

Breast Cancer Growth Prevention by Statins

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Abstract

Statins are cholesterol-lowering drugs with pleiotropic activities including inhibition of isoprenylation reactions and reduction of signals driving cell proliferation and survival responses. The objectives of this study were to examine the effects of statins on breast cancer cells, both *in vitro* and *in vivo*, and to begin to determine their mechanism of action. We evaluated the effects of statins on breast cancer cell growth, phosphoprotein signaling intermediates, survival/apoptosis regulators, cell cycle regulators, and activated transcription factors. We also examined the *in vivo* effect of statin administration in a mouse ErbB2⁺ breast cancer model. Only lipophilic statins had direct anticancer activity *in vitro*. Breast cancer cells with activated Ras or ErbB2 pathways seemed to be more sensitive than those overexpressing estrogen receptor, and this correlated with endogenous levels of activated nuclear factor κ B (NF- κ B). Key intermediates regulating cell survival by NF- κ B activation, as well as cell proliferation by the mitogen activated protein kinase cascade, were among the earliest phosphoproteins influenced by statin treatment. These early effects were followed by declines in activator protein-1 and NF- κ B activation and concordant changes in other mediators of proliferation and apoptosis. *In vivo* results showed that oral dosing of statins significantly inhibited the growth of a mouse mammary carcinoma. Lipophilic statins can exert direct anticancer activity *in vitro* by reducing proliferation and survival signals in susceptible breast cancer phenotypes. Tumor growth inhibition *in vivo* using a clinically relevant statin dose also seems to be associated with reduced tumor cell proliferation and survival. These findings provide supporting rationale for future statin trials in breast cancer patients. (Cancer Res 2006; 66(17): 8707-14)

Introduction

There is increasing interest in finding new agents with potential effectiveness for breast cancer prevention. When designing strategies for prevention, as opposed to breast cancer treatment, there are striking differences: healthy women at high risk for breast cancer are the focus of prevention, not women who are facing a risk of dying from breast cancer. In the prevention setting, we ideally look for drugs that are safe and health-promoting. One class of drugs that clearly fits these criteria is statins (1–7).

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There are intriguing epidemiologic data to suggest that statins have cancer prevention efficacy (8–12), although the epidemiologic data are mixed (8, 13–17). A recently published meta-analysis found no protective effect of statins against breast cancer (18). However, epidemiologic studies on statins and breast cancer are limited by self-reporting and dose-duration effects, and these studies to date have been designed around cardiac and vascular end points, not cancer. In addition, these studies often lack information about the type of statin used. This is important because only lipophilic statins are able to permeate the cell membrane and affect cell proliferation, survival, and motility (11). In the above-mentioned meta-analysis, four of the seven randomized clinical trials tested pravastatin, a hydrophilic statin that has been shown to lack anticancer activity both *in vitro* and *in vivo* (19).

Furthermore, data from two large retrospective studies presented at recent American Society of Clinical Oncology meetings were not included in the meta-analyses, as they had not yet been published. In a study of 40,421 individuals, Kochhar et al. (20) observed a protective benefit of statins (relative risk, 0.49), which increased with increasing duration of use. In another study of 68,071 women, Mortimer et al. (12) found that statin use resulted in a significant reduction in breast cancers among women ages >50 years. Thus, the question of whether statin use affects breast cancer risk remains unanswered.

Structurally, all statins possess a 3-hydroxy-3-methylglutaryl (HMG)-like moiety that binds with at least nanomolar affinity to sterically block substrate access to the catalytic portion of HMG-CoA reductase, thereby curtailing the production of mevalonate at the first committed step in cholesterol biosynthesis (21). Functionally, statins may also be classified by their overall lipophilicity/hydrophilicity, which is purported to distinguish statin groups capable of reducing clinical cardiac events apart from their cholesterol-lowering and anti-atherosclerotic activity (22). Hydrophilic statins, like pravastatin, are known to exhibit more selective *in vivo* distribution, being unable to penetrate membrane lipid bilayers and, thus, primarily entering hepatocytes via their organic anion transporters (21, 23, 24). In contrast, the lipophilicity of statins like lovastatin, simvastatin, and fluvastatin allows them to directly cross cell membranes and, in addition to their cholesterol-lowering hepatic effects, exert pleiotropic effects in many extrahepatic tissues (22).

Emerging interest in the use of statins as anticancer agents is based on their pleiotropic effects on various cells and tissues. Preclinical studies have shown antiproliferative, proapoptotic, anti-invasive, and radiosensitizing properties of statins. Inhibition of HMG-CoA reductase by statins leads to reduced levels of mevalonate and its downstream products, including the isoprenoid intermediates that provide lipid attachment sites for activated Ras, Rac, and Rho family members (25). Many of these downstream

products play important roles in cellular and subcellular pathways critical for cancer formation and its progression, including membrane integrity, cell signaling, cell cycle progression, immune/inflammatory reactions, stromal-epithelial cell interactions, endogenous energy metabolism, oxidative stress, and intracellular vitamin and steroid content (26, 27). Alterations of these processes in cancer cells or in the tumor microenvironment may therefore lead to inhibition of tumor initiation, growth, and metastasis.

