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ORIGINAL INVESTIGATION

Simvastatin Induces Death of Multiple Myeloma Cell Lines

Naomi Gronich, Liat Drucker, Hava Shapiro, Judith Radnay, Shai Yarkoni, Michael Lishner

ABSTRACT

Background: Accumulating reports indicate that statins widely prescribed for hypercholesterolinemia have antineoplastic activity. We hypothesized that because statins inhibit farnesylation of Ras that is often mutated in multiple myeloma (MM), as well as the production of interleukin (IL)-6, a key cytokine in MM, they may have antiproliferative and/or proapoptotic effects in this malignancy.

Methods: U266, RPMI 8226, and ARH77 were treated with simvastatin (0–30 μM) for 5 days. The following aspects were evaluated: viability (IC_{50}), cell cycle, cell death, cytoplasmic calcium ion levels, supernatant IL-6 levels, and tyrosine kinase activity.

Results: Exposure of all cell lines to simvastatin resulted in reduced viability with IC_{50}s of 4.5 μM for ARH77, 8 μM for RPMI 8226, and 13 μM for U266. The decreased viability is attributed to cell-cycle arrest (U266, G_1; RPMI 8226, G_{2M}) and cell death. ARH77 underwent apoptosis, whereas U266 and RPMI 8226 displayed a more necrotic form of death. Cytoplasmic calcium levels decreased significantly in all treated cell lines. IL-6 secretion from U266 cells was abrogated on treatment with simvastatin, whereas total tyrosine phosphorylation was unaffected.

Conclusions: Simvastatin displays significant antimyeloma activity in vitro. Further research is warranted for elucidation of the modulated molecular pathways and clinical relevance.

Key Words: myeloma, simvastatin, apoptosis, interleukin-6, calcium

Cholesterol is alternately synthesized intracellularly or derived from the plasma by receptor-mediated endocytosis of low-density lipoprotein.[1] Its biosynthesis is regulated by the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which synthesizes mevalonic acid (mevalonate).[1] Mevalonate is a key metabolite in the biosynthesis of additional sterols, which are important constituents of cell membranes. Other metabolites of mevalonate, such as isopentenyladenosine, ubiquinone, and dolichol, are involved in the progression of cell cycle and cell survival.[2,3] Furthermore, mevalonate is also a precursor of farnesyl and geranylgeranyl pyrophosphate, which are covalently attached to a large group of proteins involved in signal transduction pathways related to cell proliferation.[4,5]

Cholesterol and its precursors are required at increased concentrations by cancer cells for proliferation and progression of cell cycle.[6] Also, some proteins (such as the *p21* Ras oncogene) undergo post-translational modifications that are essential for their membrane attachment and functions in signal transduction.[7] It was shown that in malignant cells, the low-density lipoprotein cholesterol–mediated feedback control of HMG-CoA reductase is deficient, resulting in a high rate of cholesterol production and a high level of HMG-CoA reductase.[8,9] Inhibition of this key enzyme by statins in various malignant cells has been shown to inhibit cell proliferation,[9,10] modulate changes in the cell cycle[11] and morphology,[12] and induce apoptosis and cell death.[4,13] Indeed, statins have been demonstrated to inhibit neoplastic cell proliferation and/or to induce apoptosis in several tumor-derived cells in vitro, and they display synergism with chemotherapeutic agents.[14–18]

We hypothesized that because statins inhibit farnesylation of Ras that is often mutated in multiple myelomas (MMs), as well as the production of interleukin (IL)-6, a key cytokine in MMs,[19–22] they may have antiproliferative and/or proapoptotic effects in this malignancy. Indeed, the effects of lovastatin on malignant plasma cells have recently been reported,[23] yet the mode of death induced by the drug and the mechanisms of its antimyeloma effects have not been elucidated. Also, lovastatin is currently replaced by novel, more potent statins. Therefore, we set out to characterize the response of myeloma cell lines to simvastatin and the effects of the drug on several pathways that are essential to the survival of malignant plasma cells.

MATERIALS AND METHODS

Cell Cultures

U266 and RPMI 8226, purchased from the American Type Culture Collection (Rockville, MD), were cultured in

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RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum and antibiotics (Biological Industries, Beit Haemek, Israel). Epstein-Barr virus (EBV)-transformed plasma cell leukemia cell line ARH77 (kindly provided by Prof. Ben-Basat, Sheaba Medical Center, Tel-Hashomer, Israel) was sustained in media containing 20% non–heat-inactivated fetal calf serum. Twenty-four hours prior to the experiments, 3×10^6 cells were seeded in 10 mL of fresh media. Simvastatin, provided by Merck & Co. (Rahway, NJ), was dissolved to 10 mM in dimethyl sulfoxide (DMSO). Clinically achievable dose curves and IC50 were established for each cell line (0–30 μM) (0.5% DMSO).[24,25] All cell lines are referred to as MM cell lines.

