct peak in both MPM cell lines in a dose-dependent manner. A clear increase in G_2/M cells in both cell lines is also indicated. The assay was performed at 36 hours for NCI-H2452 cells and 48 hours for NCI-H2052 cells after treatment with MG132.

and invasion, mediastinal invasion in particular for thoracic MPM. New agents that restore the sensitivity of MPM cells to apoptosis induction and inhibit MPM cell invasion will be desirable candidates for treating MPM. We demonstrate in the present study that MG132 possesses the dual abilities of both inducing MPM cell apoptosis in a caspase-dependent manner and inhibiting MPM cell invasion through suppressing Rac1 activity. The two MPM cell lines used in this study derived from two major pathologic subtypes of MPM: NCI-H2052 was from an epithelial MPM, whereas NCI-H2452 was from a sarcomatoid MPM, suggesting a wide coverage of MG132's effect on major types of MPMs. Therefore, MG132, and possibly other PIs as well, may represent a new and efficient



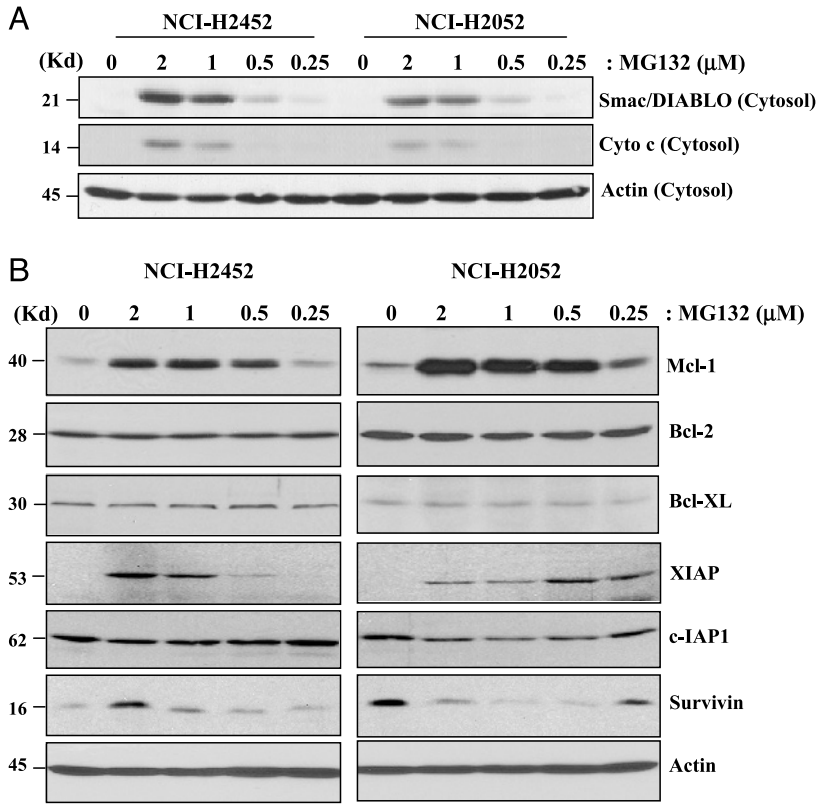


Figure 3. MG132 induces mitochondrial release of proapoptotic proteins and elevates the Mcl-1 protein in both MPM cell lines. All analyses were performed at 36 hours for NCI-H2452 cells and 48 hours for NCI-H2052 cells after treatment with MG132. (A) Western blot analysis indicates that MG132 induces mitochondrial release of the Smac and Cyto *c* proteins into the cytosol in a dose-dependent manner. Cell lysates were isolated and analyzed from the cytosol portion of each sample. (B) Western blot analysis demonstrates that, among Bcl-2 and IAP family proteins tested, the dose-dependent elevation of Mcl-1 represents the most significant change induced by MG132.

treatment that targets two critical molecular processes associated with the aggressiveness of MPM.