Breast cancer is not a homogeneous disease. Perou et al. (28) have shown that there are multiple phenotypes with associated differences in outcomes when treated. Magnetic resonance imaging studies have shown that human breast tumors exhibit various growth patterns, which may, in part, reflect these phenotypic differences (29). On presentation, breast cancers are routinely phenotyped in a more limited manner by evaluating their growth fraction (e.g., Ki67 index), estrogen and progesterone receptor expression, and ErbB2/HER2 amplification and receptor overexpression. We hypothesized that the tumor growth altering effects of statins would be dependent not only on their structural and pharmacologic differences but also on the phenotypic differences between breast cancer cell populations.

Thus, the aim of the present study was to compare the effect of four statins in common clinical use on the proliferation and molecular signaling pathways of three human breast cancer types, hormone receptor-positive/HER2-negative, hormone receptor-negative/HER2-positive, and double-negative cells. We further explored the chemoprevention potential of two commonly used lipophilic statins in an *in vivo* mouse model representing one of these breast cancer phenotypes.

Materials and Methods

Drugs, human breast cancer cell lines, and extract preparations. Purified (>99%) crystalline powders of each of the statins: fluvastatin, lovastatin, simvastatin, and pravastatin were commercially obtained (Alexis Biochemicals, San Diego, CA; LKT Laboratories, Inc., St. Paul, MN) and dissolved into refrigerated and light-protected DMSO stock solutions. The MCF-7, SKBr3, and MDA-231 human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37°C and 5% CO₂ in recommended media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (and 10 µg/mL insulin for the MCF-7), as we have previously described (30–32).

For extract preparations, treated or control cells were first harvested on ice by washing twice with cold PBS, scraping, and resuspending in 1.0-mL hypotonic buffer [20 mmol/L HEPES (pH 7.0), 10 mmol/L KCl, 1 mmol/L MgCl₂, 0.1% Triton X-100, 20% glycerol, 0.5 mmol/L DTT] containing a cocktail of protease inhibitors (Mini Complete protease inhibitors, Roche Diagnostics, Mannheim, Germany). For immunoblot assays, whole-cell extracts were prepared in solubilizing buffer (1% SDS, 20% glycerol, 100 mmol/L DTT, 50 mmol/L Tris, pH 6.8). Nuclear extracts were also prepared for transcription factor DNA-binding studies by Dounce homogenizing the harvested cells on ice, centrifuging the mixture at 3,000 rpm at 4°C (5 minutes) to collect the nuclear pellet, and then resuspending the pellet in elution buffer [20 mmol/L HEPES (pH 7.0), 10 mmol/L KCl, 1 mmol/L MgCl₂, 0.42 mol/L NaCl, 0.1% Triton X-100, 20% glycerol, 0.5mmol/L DTT] supplemented with protease inhibitors. After 20 minutes at 4°C, the solubilized pellets were centrifuged at 14,000 rpm, 4°C (10 minutes) to obtain nuclear extracts, which were stored in aliquots at –80°C. Nuclear and whole-cell extracts were assayed for total protein concentration (Bradford assay; Bio-Rad, Hercules, CA).

***In vitro* growth inhibition assays.** To quantitate *in vitro* statin growth inhibitory effects, MCF-7, SKBr3, and MDA-231 cell cultures were treated 24 hours after cell plating with 0 (vehicle) to 200 µmol/L doses of statins and cell viability was determined 5 days later using the CyQuant assay kit

(Molecular Probes, Eugene, OR). Dose-response growth inhibition curves were produced to determine specific IC₅₀ values.

Immunoblot and DNA-binding assays. All antibodies used for immunoblotting were commercially obtained (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; Abcam, Inc., Cambridge, MA; Cell Signaling Technology, Beverly, MA; Biosource, Camarillo, CA; Upstate USA, Inc., Charlottesville, VA) and specific for the following intracellular signaling, effector, and control proteins/phospho(p)-proteins: p(Ser)-IκBα, IκBα, p(Ser)-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2, p(Thr)-ERK1/2, ERK1/2, p(Ser)-signal transducers and activators of transcription (STAT)-1, p(Ser)-STAT3, p(Ser)-Akt, p(Tyr)-c-jun NH₂-terminal kinase (JNK), p(Thr)-p38, p(Ser)GSK3β, p(Ser)BAD, Bcl-2, Bcl-xL, ILP, caspase-9, caspase-3, p21^{WAF1}, cyclin D1, and β-actin. Phosphoprotein responses in MCF-7, SKBr3, or MDA-231 cells, treated with an IC₅₀ dose of statins, were determined using whole-cell extracts analyzed on immunoblot microarrays by the National Cancer Institute (NCI)/Food and Drug Administration Clinical Proteomics Program. Control, 12-, 24-, and 48-hour protein/phosphoprotein responses after SKBr3 treatment with an IC₅₀ dose of statins were determined using whole-cell extracts (15 µg protein) boiled in loading buffer [125 mmol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol], separated by electrophoresis on 4% to 12% Bis-Tris SDS gradient gels in MOPS buffer and by standard Western procedures. Bound antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat antibody against the mouse or rabbit primary immunoglobulin G (Bio-Rad) and visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

