Suppressive Effects of Statins on Acute Promyelocytic Leukemia Cells

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Abstract
The family of statins includes pharmacologic inhibitors of the 3-hydroxy-3-methylglutaryl-CoA reductase that are potent regulators of cholesterol biosynthesis. In addition to their cholesterol-lowering effects, statins inhibit cell proliferation and promote apoptosis of malignant cells in vitro, but their potential therapeutic roles in the treatment of malignancies remain to be defined. We examined the effects of statins on the growth and differentiation of acute myeloid leukemia (AML) cells. Atorvastatin and fluvastatin were found to be potent inducers of cell differentiation and apoptosis of the NB4 acute promyelocytic leukemia (APL) cell line. Such effects correlated with activation of the small G-proteins Rac1/Cdc42 and downstream engagement of the c-Jun NH2-terminal kinase pathway, whose function was found to be essential for the generation of proapoptotic responses. Importantly, different statins were found to enhance all-trans-retinoic acid (ATRA)–dependent differentiation of APL blasts and reverse resistance to the antileukemic effects of ATRA. In addition, fluvastatin exhibited growth-inhibitory properties on primary bone marrow–derived leukemic progenitors from patients with AML and enhanced the suppressive effects of ATRA on leukemic progenitor colony formation. Altogether, these studies establish that statins exhibit potent antileukemic properties in vitro and raise the possibility that combinations of statins with ATRA may be an effective approach to overcome the development of ATRA resistance by the leukemic cells. [Cancer Res 2007;67(9):4524–4532]

Introduction
Statins are pharmacologic inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, an enzyme that is responsible for the conversion of HMG-CoA to mevalonate (1). There are at least eight known members of this family that differ in their hydrophobic profile, spanning from very hydrophobic (e.g., cerivastatin) to partly hydrophobic (e.g., rosuvastatin) profiles (2). Due to their ability to prevent the formation of mevalonate, decrease cholesterol biosynthesis, and lower the serum levels of low-density lipoproteins, statins are widely used in the treatment of hypercholesterolemia and coronary artery disease in humans (3–10). The introduction of statins in clinical medicine has had a dramatic effect on the prognosis of patients with coronary artery disease and has changed the natural history of this disease. In addition to reducing cholesterol levels, statins have potent anti-inflammatory properties, and there is evidence that they exhibit antineoplastic activities as well (11, 12). Various statins have been previously shown to exhibit antiproliferative effects on tumor cells via regulatory effects on cell cycle (11–13) and to induce apoptosis of malignant cells of diverse origin (14–19). There has been particular interest on the potential role of statins as antileukemic agents, as some of them have been previously shown to exhibit antileukemic properties in vitro (14, 16, 20–22). There is also evidence that cholesterol synthesis and import contribute to protective cholesterol increments in acute myeloid leukemia (AML) cells (20), whereas cholesterol-modulating agents have been shown to kill AML cells and/or to sensitize them to chemotherapy by blocking adaptive cholesterol responses (21). Moreover, blocking protein geranylation is essential for lovastatin-induced apoptosis of human AML cells (22).

Although the effects of statins on cell cycle regulation and induction of apoptosis are well described, very little is known on the ability of these agents to induce leukemic cell differentiation. There has been some previous evidence that simvastatin (23) and lovastatin (24) promote osteoblastic differentiation while they inhibit adipocytic differentiation, but their effects on differentiation of acute leukemia cells remain to be established. In the present work, we examined the effects of new-generation statins on cells of acute promyelocytic leukemia (APL) origin. Atorvastatin, fluvastatin, and rosuvastatin were found to induce apoptosis of the NB4 APL cell line that expresses the t(15;17) translocation. Statins were also found to activate Rac1/Cdc42 and the c-Jun NH2-terminal kinase (JNK) pathway, and such activation was required for statin-dependent induction of apoptosis. Treatment of NB4 cells with statins also resulted in a time-dependent neutrophilic differentiation and growth-inhibitory effects. Importantly, statins induced granulocytic cell differentiation of all-trans-retinoic acid (ATRA)–resistant variant NB4 cell lines and reversed resistance of primary APL blasts to the effects of ATRA, raising the possibility that their combined use with retinoids may prove to be an effective approach to overcome retinoid resistance. Finally, statins were found to reverse ATRA resistance and enhance the suppressive effects of ATRA on leukemic progenitors from other AML subtypes, suggesting that combinations of statins with ATRA may be effective in other subtypes of AML that are normally resistant to ATRA.