Viability Assay

MM cells (2×10^4) were cultured 5 days with 150 μL media and simvastatin in 96-well culture plates. DMSO-treated cells served as normal controls. Viability was assessed with WST1 Cell Proliferation Reagent (#1644807; Roche, Mannheim, Germany) following the manufacturer's instructions. Absorbancy was measured by an enzyme-linked immunosorbent assay (ELISA) reader (model ''Sunrise''; Tecan, Crailsheim, Austria), and the results were plotted on a linear scale. IC_{50} was determined using linear interpolation.

Microscopy

A phase contrast microscope (Nikon Labophot, Haarlem, The Netherlands) was used for qualitative morphologic assessment of cells.

Flow Cytometry

Fluorescence was analyzed by a Coulter Flow Cytometer (EPICS-XL, Beckman Coulter, Buckinghamshire, UK). All results are expressed as a percentage of positive cells, and at least 10,000 events were counted in each fluorescence-activated cell sorter (FACS) analysis.

Cell-Cycle Analysis

For 5 days, 1.5×10^6 MM cells were cultured in six-well culture dishes with media and respective simvastatin treatment. Thereafter, the cells were harvested and analyzed for deoxyribonucleic acid (DNA) content with a DNA-Prep System Kit (#6607055; Beckman Coulter, Miami, FL) according to the manufacturer's instructions.

Cell Death Analysis

Annexin V (ANX) was employed according to the manufacturer's instructions and assayed by FACS. Autofluorescence exclusion was calibrated according to control cells (less than 5% ANX + propidium iodide $[PI]^-$ cells). Mitochondrial membrane depolarization manifested in reduced 3,3′-Dihexyloxacabocyanine iodide, 98% ($DiOC_6^3$) (#31842-6; Sigma-Aldrich, Rehovot, Israel) uptake was assessed by FACS. Cells cultured and treated in 96-well plates were sedimented (300 g), resuspended in 40 nM $DiOC_6(3)$, and incubated at 37°C in the dark for 30 minutes followed by FACS (FL1) analysis. The results are presented as the proportion of $DiOC_6(3)_{(low)}$ cell fraction.

Intracellular Calcium Ion Levels

Assessment of calcium levels in the cytosol was done employing Fluo-3-acetoxymethyl ester (Fluo-3 AM, ICN Biomedicals, Aurora, OH, USA). Again, sedimented cells were resuspended in 300 μL 10 μM Fluo 3AM, incubated at 37°C in the dark for 30 minutes followed by FACS analysis (FL1). The percentage of cells expressing low levels of fluorescence was evaluated.

Semiquantitative Reverse Transcriptase–Polymerase Chain Reaction

Total ribonucleic acid (RNA) was extracted from 1.5×10^6 MM cells cultured with simvastatin in six-well culture dishes for 5 days employing PURESCRIPT (#R-5110; Gentra, Minneapolis, MN) according to the manufacturer's specifications. SuperScript II reverse transcriptase (#18064-022; Invitrogen Life Technologies, Carlsbad, CA)) and oligo $d(T)_{15}$ primers were used for RNA transcription following standard procedures. Multiplex polymerase chain reaction (PCR) of IL-6 and β-actin was optimized at the logarithmic phase: 28 cycles of 94°C for 30 seconds, 57°C for 40 seconds, and 72°C for 30 seconds. The primers used were β-actin forward 5′′GAC CAC ACC CCT CGT AGA TGG G3′, reverse 5′GCA TAC CCC TCG TAG ATG GG3′; IL-6 forward 5′GGT ACA TCC TCG ACG GCA TCT C3′, reverse 5′GTT GGG TCA GGG GTG GTT ATT G3′. PCR products electrophoresed on a 2% agarose gel were visualized with *Gel Doc 2000* and *Multianalyst* software (Bio-Rad Laboratories, Hercules, CA).

Tyrosine Phosphorylation

Relative protein tyrosine kinase activity was assessed according to the manufacturer's instructions with a Protein Tyrosine Kinase Assay Kit (#QIA28-1EA; Oncogene Research Products, Boston, MA). Bradford assay was used for quantification of total protein and normalization of results. The results are expressed as the equivalent of c-abl units supplied with the kit as an internal control.

IL-6 Levels

Detection of secreted IL-6 in the supernatant of MM cells was done by immunoassay with a Human ELISA

IL-6 Kit (Endogen, Woburn, MA) (sensitivity < 1 pg/mL) according to the manufacturer's instructions. The results were normalized according to the total protein of each sample.

DNA Fragmentation Assay

A DNA-Generation Kit (#D-5000; GENTRA, Minneapolis, MN) was used for genomic DNA extraction from 1.5×10^6 cells cultured and treated in six-well plates. Two micrograms of DNA was electrophoresed on a 1.5% agarose gel stained with ethidium bromide and was viewed with the *Gel Doc 2000* and *Multianalyst* software.

Statistical Analysis

Student's paired and unpaired *t*-tests were employed in analysis of differences between cohorts. An effect was considered significant when a *p* value was equal to or less than .05.

