

# Statins induce mammalian target of rapamycin (mTOR)-mediated inhibition of Akt signaling and sensitize p53-deficient cells to cytostatic drugs

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## Abstract

Cholesterol-lowering statins have been shown to have anticancer effects in different models and sensitize human tumor cells to cytostatic drugs. We have investigated the effect of statins on Akt/protein kinase B signaling and the sensitizing effect of cytostatic drugs. It was found that insulin- and cytostatic drug-induced Akt phosphorylation and nuclear translocation was inhibited by pravastatin and atorvastatin in HepG2, A549, and H1299 cells in an mTOR-dependent manner. Statins also induced mTOR-dependent phosphorylation of insulin receptor substrate 1. In p53 wild-type cells (HepG2 and A549), pretreatment with statins did not sensitize cells to etoposide in concentrations which induced p53 stabilization. In line with our previous data, statins were found to attenuate the etoposide-induced p53 response. However, silencing p53 by RNA interference rescued the sensitizing effect. We also show that in a p53-deficient cell line (H1299), pretreatment with atorvastatin sensitized cells to etoposide, doxorubicin, and 5-fluorouracil and increased the level of apoptosis. Taken together, these data suggest that a mTOR-dependent, statin-induced inhibition of Akt phosphorylation and nuclear translocation sensitizes cells to cytostatic drugs. However, this effect can be counteracted in p53 competent cells by the ability of statins to destabilize p53. [Mol Cancer Ther 2006;5(11):2706–15]

## Introduction

3-Hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, statins, are potent cholesterol-lowering drugs. They also have anticarcinogenic effects in several models (1, 2), and many studies, employing a variety of human tumor cells,

have shown their antiproliferative and proapoptotic effects. In addition, low concentrations of statins have been shown to sensitize cancer cell lines for cytostatic drugs, such as 5-fluorouracil (5-FU), taxol, etoposide, doxorubicin, and cisplatin (3–8). Besides their *in vitro* effects, statins have been shown to potentiate the antitumor effect of doxorubicin in three tumor models *in vivo* (1). In a recent study, simvastatin inhibited the small cell lung cancer tumor growth *in vivo* (5). Furthermore, pravastatin was found to prolong the survival of patients with advanced hepatocellular carcinoma in a randomized trial (9). The mechanisms for these anticancer effects are still not well characterized.

We have previously shown that statins alter Mdm2 expression and the p53 response to DNA damage. Thus, pravastatin induced mTOR-dependent Mdm2 phosphorylation at Ser<sup>166</sup> in HepG2 cells (10). Phosphorylation of Mdm2 at Ser<sup>166</sup> has been shown to activate Mdm2 and enhance its ubiquitination ligase function and destabilize p53 (11–13). Our data showed that statins attenuated the p53 response induced by genotoxicity *in vitro* and in rat liver *in vivo*. These effects were associated with a low p21 response and lower level of apoptosis (10). Taken together, our previous data suggest that statins induce the phosphorylation of Mdm2 which may limit the duration and intensity of the p53 response. The significance of this finding for the possible anticancer effects of statins remains to be characterized. p53 plays a central role in the response to genotoxic stress and may trigger apoptosis (14), a function that might be crucial for chemotherapeutic efficiency in p53 competent cells.

Recent studies indicate that statins and other cholesterol-lowering drugs may influence the Akt/protein kinase B signaling pathway in cancer cells (15, 16). For example, in breast cancer cells overexpressing Akt, cerivastatin decreased the levels of pAkt (17). Another recent study showed that simvastatin could inhibit the activation of Akt in small cell lung cancer cells (5). The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the major anti-apoptotic pathways in cells (18) and Akt is constitutively active in many human tumors (19). Furthermore, many of the commonly used cytostatic drugs activate Akt in cancer cells (20). Therefore, Akt is an attractive target for cancer therapy (21, 22). Statins, which are well-characterized and established pharmaceuticals, may thus represent a potentially new and safe class of inhibitors of Akt signaling.

In the present study, we have further characterized the downstream effects of statins on mTOR signaling. We report that statins decrease the levels of pAkt in a mTOR-dependent manner and we provide evidence that an inhibition of Akt can sensitize cancer cells to cytostatic drugs. However, we also report that this effect of statins

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can be antagonized by the previously described mTOR-dependent p53 destabilizing effect of statins. We conclude that statins may effectively potentiate apoptosis induced by cytostatic drugs in p53-deficient cancer cells.

## Materials and Methods

### Cell Culture

Hepatocellular carcinoma HepG2 cells (American Type Culture Collection, Manassas, VA) were grown on collagen-coated plates (collagen type I; Sigma-Aldrich, St. Louis, MO) in MEM, with Earl's salts and L-glutamine supplemented with 1 mmol/L of sodium pyruvate, non-essential amino acids, 10% inactivated FCS, and penicillin-streptomycin. For serum starvation, HepG2 cells were cultivated in MEM at 0.5% serum for 48 hours.

Non-small cell lung cancer (NSCLC) cells, A549 and H1299, were purchased from the American Type Culture Collection. A549 is p53 wild-type and H1299 is p53-deficient cell line. A549 cells were grown in DMEM, with 1 mmol/L of sodium pyruvate, 10% inactivated calf serum and penicillin-streptomycin. H1299 cells were grown in DMEM, 1 mmol/L of L-glutamine, 10% inactivated calf serum, and penicillin-streptomycin. Serum-starved cells were cultured in medium supplemented with 0.1% serum for 24 hours. All experiments were repeated at least thrice with different batches of cells.

Pravastatin was purchased from Sigma-Aldrich and atorvastatin was provided by Pfizer (New York, NY). LY294002, doxorubicin, cisplatin, etoposide, and 5-FU were purchased from Sigma-Aldrich. These compounds and atorvastatin were dissolved in DMSO. The final concentration of DMSO added to the cells was <0.2%.

### Western Blotting

Cells were washed with PBS and lysed in IPB-7 (1 mg/mL phenylmethylsulfonyl fluoride, 0.1 mg/mL trypsin inhibitor, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and 1 mmol/L NaF). The samples were subjected to SDS-PAGE and thereafter blotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The protein bands were subsequently probed using antibodies against glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) phosphorylated at Ser<sup>9</sup> from Cell Signaling Technology (Beverly, MA); Akt, Akt phosphorylated at residues Ser<sup>473</sup> or Thr<sup>308</sup>, p21, actin, and Cdk2 were from Santa Cruz Biotechnology (Santa Cruz, CA); p53 (CM-1) was from Novocastra (Newcastle, United Kingdom), mTOR phosphorylated at residue Ser<sup>2448</sup> (Cell Signaling Technology), insulin receptor substrate 1 (IRS-1) phosphorylated at Ser<sup>636/639</sup> (Cell Signaling Technology), and cyclin D1 from Oncogene (Uniondale, NY). Proteins were visualized using an enhanced chemiluminescence procedure (Amersham Biosciences, Uppsala, Sweden). The Western Blot results were analyzed with NIH Image 1.62 software.

