

# Synergistic Interaction of Lovastatin and Paclitaxel in Human Cancer Cells<sup>1</sup>

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## Abstract

**The hydroxymethylglutaryl-CoA reductase inhibitor lovastatin is used widely to treat hypercholesterolemia and has been shown to have cell cycle-specific effects. In these studies, we have examined the effects of combining lovastatin and paclitaxel (Taxol), a microtubule-stabilizing agent, in the human leukemia K562 and HL-60 cell lines. Isobologram analysis of cytotoxicity assays revealed that there is a synergistic interaction between the two agents in both cell lines. Cell cycle analyses showed that lovastatin enhances paclitaxel-induced G<sub>2</sub>-M arrest in both cell lines. In addition, Annexin V apoptotic studies revealed that lovastatin enhances paclitaxel-induced apoptosis in HL-60 cells. Lovastatin did not affect levels of [<sup>3</sup>H]paclitaxel in cells. Whereas lovastatin induced an accumulation of unmodified Ras and caused an up-regulation of both RhoB and Rap1A, paclitaxel was found to have no effect on the isoprenylated proteins. Studies of the centromere-associated protein mitosin revealed that treatment with lovastatin and paclitaxel resulted in increased mitosin levels and that lovastatin altered the association of mitosin with condensed chromosomes. These findings provide insight into the mechanisms underlying the cell cycle effects of lovastatin and support the development of a novel therapeutic strategy directed toward altering deleterious cell proliferation.**

## Introduction

Lovastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, has traditionally been used to treat hypercholesterolemia (1, 2). However, a number of recent investigations have shown that lovastatin impairs proliferation of a wide variety of cell types (3–6). The mechanism(s) of action underlying the cytotoxic effects of lovastatin are largely unknown and continues to be the focus of active investigation. Hydroxymethyl-

glutaryl-CoA reductase inhibition not only depletes cells of mevalonic acid, a necessary precursor for the synthesis of cholesterol, but also limits the availability of isoprenyl metabolites such as farnesyl PP<sub>i</sub> and geranylgeranyl PP<sub>i</sub> for the posttranslational modification of cellular proteins, many of which are critical for cell proliferation (7). In particular, proteins of the RAS superfamily, including Ras, RhoB, and Rap1A, are highly dependent upon farnesylation and geranylgeranylation for membrane localization and biological activity. That the lovastatin-induced impairment of proliferation is reversed by mevalonic acid, but not cholesterol (8), strongly suggests that the effect is attributable to alterations in protein isoprenylation.

The cytotoxicity of lovastatin is evidenced in that lovastatin can induce cell cycle arrest *in vitro* in both G<sub>1</sub> and G<sub>2</sub>-M. The G<sub>1</sub> effect has been the most closely examined and is associated with lovastatin-induced decreases in cyclin A, D, and E levels (9) and increases in cyclin-dependent kinase inhibitors (10). Studies involving FTIs and geranylgeranyl transferase inhibitors have indicated that geranylgeranylated proteins, but not farnesylated proteins, may be required for the G<sub>1</sub> to S-phase transition (11). Lovastatin-induced G<sub>2</sub>-M arrest has also been reported in several cell lines including the T24 human bladder carcinoma cell line (12) and the L1210 mouse leukemia cell line (13). The mechanism(s) for this arrest is/are as yet unknown. Improved understanding of the basis for this arrest may enable translation of these *in vitro* phenomena to clinical applications directed toward impairing unwanted cell proliferation.

Paclitaxel (Taxol) induces a well-defined G<sub>2</sub>-M arrest and is used to treat a wide variety of cancers. On a cellular level, paclitaxel binds to and stabilizes microtubules (14, 15). In addition, paclitaxel blocks mitosis at the metaphase-anaphase transition because of interference with spindle formation (16, 17). This mitotic block is considered to be the primary mechanism for the cytotoxic effects of paclitaxel (17). Furthermore, the mitotic block induced by low concentrations (10 nM) of paclitaxel has been shown to result in apoptosis (18).

Given that both lovastatin and paclitaxel are used in the clinic and are capable of inducing cell cycle-specific effects, it is of great interest to determine whether combinations of these agents induce additive or supra-additive effects that may be harnessed for novel therapeutic applications. In this study, we evaluated the effects of combining lovastatin and paclitaxel in two human leukemia cell lines (K562 and HL-60) and investigated the mechanisms underlying the observed synergistic interaction. These results provide *in vitro* support for the novel therapeutic approach of combining lovastatin and paclitaxel for altering abnormal cell proliferation *in vivo*.

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## Materials and Methods

**Cell Cultures and Reagents.** K562 and HL-60 cells were purchased from the American Type Culture Collection (Manassas, VA). The K562 cell line is a human erythroleukemia line that was established from a patient with chronic myelogenous leukemia (19) and is generally resistant to standard chemotherapeutic agents (20). The HL-60 cell line is a human promyelocytic leukemia cell line (21) that is generally sensitive to standard chemotherapeutic agents (22). K562 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS. HL-60 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 20% heat-inactivated FCS. Cells were grown at 37°C and 5% CO<sub>2</sub> in T-75 culture flasks. Anti-RhoB, anti-Rap1A, and anti-goat IgG HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NCC-004 anti-RAS antibody (23) was kindly provided by Dr. Setsuo Hirohashi (National Cancer Center, Tokyo). Anti-lamin B antibody was obtained from Oncogene and anti-mitosis antibody was purchased from Transduction Laboratories (Lexington, KY). Antimouse and anti-rabbit HRP-linked antibodies were obtained from Amersham (Piscataway, NJ). The green fluorescent Alexa Fluor 488 goat antimouse IgG antibody was obtained from Molecular Probes (Eugene, OR).