MG132-induced apoptosis in MPM cells is caspase-dependent. As revealed in this study, MG132-induced apoptosis is accompanied by the activation of caspases 3, 7, 8, and 9; inhibition of caspase activation by the inhibitors either for wide spectrum caspases or specifically for caspases 8 and 9 resulted in the inhibition of MG132-induced apoptotic cell death. It is also believed that caspase-dependent apoptosis is mediated by the mitochondria, which are activated by the truncated Bid protein and then release both Smac and Cyto *c* into the cytosol, leading to sequential activation of caspase 9 and caspase 3/7, and eventually to apoptotic cell death.

It was noted that the Smac protein released from the mitochondria was more abundant than the Cyto *c* protein. This notion is in agreement with the findings in other types of cancer cells treated with PIs [19,20]. A recent observation in our laboratory also showed that mitochondrial release of the Smac protein happens much earlier than that of the Cyto *c* protein during MG132-induced apoptosis of lung cancer cells, supporting the view that the Smac protein is more critical than the Cyto *c* protein in initiating PI-induced apoptosis [21]. The dominance of Smac over Cyto *c* may be attributable to MG132-induced stabilization of the Smac protein. The Smac protein released from the mitochondria on apoptotic stimuli can be rapidly bound by XIAP and directed toward proteasomal degradation. In addition to its well-characterized antiapoptotic function in binding and inhibiting caspases, XIAP, c-IAP1, and c-IAP2 also target proapoptotic molecules for proteasomal degradation to protect cells from inadvertent mitochondrial damage [19,26]. Indeed, a RING finger domain in the XIAP protein possessing ubiquitin protein ligase activity was found to be essential for ubiquitination and degradation of the Smac

Figure 2. MG132 induces a caspase-dependent apoptosis in both MPM cell lines. All assays were performed at 36 hours for NCI-H2452 cells and 48 hours for NCI-H2052 cells after treatment with MG132. (A) Caspase activation assay demonstrates that MG132 activates caspases 3, 9, and 8 in a dose-dependent manner. Values are expressed as the means ± SD of three experiments. *$P < .05$ *versus* control. (B) Western blot analysis shows that MG132 treatment induces cleavage of caspases 3 and 7, Bid, and PARP in NCI-H2452 cells (B-1) and induces cleavage of the caspases 3 and 7 and PARP proteins in NCI-H2052 cells (B-2). Protein cleavage fragments are indicated by arrows. (C and D) Inhibitors, 10 μM, for broad spectrum caspases (Z-VAD-fmk), caspase 8 (Z-IETD-fmk), or for caspase 9 (Z-LEHD-fmk) inhibit MG132-induced cleavages for caspase 7 and PARP (C) as revealed by Western blot analysis and inhibit MG132-induced cell death as demonstrated by cell viability assay using WST-1 reagent (D). Protein cleavage fragments in Western blot analysis are indicated by arrows. Values from WST-1 assay are expressed as the means ± SD of three experiments. *$P < .05$ for caspase inhibitors plus MG132 *versus* Z-FA-fmk plus MG132.