### Quantification of Apoptosis

Morphologic evaluation of apoptotic cell death was done by Hoechst staining. Cells were fixed in 3.7% formaldehyde for 20 minutes. After fixation, the cells were stained with

Hoechst 33342 (Sigma-Aldrich) for 15 minutes. Nuclear morphology was then evaluated using fluorescence microscopy. Cells whose nucleus exhibited brightly stained, condensed chromatin or nuclear fragments were designated apoptotic. The percentage of cells with apoptotic morphology was determined by counting at least 1,000 cells per plate.

### Cell Viability and Cell Proliferation

The viability of cells was determined by the trypan blue exclusion assay. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay detecting the cellular mitochondrial capacity to convert MTT tetrazolium salt to formazan. Cells were incubated with the medium containing MTT (Sigma-Aldrich) for 4 hours. The cells were then lysed in DMSO. The absorbance was measured at 570 nm.

### Small Interference RNA Transfection

HepG2 cells were transfected with p53 or mTOR small interference RNA (siRNA; Cell Signaling Technology) for 24 hours according to the TranIT-TKO protocol from the manufacturer (Mirus, Madison, WI). Specific reduction of targeted p53 expression was confirmed by Western blot analysis detecting p53 (CM-1, Novocastra). Control siRNA was purchased from Santa Cruz Biotechnology.

### Immunocytochemical Staining

The HepG2 cells were fixed in 3.7% formaldehyde for 15 minutes. After fixation, the cells were stained with polyclonal antibodies against phosphorylated Akt at residues Ser<sup>473</sup> or Thr<sup>308</sup> (Santa Cruz Biotechnology). After incubation with primary antibodies overnight, secondary anti-rabbit antibodies conjugated with FITC (Dako, Glostrup, Denmark) were applied. The percentage of cells that were pAkt-positive was determined by counting at least 500 cells per plate.

### Cell Cycle Analysis

HepG2 cells were harvested by trypsinization. After fixation in 70% ice-cold ethanol for 10 minutes and incubation with RNase A (100  $\mu$ g/mL) and propidium iodide (50  $\mu$ g/mL) for 30 minutes, 10,000 cells from each sample were subjected to fluorescence-activated cell sorter scan (Becton Dickinson, Franklin Lakes, NJ) analysis.

### Statistical Analysis

Statistical analysis was conducted using Student's *t* test. The data were presented as means  $\pm$  SD. Experiments were done at least thrice with different batches of cells. Results were considered to be statistically significant at  $P \leq 0.05$ .

## Results

### Pravastatin and Atorvastatin Decrease pAkt Levels and Inhibit Cell Proliferation

Previous studies have shown that cholesterol-lowering drugs could inhibit Akt signaling in some cell types (16, 17). Therefore, the effect of statins on the constitutive level of pAkt was examined in HepG2 cells. We found that incubation of cells with pravastatin (1 and 2  $\mu$ mol/L

for 2 hours) decreased a high constitutive level of pAkt on residue Ser<sup>473</sup> (pAkt Ser<sup>473</sup>; Fig. 1A). Pravastatin treatment also inhibited the phosphorylation of Akt on residue Thr<sup>308</sup> (pAkt Thr<sup>308</sup>; data not shown). Growth factor stimulation induces the phosphorylation of Akt (23). We found that insulin induced pAkt Thr<sup>308</sup> and pAkt Ser<sup>473</sup> in serum-starved HepG2 cells after few minutes (data not shown). As shown in Fig. 1B preincubation of cells with atorvastatin (1 and 2  $\mu\text{mol/L}$ ) for 1 hour decreased the level of pAkt Ser<sup>473</sup> induced by insulin.

To determine the effect of statins on downstream proteins, we analyzed GSK3 $\beta$  phosphorylated at Ser<sup>9</sup> (pGSK3 $\beta$  Ser<sup>9</sup>), a site specifically phosphorylated by Akt (24). Insulin induced a Ser<sup>9</sup> phosphorylation, and pretreatment with pravastatin decreased pGSK3 $\beta$  Ser<sup>9</sup> levels (Fig. 1B). As shown by the densitometric analysis, the level of pAkt Ser<sup>473</sup> correlated with pGSK3 $\beta$  Ser<sup>9</sup> levels (Fig. 1B). Together, these data show that pravastatin and atorvastatin attenuate the insulin-induced Akt kinase signaling in HepG2 cells.

To study the cell specificity of the effect of statins on Akt, we also studied A549 NSCLC cells. Pravastatin and atorvastatin reduced the constitutive level of pAkt Ser<sup>473</sup> in A549 cells (data not shown) and this effect correlated with decreased cell proliferation (Supplemental data 1).<sup>1</sup> One hour of preincubation of cells with pravastatin decreased the insulin-induced Ser<sup>473</sup> phosphorylation and this decreased response was registered in cells preincubated with a single dose of pravastatin for 3, 6, and 24 hours (Supplemental data 1).<sup>1</sup> Commonly used cytostatic drugs stimulate Akt in cancer cells (20). In A549 cells, 1 hour of pretreatment with pravastatin significantly reduced the phosphorylation of Ser<sup>473</sup> induced by etoposide (Fig. 1C). LY294002 was used as a positive control. A similar effect was induced by atorvastatin (Fig. 1D). Moreover, we found that the effect of pravastatin on pAkt lasted at least 72 hours (Fig. 1E). This long-term effect was associated with lower expression of the cell cycle checkpoint protein cyclin D1, whereas no cleavage of PARP was observed (Fig. 1E).

As shown in Fig. 1F, pravastatin induced a significant inhibition of cell proliferation. Treatment with 2  $\mu\text{mol/L}$  of pravastatin decreased the amount of cells by  $26 \pm 4\%$  after 48 hours and  $33 \pm 6\%$  after 72 hours. The effect of pravastatin on cell cycle progression was examined by fluorescence-activated cell sorting analysis (Fig. 1G). Pravastatin treatment (2  $\mu\text{mol/L}$ , 48 hours) significantly increased the percentage of cells in G<sub>1</sub> (from  $63.6 \pm 5.1\%$  to  $71.1 \pm 1.2\%$ ). The percentages of cells in S phase (from  $18.8 \pm 4.2$  to  $16.4 \pm 0.2$ ) and G<sub>2</sub>-M phase (from  $17.9 \pm 1.0$  to  $12.2 \pm 1.2$ ) were concomitantly decreased. These data suggest that pravastatin blocks cells in the G<sub>1</sub> phase of the cell cycle.

