Enhanced apoptosis by pemetrexed and simvastatin in malignant mesothelioma and lung cancer cells by reactive oxygen species-dependent mitochondrial dysfunction and Bim induction

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Abstract. Pemetrexed is a multitarget antifolate currently used for the treatment of malignant mesothelioma and non-small cell lung cancer (NSCLC). Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors used primarily for hyperlidpidemia, have been studied for their antiproliferative and pro-apoptotic effects. However, the effects of simvastatin on pemetrexed-induced apoptosis have not been investigated. In this study, we investigated whether combination treatment with pemetrexed and simvastatin potentiates the apoptotic activity above that is seen with either drug alone in malignant mesothelioma and NSCLC cells. We found that the combination of pemetrexed and simvastatin induced more extensive caspase-dependent apoptosis than either drug alone in malignant mesothelioma cells (MSTO-211) or NSCLC cells (A549). In addition, reactive oxygen species (ROS) generation in cells treated with both pemetrexed and simvastatin was markedly increased compared to cells treated with either pemetrexed or simvastatin alone. Combination treatment also increased the loss of mitochondrial membrane potential, increased cytosolic release of cytochrome *c*, and altered expression of inhibitor of apoptosis proteins (IAP) and B-cell lymphoma-2 (Bcl-2) families of apoptosis related proteins. On the other hand, pretreatment with N-acetylcysteine (NAC) prevented apoptosis and mitochondrial dysfunction by pemetrexed and simvastatin. In addition, Bim siRNA conferred protection against apoptosis induced by pemetrexed and simvastatin. These results suggest that combination of pemetrexed and simvastatin potentiates their apoptotic activity beyond that of either drug alone in malignant mesothelioma and lung cancer cells. This activity is mediated through ROS-dependent mitochondrial dysfunction and Bim induction.

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Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). Non-small cell lung cancer (NSCLC) accounts for ~80% of all lung cancers with long-term survival restricted to a small subset of patients. Chemotherapy has a modest but significant impact on survival and quality of life in patients with NSCLC (2). Malignant mesothelioma is a relatively rare malignancy with a generally poor outcome. The median survival is currently 9-12 months after diagnosis. At this time, there are few effective chemotherapeutic options for treatment of malignant mesothelioma. They include cisplatin, vinorelbine, and gemcitabine (3). New strategies based on a better understanding of tumor biology may help to maximize the efficacy of current treatments.

Pemetrexed, a multitargeted antifolate cytotoxic agent, is a chemotherapeutic agent used in malignant mesothelioma and NSCLC (mostly used in non-squamous cell carcinomas) (4-6). Pemetrexed primarily inhibits thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), these are all enzymes in folate-dependent metabolic processes (7,8). Previous studies have reported that pemetrexed-induced apoptosis is associated with upregulation of p53 and inactivation of Bcl-2 (9,10), and inhibition of the intrinsic apoptosis pathway has been shown to suppress the cytotoxicity of pemetrexed (11).

Statins are a class of drugs that inhibit the rate-limiting step of the mevalonate pathway, which is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (12). Besides their lipid-lowering effect, statins have been studied for their antineoplastic properties in many solid tumor cells, including NSCLC (13,14). Statins have been also shown to sensitize cancer cell lines to cytotoxic drugs such as 5-fluorouracil (5-FU), taxol, etoposide, doxorubicin, and cisplatin (15-18). We recently demonstrated that the combination of sulindac and simvastatin augmented their apoptotic potential above that is seen with either drug alone in A549 lung cancer cells. These effects were mediated via reactive oxygen species (ROS)-dependent mitochondrial dysfunction (19).

Although pemetrexed has generally been a well-tolerated drug, its toxicity profile is not trivial. The most frequently observed adverse effects include myelosuppression, fatigue, hepatotoxicity, nephrotoxicity, pneumonitis, and mucositis (20,21). Until now, the mechanism by which pemetrexed and statin combines to induce apoptosis and inhibit the growth of mesothelioma and NSCLC cells has not been elucidated. In this study, we demonstrated the synergistic interaction of pemetrexed and simvastatin and explored the mechanisms underlying this synergy.

Materials and methods

Materials. Roswell Park Memorial Institute medium-1640 (RPMI-1640), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were obtained from Gibco BRL Co. (Grand Island, NY, USA). Pemetrexed was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). Simvastatin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide (PI), dimethyl sulfoxide (DMSO) and N-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). JC-1, a lipophilic, fluorescent dye used to detect mitochondrial membrane depolarization was obtained from Molecular Probes Co. Primary antibodies against the following targets: caspase-3, -8 and -9, poly(ADP-ribose) polymerase (PARP), Puma, Bim, Mcl-1, Bcl-XL, and XIAP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against heme oxygenase-1 (HO-1), MnSOD, survivin, VDAC, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to cytochrome *c* were obtained from Pharmingen (San Diego, CA, USA). Anti-rabbit IgG-conjugated horseradish peroxidase (HRP) antibodies and enhanced chemiluminescence (ECL) kits were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell culture and viability test. MSTO-211 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and A549 human lung cancer cells were obtained from the Korean Cell Line Bank (Seoul, Korea). These cell lines were grown in RPMI-1640 containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37˚C and maintained in log phase growth.