**MTT<sup>3</sup> Assay.** Cells were seeded ( $5-8 \times 10^4$  cells in 150  $\mu$ l per well) in 96-well flat-bottomed plates. Cells were incubated with lovastatin (kindly provided by Merck) and paclitaxel (Sigma Chemical Co., St. Louis, MO) at 37°C and 5% CO<sub>2</sub>, and the MTT (Sigma) assay was performed as described previously (24). The absorbance for control cells was defined as an MTT activity of 100%.

## Flow Cytometry

**Annexin V Staining.** Following the incubation of cells with drugs for 24 h, a total of  $6 \times 10^5$  cells/sample was costained with Annexin V and PI according to the manufacturer's protocol (ApoAlert Annexin V-FITC Apoptosis kit; Clontech, Palo Alto, CA).

**PI Staining.** Cells were incubated with lovastatin and/or paclitaxel for 24 h. After being washed with  $1 \times$  PBS,  $1 \times 10^6$  cells/sample were pelleted and 1 ml of ice-cold hypotonic PI solution [50  $\mu$ g/ml PI (Sigma) in 0.1% sodium citrate solution] was added with subsequent vortexing.

Flow cytometry was performed with a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cellquest V3.3 software (Becton Dickinson) was used for the acquisition and analysis of data. Forward scatter and orthogonal scatter were collected by using linear amplification. Annexin V-FITC and PI fluorescence were collected by using log amplification. Ten thousand events were collected in listmode. A bitmap gate was placed around the cell population on the basis of forward and orthogonal light scatter to eliminate small debris and aggregates. The bitmap

was large enough so that apoptotic cells were not eliminated. Cells satisfying the bitmap gate were analyzed using quadrant statistics in an Annexin V-FITC *versus* PI dual parameter histogram. For cell cycle experiments, a bitmap was drawn around single-cell events on the basis of pulse width and area of the PI fluorescence signal. A total of 10,000 events were collected in listmode that satisfied the bitmap. The pulse area histogram that resulted from the single-cell gate was subsequently analyzed with Modfit LT V2.0 (Verity Software House, Inc.) cell cycle analysis software.

**Cellular [<sup>3</sup>H]Paclitaxel Levels.** Cells were seeded ( $2 \times 10^5$  cells in 200  $\mu$ l per well) in 96-well round-bottomed plates and incubated with [<sup>3</sup>H]paclitaxel (20 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) and lovastatin for 24 h. Cells were subsequently harvested onto filter paper (Brandel, Gaithersburg, MD) and counted via liquid scintillation counting.

**Western Blot Analysis.** Cells ( $5 \times 10^6/5$  ml) were incubated with lovastatin and/or paclitaxel and/or mevalonate [ $\Delta^2$ -mevalonic acid lactone (Sigma) was converted to mevalonate prior to use] for 24 h. Cells were then collected, washed with PBS, and lysed in radioimmunoprecipitation assay buffer [0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton (v/v) X-100, and 0.05 M Tris-HCl] containing protease inhibitors (phenylmethylsulfonyl fluoride and aprotinin) and sodium orthovanadate. Protein content was determined using the Lowry method (25). Equivalent amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with the appropriate antibodies. Blots probed for RhoB, Rap1A, lamin B, and mitosis were detected with HRP-linked secondary antibodies and Amersham Pharmacia Biotech enhanced chemiluminescence Western blotting reagents, according to manufacturer's protocols. Blots probed for Ras were incubated with biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA) and avidin-linked HRP (Vectastain ABC; Vector). Incubation of the membrane with 3,3'-diaminobenzidine (Sigma) and H<sub>2</sub>O<sub>2</sub> in 10 mM Tris (pH 7.5) allowed visualization of the Ras bands.

**Immunofluorescence Microscopy Studies.** K562 cells were treated with lovastatin and/or paclitaxel for 24 h. Cells were prepared for both whole-cell studies and for metaphase spread analyses. For whole-cell studies, cells were washed in PBS, cytospun onto glass microscope slides, and fixed with 2% paraformaldehyde. Following blocking (10% goat serum, 3% BSA, 0.1% Triton X-100, and PBS), slides were incubated with primary antibody at 4°C overnight with subsequent incubation with secondary antibody (Alexa Fluor 488 goat antimouse IgG) for 2 h. Cells were counterstained with PI. For the metaphase spread preparation, cells were incubated in hypotonic solution (0.075 M KCl) at 37°C for 30 min and then fixed in a 3:1 methanol:acetic acid solution. Nuclei were then dropped onto glass slides and stained as for the whole-cell preparations, except that the paraformaldehyde fixation step was eliminated. Microscopy was performed using a Bio-Rad MRC-1024 laser scanning confocal microscope with a 60 $\times$  objective.

**Isobologram Analysis.** Data from the MTT assays were analyzed using CalcuSyn software (Biosoft). The software

<sup>3</sup> The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAPK, mitogen-activated protein kinase; HRP, horseradish peroxidase; CI, combination index; PI, propidium iodide; FTI, farnesyl transferase inhibitor.

analyzes drug interactions based on the method of Chou and Talalay (26).