The DNA binding of activated nuclear factor κB (NF-κB) and activator protein-1 (AP-1) transcription factor complexes isolated from control and statin-treated breast cancer cell nuclear extracts was measured by electrophoretic mobility shift assay, as we have previously described (32, 33). In brief, ³²P-labeled oligonucleotide probes bearing consensus κB or AP-1 binding elements were incubated with nuclear extracts and the resulting DNA-protein complexes were resolved by electrophoresis using native 5% polyacrylamide gels. The gels were dried on filter papers and then exposed overnight to X-ray film at –80°C with an intensifying screen. Quantitative p50 and p65 NF-κB DNA binding were also determined using ELISA-based Trans-AM assays (ActiveMotif, Carlsbad, CA), as previously described (32). For these ELISA-based DNA-binding assays, a duplexed NF-κB oligonucleotide containing the same κB consensus sequence described for the electrophoretic mobility shift assay above is attached to the surface of 96-well plates. Activated NF-κB in nuclear extracts, which is first bound to the attached oligonucleotide, is specifically and quantitatively detected by subsequent incubation with p50- or p65-specific antibody, followed by an enzyme (HRP)-linked secondary antibody for colorimetric (A_{450nm}) scoring.

Statin effects on *in vivo* mammary tumor (MCNeuA) growth. Eight- to ten-week-old female transgenic FVB-NeuN mice (N202; ref. 34), hereafter denoted *neuTg*, were used in this study. Animal experiments were done under formal approval by the Institutional Committee for Animal Research. The MCNeuA cell line used in these *in vivo* studies is a HER2/*neu*⁺, estrogen receptor-negative mouse mammary carcinoma line established from a spontaneous tumor that arose in a female *neuTg* mouse (35).

On day 0, groups of 10 mice were injected with 10⁵ MCNeuA tumor cells s.c. on the left flank. Beginning on day 0, simvastatin or fluvastatin was given orally in the animals' drinking water *ad libitum*. In experiment 1, a final simvastatin dosage of 1 to 2 mg/kg/d was calculated by measuring daily water intake. This dose of simvastatin was chosen based on previous preclinical studies and was expected to be nontoxic. In experiment 2, a final statin (simvastatin or fluvastatin) dosage of 15 to 16 mg/kg/d was achieved. This higher dose was chosen to be equivalent to a human dose of 80 mg/d (based on body surface area equivalency). Tumor growth was monitored by caliper measurements and tumor volumes were estimated by the following formula: tumor volume (mm³) = (length × width²) × (π/6). Mean tumor volumes in control and treated groups of mice were compared using Student's *t* test. At the end of each study, all groups of mice were euthanized and their tumors were excised, weighed, and used for immunohistochemical analyses.

Table 1. Breast cancer cell phenotypes and statin growth inhibitory activities

	ER	ErbB2	Ras	NF- κ B
MDA-231	–	–	mut-K	+++
SKBr3	–	+++	–	++
MCF-7	+++	–	–	+
IC ₅₀ values (μ mol/L)				
	Fluvastatin	Simvastatin	Lovastatin	Pravastatin
MDA-231	<1.0	<1.0	2	>200
SKBr3	35	26	49	>200
MCF-7	85	91	138	>200

NOTE: The three human breast cancer cell lines used in this study differ with respect to their expression of estrogen receptor (ER), ErbB2, mutated Ras, and NF- κ B. Proliferation assays were done after the addition of statins (fluvastatin, simvastatin, lovastatin, or pravastatin) at varying doses (0-200 μ mol/L) to these breast cancer cells lines for 5 days. Dose-response curves were generated from these assays and IC₅₀s were calculated.

Immunohistochemistry. Frozen tumor tissue was cut at 4 to 5 μ m, dried at room temperature, and fixed in acetone at 4°C for 10 minutes. Immunohistochemical stains were done with the rabbit anti-cleaved caspase-3 polyclonal antibody, 1:200 dilution (Cell Signaling Technology, Beverly, MA); rat anti-Ki67 antibody, 1:25 dilution (Dako, Carpinteria, CA); and rabbit anti-CD31 antibody, 1:20 dilution (PharMingen, San Diego, CA) using routine avidin-biotin immunoperoxidase technique (Vectastain Elite Kit, Vector Labs, Burlingame, CA). Before incubation with the primary antibody, the tissue sections were treated with 3% hydrogen peroxide and normal goat serum (Vector Labs). After incubation with the primary antibody, tissue sections were incubated with a secondary biotinylated goat anti-rabbit (Vector Labs) followed by avidin-biotin immunoperoxidase. The sections were visualized with diaminobenzidine chromogen (Sigma-Aldrich, St. Louis, MO) and counterstained with hematoxylin (Thermo Shandon, Pittsburgh, PA). Cells with detectable staining above background levels (negative control without primary antibody) were scored as positive.