Cell viability was determined by measuring the mitochondrial conversion of MTT to formazan, which was measured spectrophotometrically. After cells were treated with the specified study drugs, MTT was added to the cell suspension for 4 h. After three washes with phosphate-buffered saline (PBS; pH 7.4), the insoluble formazan product was dissolved in dimethyl sulfoxide (DMSO). The optical density (OD) of each well was measured using a microplate reader (Titertek Multiskan; Flow Laboratories, North Ryde, New South Wales, Australia) at 590 nm. The OD resulting from formazan production in control cells was considered as 100% cell viability, and all other measurements were expressed as a percentage of the control cell value.

Annexin V assay for the assessment of apoptosis. MSTO-211 and A549 cells undergoing early/late apoptosis were analyzed by Annexin V-FITC and PI staining. Cells in the exponential growth phase ($2.5x10^5$ cells) were seeded in 35-mm^2 dishes. Cells were left untreated or incubated with specified drugs for the indicated times at 37˚C. Both adherent and floating cells were collected and analyzed by the Annexin V assay, according to the manufacturer's instructions. Pelleted cells were briefly washed with PBS and resuspended in annexin binding buffer. Cells were then incubated with Annexin V-FITC and PI for 15 min at room temperature. After incubation, the stained cells were analyzed using a fluorescence-activated cell sorting (FACS)Calibur system equipped with CellQuest software (Becton-Dickinson, San Jose, CA, USA). Cells with no drug treatment were used as controls.

Measurement of the mitochondrial membrane potential ($\Delta\Psi_m$). MSTO-211 and A549 cells were harvested at the indicated treatment times, washed with PBS, and then stained with 10 μg/ml JC-1 at 37˚C for 30 min. After a brief wash with PBS, cells were immediately analyzed using a FACSCalibur system equipped with CellQuest software. At low concentrations, JC-1 exists mainly in a monomeric form, emitting green fluorescence (emission maximum at ~530 nM), whereas at higher concentrations it forms aggregates, known as J-aggregates, which emit orange-red fluorescence (emission maximum at ~590 nM).

Measurement of reactive oxygen species (ROS). To measure intracellular ROS, cells were incubated with 10 μmol/l 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes, Eugene, OR, USA) at 37˚C for 30 min. Cells were then washed, scraped gently, resuspended in PBS, and kept on ice for immediate analysis by FACSCalibur flow cytometry using an argon laser (488 nm) for excitation. Green fluorescence due to trapped DCF inside the cells was collected and plotted on a log scale. Data were acquired and analyzed with the CellQuest program. To measure mitochondria-derived ROS, the mitochondria-targeted, peroxide ion (O_2^-) sensitive, hydroethidine analog probe MitoSOX (Invitrogen Life Technologies, M36008) was used to determine relative O_2^- levels. Cells were incubated with 5 μM MitoSOX for 10 min in RPMI-1640, washed twice with PBS, and analyzed with FACSCalibur flow cytometry.

Western blotting. Cells were harvested and lysed using radioimmunoprecipitation assay buffer [50 mM Tris-Cl (pH 7.4), 1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin and leupeptin

and 1 mM Na_3VO_4]. After centrifugation at 12,000 x g for 30 min, the supernatant was collected, and the protein concentration was determined by the method of Bradford (Bio-Rad protein assay). Equal amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and subsequently transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in TBS-T [25 mM Tris (pH 7.6), 138 mM NaCl, and 0.05% Tween-20] for 1 h and probed with primary antibodies (at 1:1,000-1:5,000). After a series of washes, membranes were further incubated with secondary antibody (at 1:2,000-1:10,000) conjugated with HRP. Detection of the immunoreactive signals was carried out using an ECL detection system.

Preparation of cytosolic and mitochondrial fractions. Cytosolic and mitochondrial fractions were prepared as described previously (22) with modifications. Cells were harvested, washed with ice-cold PBS, and then incubated with 500 μM buffer A [250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A] on ice for 30 min. Cells were then disrupted by 20 passages through a 26-gauge needle and centrifuged at 750 x g for 10 min. The supernatant was centrifuged at 10,000 x g for 25 min. After centrifugation, the cytosolic fraction was frozen at 70˚C. The pellet containing mitochondria was washed with ice-cold buffer A and then resuspended with cell lysis buffer. The resuspended pellet was incubated on ice for 30 min and then centrifuged at 10,000 x g for 25 min. The supernatant thus collected represented the mitochondrial fraction of cells.

Gene silencing. Transcriptional expression of Bim was specifically suppressed by the introduction of 21-nucleotide duplex small interfering RNA (siRNA), which targets nucleotides of Bim mRNA coding sequence (23). Cells (10^5 cells/well) were plated in 6-well plates and transiently transfected with 50 nM per well of Bim siRNA (Cell Signaling Technology) mixed with the X-tremeGENE siRNA transfection reagent (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Silencer Negative Control siRNA (Roche Applied Science) was used as a negative control and introduced into the cells using the same protocol.