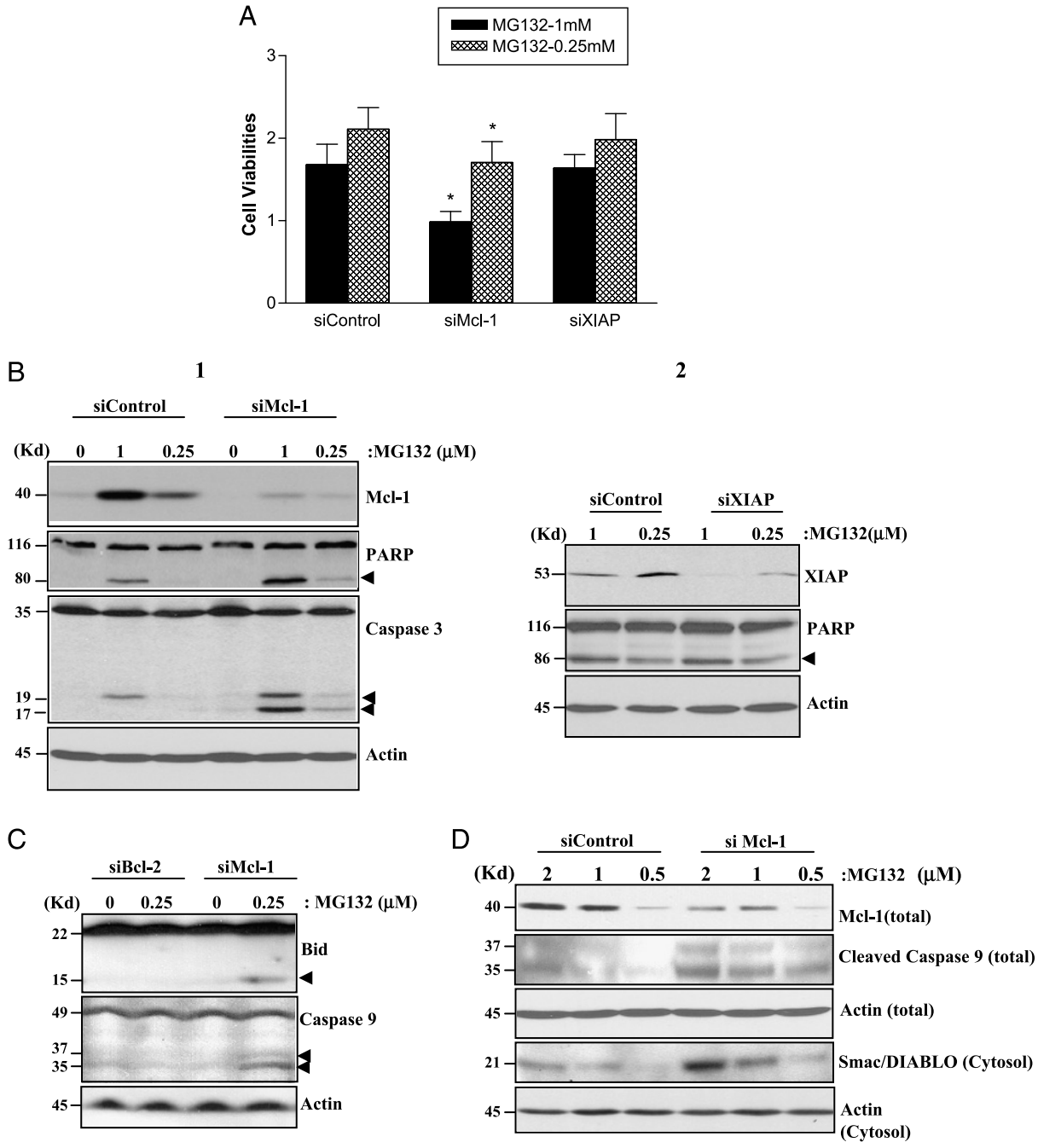


Figure 4. Mcl-1 siRNA sensitizes NCI-H2052 cells to MG132-induced apoptosis. All analyses were performed at 48 hours after treatment with MG132. Cell viability assay using WST-1 reagent shows that Mcl-1 siRNA, but not XIAP siRNA, significantly sensitizes MPM cells to MG132-induced cell death (A). Values are expressed as means ± SD of two experiments. *$P < .05$ for siMcl-1 *versus* siControl. Western blot analysis indicates that both Mcl-1 and XIAP siRNAs reduce the expression of each protein (B-1 and B-2). However, it is Mcl-1 siRNA (B-1), but not XIAP siRNA (B-2), that increases MG132-induced cleavage for PARP and caspase 3 (B-1). Mcl-1 siRNA also enhances Bid and caspase 9 protein cleavages with increased mitochondrial release of the Smac protein (C and D). Protein cleavage fragments in Western blot analysis are indicated by arrows.

protein [26]. Therefore, given that MG132 is a strong proteasome inhibitor, the caspase activation observed in MG132-treated MPM cells is likely achieved at least in part through Smac stabilization.