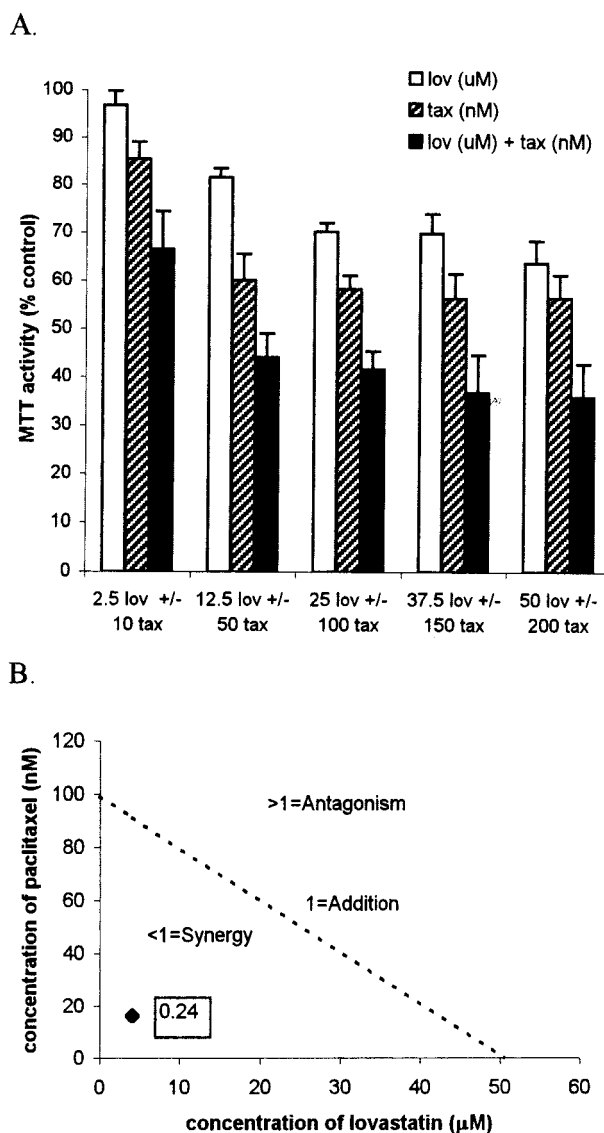
**Statistical Analysis.** Data from the cell cycle and cellular [ $^3\text{H}$ ]paclitaxel experiments were analyzed using the Student's *t* test.

## Results

**Synergistic Interaction between Paclitaxel and Lovastatin.** MTT assays were performed to evaluate the potential cytotoxic effects of combining lovastatin and paclitaxel. As shown in Fig. 1A, K562 cells were incubated with incrementally increasing concentrations of lovastatin and/or paclitaxel for 72 h. Whereas either lovastatin or paclitaxel alone inhibited MTT activity in a concentration-dependent manner, the effect was greater when the two agents were combined. This effect was evident even at the lowest concentrations (2.5  $\mu\text{M}$  lovastatin and 10 nM paclitaxel). To determine whether the combination of lovastatin and paclitaxel in K562 cells results in synergistic cytotoxic effects, isobologram analysis was performed. In this analysis, there is synergy when the CI is  $<1.0$ , additivity when the CI is equal to 1.0, and antagonism when the CI is  $>1.0$ . As shown in Fig. 1B, there is a synergistic interaction between lovastatin and paclitaxel in K562 cells. To determine whether this interaction existed in other cell lines, MTT assays were performed in the HL-60 cells. Because of the increased sensitivity of this cell line to paclitaxel, a shorter incubation period (48 h) and smaller concentration range (2–10 nM) of paclitaxel was used. As shown in Fig. 2A, a combination of lovastatin and paclitaxel resulted in a marked inhibition of MTT activity. Isobologram analysis of this data revealed that the two drugs interact in a synergistic manner (Fig. 2B). In all of the MTT assays, fixed ratios of the two drugs were used so that the data could be analyzed via the method of Chou and Talalay (26). Thus, there is a synergistic interaction between lovastatin and paclitaxel in both the K562 and HL-60 cells.

### Lovastatin Enhances Paclitaxel-induced $G_2$ -M Arrest.

Paclitaxel, through its stabilizing effects on microtubules, induces  $G_2$ -M arrest (16). To determine whether lovastatin was interacting with paclitaxel to enhance  $G_2$ -M arrest, flow cytometric cell cycle analyses were performed following the PI staining of nuclei. Fig. 3 shows the results from a representative experiment ( $n = 5$ ) in which K562 cells were incubated for 24 h with low concentrations of either paclitaxel (10 nM) or lovastatin (10  $\mu\text{M}$ ). Either agent alone resulted in a modest  $G_2$ -M arrest ( $P < 0.01$ ). However, the combination of lovastatin with paclitaxel resulted in a  $G_2$ -M arrest greater than those caused by either agent alone ( $P < 0.01$ ). This effect was maximal at the combination of 10  $\mu\text{M}$  lovastatin and 10 nM paclitaxel but was also observed for other combinations (data not shown). As a control, cells were also coincubated with 5 mM mevalonate, the product of the reaction inhibited by lovastatin. Coincubation with mevalonate prevented the supra-additive effect of lovastatin and paclitaxel. The cell cycle effects of lovastatin and paclitaxel were also examined in the HL-60 cells (Fig. 4). Whereas incubation with 10  $\mu\text{M}$  lovastatin alone had little effect on the percentage of cells in  $G_2$ -M, a concentration-dependent response was seen with increasing concentrations of paclitaxel (5, 10, and