Statistical analysis. Each experiment was performed at least 3 times, and all values were expressed as the mean ± SD of triplicate samples. The Student's t-test was used to determine the statistical significance of the results. Values of $p<0.05$ were considered statistically significant.

Results

Effect of pemetrexed and simvastatin, alone and in combination on the growth of malignant mesothelioma and lung cancer cells. MSTO-211 and A549 cells were treated with different concentrations of simvastatin in the absence or presence of pemetrexed, and viability was measured by the MTT assay. As shown in Fig. 1, the combination of pemetrexed and simvastatin produced a synergistic inhibitory effect on the growth of both MSTO-211 and A549 cells. Simvastatin inhibited cell growth in a dose-dependent fashion in the presence of pemetrexed.

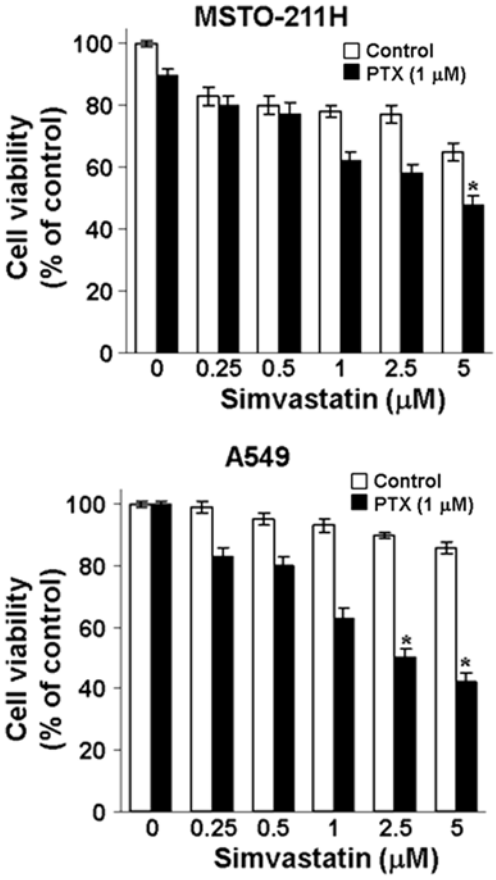

Figure 1. Inhibitory effects of combination treatment with pemetrexed and simvastatin on cell growth of MSTO-211 and A549 cells. Cells were treated with different concentrations of simvastatin in the absence or presence of 1 μM pemetrexed for 48 h, and viability was then measured by the MTT assay. The viability of control cells was set at 100%, and the cell survival of treated cells relative to the control cells is presented. The data represent the mean ± SD of three independent experiments. *$p<0.05$ compared to the control.

Combination of pemetrexed and simvastatin enhances caspase-dependent apoptosis. To examine whether the observed growth inhibition was due to enhanced apoptosis, the proportion of apoptotic cells was determined using Annexin V-PI staining. Annexin V staining showed that the combination of pemetrexed and simvastatin significantly enhanced apoptosis compared with either drug alone in MSTO-211 and A549 cells (Fig. 2A).

To further elucidate the mechanism of apoptosis induced by pemetrexed and simvastatin, cell lysates were evaluated by immunoblotting (Fig. 2B). Our results showed that the combination of pemetrexed and simvastatin enhanced the expression of the processed 85-kDa isoform of PARP, which is known to play a major role in circumventing the apoptosis process. Moreover, the combination of pemetrexed and simvastatin led to a marked increase in the expression of caspase-3, -8 and -9. These results indicate that pemetrexed and simvastatin enhanced caspase-dependent apoptosis in MSTO-211 and A549 cells.