Necrosis, Ki67 labeling, CD31 vascular staining, and cleaved caspase-3 staining patterns were determined by evaluating 20 intermediate-power (\times 100) or high-power (\times 400) fields, chosen from both the periphery and

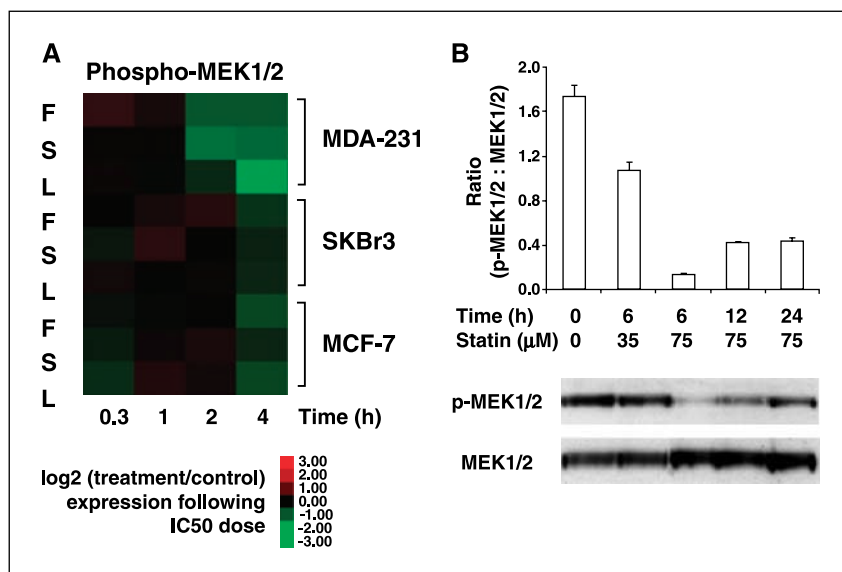
central tumor areas by scanning at low magnification. Staining patterns are denoted as peripheral, central, or diffuse if the staining was noted only in the periphery of the tumor, the center of the tumor, or evenly distributed throughout the tumor, respectively.

Calculation of a Ki67 labeling index was determined by assessing 1,000 cells in well-labeled areas, as determined by scanning at low magnification. Actual counts were made at \times 400 magnification. Nuclei with any detectable staining above background levels (negative control without primary antibody) were scored as positive.

Results

Direct anticancer activity of lipophilic statins against specific breast cancer phenotypes. Table 1 shows the phenotypic characteristics of the human breast cancer cell lines used to assess *in vitro* responses to the hydrophilic statin pravastatin and three lipophilic statins: fluvastatin, lovastatin, and simvastatin. Chosen for their spectrum of breast cancer phenotypes with respect to

Figure 1. Inhibition of phospho-MEK1/2: earliest signaling response common to breast cancer cells inhibited by statins. **A**, human breast cancer cell lines were exposed to IC₅₀ doses of statins and screened by phosphoprotein immunoblot microarrays. Each time-dependent result is expressed as log₂ (treatment/control) change in the microarray determined p-MEK1/2 value. F, fluvastatin; S, simvastatin; L, lovastatin. **B**, time- and dose-dependent decline in p-MEK1/2 following simvastatin treatment of MCNeuA mouse mammary tumor cells assessed by immunoblotting.



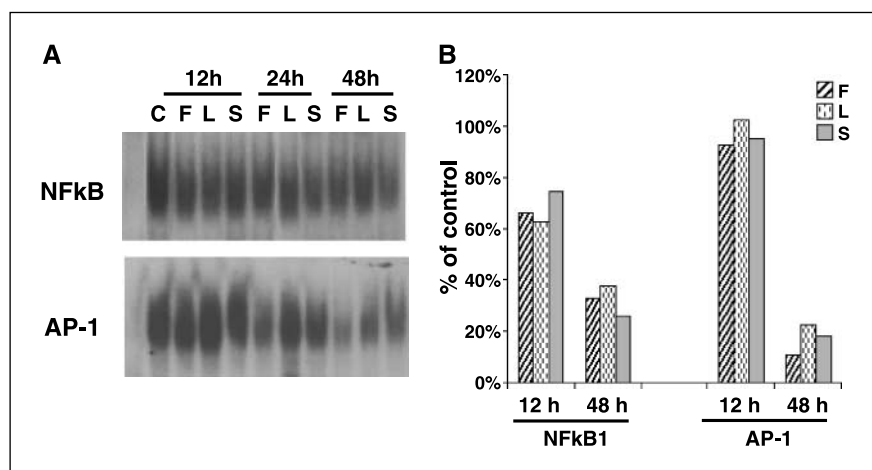


Figure 2. Statin effects on NF-κB and AP-1 activity in SKBr3 cells. *A*, changes in the SKBr3 nuclear DNA-binding activities of NF-κB and AP-1 transcription factor complexes occurring 12 to 48 hours following IC₅₀ dose statin treatments. *B*, densitometry measurements of NF-κB and AP-1 complexes after 12 and 48 hours of treatment with statins. *Columns*, percent of control, untreated cells.

activation of estrogen receptor (MCF-7), ErbB2/HER2 (SKBr3), and mutated Ras (MDA-231) tumorigenic pathways, the table illustrates that these breast cancer lines also possess a range in endogenous NF-κB activity, an antiapoptotic survival pathway effector and transcription factor complex (containing differentially activated p50 and p65 subunits) recently recognized as identifying poor-risk subsets of early-stage breast cancers (32). As shown in Table 1, the hydrophilic statin pravastatin has no demonstrable growth inhibitory activity at doses up to 200 μmol/L, and this complete inactivity was also confirmed in additional cell culture studies evaluating various intracellular responses to statins (data not shown). In contrast, the lipophilic statins (fluvastatin, lovastatin, and simvastatin) showed significant growth inhibitory activity as

quantified by their IC₅₀ values. Of note, the breast cancer lines with activated Ras or ErbB2 pathways seemed to be more sensitive to the lipophilic statins than that overexpressing estrogen receptor; and their overall pattern of statin responsiveness seems to correlate best with their endogenous level of activated NF-κB.