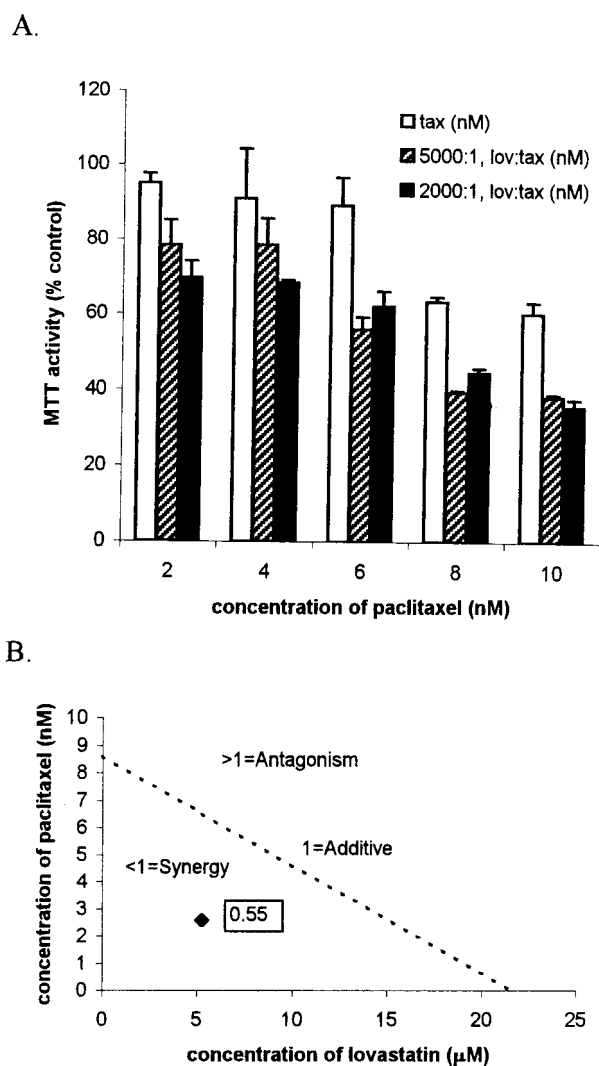


**Fig. 1.** The synergistic cytotoxic effects of lovastatin and paclitaxel in K562 cells. **A**, the effects of lovastatin and paclitaxel on MTT activity in K562 cells after a 72-h incubation. The concentrations of lovastatin alone ( $\mu\text{M}$ ), paclitaxel alone (nM), and the two agents in combination are labeled on the X axis beneath each set of combinations. The MTT activity of the treated cells is expressed as a percentage of the MTT activity of the untreated cells for each time point. Data are presented as the mean  $\pm$  SD ( $n = 6$ ). **B**, isobologram analysis of the combination of lovastatin and paclitaxel shows a synergistic interaction in K562 cells. The  $\blacklozenge$  represents the concentrations of both drugs that result in a MTT activity that is 60% of control ( $\text{IC}_{40}$ ). The  $\cdots$  of additivity is also shown. The boxed number is the CI, which was calculated using CalcuSyn software.

20 nM). Coincubation with lovastatin increased the  $G_2$ -M indices of cells treated with low (5 or 10 nM) concentrations of paclitaxel. Thus, lovastatin enhances paclitaxel-induced  $G_2$ -M arrest in both cell lines, although to a greater degree in the K562 cells.

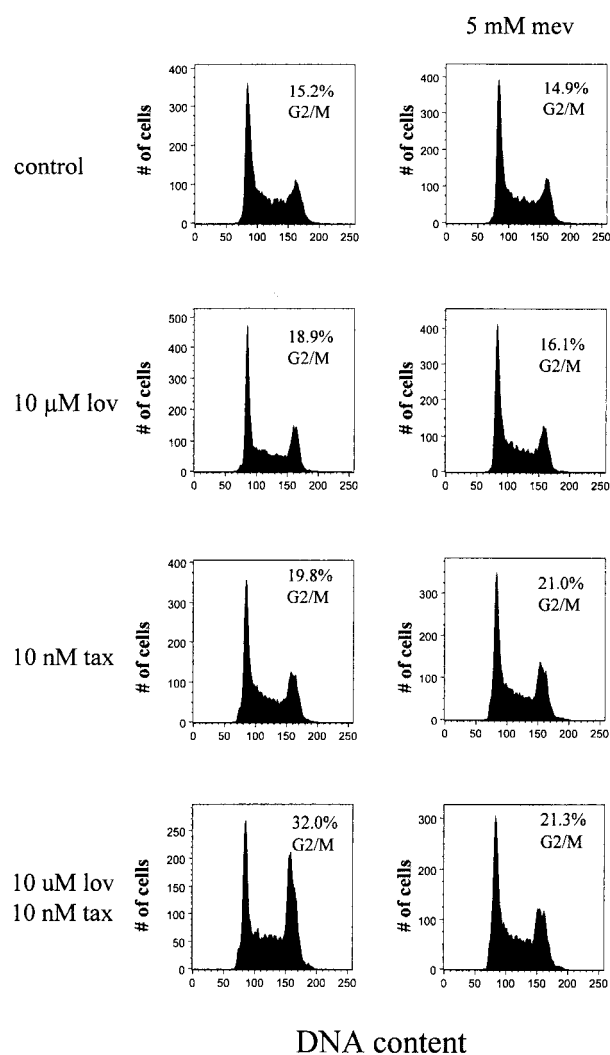
### Lovastatin Enhances Paclitaxel-induced Apoptosis.

Whereas lovastatin also enhanced paclitaxel-induced  $G_2$ -M arrest in HL-60 cells, the magnitude of the effect was less



**Fig. 2.** The synergistic cytotoxic effects of lovastatin and paclitaxel in HL-60 cells. **A**, the effects of lovastatin and paclitaxel on MTT activity in HL-60 cells after a 48-h incubation. The effects of paclitaxel alone as well as in combination with lovastatin in two different fixed ratios are shown. The MTT activity of the treated cells is expressed as a percentage of the MTT activity of the untreated cells for each time point. Data are presented as the mean  $\pm$  SD ( $n = 4$ ). **B**, isobologram analysis of the combination of lovastatin and paclitaxel shows a synergistic interaction in HL-60 cells. The  $\blacklozenge$  represents the concentrations of both drugs that results in a MTT activity that is 70% of control ( $\text{IC}_{50}$ ). The ---- of additivity is also shown. The boxed number is the CI, which was calculated using CalcuSyn software.

than in the K562 cells. Because HL-60 cells have been shown to undergo apoptosis in response to paclitaxel (27), Annexin V flow cytometric experiments were performed to determine whether lovastatin also increased paclitaxel-induced apoptosis. The change in location of phosphatidylserine in the cell membrane during apoptosis can be detected with Annexin V (28). Costaining with Annexin V and PI allows differentiation of viable cells (Annexin V-negative, PI-negative) from early apoptotic cells (Annexin V-positive, PI-negative) and late apoptotic/necrotic cells (Annexin V-positive, PI-positive). HL-60 cells were incubated for 24 h with varying concentrations of paclitaxel (0, 5, 10, and 20 nM) with