Among the antiapoptotic proteins of both IAP and Bcl-2 families tested, Mcl-1 (or Mcl-1_L) proved to be the major survival molecule to regulate MG132-induced apoptosis in MPM cells. Both Mcl-1 of the antiapoptotic Bcl-2 family proteins and XIAP of the IAP family proteins displayed protein elevation in two MPM cell lines in response to MG132 treatment. However, Mcl-1 demonstrated a much more consistent and higher protein elevation than XIAP. Moreover, it is Mcl-1 siRNA, but not XIAP siRNA, that significantly enhanced MG132-induced apoptosis in MPM cells. The concomitant elevation of the Mcl-1 protein in MPM cells is believed to be the consequence of MG132-induced proteasome inhibition as the Mcl-1 protein is also subjected to proteasome-mediated degradation [27,28]. Multiple mechanisms have been proposed for the protection by Mcl-1

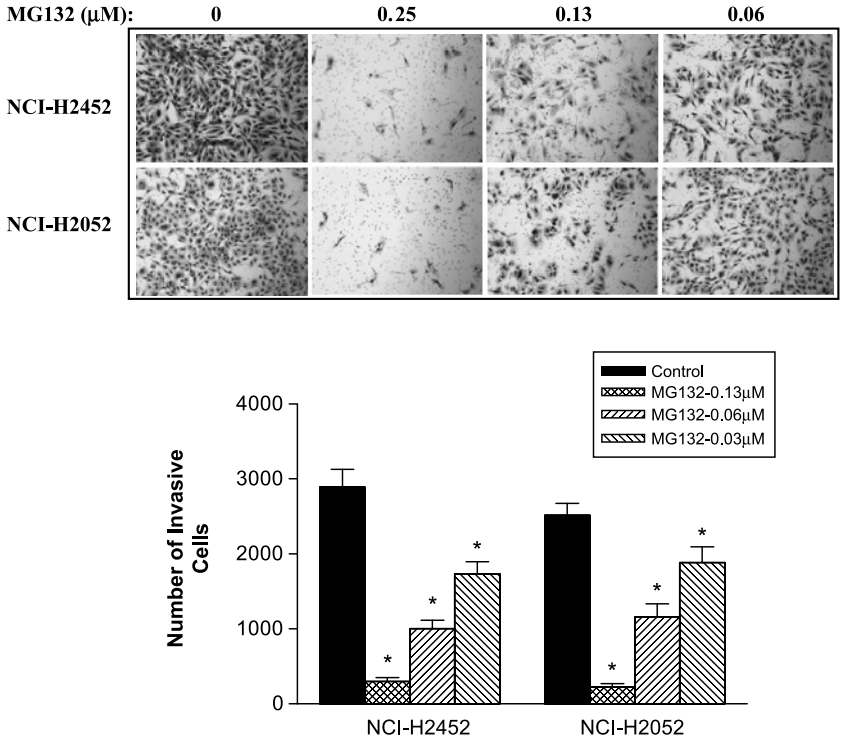


Figure 5. Subapoptotic doses of MG132 inhibits MPM cell invasion. A total of 1×10^5 cells were seeded in each Matrigel-coated transwell and incubated for 48 hours with different concentrations of MG132. Invasive cells at the lower side of each transwell membrane were fixed and stained by Diff-Quick solutions and quantified. The quantitative values expressed as the means ± SD of five microscopic fields are representative of two separate experiments. *$P < .05$ *versus* control.