Statin-induced changes in intracellular mediators and effectors of proliferation and apoptosis. Human breast cancer cell lines MDA-231, SKBr3, and MCF-7 were exposed to IC₅₀ doses of fluvastatin, lovastatin, or simvastatin and screened by immunoblot microarrays. Changes in two particular upstream phosphoproteins that regulate proliferation and survival/apoptosis pathways were noted. Within 4 hours of statin exposure, all three cell lines showed a 2- to 8-fold decline in the key p-MEK1/2

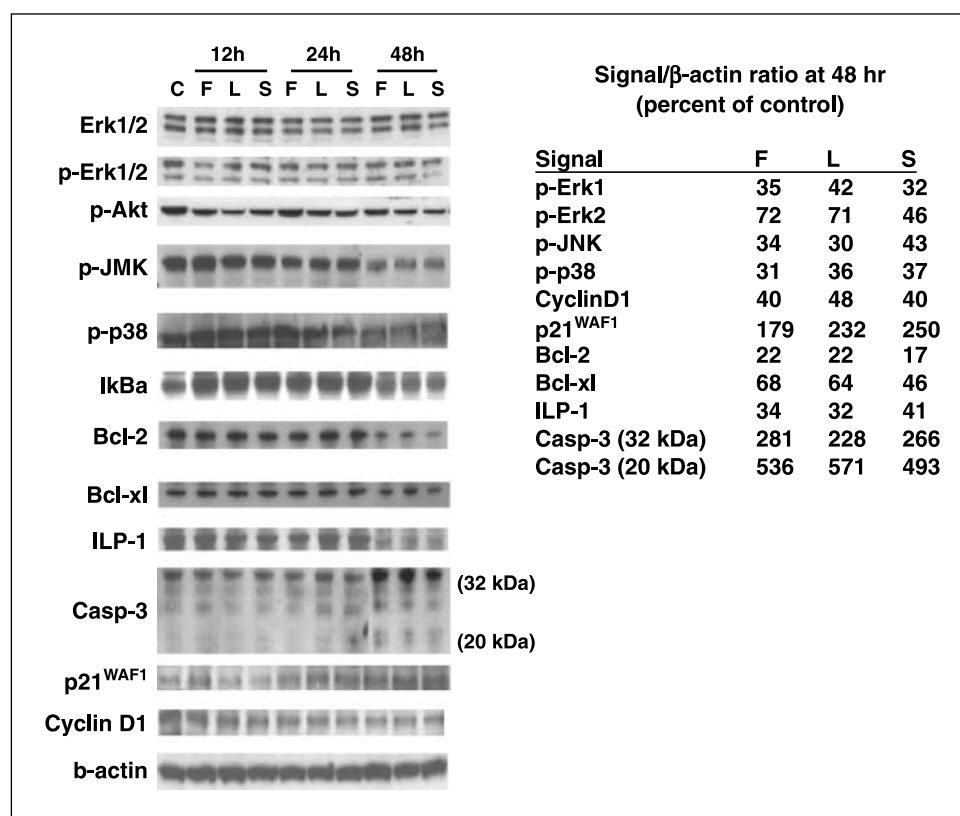


Figure 3. Statin effects on SKBr3 proliferation and survival signals. *Left*, immunoblot comparison of statin-induced changes in SKBr3 proteins, as indicated, occurring between 12 and 48 hours after treatment with equitoxic (IC₅₀) statin doses and relative to vehicle-treated control levels (C). *Right*, densitometry measurements of immunoblots at 48 hours of statin treatment. Ratios of the specific signal to the β-actin signal were calculated from the densitometry data; *columns*, percent of control cells.

Table 2. Effects of simvastatin and fluvastatin on MCNeuA mammary tumor formation

Expt 1	Tumor volume at day 27 (mm ³)	Tumor weight (g)
Control	1,019 ± 29	0.61 ± 0.04
Simvastatin (1-2 mg/kg/d, p.o.)	518 ± 36 (<i>P</i> < 0.001*)	0.30 ± 0.03 (<i>P</i> < 0.001*)
Expt 2	Tumor volume at day 35 (mm ³)	Tumor weight (g)
Control	1,876 ± 203	1.03 ± 0.04
Simvastatin (15-16 mg/kg/d, p.o.)	1,013 ± 137 (<i>P</i> = 0.004*)	0.60 ± 0.03 (<i>P</i> < 0.001*)
Fluvastatin (15-16 mg/kg/d, p.o.)	821 ± 111 (<i>P</i> < 0.001*)	0.45 ± 0.03 (<i>P</i> < 0.001*)

NOTE: Values represent mean ± SE. MCNeuA cells were injected s.c. into female *neuTg* mice on day 0. Statins were administered in the drinking water from days 0 to 27 (expt 1) or days 0 to 35 (expt 2). At the end of the experiments, tumors were excised, weighed, and used for immunohistologic examination.