RESULTS

Simvastatin Reduced Viability of MM Cell Lines

Initially, we tested if simvastatin has any effect on the visibility of MM cell lines. The specific cell lines were chosen because of their different drug sensitivities and known molecular variability (described in the Discussion section). The cell lines were exposed for 5 days to a wide concentration range of simvastatin (0–30 μM). Viability was assessed with the WST1 assay that measures the metabolic activity of viable cells and is commonly used to evaluate the cytotoxic effects of drugs on cancer cells in vitro. The assay output values of absorbance were normalized according to solvent-only (DMSO) treated cells. The experiment was repeated in duplicates four separate times, and the results are presented as mean ± standard error

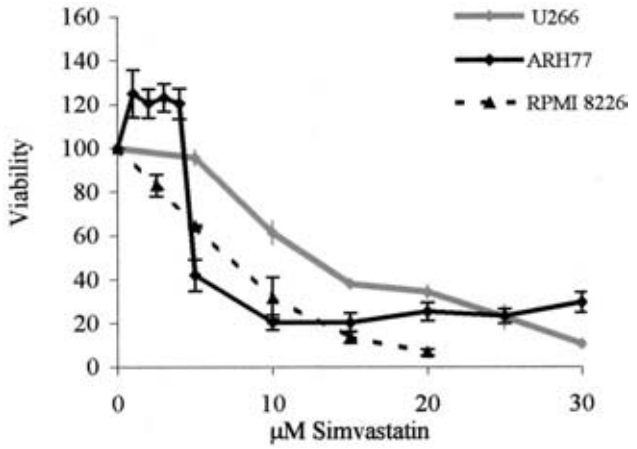
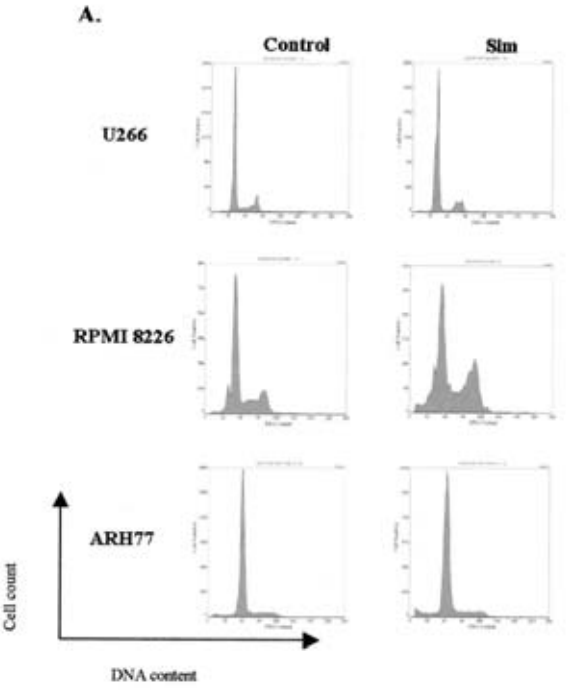


FIGURE 1 Simvastatin reduces the viability of multiple myeloma cell lines in a dose-dependent manner. The graph depicts the dose response of U266, RPMI 8226, and ARH77 to various doses of simvastatin as assayed after a 5-day exposure with WST1 Cell Proliferation Reagent (Roche). The viability is expressed as the mean relative percentage of the control cells (ie treated with dimethyl sulfoxide only) ± standard error. The experiments were conducted four separate times in hexaplicates.