### **Etoposide and Insulin-Induced Nuclear Translocation of Akt Is Inhibited by Statins in an mTOR-Dependent Manner**

Nuclear translocation of Akt is crucial for its activity (25). To test whether statins could affect the nuclear translocation of Akt, immunocytochemical methods were employed. The data in Fig. 2A shows that in unstimulated HepG2 cells and in pravastatin-treated cells, no nuclear staining for pAkt Thr<sup>308</sup> was observed and pAkt was mainly detected in the cytoplasm. In insulin- or etoposide-treated cells, a nuclear localization of pAkt Thr<sup>308</sup> was induced (Fig. 2A). This rapid translocation of pAkt Thr<sup>308</sup> to the nucleus is in line with a previous report (26). However, cells pretreated with pravastatin (2  $\mu\text{mol/L}$ , 1 hour) did not show a nuclear translocation of pAkt Thr<sup>308</sup> (Fig. 2A). We also examined the effect of pravastatin on insulin-induced nuclear translocation employing pAkt Ser<sup>473</sup> antibody, and the same effects were seen (data not shown). These effects were also registered in H1299 NSCLC cells (Fig. 2B) and in A549 cells (Fig. 2C). As shown in Fig. 2D, the number of pAkt-positive cells was decreased after 5 minutes of pretreatment with atorvastatin and that effect lasted at least 45 minutes.

Our previous data show that the phosphorylation of mTOR is induced by pravastatin in HepG2 cells (10). Figure 3A shows that a specific inhibitor of mTOR, rapamycin, abrogated the atorvastatin-induced cytoplasmic localization of pAkt Thr<sup>308</sup> in A549 cells. Rapamycin alone did not have any effect on pAkt (Fig. 3A). As shown in Fig. 3B, the same effect was registered employing the pAkt Ser<sup>473</sup> antibody, and rapamycin significantly prevented the effect of atorvastatin on pAkt (Fig. 3B). Our preliminary data indicate that silencing mTOR with siRNA abrogates the pravastatin-induced cytoplasmic localization of pAkt Thr<sup>308</sup> in HepG2 cells (data not shown). Figure 3C and D shows that statin treatment induces pIRS-1 phosphorylation at residue Ser<sup>636/639</sup> in HepG2 and H1299 cells. Statin and insulin treatment induced additive IRS-1 phosphorylation at residue Ser<sup>636/639</sup> (Fig. 3E), and this effect was inhibited by rapamycin (Fig. 3F). IRS-1 phosphorylation has been shown to mediate mTOR-induced inhibition of Akt signaling (27) and treatment with rapamycin inhibited IRS-1 phosphorylation and abrogated the atorvastatin-induced inhibition of Akt. These data suggest that pharmacologically relevant concentrations of statins can inhibit the activity of Akt induced by etoposide in hepatocellular carcinoma cells as well as in lung cancer cells in a mTOR-dependent way.

### **LY294002 Enhances the Statin-Induced Antiproliferative Effect and Induces Apoptosis**

The effect of PI3K inhibitor LY294002 (28) on the antiproliferative effect of atorvastatin was examined. In these experiments, low concentrations of LY294002 were used. As shown in Fig. 4A, treatment of cells with the combination of atorvastatin (1  $\mu\text{mol/L}$ ) and LY294002 (2.5  $\mu\text{mol/L}$ ) significantly decreased the level of pAkt and induced a synergistic growth inhibition of HepG2 cells (Fig. 4B). This effect was associated with a significant

<sup>1</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

increase in apoptosis (Fig. 4C). A similar effect was registered in p53-deficient H1299 cells. H1299 cells were more resistant to LY294002 alone, but the synergistic effect on cell proliferation was induced by the combination (Fig. 4D). Also, in this case, a significant increase in apoptosis was induced (Fig. 4E).

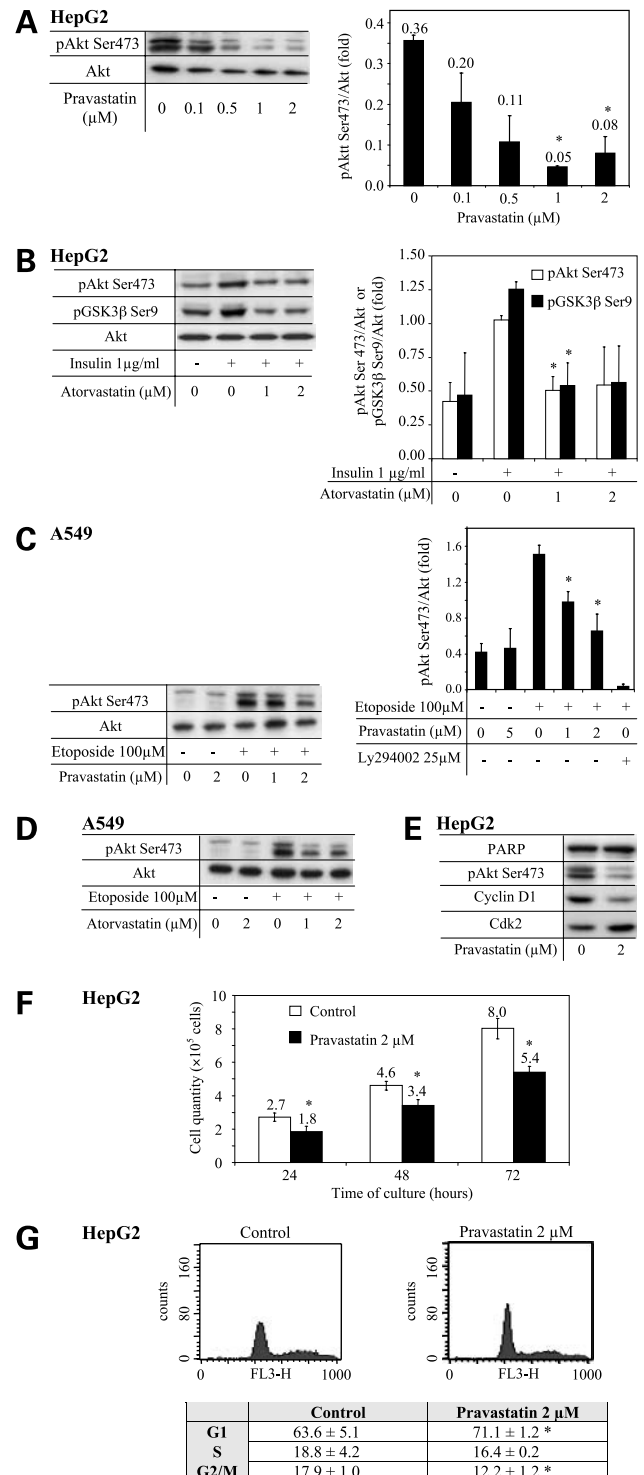
### p53 Stabilization Counteracts the Pravastatin-Induced Effect