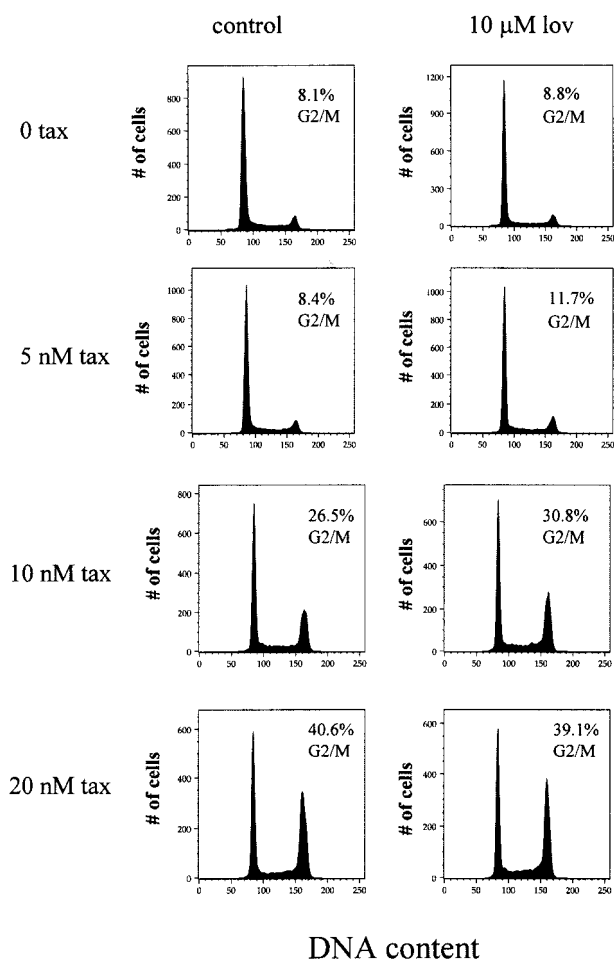


**Fig. 3.** Lovastatin enhances paclitaxel-induced G<sub>2</sub>-M arrest in K562 cells. K562 cells were treated for 24 h with lovastatin (*lov*) and/or paclitaxel (*tax*) and/or mevalonate (*mev*), followed by PI staining of the nuclei and analysis by flow cytometry. The percentages of cells in the G<sub>2</sub>-M phase are shown for each treatment.

or without 10  $\mu\text{M}$  lovastatin. As shown in Fig. 5 and Table 1, the addition of lovastatin increased the percentages of both early (*bottom right quadrant*) and late (*top right quadrant*) apoptotic cells as compared with paclitaxel alone. Coincubation of lovastatin with 5 nM paclitaxel doubled the percentage of apoptotic cells. These results were confirmed with whole-cell PI flow cytometric experiments in which the percentage of cells with subdiploid amounts of DNA were determined (data not shown). Thus, the data from Fig. 4 and 5 show that addition of lovastatin to lower concentrations of paclitaxel enhances both paclitaxel-induced G<sub>2</sub>-M arrest and apoptosis, whereas at higher concentrations of paclitaxel (20 nM), the predominant effect of lovastatin is to increase the induction of apoptosis in HL-60 cells.

#### Lovastatin Does Not Alter Cellular Paclitaxel Levels.

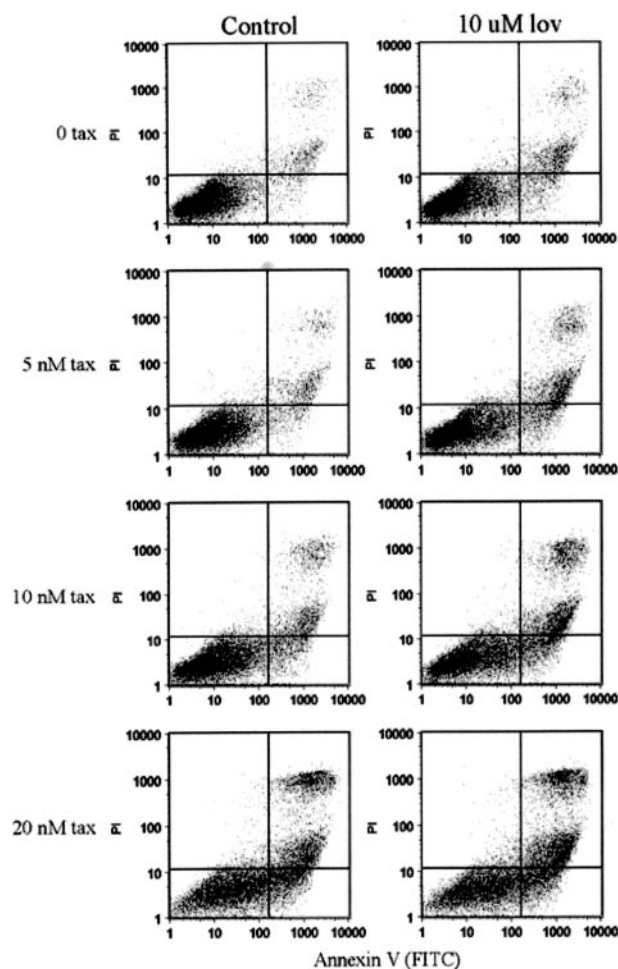
To determine whether lovastatin was affecting the level of paclitaxel in cells, K562 cells were incubated with [<sup>3</sup>H]pacli-



**Fig. 4.** Lovastatin enhances paclitaxel-induced G<sub>2</sub>-M arrest in HL-60 cells. HL-60 cells were treated for 24 h with lovastatin (*lov*) and/or paclitaxel (*tax*), followed by PI staining of the nuclei and analysis by flow cytometry. The percentages of cells in G<sub>2</sub>-M phase are shown for each treatment.

taxel (1 or 10 nM; 20 Ci/mmol) in the presence or absence of lovastatin (5 or 50 μM) for 24 h. Levels of cellular [<sup>3</sup>H]paclitaxel were then quantified by liquid scintillation counting. As Fig. 6 shows, lovastatin did not significantly alter the level of cellular [<sup>3</sup>H]paclitaxel. Thus, the interaction between lovastatin and paclitaxel was not attributable to an effect by lovastatin on cellular paclitaxel levels.