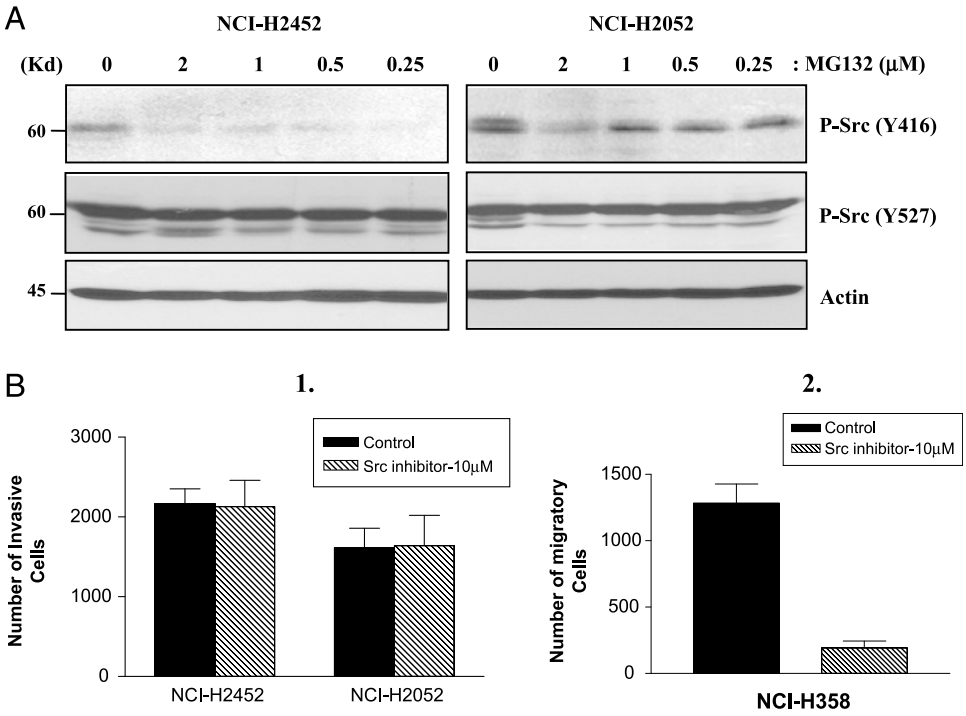


Figure 6. MPM cell invasion is independent of Src protein activity. (A) Western blot analysis reveals that apoptotic doses of MG132 reduce phosphorylation of Src at Tyr 416 residue in both MPM cell lines. (B) Migration or invasion assays demonstrate that 10 μM Src inhibitor I, which dramatically reduces migration of NCI-H358 cells, exhibits no effect on invasion of both MPM cell lines. A total of 2×10^5 NCI-H358 cells were used in the migration assay, and the migratory cells were stained at 24 hours after treatment with Src inhibitor I. A total of 1×10^5 cells of each MPM cell line were used in a Matrigel invasion assay, and the invasive cells were stained at 36 hours after treatment with Src inhibitor I.