The effect of pravastatin on the antiproliferative effect of etoposide was studied in HepG2 cells. Etoposide induced a dose-dependent inhibition of cell proliferation (5%, 44%, 55%, and 64% inhibition by 0.1, 1, 10 and 100  $\mu\text{mol/L}$  etoposide, respectively). As shown in Fig. 5A, 1 hour of pretreatment with pravastatin potentiated the etoposide-induced inhibition of cell proliferation. However, this effect was only detected in the lower concentration range (0.1 and 1  $\mu\text{mol/L}$ ). No significant effects of pravastatin were observed at higher concentrations of etoposide (Fig. 5A).

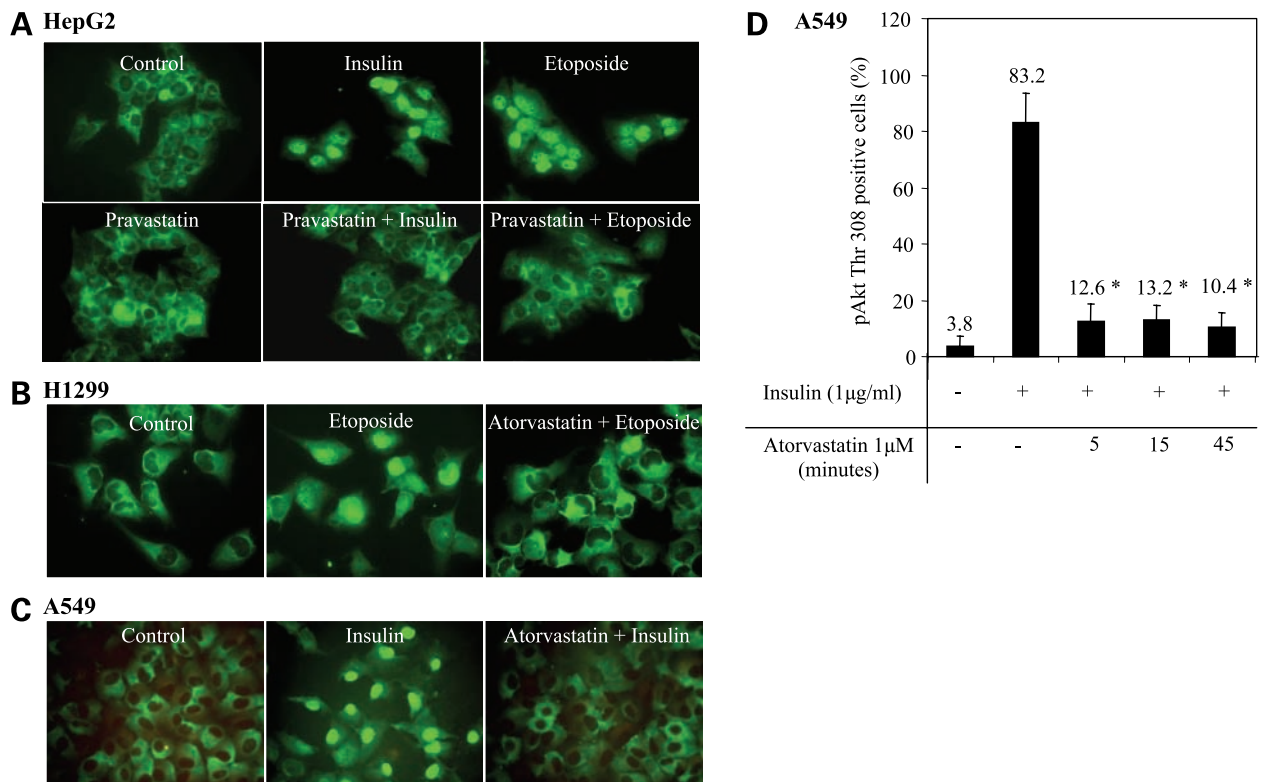
Statins can attenuate the p53 response to DNA damage (10) and the effect of pravastatin on etoposide-induced p53 stabilization was studied. As shown in Fig. 5B, etoposide induced a dose-dependent stabilization of both p53 and its downstream target, p21. Clear p53 stabilization was induced at 10  $\mu\text{mol/L}$ , whereas no significant p53 or p21 stabilization was observed at lower concentrations. Thus, the p53 response was not activated by etoposide concentrations that were potentiated by pravastatin (Fig. 5B). This suggests that the ability of pravastatin to increase the antiproliferative effect of etoposide could be dependent of the p53 status of the cells. We next studied whether pravastatin influences the p53 stabilization induced by etoposide. As shown in Fig. 5C, preincubation of cells with pravastatin lowered both the p53 and p21 responses induced by etoposide. In this experiment, siRNA p53 transfection was used as a control (Fig. 5C). These results support previous conclusions that pravastatin inhibited the

p53 response induced by etoposide. Thus, it can be anticipated that this effect might counteract the ability of pravastatin to enhance the efficiency of etoposide.

The role of p53 for the pravastatin-sensitizing effect of etoposide was studied by transfecting cells with siRNA p53. As can be seen in Fig. 5D, the antiproliferative effect of



**Figure 1.** Pravastatin and atorvastatin decrease constitutive, insulin- and etoposide-induced phosphorylations of Akt and inhibits proliferation. **A** and **B**, HepG2 cells were incubated with pravastatin at the concentrations indicated for 2 h. Samples were then analyzed by Western blotting, employing antibodies for Akt and pAkt Ser<sup>473</sup>. **A**, columns, mean ratio from densitometric analyses of pAkt/Akt from three different experiments; bars,  $\pm$ SD. **B**, serum-starved cells were incubated with atorvastatin for 1 h in the concentrations indicated and thereafter with insulin for 15 min. Columns, mean ratio from densitometric analyses of pAkt Ser<sup>473</sup>/Akt and pGSK3 $\beta$  Ser<sup>9</sup>/Akt from three independent experiments; bars,  $\pm$ SD; \*,  $P \leq 0.05$ , significantly different from values without atorvastatin. **C** and **D**, serum-starved A549 cells were incubated with pravastatin or with atorvastatin for 1 h in the concentrations indicated and thereafter with etoposide for 2 h. **C**, columns, mean ratio from densitometric analyses of the pAkt/Akt ratio from three different experiments; bars,  $\pm$ SD; \*,  $P \leq 0.05$ , values significantly different from controls. **E**, HepG2 cells were incubated with pravastatin for 72 h. Samples were analyzed by Western blotting employing antibodies for Akt, pAkt Ser<sup>473</sup>, pAkt Thr<sup>308</sup>, pGSK3 $\beta$  Ser<sup>9</sup>, PARP, and cyclin D1. Cdk2 was used as a loading control. **F**, cells were treated with pravastatin (2  $\mu\text{mol/L}$ ) for the times indicated. The number of cells was quantified using a trypan blue exclusion assay. Columns, means of three independent experiments; bars,  $\pm$ SD. **G**, cell cycle distribution of cells treated with pravastatin (2  $\mu\text{mol/L}$ ) for 48 h were analyzed by flow cytometry; \*,  $P \leq 0.05$ , values significantly different from controls.