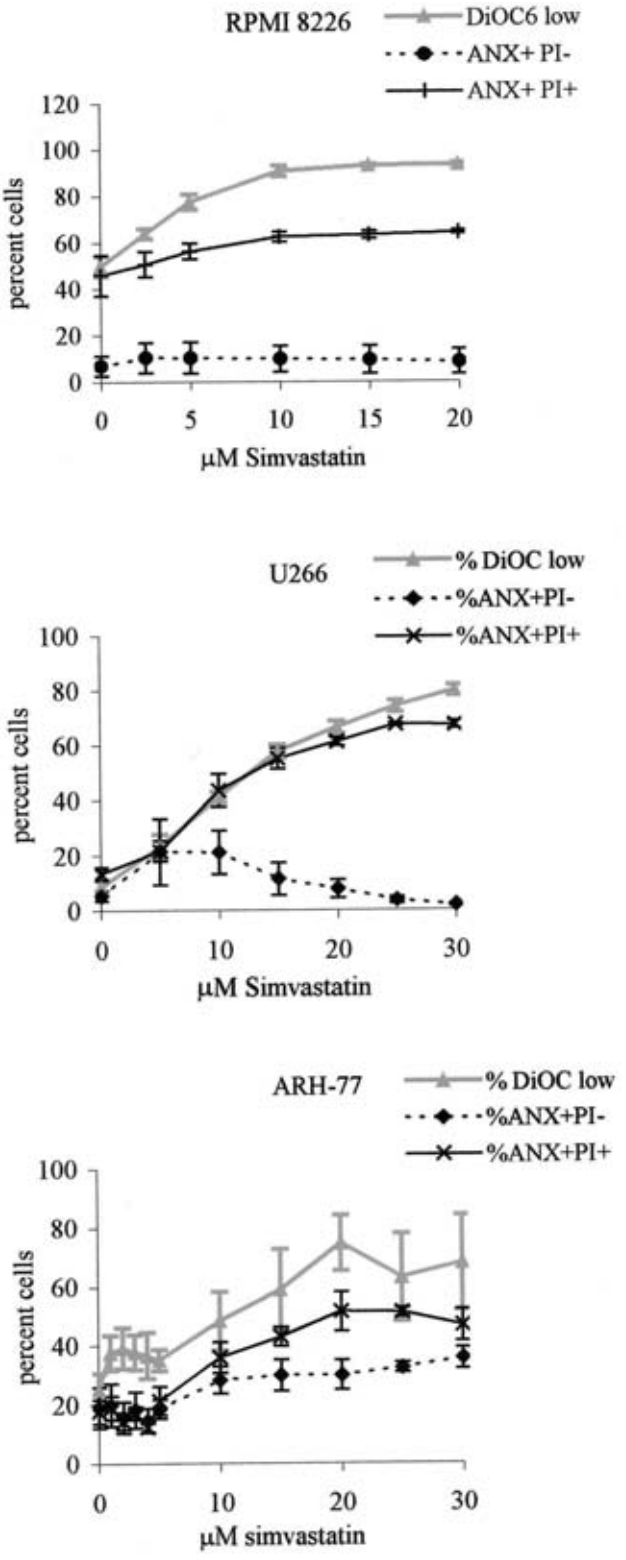


B.

Cell lines	Phase	Control	Sim
U266	*G1*	66	88
	S	25	5
	G2M	9	9
RPMI 8226	*G1*	60	43
	S	28	28
	G2M	12	29
ARH77	*G1*	82	81
	S	15	14
	G2M	2	5

FIGURE 2 Simvastatin (Sim) induces cell-cycle arrest in U266 and RPMI 8226. *A* depicts an exemplary flow scan of simvastatin-treated and untreated cells. The doses of simvastatin were U266, 13 μM; RPMI 8226, 8 μM; and ARH77, 4.5 μM. Cells were perforated and stained with propidium iodide (PI), which intercalates with deoxyribonucleic acid (DNA). PI fluorescence is proportional to the amount of DNA per cell and is indicated on the x-axis, whereas the number of cells corresponding to a given fluorescence is indicated on the y-axis. *B*, A table presenting values of respective cell proportions (%) in each cycle phase. These values are representative of at least two separate experiments. The fluorescence-activated cell sorter analysis included 10,000 events.

(Figure 1). All three cell lines, regardless of their variance in drug resistance, responded to simvastatin treatment in a dose-dependent reduced viability. IC50 values were calculated by linear interpolation and established at 4.5 μM, 8 μM, and 13 μM for ARH77, RPMI 8226, and U266, respectively.



Simvastatin Caused Cell-Cycle Arrest of U266 and RPMI 8226

We further tried to ascertain whether the decrease in viability on simvastatin treatment was attributed to a growth arrest, an induction of cell death, or a combination of both. Flow cytometric analysis of simvastatin-treated cell lines showed a variable response of cell cycle, with U266 and RPMI 8226 displaying an arrest (Figure 2). U266 displayed an accumulation of cells in G_1, RPMI 8226 responded to simvastatin administration with a G_{2M} cell-cycle arrest, and no significant changes in cell cycle were observed in simvastatin-treated ARH77 cells.

Simvastatin Induced Death of MM Cell Lines

We next examined if simvastatin caused the death of myeloma cells. Analysis of phosphatidylserine (PS) exposure as an early marker of apoptosis combined with PI inclusion indicative of necrosis demonstrated a dose-dependent death of all three cell lines included in our study (Figure 3). Examination of cell morphology following simvastatin treatment exhibited chromatin condensation, formation of apoptotic bodies, and cellular shrinkage in ARH77 cells, whereas U266 cells were characterized by nuclear spillage and no shrinkage of cells. The morphologic characteristics of both apoptosis and necrosis characterized the RPMI 8226 cell population after simvastatin treatment (Figure 4).

DNA fragmentation analysis, indicative of caspase activation, corroborated the morphologic evidence with a typical ladder displayed by electrophoresis of ARH77 DNA, no ladder in the U266 sample, and a combination of fragmented and degraded DNA in the RPMI 8226 DNA (Figure 5).

In an attempt to evaluate mitochondrial involvement in simvastatin-induced death of MM cell lines, we studied its membrane potential (Ψ) based on mitochondrial permeability to $DiOC_6(3)$ (see Figure 3). Apoptotic death involving mitochondrial pathways is characterized by

FIGURE 3 Simvastatin induces death of multiple myeloma cell lines. Cell death was determined based on the presence of phosphatidylserine indicative of apoptosis Annexin V (ANX) + propidium iodide [PI] $-$), compromised dye exclusion characteristic of necrosis (ANX + PI+), and reduced $DiOC_6(3)$ uptake marking depolarization of mitochondrial membrane [$DiOC_6(3)_{(low)}$]. Each graph depicts the dose response of a single cell line treated with simvastatin, with the x-axis indicating simvastatin concentrations and the y-axis showing the proportion of cells (%) positive for the assayed parameter. The experiments were conducted at least three times in hexaplicates, and the values are the calculated mean $\pm$ standard error.