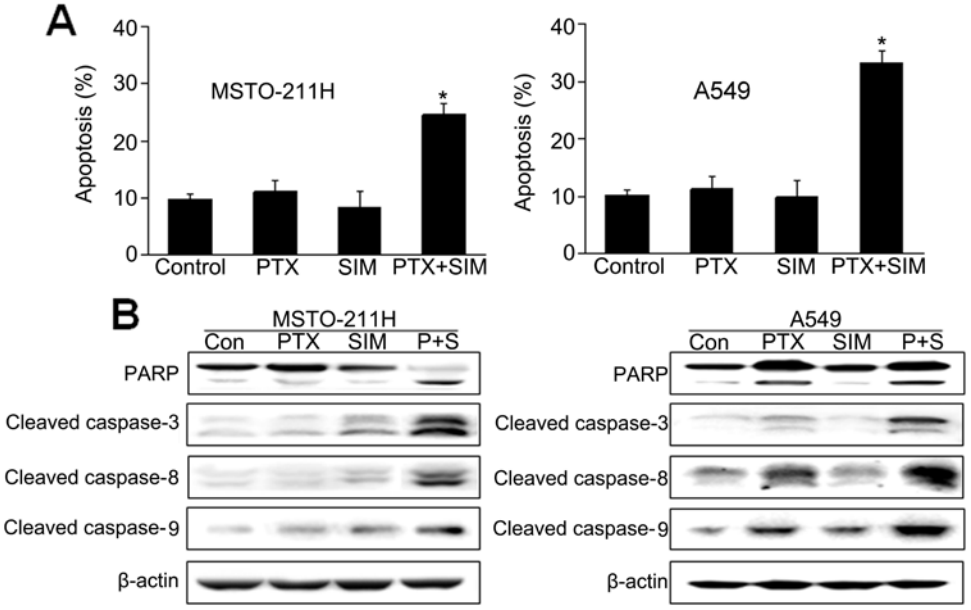

Figure 2. Effect of pemetrexed and simvastatin on caspase-dependent apoptosis. (A) Cells were incubated with 1 μM pemetrexed and/or 5 μM simvastatin for 48 h, and apoptosis was evaluated by green fluorescent protein-Annexin V + propidium iodide. The percentage of Annexin V and propidium iodide-positive cells was quantified. (B) Cells were treated with pemetrexed and simvastatin, alone and in combination for 48 h. Then the cells were lysed, and the cell lysate was subjected to 12% SDS-PAGE to measure the expression of PARP and caspase-3, -8 and -9.

Combination of pemetrexed and simvastatin enhances intracellular ROS production. We also investigated the upstream regulatory mechanisms leading to the induction of apoptosis by the combination of pemetrexed and simvastatin. Intracellular ROS generation was assessed by flow cytometry using the total ROS marker H_2DCFDA and the mitochondrial superoxide marker MitoSOX RED. The results demonstrate that ROS generation in MSTO-211 and A549 cells treated with both pemetrexed and simvastatin increased markedly compared to ROS generation in cells treated with pemetrexed or simvastatin alone (Fig. 3A). FACS analysis using MitoSOX revealed that intracellular O_2^- levels increased significantly, which correlated well with the onset of total ROS production. It is possible that the combination treatment increased cellular oxidative stress. We then investigated whether combined treatment with pemetrexed and simvastatin affected two markers for oxidative stress: inducible HO-1 and MnSOD (Fig. 3B). The combination of pemetrexed and simvastatin resulted in enhanced expression of HO-1 and MnSOD compared to MSTO-211 and A549 cells treated with either pemetrexed or simvastatin alone.

Combination of pemetrexed and simvastatin leads to mitochondrial dysfunction. We also investigated components upstream of caspase-3 in apoptotic signaling. Markers of mitochondrial dysfunction, including $\Delta\Psi_m$ transition and cytosolic release of cytochrome *c*, were evaluated in cells treated with pemetrexed and simvastatin. JC-1 has been widely used for the detection of apoptosis by measuring mitochondrial depolarization. As shown in Fig. 4A, JC-1 monomer level was enhanced in MSTO-211 and A549 cells treated with the drug combination. Since the loss of $\Delta\Psi_m$ results in cytochrome *c* release into the cytosol, cytochrome *c* levels were evaluated by western blotting in both mitochondrial and cytosolic fractions (Fig. 4B). Combination treatment with pemetrexed and simvastatin was associated with an increased level of cytochrome *c* in the cytosolic fraction over that seen with either agent alone and a corresponding decrease in levels in the mitochondrial fraction.

Combination of pemetrexed and simvastatin induces changes in IAP and Bcl-2 families. Members of the IAP and Bcl-2 families are important regulators of the mitochondrial apoptotic pathway. To identify the molecular mechanism underlying apoptosis induced by combined treatment with pemetrexed and simvastatin, we examined the expression levels of the IAP (XIAP and survivin), anti-apoptotic (Mcl-1 and Bcl-xL), and pro-apoptotic (Bim and Puma) Bcl-2 families, by immunoblot analysis in MSTO-211 and A549 cells treated with pemetrexed and/or simvastatin for 36 h. As shown in Fig. 4C, treatment of MSTO-211 and A549 cells with pemetrexed and simvastatin resulted in a significant decrease in XIAP and survivin levels relative to treatment with either drug alone. In addition, combination treatment with pemetrexed and simvastatin decreased the expression of the anti-apoptotic factors Mcl-1 and Bcl-xL, and increased the expression of pro-apoptotic factors Bim and Puma.

Pretreatment with NAC prevents apoptosis induced by pemetrexed and simvastatin. We next tested the effect of the free radical scavenger NAC in pemetrexed and simvastatin-treated MSTO-211 and A549 cells. Cells were pretreated with NAC, followed by the addition of pemetrexed and simvastatin for 24 h. As shown in Fig. 5A, the enhancement of ROS generation by combination treatment with pemetrexed and simvastatin was abrogated by NAC. Moreover, NAC markedly inhibited the effects of combination therapy on cell viability, as evaluated by the MTT assay (Fig. 5B).