**Figure 2.** Etoposide- and insulin-induced nuclear translocation of pAkt is inhibited by statin in HepG2, H1299, and A549 cells. **A**, serum-starved HepG2 cells were incubated with pravastatin (2 µmol/L) for 1 h and thereafter with insulin (1 µg/mL) for 15 min or with etoposide (100 µmol/L) for 2 h. **B**, serum-starved H1299 cells were incubated with atorvastatin (1 µmol/L) for 1 h and thereafter with etoposide (100 µmol/L) for 2 h. Serum-starved A549 cells were treated with atorvastatin (1 µmol/L) for 15 min (**C**) or for the times indicated (**D**), and thereafter with insulin (1 µg/mL) for 15 min. Cells were stained for pAkt Thr<sup>308</sup>. **D**, the results are expressed as the percentage of cells with nuclear staining for pAkt. *Columns*, means of positive cells from three independent experiments; *bars*, ±SD; \*,  $P \leq 0.05$ , values significantly different from insulin.

the combination of etoposide and pravastatin was evident in transfected cells, whereas no significant effect was registered in p53-expressing cells. siRNA p53 transfection had a minor effect on the antiproliferative effect of etoposide (data not shown). These data indicate that the effect of statins on cytostatic efficiency is dependent on the cellular expression of p53.

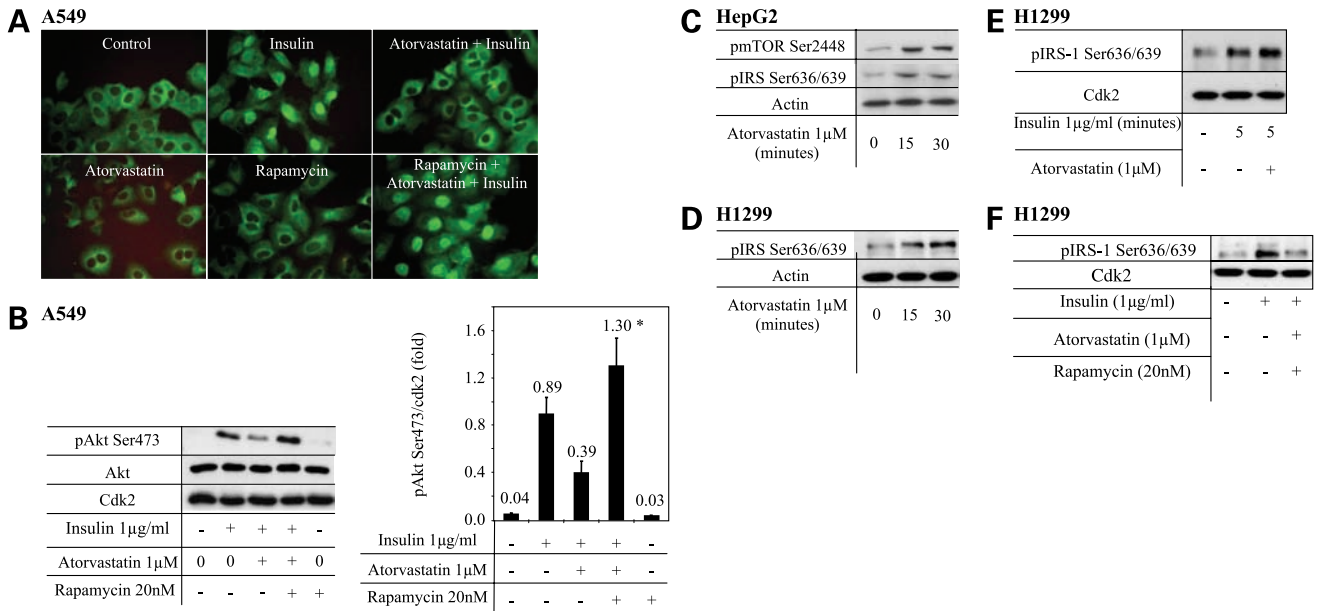
#### Atorvastatin Enhances the Efficiency of Etoposide in p53-Deficient H1299 Cells

To further elucidate the role of p53 status for the effect of statins on the cytostatic efficiency of etoposide, p53 wild-type A549 cells and p53-deficient H1299 cells were studied. Treatment with etoposide induced a dose-dependent inhibition of cell proliferation in both cell lines (Fig. 5E and F). In A549 cells, 0.5, 1, and 10 µmol/L decreased the proliferation by 44%, 51%, and 64%, respectively (Fig. 5E), whereas the proliferation of H1299 cells was decreased by 23%, 33%, and 49%, respectively (Fig. 5F). Clearly, A549 cells were more sensitive to etoposide than the H1299 cells. In the same way as in HepG2 cells (Fig. 5A), atorvastatin significantly increased the antiproliferative effect induced by the lowest dose of etoposide tested in A549 cells (Fig. 5E). No increase of efficiency was observed for higher concentrations, which stabilized p53 (as shown in Fig. 5G).

However, in the H1299 cells (Fig. 5F), a significant increase in efficiency was observed for the whole range of etoposide concentrations (30%, 20%, and 22% for 0.5, 1, and 10 µmol/L of etoposide, respectively). The different results obtained with the two cell lines further support the notion that the effect of statins is dependent of the p53 status of the cells. Figure 5G and H show the p53 response in A549 cells and the attenuation induced by atorvastatin.