**Effects of Lovastatin and Paclitaxel on Isoprenylated Proteins.** Lovastatin, and more recently, paclitaxel (29) have both been reported to impair protein isoprenylation. We were, therefore, interested in determining the degree to which combinations of these agents alter protein isoprenylation as a possible mechanism for their synergistic interaction with regard to proliferation, G<sub>2</sub>-M arrest, and apoptosis. Cytoplasmic (Ras, RhoB, and Rap1A) and nuclear (lamin B) isoprenylated proteins were examined. These proteins are preferentially farnesylated [Ras (30) and lamin B (31)], preferentially geranylgeranylated [Rap1A (32)], or can be either farnesylated or geranylgeranylated [RhoB (33)]. Fig. 7 shows Western blots of K562 cells that have been incubated with



**Fig. 5.** Lovastatin enhances paclitaxel-induced apoptosis in HL-60 cells. HL-60 cells were treated for 24 h with lovastatin (*lov*) and/or paclitaxel (*tax*). Cells were costained with Annexin V and PI and analyzed by flow cytometry. Cells in the *bottom left quadrant* (Annexin V-negative, PI-negative) are viable, whereas cells in the *bottom right quadrant* (Annexin V-positive, PI-negative) are in the early stages of apoptosis, and cells in the *top right quadrant* (Annexin V-positive, PI-positive) are in later stages of apoptosis and necrosis.

**Table 1** Quantification of Annexin V apoptosis experiment

HL-60 cells were incubated with lovastatin (*lov*) and/or paclitaxel (*tax*) for 24 h. Control cells were incubated without drugs. Quadrant statistics were determined using an Annexin V-FITC vs. PI dual parameter histogram.

	Bottom left quadrant (viable)	Bottom right quadrant (early apoptotic)	Top right quadrant (late apoptotic/necrotic)
Control	89.5	2.99	6.50
10 μM lov	84.0	5.05	9.83
5 nM tax	87.9	3.70	7.35
5 nM tax + 10 μM lov	76.7	8.39	13.6
10 nM tax	76.4	8.63	13.4
10 nM tax + 10 μM lov	63.6	11.9	22.5
20 nM tax	52.7	16.8	27.8
20 nM tax + 10 μM lov	46.3	16.9	34.0

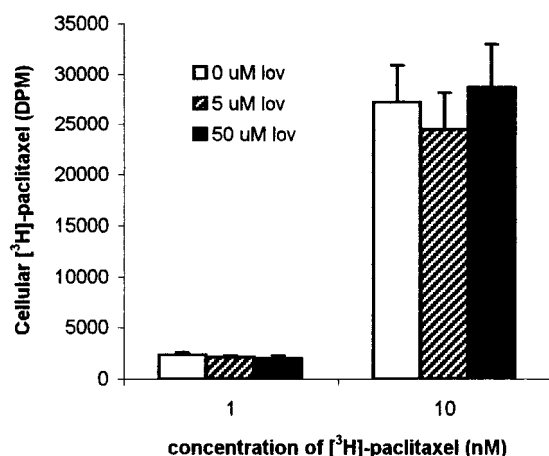


Fig. 6. Lovastatin does not alter cellular [ $^3\text{H}$ ]paclitaxel levels. K562 cells were incubated with [ $^3\text{H}$ ]paclitaxel with or without lovastatin for 24 h. Cells were subsequently harvested and counted via liquid scintillation counting. Data are presented as the mean  $\pm$  SD ( $n = 6$ ). Significant differences between samples treated without or with lovastatin were assessed using the Student's  $t$  test, and  $P$ s were found to be  $>0.05$ .

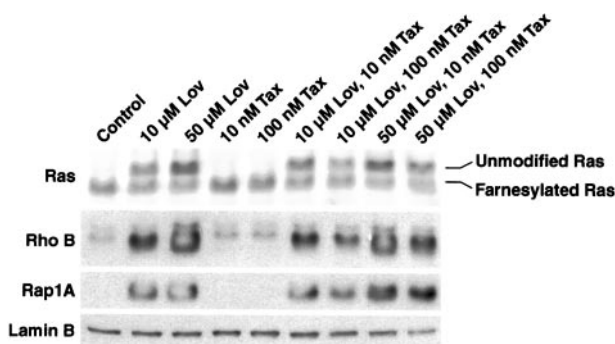


Fig. 7. The effects of lovastatin and paclitaxel on isoprenylated proteins in K562 cells. K562 cells were incubated with lovastatin and/or paclitaxel for 24 h. These Western blots were developed as described in "Materials and Methods." Each lane contains an equivalent amount of protein from cell lysate and is labeled with the concentration of lovastatin (*lov*) or paclitaxel (*tax*) used. The control cells were incubated without drugs.

paclitaxel and lovastatin. We and others (34, 35) have shown that treatment with lovastatin results in the accumulation of unmodified Ras, seen as a more slowly migrating band in Western blots. This effect is evident in cells incubated with 10 or 50  $\mu\text{M}$  lovastatin (Lanes 2 and 3). Paclitaxel (10 or 100 nM) had no effect on Ras processing as only the band representing modified Ras was seen. Furthermore, paclitaxel did not affect the inhibition of Ras processing by lovastatin when cells were treated with both agents. We found that the predominant effect induced by lovastatin on RhoB and Rap1A was to markedly increase the total levels of those proteins. Treatment with paclitaxel did not alter RhoB protein levels as compared with control and did not significantly alter the effects of lovastatin when used in combination. Under control or paclitaxel-treated conditions, Rap1A could not be detected. However, treatment with lovastatin induced an increase in Rap1A, an effect that was not altered by coincu-