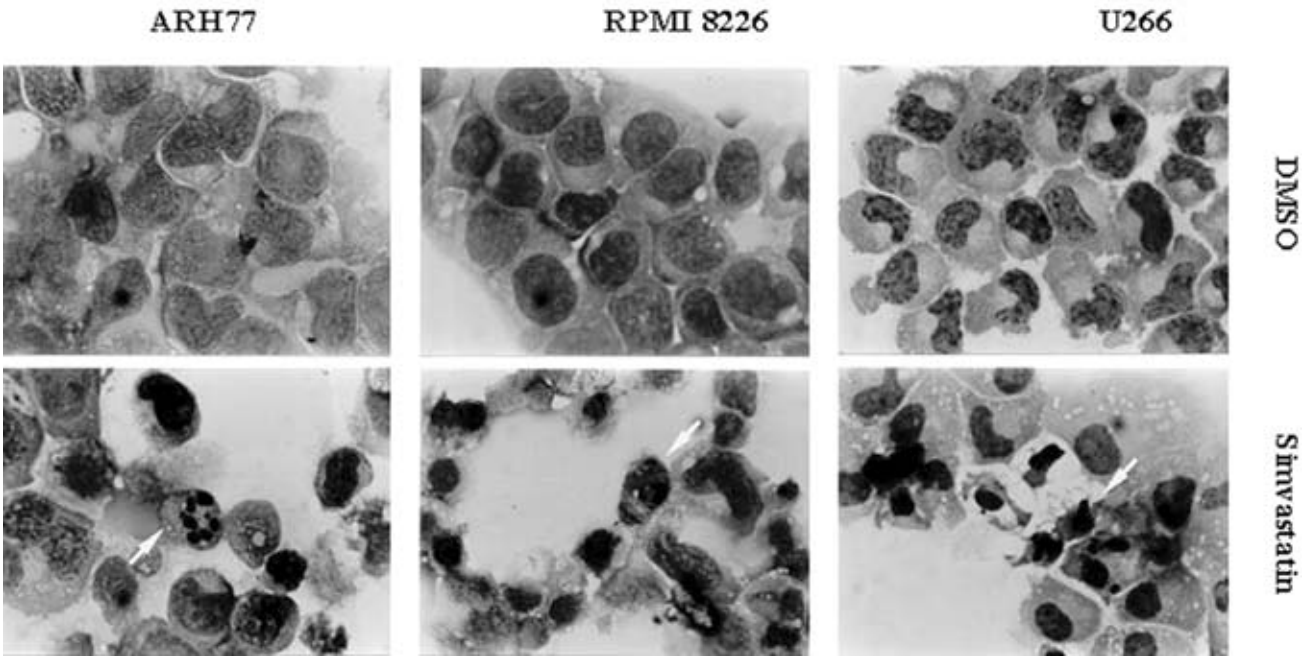


FIGURE 4 Diverse influences of simvastatin on the morphology of multiple myeloma cell lines. The figure shows a representative picture of simvastatin-treated myeloma cell lines and respective controls. The white arrows depict an ARH77 cell displaying nuclear condensation and fragmentation combined with intact membranes characteristic of apoptosis, an RPMI 8226 cell with breach of nuclear membrane integrity indicative of necrosis, and a U266 cell with condensed nuclei but no fragmentation. DMSO = dimethyl sulfoxide.

decreased transmembrane Ψ early in the process and, as a result, reduced $DiOC_6(3)$ permeability and lower fluorescence. A significant increase of the proportion of $DiOC_6(3)_{(low)}$ cells was evident in the ARH77, RPMI 8226, and U266 cell lines treated with 5 μM simvastatin compared with respective controls of cells exposed to