#### Atorvastatin Sensitizes p53-Deficient H1299 Cells to Etoposide, Doxorubicin, 5-FU, and Cisplatin

To determine whether statins enhance the efficiency of other p53-inducing cytostatic drugs in p53-deficient H1299 cells, we tested doxorubicin, 5-FU, and cisplatin. Cells were pretreated with atorvastatin for 1 hour and thereafter exposed to the drugs for 48 hours. As expected, atorvastatin potentiated the effect of all three cytostatics over a broad concentration range. Thus, pretreatment with atorvastatin combined with 1 µmol/L of 5-FU or cisplatin induced a similar inhibition of cell proliferation as 10 µmol/L of 5-FU or cisplatin alone (Supplemental data 2).<sup>1</sup> This indicated a 10 times increased efficiency of the drugs. Pretreatment with atorvastatin combined with 5 nmol/L of doxorubicin induced a similar inhibition of cell proliferation as 15 nmol/L of doxorubicin alone.



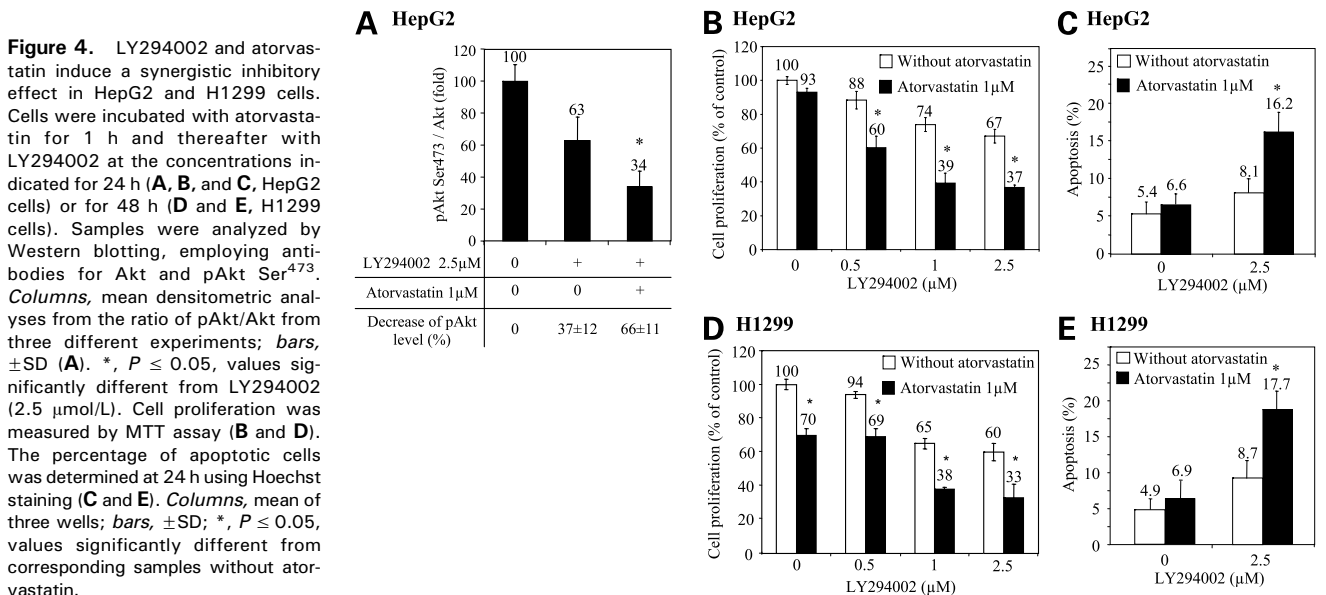
**Figure 3.** Statin-induced attenuated Akt response is prevented by mTOR inactivation. **A, B,** and **F,** serum-starved A549 cells were incubated with rapamycin (20 nmol/L) for 1 h and thereafter with atorvastatin (1 µmol/L) for 1 h. Cells were then treated with insulin (1 µg/mL) for 15 min. HepG2 cells (**C**) and H1299 (**D**) cells were treated with atorvastatin for the times indicated. **E,** serum-starved H1299 cells were treated with atorvastatin for 5 min and then with insulin for 5 min. Cells were stained for the pAkt Thr<sup>308</sup>. Samples were analyzed by Western blotting employing antibodies for pAkt Ser<sup>473</sup>, pmTOR Ser<sup>2448</sup>, pIRS Ser<sup>636/639</sup>, and Cdk2. *Columns*, mean densitometric analysis of the ratio of pAkt Ser<sup>473</sup>/Cdk2 from three independent experiments; *bars*, ±SD; \*, *P* ≤ 0.05, values significantly different from insulin + atorvastatin.

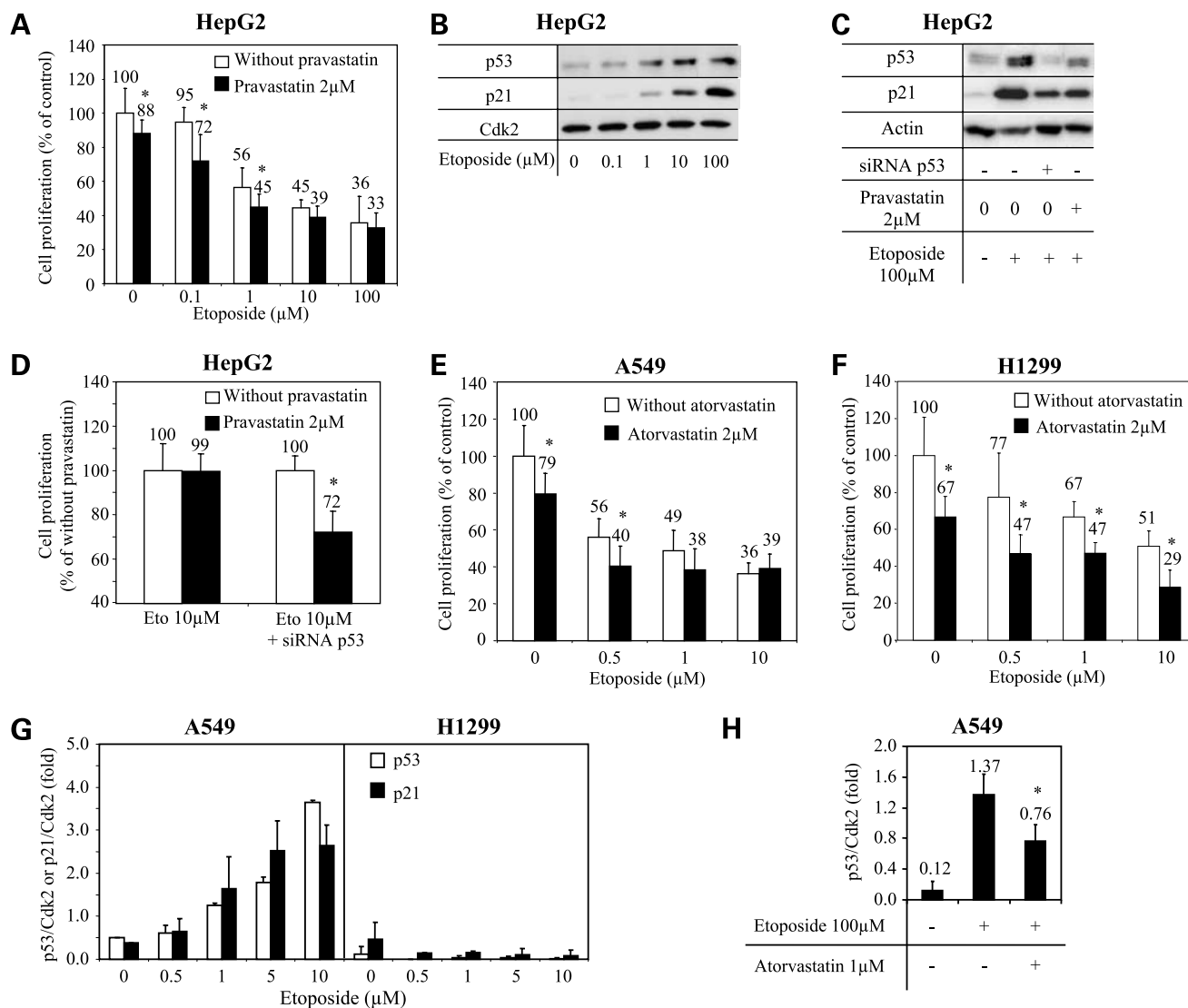
The effect of atorvastatin on proliferation was studied in H1299 cells. Forty-eight hours of incubation with 1 to 4 µmol/L of atorvastatin inhibited cell proliferation dose-dependently (Fig. 6A). We next studied the effect of pretreatment with atorvastatin on apoptosis. As shown in Fig. 6B, the combination of 10 µmol/L of etoposide and 1 µmol/L of atorvastatin significantly decreased cell proliferation and increased the level of apoptosis. Similar