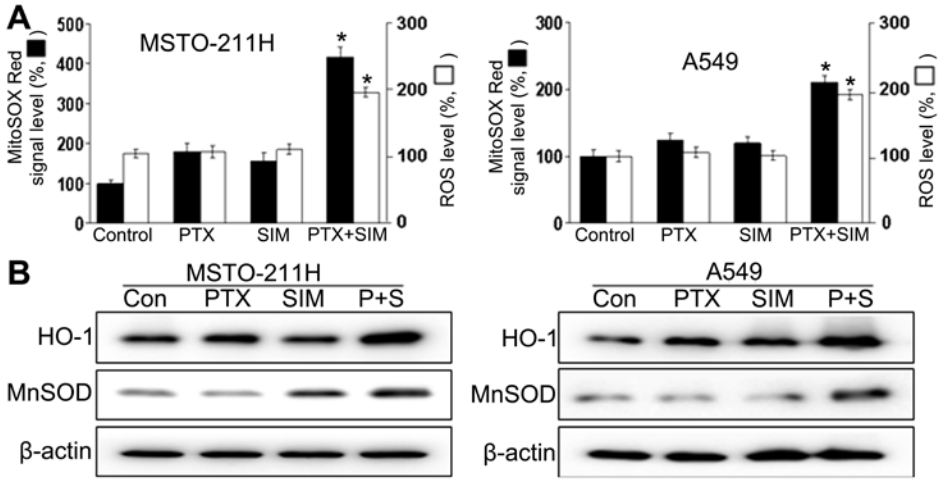

Figure 3. Contribution of ROS generation to the activity of combination therapy in MSTO-211 and A549 cells. (A) Cells were treated with 1 μM pemetrexed and/or 5 μM simvastatin for 48 h and then loaded with H_2DCFDA (white bars) and MitoSOX Red (black bars). Fluorescence measurements were carried out using a FACSCalibur flow cytometer. The data represent the mean ± SD of three independent experiments. $^*p<0.05$ compared to control. (B) Cells were treated with pemetrexed and simvastatin, alone and in combination for 48 h. The cells were lysed and the cell lysate was subjected to 12% SDS-PAGE to measure the expression of HO-1 and MnSOD.