*Student's *t* test; statin treatment versus control.

intermediate of the Ras-Raf-MEK-ERK cascade thought to drive cell proliferation (Fig. 1A). Similarly, treatment of the mouse mammary carcinoma cell line MCNeuA with simvastatin for 6 hours resulted in a dose-dependent decrease in p-MEK1/2 as determined by Western blot analysis (Fig. 1B). This decrease seemed to be transient, as p-MEK1/2 levels began to increase at 12 and 24 hours. Statin-induced alterations in IκBα, which regulates NF-κB activation and cell survival, were also observed on the immunoblot microarrays (data not shown).

SKBr3 cells treated with statins were further evaluated with respect to a variety of protein/phosphoprotein intracellular responses. After 12-hour statin exposure and with no apparent effect on cell number or viability at this early time point, there was a 25% to 30% decline detectable in activated NF-κB levels, which progressed to an ~70% decrease in activity by 48 hours (Fig. 2). AP-1 DNA binding showed an 80% to 90% decline by 48 hours, but the onset of this decline in AP-1 levels was delayed relative to that of NF-κB DNA-binding activity (Fig. 2). Immunoblots showing multiple mediators and effectors of cell proliferation and survival indicate that other consistent and early (12 hours) changes in response to statins are limited to a significant increase in the level of the NF-κB inhibitor, IκBα, as well as a slight decline in cyclin D1 protein levels (Fig. 3). By 48 hours, there are significant statin-induced declines in various MAP kinase proteins (p-ERK1/2, p-JNK, and p-p38) as well as a further reduction in cyclin D1 associated with increased levels of p21. Concomitant with reduced levels of several antiapoptotic factors (Bcl-2, Bcl-xl, and ILP-1), there is evidence of increased 32-kDa caspase-3 and its 20-kDa degradation product (Fig. 3). Interestingly, p-Akt levels show little if any consistent change following statin exposure.

Statin inhibition of *in vivo* mammary tumor (MCNeuA) growth by reduction of tumor proliferation index. We evaluated the ability of two lipophilic statins to inhibit mammary tumor growth after s.c. inoculation of MCNeuA cells in female *neuTg* mice. MCNeuA cells (ErbB2/HER2⁺, estrogen receptor negative) are sensitive to simvastatin and fluvastatin *in vitro*, with IC₅₀ values of ~35 and 20 μmol/L, respectively (data not shown). A significant antitumor effect was evident in mice receiving 1 to 2 mg/kg/d of simvastatin orally (Table 2, expt 1). This dose, based on body surface area comparisons, is approximately equivalent to a 5 to 10 mg/d oral dose of simvastatin in humans. In a second

experiment, simvastatin or fluvastatin was administered orally at a higher dose (15-16 mg/kg/d). This dose is approximately equivalent to an 80 mg/d oral dose in humans. As shown in Table 2 (expt 2) and Fig. 4, both statins significantly inhibited tumor growth *in vivo*, with fluvastatin being slightly more effective than simvastatin.

Tumors were obtained from simvastatin-treated and untreated control mice for immunohistochemical analyses. Fresh-frozen H&E-stained sections obtained from seven of nine control mouse tumors showed a central pattern of necrosis, whereas all 10 statin-treated tumors showed extensive central necrosis. No significant differences in CD31 vascular staining patterns were observed between treated and untreated tumors. All tumors showed an average of five CD31-positive vessels per high-power field and all areas of the tumors showed an equivalent diffuse CD31 staining pattern. Cleaved caspase-3 was used to assess treatment-induced changes in tumor apoptosis. Most of the control tumors (seven of nine) had low numbers (<25%) of cleaved caspase-3-stained cells.

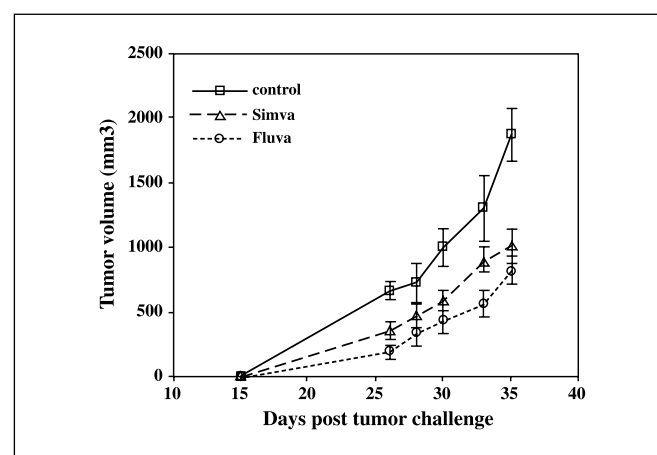


Figure 4. Statin induced *in vivo* growth inhibition of mouse mammary tumors. Mice were implanted s.c. with 10⁵ MCNeuA tumor cells (day 0); statin-treated mice received fluvastatin or simvastatin at 15 to 16 mg/kg/d orally in their drinking water (days 0-35). Tumor volumes were significantly smaller in the fluvastatin-treated mice compared with controls on days 26, 30, 33, and 35 (*P* < 0.001, *P* < 0.01, *P* < 0.01, and *P* < 0.001, respectively; Student's *t* test) and in the simvastatin-treated mice versus controls on days 26, 30, and 35 (*P* < 0.02, *P* < 0.025, and *P* < 0.005, respectively; Student's *t* test).