results were also registered for doxorubicin (Fig. 6C) and for 5-FU (Fig. 6D). Taken together, these results indicate that statins potentiate the effect of commonly used cytostatic drugs in p53-deficient cells.

**Discussion**

Our data show that in micromolar concentrations, statins inhibit phosphorylation and nuclear translocation of Akt in





**Figure 5.** p53 stabilization counteracts the pravastatin-induced increased etoposide efficiency. HepG2 cells were incubated with pravastatin for 1 h and thereafter with etoposide at the concentrations indicated for 48 h. Cell proliferation was measured by MTT assay (**A**). Cells were incubated with etoposide at the concentrations indicated for 24 h. Samples were analyzed by Western blotting employing antibodies for p53, p21, and Cdk2 (**B**). Cells were transfected with siRNA against p53 for 24 h. Cells were then pretreated with pravastatin for 1 h and thereafter with etoposide for 24 h. Samples were analyzed for p53 and p21 levels (**C**). After 48 h, cell proliferation was estimated using MTT assay (**D**). Wild-type p53 A549 cells (**E**) and p53-deficient H1299 cells (**F**) were incubated with atorvastatin for 1 h and thereafter with etoposide for 48 h at the concentrations indicated. Cell proliferation was estimated by MTT assay. *Columns*, mean of six wells; *bars*,  $\pm$ SD. **G**, cells were incubated with etoposide at the concentrations indicated for 24 h. *Columns*, mean from densitometric analysis of the p53/Cdk2 and p21/Cdk2 ratio; *bars*,  $\pm$ SD. **H**, A549 cells were incubated with atorvastatin (1  $\mu\text{mol/L}$ ) for 1 h and thereafter with etoposide (100  $\mu\text{mol/L}$ ) for 24 h. *Columns*, mean from densitometric analysis of the p53/Cdk2 ratio from three experiments; *bars*,  $\pm$ SD; \*,  $P \leq 0.05$ , values significantly different from samples without statin.

HepG2 cells and in NSCLC cells in an mTOR-dependent manner. We found that high constitutive levels of Akt as well as insulin- and cytostatic drug-induced activation of Akt were inhibited by statins, and that these effects were restored by blocking mTOR. We also present data showing a synergistic effect between statins and PI3K inhibitors and that statins sensitize p53-deficient cells to cytostatic drugs.

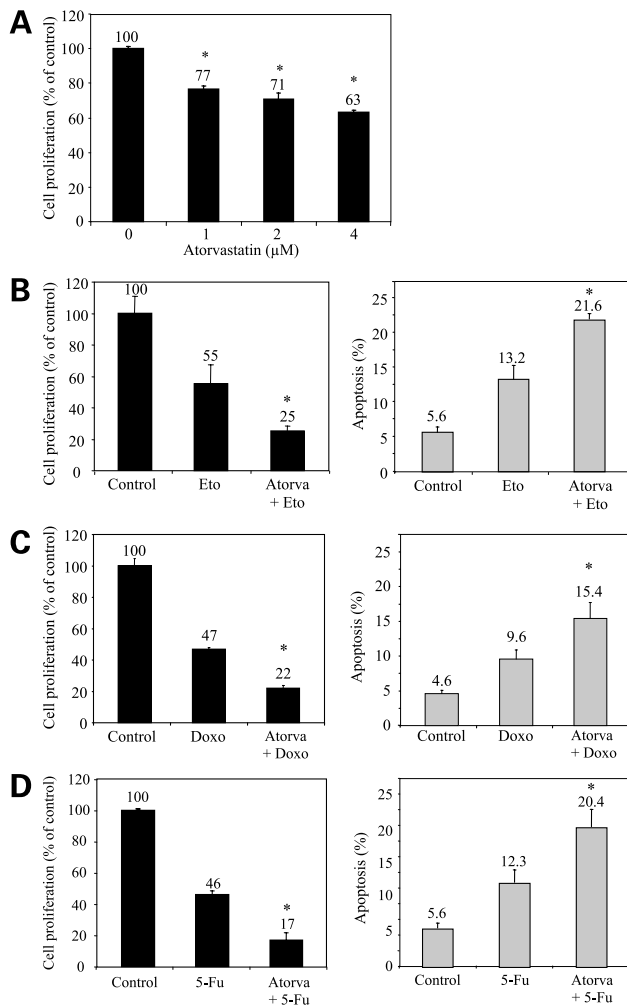
Statin-induced Akt inhibition correlated with decreased phosphorylation of GSK3 $\beta$  on Ser<sup>9</sup> and with the inhibition

of cell proliferation. Cells were blocked in G<sub>1</sub> phase without any signs of apoptosis. In all three cell lines tested, HepG2, A549, and H1299, treatment with atorvastatin (1–4  $\mu\text{mol/L}$ ) led to a 40% inhibition of growth. We also found that pretreatment with statins prevented nuclear translocation of Akt induced by insulin or etoposide. This effect was documented by employing antibodies specific for both Ser<sup>473</sup> and Thr<sup>308</sup> phosphorylated Akt. It has been shown that following activation through phosphorylation of Ser<sup>473</sup>