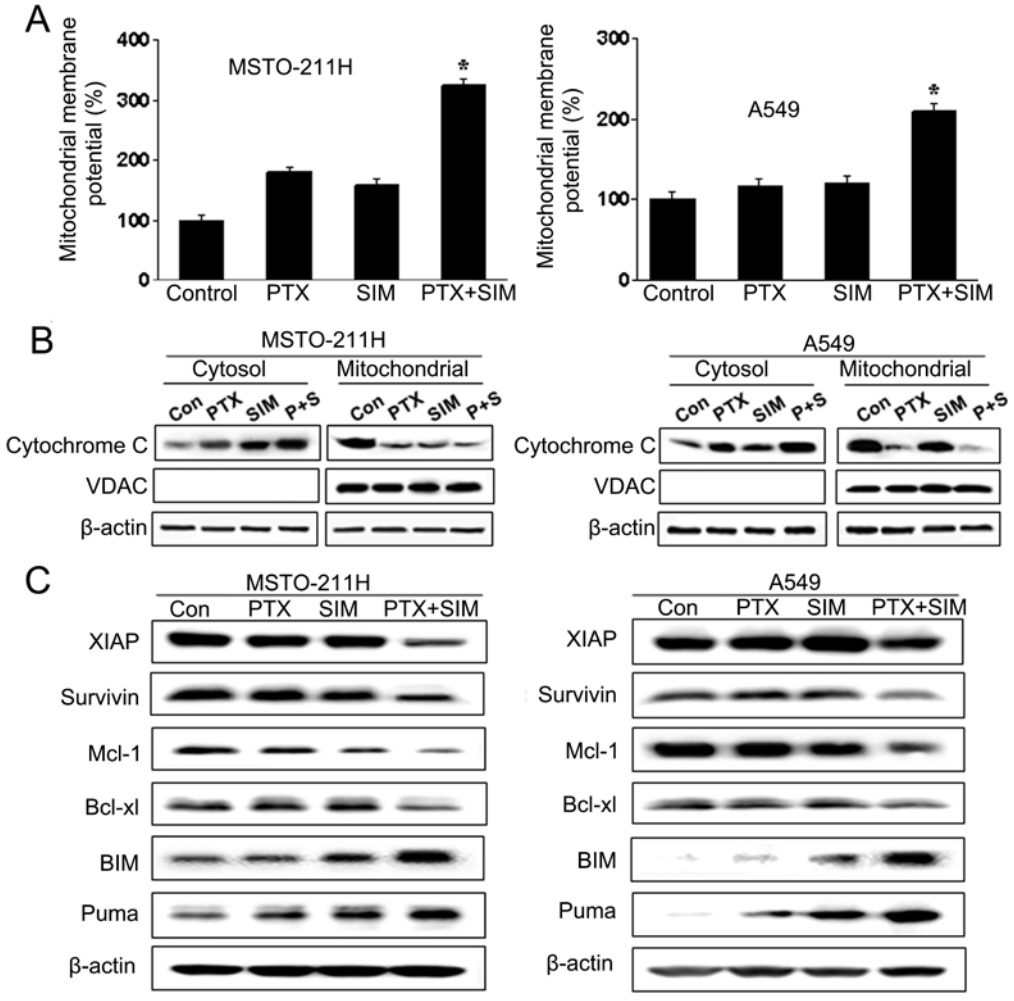

Figure 4. Mitochondrial dysfunction induced by treatment with pemetrexed and/or simvastatin in MSTO-211 and A549 cells. (A) Cells were treated with 1 μM pemetrexed and/or 5 μM simvastatin for 24 h, then stained with 10 μg/ml of JC-1, and analyzed by flow cytometry. Percentage of JC-1 monomer in control cells was set at 100%, and $\Delta\Psi_m$ transition relative to that of the control is presented. Each panel is representative of three identical experiments. (B) Cells were incubated with 1 μM pemetrexed and/or 5 μM simvastatin for 36 h. The cell lysate was fractionated into cytosolic and mitochondrial portions, and proteins were separated on 15% SDS-PAGE for cytochrome *c* immunoblotting. The purity of mitochondrial fraction was verified by western blotting with anti-VDAC antibody. (C) Cells were treated with pemetrexed and simvastatin, alone and in combination, for 48 h, and the cell lysate was subjected to 15% SDS-PAGE to measure the expression of the IAP (XIAP and survivin), anti-apoptotic (Mcl-1, Bcl-xL), and pro-apoptotic (Bim, Puma and Bid) Bcl-2 families. Immunoblots are representative of at least two independent experiments.