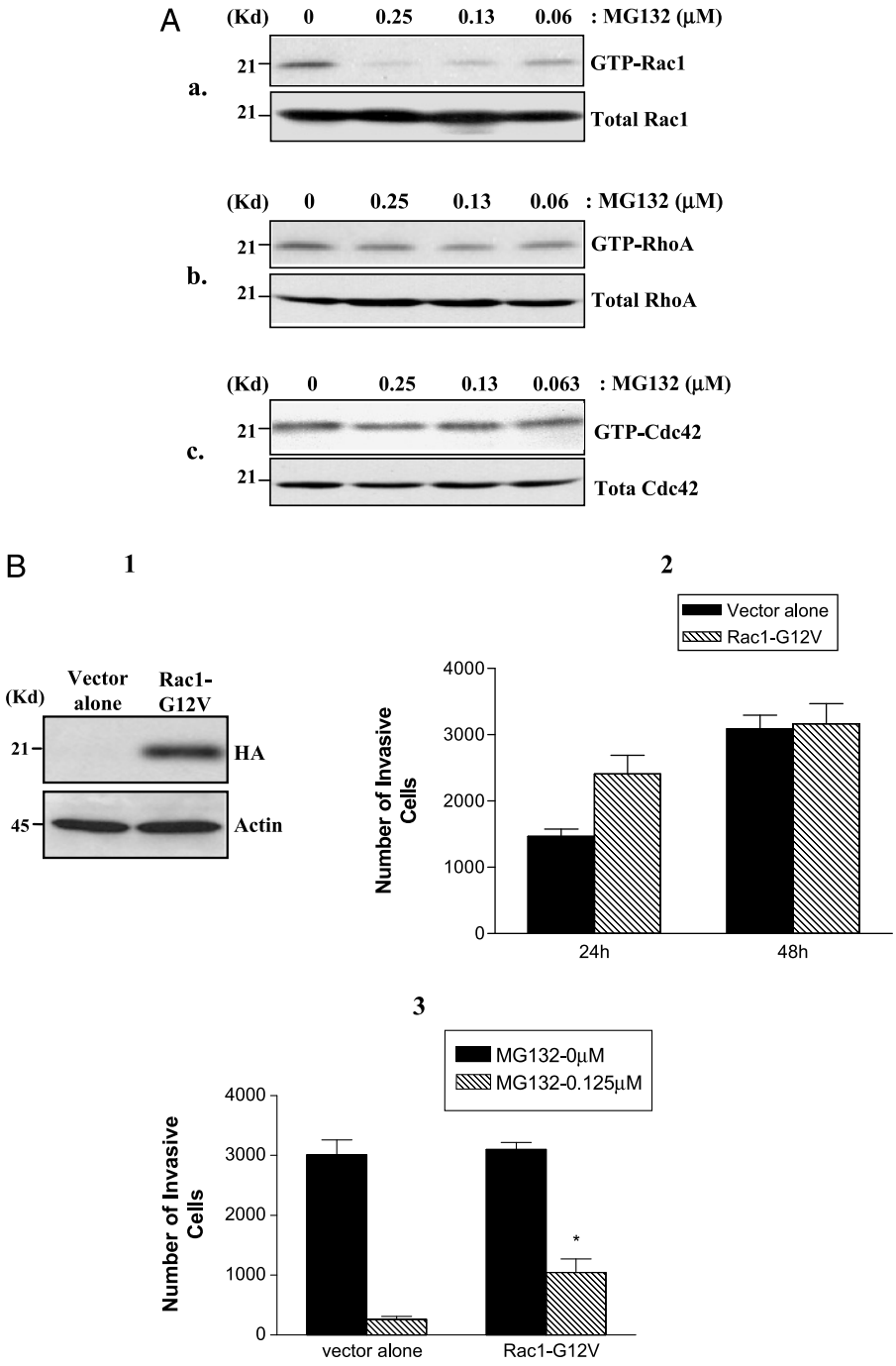


Figure 7. Rac1 activity is associated with MPM cell invasion and serves as a target for MG132 in its inhibition of MPM cell invasion. (A) Western blot analysis after pull-down assays shows that, among Rac1, Cdc42, and RhoA, the GTP-bound Rac1 protein was most significantly reduced by subapoptotic doses of MG132 in a dose-dependent manner. (B) Expression of the Rac1-G12V protein, confirmed by Western blot analysis using an anti–HA antibody (B-1), promotes invasion of NCI-H2452 cells as assayed at 24 hours (B-2) and reduces the induction of MG132-inhibited MPM cell invasion (B-3). Values expressed as the mean ± SD of five microscopic fields are representative of two separate experiments. *$P < .05$ for Rac1-G12V *versus* vector alone.

from apoptotic damage. For example, Mcl-1 inhibits death receptor–mediated apoptosis by interacting with the truncated Bid protein causing inhibition of Cyto *c* release from the mitochondria, indicating that Mcl-1 acts as an antiapoptotic molecule in parallel or downstream of Bid protein activation [29]. However, the finding that Mcl-1 siRNA enhances MG132-induced Bid protein cleavage suggests that Mcl-1's action in MPM cells is, in contrast, upstream of Bid protein activation probably through a positive feedback mechanism [21]. Therefore, while MG132 activates multiple caspases to drive apoptotic cell death, it also elevates Mcl-1, most likely through inhibiting proteasomes, which then restrains such apoptotic response.