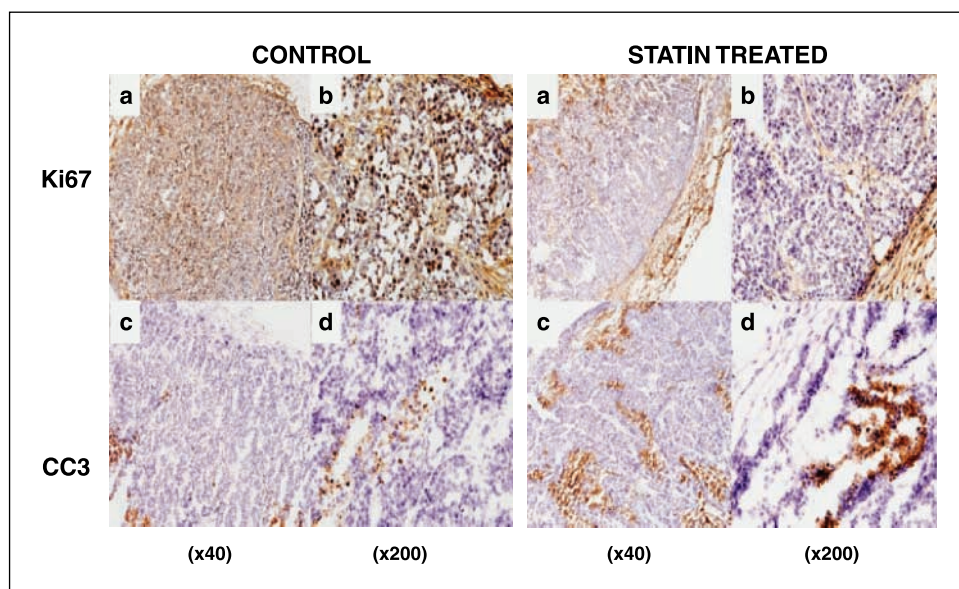


Figure 5. Reduction in MCNeuA tumor proliferation (Ki67) associated with increase in tumor apoptosis [cleaved caspase-3 (CC3)] following simvastatin treatment. Tumors were excised from control and simvastatin-treated mice for immunohistochemical analyses. Antibodies specific for Ki67 and caspase-3 cleavage product (cleaved caspase-3) were used to determine statin-induced changes in tumor proliferation and apoptosis, respectively. Low- and high-power magnifications are indicated ($\times 40$, $\times 200$). Statin-treated tumors (*right*) show decreased Ki67 staining and increased cleaved caspase-3 staining compared with control tumors (*left*).

In contrast, the majority of the treated tumors (9 of 10) showed greater staining intensity and a greater proportion of cleaved caspase-3-stained tumor cells (Fig. 5). Ki67 staining was used to assess tumor proliferation. Control tumors all showed diffuse Ki67 positivity throughout the tumor. In contrast, statin-treated tumors showed reduced Ki67 labeling, particularly in the peripheral regions of each tumor mass (Fig. 5). Mean Ki67 labeling index of the control tumors was 472 ± 73 , whereas the mean Ki67 labeling index of the treated tumors was reduced to 223 ± 37 ($P < 0.0001$).

Discussion

More than 10 million Americans currently use statins to lower their low-density lipid cholesterol blood levels, as there is incontrovertible clinical evidence that statins of all types significantly reduce coronary artery disease morbidity and mortality (1, 3–7, 15). Normal cells, including hepatocytes, respond to statin inhibition of HMG-CoA reductase activity with a feedback up-regulation of reductase gene expression along with up-regulation of other sterol- and lipid-synthesizing gene programs, including low-density lipid receptor, of which up-regulation directly accounts for the low-density lipid cholesterol-lowering effects of statins (21). Cancer cells usually exhibit constitutively elevated levels of HMG-CoA reductase and low-density lipid receptor, presumably to satisfy their increased need for isoprenoids and lipids, and potentially making them more sensitive than normal cells to the isoprenoid-depleting effects of statins (23, 36).