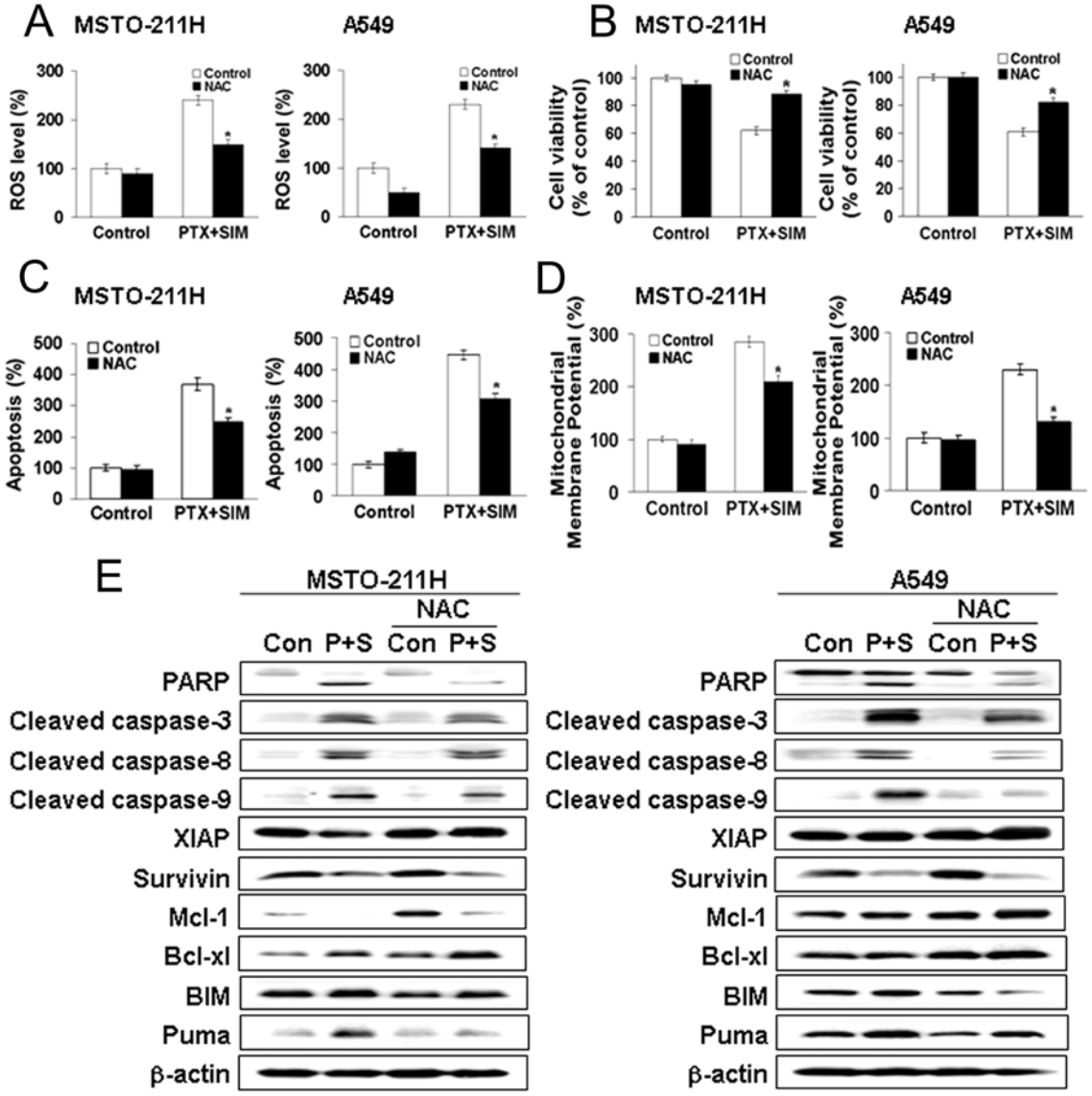

Figure 5. Effect of NAC on pemetrexed and simvastatin-induced apoptosis. (A) ROS generation. Cells were treated with pemetrexed and simvastatin in the presence or absence of 10 mM NAC for 24 h, and then loaded with 10 μM carboxy-H_2DCFDA. Fluorescence measurements were carried out with a FACSCalibur flow cytometer. Representative data are shown. Numbers indicate the percentage of DCF fluorescence. (B) Cells were treated with pemetrexed and simvastatin in the presence or absence of NAC for 24 h, and cell viability was determined by MTT assay. The data represent the mean ± SD of three independent experiments. $^*p<0.05$ compared to the control. (C) Apoptosis was evaluated by green fluorescent protein-Annexin V + propidium iodide. Percentage of Annexin V and propidium iodide-positive cells are indicated for each test condition. (D) Cells were treated with pemetrexed and simvastatin in the presence or absence of 10 mM NAC for 36 h. Cells were stained with 10 μg/ml of JC-1 and analyzed by flow cytometry. Each panel is representative of three identical experiments. (E) The cell lysate was subjected to 12% SDS-PAGE to measure the expression of indicated antibodies. Data are representative of two independent experiments.

Our results indicate that elevated ROS may be necessary for the potentiation of cell death in pemetrexed plus simvastatin-treated cells. To determine whether elevated ROS participated in the apoptosis induced by the combination of pemetrexed and simvastatin, the proportion of apoptotic cells was determined by Annexin V-PI staining (Fig. 5C). Annexin V-positive cells were increased in MSTO-211 and A549 cells treated with the drug combination. Pretreatment with NAC markedly reduced this increase.

We also observed that JC-1 monomers were increased in MSTO-211 and A549 cells treated with the drug combination, and the loss of $\Delta\Psi_m$ was significantly reduced in cells pretreated with NAC (Fig. 5D). Western blot analysis of MSTO-211 and A549 cell lysates (Fig. 5E) showed that the combination of pemetrexed and simvastatin enhanced the expression of cleaved PARP, caspase proteins, Bim, and Puma, decreased the expression of XIAP, survivin, Mcl-1, and Bcl-xL. Pretreatment with NAC blocked these effects. Together, these findings indicate that ROS generation played a primary role in apoptosis induced by pemetrexed and simvastatin.

Combination of pemetrexed and simvastatin induces apoptosis by upregulation of Bim expression. Previous studies revealed that the expression of Bim, a pro-apoptotic protein, was significantly induced by statins or gefitinib in lung cancer (24,25). To determine the role of Bim in apoptosis induced by

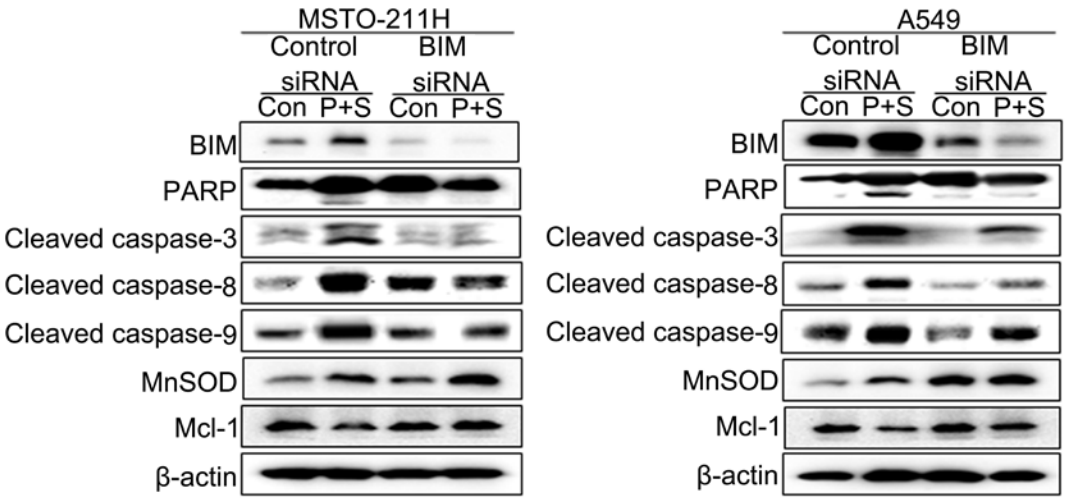

Figure 6. Effect of Bim depletion on apoptosis induced by pemetrexed and simvastatin. MSTO-211 and A549 cells were transfected with siRNA specific for Bim. Bim protein expression was assessed by immunoblot analysis at 48 h after transfection. Scrambled RNA containing the same number of each nucleotide as the Bim siRNA was used as the transfection control. Transfected cells incubated in complete medium with or without 1 μM pemetrexed and 5 μM simvastatin for 36 h. Cell lysates were then prepared and subjected to immunoblot analysis with indicated antibodies.

pemetrexed and simvastatin, we decreased the level of Bim expression by introducing siRNA for Bim. We then examined the effect of Bim siRNA-transfected cells on apoptosis induced by pemetrexed and simvastatin. We spread an equal number of viable Bim siRNA-transfected and non-silencing siRNA-transfected cells at 48 h after siRNA transfection. After an additional 48-h incubation, the cells were treated with pemetrexed and simvastatin for 36 h, and the cell lysate was used to carry out western blotting. Both Bim siRNA and the pemetrexed-simvastatin combination decreased the expression of cleaved PARP, caspase-3, -8 and -9. Together, these data indicate that the induction of apoptosis by pemetrexed and simvastatin is due, at least in part, to the upregulation of Bim.