Inhibition of MPM cell invasion by MG132 represents a newly discovered anticancer activity of PIs. Such activity is independent of its proapoptotic activity because it can be achieved by subapoptotic doses of MG132. Several signaling pathways, such as c-Met/HGF and Src signaling, have been reported to mediate different aspects of

MPM's tumorigenicity [30–32]. However, based on our analyses, both Src and c-Met/HGF are less likely to be directly involved in invasion of both MPM cell lines tested in this study. Both MPM cell lines are not responsive to a c-Met inhibitor in terms of cell proliferation and migration, although NCI-H2452 cell line expresses a high level of the c-Met protein [30,31]. Although apoptotic doses of MG132 can reduce Src activation as evidenced by the reduced phosphorylation of Src at Tyr416 in Western blot analysis, the subapoptotic dosages of MG132 that can inhibit MPM cell invasion exhibited no effect on Src activation. In addition, an Src-specific inhibitor, which inhibits migration of a human NSCLC cell line, showed no effect on both migration and invasion of MPM cells. The insensitivity of both MPM cell lines to the inhibition by both c-met and Src inhibitors suggests that other signaling molecules other than c-Met/HGF and Src are more involved in mediating MPM cell invasion and may be a target of MG132 in inhibiting MPM cell invasion.

Rac1 is believed to be a target of MG132 in its inhibition of MPM cell invasion. Increased Rac1 activity has been tightly associated with enhanced cell motility and invasion [25]. However, its involvement in MPM cell invasion has not been addressed. Among the small GTPase Rho subfamily proteins tested in this study, only Rac1 showed a significant reduction in GTP-bound form in response to MG132 treatment as seen in a pull-down assay. Also, the Rac1 protein level seems to be higher than both RhoA and Cdc42 in MPM cells. Most importantly, transfection of a constitutively active Rac1 mutant can further promote MPM cell invasion and partially block MG132-induced inhibition of MPM cell invasion. These findings support that the Rac1 protein is involved in MPM cell invasion and serves as a target of MG132 although it is not the only target. Rac1 activity is regulated by Tiam1, Rac1's specific guanine nucleotide exchange factor, which activates Rac1 and promotes tumor cell invasion by converting GDP-bound Rac1 into GTP-bound Rac1 [33], and by Rac1 specific Rho GTP activating protein (RhoGAP), yet to be identified, which inactivates Rac1 by catalyzing GTP-bound Rac1 into GDP-bound Rac1. Activated Rac1 is reported to be degraded by proteasomes in certain types of cells undergoing special treatment [34]. However, the reduction of Rac1 activity in MG132-treated MPM cells is unlikely to be the primary consequence of proteasome inhibition, rather it may be achieved through activation of Rac1's RhoGAP or inactivation of Tiam1 by yet unknown mechanisms.

In summary, the present study demonstrates that proteasome inhibitor MG132 may represent a promising new treatment of MPM, which induces apoptosis as well as inhibits invasion of MPM cells. Because the concomitant increase of Mcl-1 protein inhibits MG132-induced apoptosis, other treatments for down-regulating Mcl-1 expression are needed to synergize with MG132 in treating MPM. Mcl-1 siRNA, Mcl-1 small molecule inhibitor, or inhibitors of other signaling molecules, such as the inhibitor of STAT3, are potentially efficient cotreatments with MG132. Indeed, Mcl-1 siRNA has proved to be effective in enhancing MG132-induced apoptosis in this study. STAT3 acts as a survival protein by up-regulating Mcl-1, c-Myc, Cyclin D1, and Bcl-X_L at the transcriptional level in tumor cells [35]. Further investigation of the synergy between PIs and different inhibitors of signaling pathways will provide new insights into developing more efficient PI-based combination therapies for MPM treatment. In addition, further investigation of Rac1 in MPM cell invasion may help develop new diagnostic markers to measure the invasion potential of MPM cells and to guide the usage of PIs in treating MPM.

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Disclaimer

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