As recently reviewed, there is an expanding body of evidence supporting the ability of some statins to exert direct antiproliferative and pro-apoptotic effects on various types of human cancer cells (11). The lack of any detectable effect on our breast cancer panel following *in vitro* treatment with pravastatin at doses up to 200 $\mu\text{mol/L}$ is likely due to its hydrophilic nature and reduced ability to effectively penetrate lipid membranes (22, 24), and is consistent with its reported inability to inhibit leukocyte HMG-CoA reductase or LFA-1 integrin function (37). In contrast, micromolar doses of the lipophilic statins (fluvastatin, lovastatin, and simvastatin) were all able to inhibit proliferation and intracellular signaling of MDA-231, SKBr3, and MCF-7 cells, with the Ras

(MDA-231) and ErbB2 (SKBr3) transformed cells seeming to be significantly more sensitive than estrogen receptor-overexpressing (MCF-7) breast cancer cells. Only three statins, lovastatin (NSC-633781), simvastatin (NSC-633782), and mevastatin (NSC-281245), are among the >100,000 compounds screened since the early 1990s for anticancer activity by the NCI Developmental Therapeutics Program (NCI/DTP) against their 60 human cancer cell line panel.⁵ This NCI/DTP panel includes two of the three breast cancer cell lines used in this study (MCF-7 and MDA-231); as compared with the range of breast cancer IC₅₀ values we observed for fluvastatin, lovastatin, and simvastatin (Table 1), the NCI/DTP cell line screen yielded average cytotoxicity (LC₅₀) values of 100 $\mu\text{mol/L}$ for mevastatin, 29 $\mu\text{mol/L}$ for lovastatin, and 25 $\mu\text{mol/L}$ for simvastatin. Their self-organizing map-based cluster analysis (3d MIND⁶; ref. 38) has suggested that among 20,000 structurally unrelated compounds, mevastatin, lovastatin, and simvastatin share anticancer activity most similar to a few unusual kinase inhibitors, particularly cyclin-dependent kinase inhibitors like staurosporines, rapamycins, and cephalostatins (38).

The observed *in vitro* responsiveness of our breast cancer cell lines to lipophilic statins correlated best with pretreatment levels of activated NF- κ B, a transcription factor complex known to regulate both cell survival and proliferation, including induction of cyclin D1 in mammary epithelial cells (39, 40). NF- κ B activity is up-regulated on exposure to various cytokines and growth factors, in response to Ras and Raf mutations, or activated ErbB2 and other tyrosine kinases, and this NF- κ B up-regulation is mediated by Ras-Raf-MEK-ERK and MEK-I κ B kinase-I κ B signaling pathways (40–44). That these basic signaling pathways converge on NF- κ B activation to regulate cancer cell proliferation and survival is consistent with our observation that the earliest (<4 hours) intracellular response of our breast cancer cell lines to an equitoxic dose of fluvastatin, lovastatin, or simvastatin involved down-regulation of p-MEK1/2 and up-regulation of the NF- κ B inhibitor

⁵ <http://www.dtp.nih.gov>.

⁶ <http://spheroid.ncicrf.gov>.

p-I κ B α . Within 12 hours of exposure to statins, SKBr3 cells showed significant declines in nuclear NF- κ B activation and total cyclin D1. By 48 hours, these statin-treated cells exhibited a broader spectrum of cell signaling changes, including down-regulation of p-JNK and p-p38, loss of AP-1 activation, increase in p21^{WAF1}, and loss of cell survival mediators (Bcl-2, Bcl-xl, and ILP-1) in association with the appearance of caspase-3 and caspase-9 cleavage products. Given the key upstream role of Ras and Raf kinases in activating MEK1/2, and its central position in directing proliferation and survival responses downstream of ERK and NF- κ B activated pathways, it is reasonable to conclude that the direct statin effects observed on breast cancer cells resulted from their initial inhibition of HMG-CoA reductase, causing a rapid intracellular depletion of isoprenoids sufficient to impair inner membrane attachment and function of critical Ras, Rac, or Rho family members. Although not assessed in this study, statin inhibition of the geranylgeranylation of Rho family members has also been shown to prevent the invasive and metastatic properties of malignant cells (45).

Using the ErbB2 transformed MCNeuA mouse mammary cancer model, daily oral intake of simvastatin or fluvastatin produced significant *in vivo* antitumor effects. Immunohistochemical studies on these MCNeuA tumors demonstrated that this *in vivo* antitumor effect was due to a statin-induced decline in tumor cell proliferation (decreased Ki67 staining) and survival (increased cleaved caspase-3 staining).

A 40-mg oral dose of simvastatin or fluvastatin results in a serum C_{max} of 10 to 34 ng/mL (24-81 nmol/L) and 448 ng/mL (~1.0 μ mol/L), respectively (46). It is unclear why an oral statin dose

known to achieve low- or sub-micromolar statin blood levels has *in vivo* antitumor activity when much higher micromolar doses of statins are needed *in vitro* to arrest tumor cell proliferation and induce apoptosis. It is possible that prolonged *in vivo* dosing may allow statin accumulation in the tumor to micromolar concentrations. Alternatively, statins may have an inhibiting effect on essential stromal-epithelial interactions in the tumor, interactions which are not detectable studying cultured cell lines. Ongoing preclinical studies are evaluating statin effects on tumor paracrine and inflammatory reactions.

Based on these preclinical findings, we are conducting a pilot randomized clinical trial of the effects of statins on early breast tumors (ductal carcinoma *in situ* and stage I breast cancer). The design of this trial tests the dose-dependent effect of statins in the perioperative setting of newly diagnosed preinvasive breast tumors 3 to 6 weeks before tumor resection. It will allow the measurement of various tumor and serum biomarkers influenced by statins. Preclinical rationale supported by pilot clinical data will fuel large-scale clinical evaluation of safe, health-promoting agents like statins to discover their cancer-preventing potential.

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