Discussion

In the present study, we demonstrated the synergistic effect of the combination of pemetrexed and simvastatin on apoptosis of MSTO-211 malignant mesothelioma cells and A549 lung cancer cells compared to the use of either agent alone. These findings suggest that a combination of these two agents can potentially kill malignant mesothelioma and lung cancer cells more effectively and with fewer side effects than either drug alone, thereby providing a rationale for combining these drugs for the treatment of malignant mesothelioma and lung cancer.

Previous studies have examined the effects of pemetrexed on various human tumor cells including malignant mesothelioma and NSCLC. Pemetrexed has demonstrated clinical activity, either alone or in combination with the platinum compounds, vinorelbine, and gemcitabine, in a broad array of solid tumors (26). Studies on the mechanism of action of pemetrexed have shown that it inhibits cell proliferation and induces apoptosis in cancer cells (9). One of the most important approaches for developing improved cancer therapies is to understand the mechanisms by which successful therapies induce apoptosis. To our knowledge, no mechanistic studies have been conducted on the combination treatment of pemetrexed and simvastatin in malignant mesothelioma and lung cancer cells.

Mitochondria play a central role in cellular metabolism and are a major source of ROS in cells (27,28). Several studies have reported that mitochondrial morphology changes during apoptosis, resulting in the appearance of small, round mitochondrial fragments (29,30). Mitochondria play a major role in many apoptotic responses by coordinating caspase activation through cytochrome *c* and Bcl-2 family proteins (31-33). Our results demonstrate the release of cytochrome *c* from the mitochondria into the cytosol. This leads to activation of caspase-9, and subsequently, the activation of caspase-3. In addition, cleavage of PARP, a downstream target in this pathway, occurs during pemetrexed and simvastatin-induced apoptosis in malignant mesothelioma and lung cancer cells.

Although ROS are essential to cell survival, elevated levels of ROS result in slowed growth, cell cycle arrest, and apoptosis (34). Many chemotherapeutic strategies have been designed to significantly increase cellular ROS levels in order to induce irreparable tumor cell damage and death. In our earlier study, we investigated the role of simvastatin-induced apoptosis in lung A549 cells by mitochondrial ROS production (19). Buque *et al* (35) reported that an increase in intracellular ROS and p53 was required for pemetrexed-induced cytotoxicity in melanoma cells.

Increased ROS initiates a wide range of irreversible oxidative damage in the mitochondria. This in turn can lead to alteration in mitochondrial membrane potential (36). Accordingly, we investigated the possibility that ROS plays a role in pemetrexed and simvastatin-induced ROS generation in malignant mesothelioma and lung cancer cells. We demonstrated that, compared to individual treatments, combination treatment with pemetrexed and simvastatin increased ROS levels, suggesting that the combination of these drugs produces higher ROS levels.

If ROS were indeed involved in apoptosis, ROS quenchers, such as antioxidants, would be anticipated to prevent apoptosis.

Moreover, we found that pemetrexed and simvastatin-induced apoptosis, mitochondrial dysfunction, and caspase activation were greatly reduced by pretreatment with NAC. These results suggest that, in this model system, ROS generation has a primary role in the induction of apoptosis by pemetrexed and simvastatin.

$\Delta\Psi_m$ occurred during pemetrexed and simvastatin-induced apoptosis that also resulted in several changes in IAP and Bcl-2 family proteins that may promote apoptosis. To better understand the contribution of these proteins to the sensitivity of malignant mesothelioma and lung cancer to pemetrexed and simvastatin-induced apoptosis, we compared their expression levels. Our study revealed that pemetrexed and simvastatin induced downregulation of XIAP, survivin, Mcl-1 and Bcl-xL and upregulation of Bim and Puma. We also found that siRNA-mediated knockdown of Bim reduced the expression of apoptotic related proteins. Taken together, our results indicate that the upregulation of Bim may have contributed, at least in part, to pemetrexed and simvastatin-induced apoptosis.

In conclusion, we demonstrated that combination treatment with pemetrexed and simvastatin potentiated their apoptotic activity over that seen with either drug alone in malignant mesothelioma and lung cancer cells. These effects were mediated through mitochondrial dysfunction, by triggering ROS production, and by Bim induction. Taken together, these results indicate that the combination of pemetrexed and simvastatin may be a clinically promising therapy for the treatment of malignant mesothelioma or NSCLC. Our study elucidated a possible mechanism of action for the pemetrexed and simvastatin combination in effecting cell death in malignant mesothelioma and lung cancer cells. However, further studies are required including *in vivo* xenograft models of malignant mesothelioma and lung cancer before embarking on human studies.

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