Materials and Methods

Cells lines and reagents. The NB4 human APL cell line and the RA-resistant variants NB4.007/6 and NB4.300/6 were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Atorvastatin and rosuvastatin were purchased from 21CEC Pharmaceuticals Ltd. Fluvastatin was provided by Novartis. ATRA was purchased from Sigma.
ATP) and suspended in 30 h.

Tyr204), and stress-activated protein kinase (SAPK)/JNK (Thr 183/Tyr185) were analyzed by SDS-PAGE, and the phosphorylated form of c-Jun was absent in the mixture, and after 30 min of incubation at room temperature, the p21Waf1/Cip1 were purchased from Santa Cruz Biotechnology. An antibody (JNKI) was purchased from Axxora. Polyclonal antibodies against p38 and anti-JNK1 antibody was purchased from Santa Cruz Biotechnology and was obtained from Cell Signaling Technology. Polyclonal antibodies against Erk1/2 and SAPK/JNK were obtained from Cell Signaling Technology. An antibody against human cleaved poly(ADP-ribose) polymerase (PARP; Asp214) was purchased from Calbiochem. Antibodies against cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, and cleaved PARP were purchased from Cell Signaling Technology. Cleaved PARP was resolved by 10% SDS-PAGE gel and detected by Western blotting using an enhanced chemiluminescence method.

Cell lysis and immunoblotting. Cells were treated with different statins for the indicated times at a final concentration of 10 μmol/L, unless otherwise indicated, and were subsequently lysed in SDS-PAGE sample buffer. Plasma membranes were isolated from the peripheral blood or bone marrow of patients with AML, after obtaining informed consent approved by the Institutional Review Board of Northwestern University.

In vitro kinase assays. Cells were incubated in the presence or absence of statins for the indicated times. The cells were subsequently lysed in PLB (27, 28), and lysates were immunoprecipitated with an antibody against JNK1 using protein G-Sepharose. The immunoprecipitated complexes were subsequently washed thrice with PLB containing 0.1% Triton X-100 and twice with kinase buffer (25 mmol/L HEPES, 25 mmol/L MgCl2, 25 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na3VO4, and 20 μmol/L ATP) and resuspended in 30 μL of kinase buffer containing 1 μg c-Jun fusion protein, used as an exogenous substrate; 10 μL [γ-32P]ATP was added to the mixture, and after 30 min of incubation at room temperature, the reaction was terminated by the addition of SDS sample buffer. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of c-Jun was detected by autoradiography.

Rac1 and Cdc42 activation assays. The activation of Rac1 and Cdc42 were determined as in our previous studies (28). Briefly, a pGEX–T3 construct encoding for the GTPase binding domain of human Pak1 (provided by Dr. Gary Bokoch, Scripps Research Institute, La Jolla, CA) was expressed in Escherichia coli as a glutathione S-transferase fusion protein (GST-PBD). The cells were treated with statins as indicated and lysed in PLB. Cell lysates were incubated with GST-PBD, and bound proteins were separated by SDS-PAGE and immunoblotted with antibodies against either Rac1 or Cdc42 (purchased from BD Biosciences Pharmingen) to detect GTP-bound Rac1 or GTP-bound Cdc42, respectively.

Cell proliferation assays. Cells were seeded in flat-bottomed 96-well plates at a final concentration of 2.5 × 104 per mL, in the presence or absence of the indicated doses of statins and ATRA, and incubated at 37°C for 4 days. Cell proliferation was assessed using a methyl thiazolyl tetrazolium assay system as described previously (29).