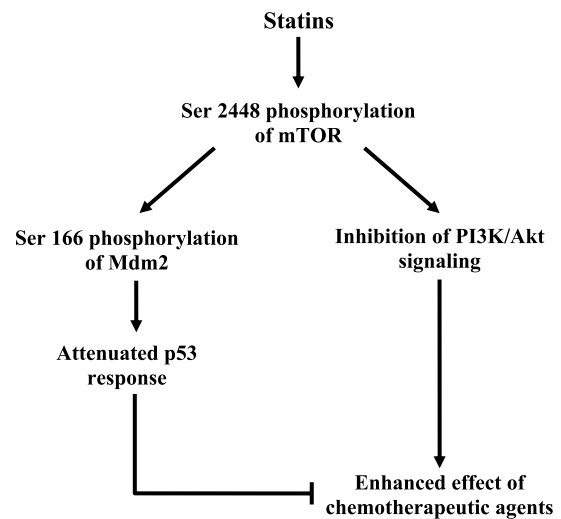


and Thr<sup>308</sup>, Akt detaches from the plasma membrane and translocates to the nucleus, and that this translocation is crucial for its mitogenic activity (25). Several nuclear targets for Akt involved in the cell cycle regulation, such as p27 and FOXO, have been identified (29). Exclusion from the nucleus may thus restrain the kinase activity on targets involved in the regulation of the cell cycle.

Previously, we showed that mTOR was phosphorylated and activated by statins (10). mTOR has been described to initiate a negative feedback loop which attenuates the ability of insulin to activate Akt and may play a role in insulin resistance (27, 30–33). Here, we show that inhibition of mTOR abrogated the statin-induced inhibition of pAkt. Thus, inhibition of mTOR, by rapamycin or siRNA, rescued the nuclear localization of pAkt. These data



**Figure 6.** Atorvastatin sensitizes p53-deficient H1299 cells for cytostatic drugs. Cells were incubated with atorvastatin at the concentrations indicated (A) or with 1 μmol/L of atorvastatin for 1 h and thereafter with 10 μmol/L of etoposide (B), 15 nmol/L of doxorubicin (C), or 15 μmol/L of 5-FU (D). Cell proliferation was estimated by MTT assay after 48 h and the percentage of apoptotic cells was quantified using Hoechst staining after 24 h. Columns, mean of three dishes; bars, ±SD; \*,  $P \leq 0.05$ , values significantly different from drugs without atorvastatin.



**Figure 7.** Statin-induced effects on p53 and Akt signaling.

implicate mTOR in the attenuated pAkt response. Moreover, statin-induced effects on mTOR phosphorylation correlated with the phosphorylation of IRS-1, suggesting a role for IRS-1 and its phosphorylation on Ser<sup>636/639</sup>. This phosphorylation has been shown to mediate mTOR-induced inhibition of Akt signaling by a negative feedback loop and to terminate insulin signaling (27), and may thus have down-regulated Akt signaling in our experiments. Further studies will characterize the effect of statins on mTOR/Akt signaling pathway in more detail.

We found that combined treatment of cells with atorvastatin and the PI3K inhibitor, LY294002, induced synergistic effects on cell proliferation and apoptosis in p53 wild-type and p53-deficient cells. Thus, low nontoxic concentrations of LY294002 significantly potentiated the effect of atorvastatin and elicited apoptosis. This synergistic effect suggests that statins act by inhibiting the PI3K/Akt pathway. The combination of statins and LY294002 might bring the level of Akt activity below a threshold level that maintains cell survival (34). Alternatively, different isoforms of Akt may have overlapping roles (35, 36) and LY294002 and statins might inhibit different isoforms of Akt.

Inhibition of Akt has been shown to sensitize cancer cell lines to chemotherapeutic agents (37–39), and statins have been shown to increase the efficiency of 5-FU, cisplatin, doxorubicin, etc. (1). However, p53 stabilization may play a central role in the toxicity of these genotoxic drugs (40–42), and our previous data showed that statins could attenuate the p53 response to DNA damage (10). Therefore, the influence of p53 on the statin-induced sensitizing effect was studied and we found evidence that the effect of statins on p53 abrogated the effect mediated by Akt inhibition. Thus, in p53-containing HepG2 and A549 cells, pretreatment with statins sensitized cells to low concentrations of etoposide, but not to high concentrations, which induced p53 and p21. We also showed that pretreatment with pravastatin



attenuated the etoposide-induced p53 response and that silencing p53 with siRNA rescued the sensitizing effect at high concentrations of etoposide. Together, these data indicate that an Akt-mediated sensitizing effect of statins can be counteracted by an attenuation of the p53 response.

A comparison between p53 wild-type A549 cells and p53-deficient H1299 NSCLC cells clearly show that a more robust additive effect of statins was induced in p53-negative cells. Thus, pretreatment with atorvastatin sensitized H1299 cells to etoposide, doxorubicin, 5-FU, and cisplatin in all concentrations tested. H1299 cells were more resistant to etoposide alone than A549 cells, but preincubation with 1  $\mu\text{mol/L}$  of atorvastatin decreased the  $\text{IC}_{50}$  for etoposide from 10 to 0.5  $\mu\text{mol/L}$ . Together, these data suggest that statins potentiate the effect of commonly used cytostatic drugs in p53-deficient cells.

The effects of statins on mTOR, p53, and PI3K/Akt signaling are summarized in Fig. 7. Some previous studies have reported synergistic effects between cytostatic drugs and statins (5–8). However, in most of these studies, mutated or p53-null cell lines were used, and we show that this characteristic could be critical for the cell-killing effects. In a clinical trial on advanced human hepatocellular carcinoma, a synergistic effect between pravastatin and 5-FU was reported (9). Interestingly, in this study, >80% of the patients were infected by hepatitis C virus, which is associated with frequently mutated p53, especially in late stages of the disease (43, 44). This may explain the favorable interaction.

In summary, we have shown that statins inhibit Akt signaling in a mTOR-dependent manner in HepG2 and NSCLC cells. The activation of Akt plays a role in inducing chemoresistance in cancer cells and selective Akt inhibitors are under development for clinical use. The first generation of Akt inhibitors, such as LY294002, has serious side effects and high toxicity (20), and statins may represent potentially new and safe Akt inhibitors. However, we find that the ability of statins to sensitize cells for apoptosis induced by p53-stabilizing drugs is dependent on the p53 status of the cells.

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