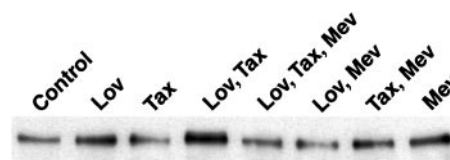
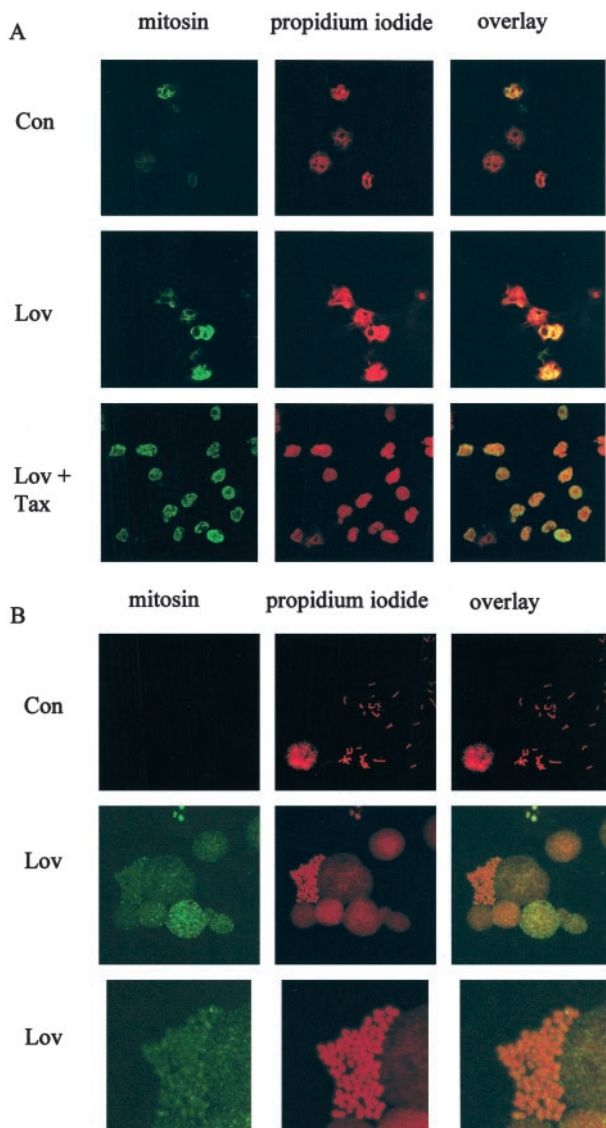


Fig. 8. The combination of lovastatin and paclitaxel increases mitosis protein levels. K562 cells were incubated for 24 h with 10  $\mu\text{M}$  lovastatin (*lov*), 10 nM paclitaxel (*tax*), and/or 5 mM mevalonate (*mev*). These Western blots were developed as described in "Materials and Methods." Each lane contains an equivalent amount of protein from cell lysate. The control cells were incubated without drugs.

bation with paclitaxel. Neither lovastatin nor paclitaxel was found to change the level of lamin B protein. Thus, we found no evidence to suggest that paclitaxel either affects isoprenylation or alters the effects of lovastatin on isoprenylated proteins.

**Lovastatin and Paclitaxel Alter Mitosis Protein Levels and Localization.** Because the centromere-associated protein mitosis (CENP-F; Ref. 36) has been demonstrated to be isoprenylated (37), we examined the effects of combining lovastatin and paclitaxel on its expression as a possible mechanism to explain the synergistic effects between the two agents. As shown in Fig. 8, treatment with 10  $\mu\text{M}$  lovastatin for 24 h increased the level of mitosis protein in K562 cells. Interestingly, whereas paclitaxel (10 nM) only modestly increased mitosis levels, the combination of lovastatin and paclitaxel markedly increased the level of mitosis protein. As a control, cells were also coincubated with 5 mM mevalonate, which returned the amount of mitosis back to control levels in lovastatin- or paclitaxel/lovastatin-treated cells. The coincubation of mevalonate with paclitaxel did not alter mitosis levels compared with paclitaxel alone. Thus, we found that the combination of lovastatin and paclitaxel affected mitosis levels more than with either drug alone and that this effect could be prevented by coincubation with mevalonate.

To determine whether lovastatin and/or paclitaxel, in addition to altering mitosis protein levels, also altered mitosis localization, a series of immunofluorescent microscopy studies were performed. K562 cells were treated for 24 h with 10  $\mu\text{M}$  lovastatin and/or 10 nM paclitaxel. As is shown in Fig. 9A, under the control conditions only a fraction of the cells stain positively for mitosis. This is consistent with reports that mitosis is predominantly expressed during the  $G_2$  phase and mitosis and is rapidly degraded following mitosis (38). Treatment with lovastatin increased the number of interphase cells staining positively for mitosis and this effect was further enhanced by coincubation with paclitaxel. Cells treated with paclitaxel alone had similar staining patterns as the control cells (data not shown). Cells were counterstained with PI to visualize the nuclei, and overlays of the images show that mitosis is localized to the nuclei, both under control conditions and in cells treated with lovastatin and paclitaxel. To further investigate the effects of the agents on mitosis localization, slides were prepared using the method used for genetic karyotype analysis, allowing for the visualization of individual condensed chromosomes. As shown in Fig. 9B, under the control conditions mitosis could not be detected in



**Fig. 9.** Indirect immunofluorescent studies of mitosin in K562 cells. K562 cells were incubated for 24 h with 10  $\mu\text{M}$  lovastatin (*lov*) and/or 10 nM paclitaxel (*tax*). The control cells (*con*) were incubated without drugs. **A**, the effects of lovastatin and paclitaxel on mitosin in whole-cells. Cells were fixed, stained with anti-mitosin antibody and Alexa Fluor 488-conjugated antimouse antibody, and counterstained with PI. Indirect immunofluorescence microscopy was performed with a laser-scanning confocal microscope. **B**, lovastatin disrupts the localization of mitosin to centromeres. Shown are condensed chromosomes and intact nuclei from control cells and lovastatin-treated cells. The *bottom panels* represent a magnified view of the chromosomes shown from the lovastatin-treated cells.

the condensed chromosomes. However, in cells treated with lovastatin, we observed staining for mitosin along the entire length of the chromosomes. This was confirmed by the overlay image, which shows colocalization of mitosin with the PI-stained chromosomes. Similar results were observed in cells treated with both agents, whereas paclitaxel-treated cells stained similarly to the control cells (data not shown). Slides incubated without primary antibody demonstrated

that there was not nonspecific binding of the fluorescently-labeled secondary antibody (data not shown). Thus, whereas treatment with lovastatin did not alter the normal nuclear localization of mitosin, it did alter the localization of mitosin on condensed chromosomes.