Flow cytometric analysis. Flow cytometric studies were done as in our previous studies (30). Briefly, NB4 cells were treated with statins and ATRA for the indicated times. Induction of cell differentiation was determined by flow cytometric analysis after labeling the cells with phycocyrrhitin-conjugated monoclonal CD11b or isotype-specific control IgG purchased from BD Biosciences Pharmingen. Apoptosis was evaluated by propidium iodide/Annexin V staining. For the experiments in which the effects of the JNKI peptide inhibitor on atorvastatin-induced apoptosis were examined, the cells were preincubated for 60 min in the absence or presence of JNKI peptide (10 μmol/L) and subsequently treated for 48 h with atorvastatin (5 μmol/L), in the continuous presence or absence of the JNK-inhibitory peptide. The JNK-inhibitory peptide was re-added to the cultures at the same dose after 24 h. The induction of apoptosis was evaluated at 48 h by flow cytometry using Annexin V/propidium iodide staining.

Hematopoietic progenitor cell assays. Bone marrow or peripheral blood from patients with AML were collected after obtaining informed consent approved by the Institutional Review Board of Northwestern University. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation and used for clonogenic assays in methylcellulose as in our previous studies (28). The cells were cultured in the presence or absence of ATRA (0.5 μmol/L), fluvastatin (3 μmol/L), with the combination of these agents. Leukemic granulocyte-macrophage colony-forming unit (CFU-GM) colonies were scored on day 14 of culture.

Results

We initially examined the effects of new-generation statins on cells of APL origin. For such experiments, we used the NB4 APL cell line that has the t(15;17) translocation and expresses the abnormal PML-RARα fusion protein. As it has been previously reported that lovastatin-induced apoptosis correlates with inhibition of the MAPK kinase Mek/Erk cascade in acute leukemia (14), we initially determined the effects of statins on the activation of different MAPK signaling cascades in these cells. When NB4 cells were treated with atorvastatin, there was time-dependent down-regulation of phosphorylation of Erk1/2 (Fig. 1A), which was observed after prolonged treatment of the cells (24 h), but not at earlier time points (Fig. 1A). Similar results were seen in experiments using fluvastatin or rosvastatin (data not shown). Treatment of cells with different statins did not affect the phosphorylation/activation of the p38 MAPK, indicating that this MAPK cascade is not engaged by statins (Fig. 1B). On the other hand, treatment of NB4 cells with atorvastatin or fluvastatin induced phosphorylation of JNK (JNK1 and JNK2/3), although such phosphorylation/activation was less intense than chemical stress–induced activation (Fig. 1C). Moreover, treatment of the cells with either atorvastatin or fluvastatin induced activation of the kinase domain of JNK1, as evidenced in immune complex kinase experiments using c-Jun as an exogenous substrate (Fig. 1C). In other experiments, we determined whether different statins induce activation of the Rac1 and Cdc42 GTPases, which have been recently shown to act as upstream effectors of lovastatin-activated JNK in mouse macrophages (32). As shown in Fig. 1D, atorvastatin and fluvastatin induced activation of both Rac1 and Cdc42, strongly suggesting that these small G-proteins are activated in a statin-inducible manner in APL cells, to regulate JNK activation. Altogether, these studies showed that in APL cells, different statins block activation of the Mek/Erk pathway, while they induce activation of JNK, and such engagement is likely regulated by upstream engagement of the small G-proteins Rac1 and Cdc42.

In subsequent studies, we evaluated the effects of atorvastatin on the induction of apoptosis in NB4 cells. In initial experiments using atorvastatin, we found that this statin induces apoptosis in a time- and dose-dependent manner (Fig. 2A). Similarly, apoptosis of NB4 cells was inducible when fluvastatin was used (Fig. 2B). Importantly, such apoptosis was also observed in the variant cell lines NB4-007/6 (Fig. 2C) and NB4-306 (Fig. 2D), which are resistant to the effects of ATRA (33). In addition, treatment of NB4 cells with either atorvastatin or fluvastatin resulted in PARP cleavage (Fig. 3A), reflecting statin-dependent caspase activation. To determine whether engagement of JNK by statins is required for PARP cleavage/induction of apoptosis, the effects of SP600125, a specific inhibitor of JNK kinase, were determined. The induction of PARP cleavage by either atorvastatin or fluvastatin was reversed by
pretreatment of the cells with SP600125 (Fig. 3B, (a)) and was seen when doses of the inhibitor of either 10 or 20 μmol/L were used (Fig. 3B, (b)). On the other hand, the pharmacologic inhibitor of p38, SB203580, or the Mek/Erk inhibitor PD98059 had no effects on such statin-dependent PARP cleavage (Fig. 3C). Pharmacologic inhibition of JNK activation also reversed statin-induced apoptosis of NB4 cells, as determined by Annexin V/propidium iodide staining (Fig. 3D, left). To definitively establish the requirement for JNK in the induction of statin-induced apoptosis, an approach involving inhibition of JNK activity via use of a JNK-inhibitory peptide (34) was employed. The induction of apoptosis in response to atorvastatin treatment was partially blocked when cells were treated with the cell-permeable JNK-inhibitory peptide (Fig. 3D, right), consistent with a requirement for JNK kinase activity in this process. Thus, activation of the JNK pathway by statins seems to play a key role in the induction of apoptosis and PARP cleavage.