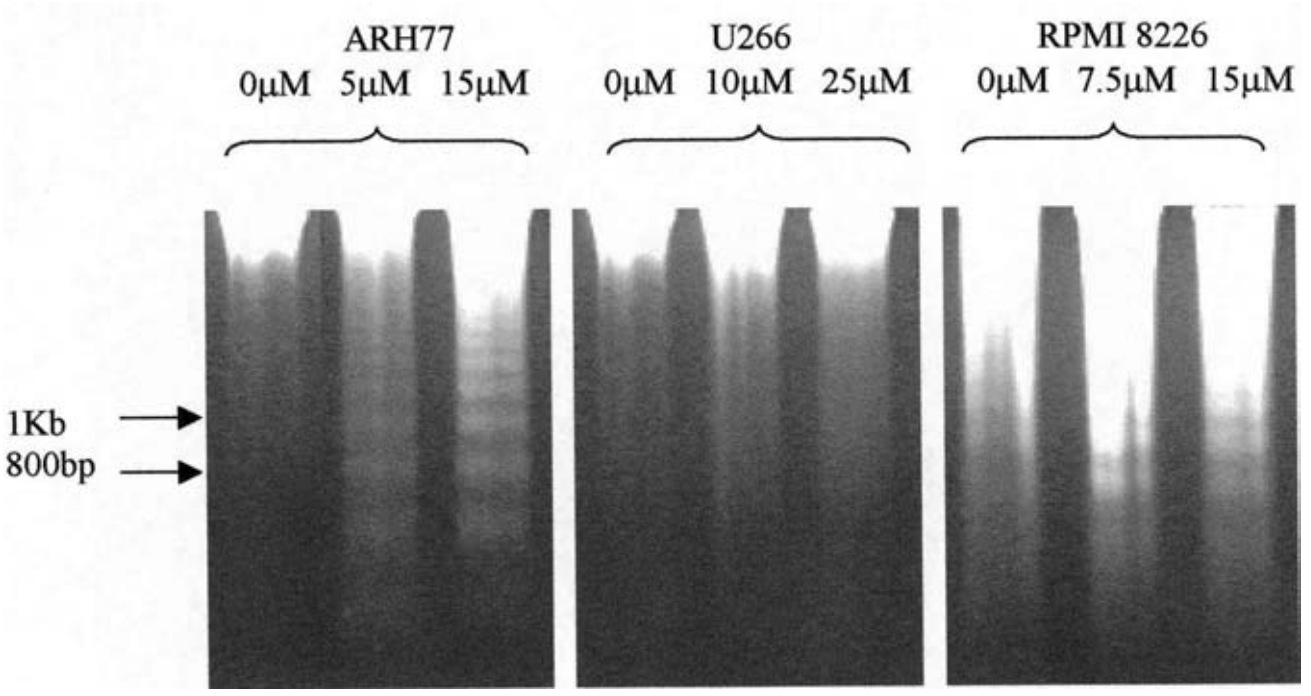


FIGURE 5 Simvastatin induces deoxyribonucleic acid (DNA) fragmentation in ARH77 and combined fragmentation and degradation of RPMI 8226 DNA. Genomic DNA was extracted from multiple myeloma cell lines treated with simvastatin and untreated. Two μg DNA was electrophoresed on a 1.5% agarose gel stained with ethidium bromide and was visualized with *Gel Doc 2000* and *Multianalyst* software (Bio-Rad). Respective cell lines and simvastatin doses are indicated, and arrows indicate DNA marker sizes.

DMSO solvent only ($p < .05$). These findings indicate that simvastatin causes the death of MM cell lines, involving both apoptotic and necrotic mechanisms.

Simvastatin Reduced IL-6 in U266 Supernatant

U266 cells are characterized by an autocrine production of IL-6 readily measured in the supernatant of cultured cells. We set out to determine if simvastatin attenuates IL-6 secretion in MMs as well. U266 cells were cultured for 5 days in 96-well plates with either DMSO or 10 μM simvastatin ($\sim IC_50$ dose). The experiment was conducted four times in quadruplicates. Indeed, following simvastatin treatment, no IL-6 was detected in supernatants compared with 4.27 pg (normalized value; described in Materials and Methods) in solvent-treated cells ($p < .01$). No change in the messenger ribonucleic acid (mRNA) levels of IL-6 was found.

Simvastatin Has No Detectable Effect on Total Tyrosine Phosphorylation

To determine if simvastatin generally attenuates cellular tyrosine phosphorylation, we used an activity assay. We found no significant changes in activity levels because of simvastatin treatment (Figure 6). It is interesting to note that whereas similar tyrosine phosphorylation levels characterize U266 and RPMI 8226, ARH77 displays a much enhanced activity level.

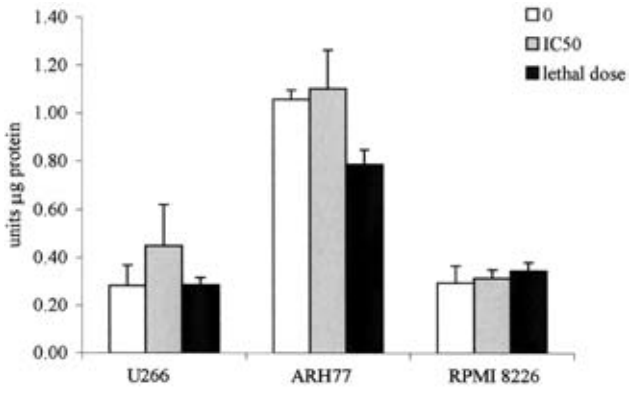


FIGURE 6 Simvastatin does not induce changes in the total tyrosine phosphorylation of multiple myeloma cell lines. The graph shows the mean ± standard error values of total tyrosine phosphorylation assayed in three different experiments conducted in quadruplicates with a Protein Tyrosine Kinase Assay Kit (see Materials and Methods). Each cell line was treated with its calculated IC50 dose (U266, 13 μM; ARH77, 4.5 μM; RPMI 8226, 8 μM), an excessive dose (U266, 25 μM; ARH77, 15 μM; RPMI 8226, 15 μM), and a control (dimethyl sulfoxide). The y-axis indicates the number of activity units per μg protein. The activity units are expressed as the equivalent of Abl tyrosine kinase activity, which is defined as the amount required to catalyze the transfer of 1 pmol phosphate to peptide substrate in 1 minute at 30°C.