### Discussion

Our results demonstrate an interaction between lovastatin and paclitaxel in several cellular processes. In both the K562 and HL-60 cell lines, lovastatin was found to enhance paclitaxel-induced cytotoxicity in a synergistic manner (Fig. 1 and 2). In addition, lovastatin enhanced paclitaxel-induced  $G_2$ -M arrest in both cell lines and paclitaxel-induced apoptosis in the HL-60 cells (Fig. 3–5). These interactions were not attributable to an effect by lovastatin on cellular paclitaxel levels (Fig. 6) or to a general effect by paclitaxel on isoprenylated proteins (Fig. 7). However, we found evidence to suggest that alterations in the expression of the isoprenylated centromere-associated protein mitosin might be involved (Fig. 8 and 9).

Because of a report that indicated that paclitaxel could alter protein isoprenylation (26), we investigated the effects of lovastatin and/or paclitaxel treatment on several isoprenylated proteins. Importantly, we found no evidence to suggest that paclitaxel alters isoprenylation in our cell lines, consistent with the findings of Moasser *et al.* (39). In addition, paclitaxel did not affect the effects of lovastatin on isoprenylated proteins. Whereas the predominant effect of lovastatin on Ras was an accumulation of unmodified protein, the major effect of lovastatin on RhoB and Rap1A was to significantly increase protein levels. Although there are as yet no published reports of this phenomenon, these findings are highly reproducible and are particularly interesting because of the function of Rap1A. Rap1A has been shown to inhibit Ras-mediated signaling by competing with Ras for Raf-1 (40), however, unlike Ras, Rap1A is unable to activate Raf-1. Our results indicate that lovastatin can inhibit Ras-mediated signaling by at least two mechanisms: inhibiting Ras farnesylation and increasing the expression of the Ras-inhibitory protein Rap1A. We have shown that lovastatin does inhibit signaling downstream of Ras by demonstrating a decrease in MAPK phosphorylation (24). Interestingly, paclitaxel has been shown to induce the phosphorylation of Raf-1 (41), and high Raf-1 kinase activity has been associated with protection against paclitaxel-induced cytotoxicity (42). Paclitaxel-induced apoptosis may be a consequence of bcl-2 hyperphosphorylation (43) and may be dependent on MAPK pathways (44). Thus, lovastatin-induced inhibition of the Ras-Raf-1-MAPK signaling pathway may be one mechanism by which lovastatin increases paclitaxel-induced cytotoxicity and apoptosis.

The role of protein isoprenylation is generally considered to be involved with enabling the membrane localization of small molecular weight proteins. For example, inhibition of isoprenylation of the Ras protein prevents localization to the membrane and, therefore, prohibits the ability of Ras to interact with other members of its signal transduction pathway. Mitosin, on the other hand, is a  $M_r$  350,000 protein that associates with other proteins in the kinetochore. Recently,

Thissen *et al.* (45) reported that Ki-Ras associates with microtubules in an isoprenylation-dependent manner. Ashar *et al.* (37) have also reported that FTI treatment disrupts microtubule binding of another farnesylated kinetochore protein, CENP-E. Whereas mitosin has been shown to be in the outer kinetochore plate, and the outer kinetochore plate is believed to interact with microtubules (46), mitosin has not been shown to directly bind microtubules. Thus, isoprenylation of mitosin may be serving a novel purpose that may include facilitating interactions with CENP-E and other kinetochore proteins. Additionally, because there is evidence that mitosin can homodimerize (47), and because this dimerization is highly dependent on the COOH terminus, it may be that isoprenylation facilitates this interaction.

Ashar *et al.* (37) have also reported that treatment with a FTI did not alter the pattern of staining of CENP-F/mitosin in the nuclei of prometaphase cells. They concluded that treatment with a FTI did not alter mitosin localization to the kinetochore. Our studies demonstrate that lovastatin increases mitosin levels and alters mitosin chromosomal localization in a metaphase spread (Fig. 9B). The significance of this interesting finding is as yet unknown. Lovastatin, in contrast to FTIs, has the potential to impair both posttranslational farnesyl and geranylgeranyl modifications. As for some other isoprenylated proteins (e.g., RhoB) in the presence of a FTI, mitosin may alternatively be geranylgeranylated. This geranylgeranylation may be responsible for the observation by Ashar *et al.* that mitosin remains associated with kinetochores following FTI treatment.

The mechanisms of action by which lovastatin alters the cell cycle and whether lovastatin blocks a specific stage in mitosis are as yet described incompletely. Our studies, which demonstrate a synergistic interaction between lovastatin and paclitaxel, more directly implicate nuclear targets for lovastatin-induced cell cycle arrest at G<sub>2</sub>-M. Specifically, lovastatin-induced alterations in mitosin expression and localization may underlie its disruption of the mitotic cycle. The results of our current investigation and the widespread use of lovastatin and paclitaxel in the clinic support the application of this combination in early clinical trials, especially given that the concentrations of lovastatin and paclitaxel used in these *in vitro* studies are pharmacologically achievable in humans (48, 49).

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