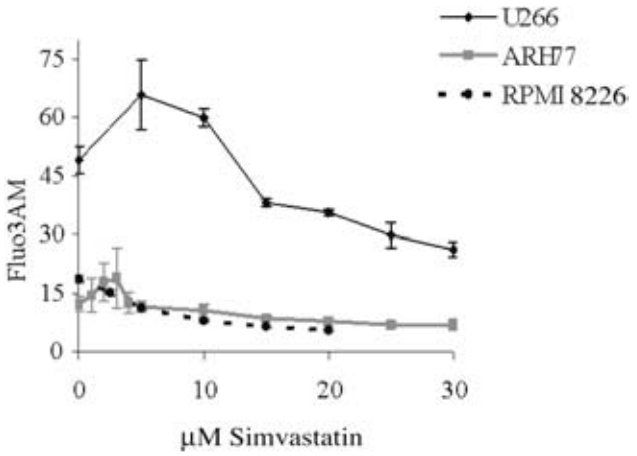


FIGURE 7 Simvastatin reduces intracellular calcium ion levels in multiple myeloma cell lines. The graph demonstrates dose-dependent changes in Ca levels in response to a 5-day simvastatin treatment. Values are mean ± standard error of three experiments carried out in hexaplicates. The x-axis indicates simvastatin concentrations, whereas the y-axis displays fluorescence of Fluo3AM in arbitrary units. The calcium levels at 30 μM simvastatin were significantly different than untreated controls in each of the respective cell lines ($p < .05$).

Simvastatin Administration Induces Changes in Intracellular Calcium Ion Levels

The intracellular calcium ion levels on 5 days of simvastatin treatment were measured. Dose-dependent changes in Ca^{2+} were recorded in all assayed cell lines (Figure 7). An increase was observed at simvastatin doses of 5 μM or less in U266 and ARH77. Higher doses of simvastatin caused a decline in free calcium that reached statistical significance only at 30 μM ($p < .05$). RPMI 8226 displayed only decreasing levels of cytoplasmic Ca^{2+} as a response to increased concentration of simvastatin.

DISCUSSION

In the ongoing search for cancer treatment, the targeting of specific molecular pathways is a promising field of research. HMG-CoA reductase, the rate-limiting step of the mevalonate pathway, personifies such a potential target. Malignant cells are highly dependent on the sustained availability of the end products of this pathway,[26,27] and, in effect, deregulated or elevated activity of HMG-CoA reductase has been shown in different tumors.[28–31] In vitro studies have shown that statins actually blocked tumor cell proliferation by growth arrest and induced apoptosis.[32] Testing different metabolites of this pathway indicates that the loss of geranylgeranylated proteins is partially accountable for the effect of statins.[33,34] Experimental evidence indicates that statins attenuate various pathways, including modulation of Ras by inhibition of its isoprenylation and decreasing secretion of IL-6.[19–21,35] Based on the importance of these signaling modalities

for malignant plasma cell proliferation and survival, we hypothesized that statins may potentially regulate MM cells growth and continued existence. Indeed, our research demonstrated an antimyeloma activity of simvastatin manifested primarily by markedly reduced viability in all three MM cell lines with no indication of resistance despite variable modes of cell death.

Reduced viability could be partially attributed to growth arrest in U266 and RPMI 8226. An accumulation of U266 cells in G_1 was in concordance with most reports describing the effect of statins on transformed cell lines and the necessity of HMG-CoA function for entrance into the S phase.[8,36,37] It has been shown in breast and prostate carcinoma cells that this G_1 arrest by statins is associated with up-regulation of cyclin-dependent kinase inhibitors $p21^{Cip1}$ and/or $p27^{Kip1}$.[38,39] Indeed, $p27^{Kip1}$, known to negatively regulate G_1 progression, is highly expressed in U266 cells,[40] thus possibly conferring on these cells the capacity to execute a G_1/S growth arrest. In contrast with U266, a depressed activity of $p27^{Kip1}$ was demonstrated in RPMI 8226 cells,[41] which may lend support to our results, namely, a simvastatin-induced G_2 arrest. Indeed, despite the prominence of G_1 arrest with statin exposure, there have been previous reports of G_2 cell-cycle arrests as well.[42–44] Publications have reported that the cell death mode induced after growth arrest is often of necrotic form.[45,46] In concordance, RPM I8226 and U266 both exhibited manifestations of necrosis, whereas ARH77, which did not manifest a growth arrest, was characterized with an apoptotic mode of cell death. Specifically, surface expression of phosphatidylserine residues and DNA fragmentation, both marking an apoptotic mode of death, were prominent in ARH77 cells. RPMI 8226 treated with simvastatin displayed a combination of apoptotic (fragmentation) and necrotic (degradation and decreased dye exclusion) characteristics, indicating a compound activation of cellular pathways. In U266 cells, despite a condensation of the nucleus, there was no fragmentation of the DNA.

The diverse cell death modes are not surprising because there is a prominent variability between these cell lines: U266 is resistant to Fas-mediated apoptosis and is characterized by an autocrine loop of IL-6 signaling, overexpression of $BclX_L$, and constitutive expression of STAT3. It is also characterized by wild-type Ras and a mutant *TP53*. In contrast, RPMI 8226 is Ras mutated and overexpresses c-myc. ARH77 is an EBV-transformed plasma cell leukemia lacking IL-6 receptors. A similar phenomenon of diverse responses to the same stimulus was previously reported in which a loss of mitochondrial transmembrane potential and apoptosis were demonstrated in RPMI 8226 but not in U266 cells following stimulation with imexon.[47]

An additional aspect of cell injury was demonstrated in all three cell lines treated with simvastatin and constituted a mitochondrial insult manifested in reduced $DiOC_6(3)$ uptake, indicating a decreased trans–mitochondrial membrane potential,[48,49] which occurs in both apoptotic and necrotic modes of death.[50,51] Taken together, we conclude that all three cell lines responded to simvastatin by prominent reduction of viability and death overriding their different characteristics. These findings are in accordance with reports that showed reduced viability of myeloma cell lines and myeloma cells derived from patients on treatment with lovastatin.[23] However, our findings imply that the mode of death induced by statins is not entirely apoptotic but is rather variable and compound, involving necrotic pathways as well.

To better characterize the mechanism of statin-induced MM cell death, we studied simvastatin's effect on the major survival agents in myeloma, that is, IL-6.[52] Former studies have shown decreased IL-6 secretion with reduced mRNA expression in vitro after statin incubation in primary endothelial cells[35,53] and human mesangial cells.[20] In vivo, decreased IL-6 was measured in pouche-bearing mice treated with statins.[21] U266 is the only cell line of the three that has an autocrine IL-6 loop. RPMI 8226 and ARH77 do not secrete detectable amounts of IL-6,[54] and prior studies have found IL-6 mRNA in RPMI 8226 and ARH77 cells only by sensitive PCR methods.[55] Therefore, we studied the effect of simvastatin on IL-6 secretion and mRNA transcription in U266 cells only. We noted statistically significant decreased levels of secreted IL-6 in culture media following incubation with simvastatin, yet no change was demonstrated in the mRNA level. This may be explained by attenuation of a post-translational modification necessary for IL-6 secretion, such as glycosylation and/or phosphorylation.[56,57] Reduced IL-6 in the milieu of MM cells has been previously determined as sufficient for inhibition of myeloma cell growth and induction of cell death.[22]

Important MM growth signals, IL-6 included, activate tyrosine kinase–dependent pathways. The Ras-Raf-MEK (MAP kinase) pathway, for example, plays a critical role in signaling for cell proliferation and differentiation. Moreover, in U266 and RPMI 8226, a constitutive expression of some tyrosine kinases was demonstrated.[55] Given that simvastatin reduced IL-6 levels, it might have been expected to result in diminished signaling as well. Yet, in our work, we did not detect any change in tyrosine phosphorylation levels after simvastatin treatment. The measurement of tyrosine phosphorylation was done by assessment in vitro of MM cell extract phosphorylation of a synthetic substrate. It should be taken into consideration that this general test of phosphorylation does not exclude the possibility of simvastatin-induced modulation of individual tyrosine kinases masked by the general activity assessed. An additional option is that serine-threonine phosphorylation pathways such as PI3K/Akt are primarily affected. Future studies will be implemented to examine this option. Intracellular Ca^{2+} levels are important for a variety of processes, including activity of

enzymes required for cell growth and apoptosis.[56] Cytosolic Ca^{2+} originates from two sources: release from intracellular stores and influx of extracellular Ca^{2+} across the plasma membrane. Studies that determined intracellular Ca^{2+} levels in various cells after prolonged statin exposure have shown reduced Ca^{2+} level as a result of both depressed crossmembranal influx and decreased intracellular release (vascular smooth muscle cells, U937 and HL-60).[57–62] Indeed, products of the mevalonate pathway, such as some of the G proteins, are involved in intracellular Ca^{2+} homeostasis. Complying with these reports, we found a statistically significant decrease in intracellular Ca^{2+} concentration after statin exposure. This effect was observed only in high simvastatin doses in the three cell lines. Lower simvastatin doses induced nonsignificant increases in the Ca^{2+} levels of U266 and ARH77 cell lines.

In summary, we have proven that simvastatin has significant antimyeloma activity manifested in growth arrest and cell death. We have shown that simvastatin affects IL-6 secretion in U266 and Ca^{2+} levels in all assayed cell lines. We propose that these results delineate cholesterol biosynthesis as an eligible drug target in myeloma control, which warrants further research into the precise mechanism and crucial molecules involved. Furthermore, combination of the relatively nontoxic statins with cytotoxic treatment may promote anticancer activity while avoiding dose-limiting side effects. The effects of such combinations and their mechanisms are currently being evaluated in our laboratory.

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