Figure 1. Engagement of MAPK cascades by statins. A, top, NB4 cells were incubated in the presence or absence of DMSO (control) or atorvastatin (10 μmol/L), for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of Erk1/2. Bottom, the same blot was then stripped and reprobed with an anti-Erk1/2 antibody to control the protein loading. B, top, NB4 cells were incubated in the presence or absence of DMSO (control), atorvastatin, fluvastatin, or rosuvastatin (5 μmol/L) for the indicated times, or exposed to anisomycin (1 μg/mL) for 30 min, as a positive control. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form the p38 kinase. Bottom, the same blot was then stripped and reprobed with an anti-p38 antibody to control the protein loading. C, top left, NB4 cells were incubated in the presence or absence of atorvastatin or fluvastatin (10 μmol/L), or anisomycin (1 μg/mL) used as a positive control, for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated forms of JNK. Bottom left, the same blot was then stripped and reprobed with an anti-JNK antibody to control for protein loading. Top right, NB4 cells were treated in the presence or absence of atorvastatin or fluvastatin (10 μmol/L) for the indicated times, or exposed to anisomycin (1 μg/mL) for 30 min, as a positive control. Cell lysates were immunoprecipitated (IP) with an antibody against JNK1 or control non-immune rabbit immunoglobulin (RiGg). The immunoprecipitates were then subjected to in vitro kinase assays, using c-Jun as an exogenous substrate. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of c-Jun was detected by autoradiography. Bottom right, the blot was subsequently immunoblotted with an anti-JNK1 antibody. D, top left, NB4 cells were treated with fluvastatin for the indicated times, and cell lysates were bound to either GST-PBD or GST alone as indicated. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-Rac1 antibody to detect the GTP-bound form of Rac1. Middle left, the same blot was then stripped and reprobed with an anti-Cdc42 antibody to detect GTP-bound Cdc42. Bottom left, the blot was then stripped and reprobed with an anti-GST antibody. D, Top right, NB4 cells were treated with atorvastatin or fluvastatin for 24 h as indicated, and cell lysates were bound to either GST-PBD or GST alone as indicated. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-Rac1 antibody to detect the GTP-bound form of Rac1. Middle right, the same blot was then stripped and reprobed with an anti-GST antibody.
whereas the p38 and Mek/Erk pathways do not participate in the regulation of such events.

An important mechanism by which ATRA induces antileukemic responses in APL is the induction of granulocytic differentiation of promyelocytes and leukemic blasts (35–37). We examined whether statins can induce differentiation of leukemia cells of promyelocytic origin. Treatment of NB4 cells with either atorvastatin or fluvastatin induced differentiation of NB4 cells, evidenced by the

**Figure 2.** Induction of apoptosis by statins in NB4 cells and NB4 variant cell lines. A, NB4 cells were treated with DMSO (control) or atorvastatin for the indicated times, at the indicated concentrations. The induction of apoptosis was evaluated by flow cytometry using Annexin V/propidium iodide staining. Columns, mean % early and late apoptotic cells of two independent experiments; bars, SE. B to D, NB4 (B), NB4 007/6 (C), or NB4 306 (D) cells were incubated for 48 h in the absence or presence of DMSO (control), atorvastatin (2 μmol/L), or fluvastatin (2 μmol/L). The induction of apoptosis was evaluated by flow cytometry using Annexin V/propidium iodide staining. Columns, mean % early and late apoptotic cells four (B), three (C), and two experiments (D); bars, SE. For the experiments in (B), paired two-tailed t test analysis showed a two-tailed P = 0.00257 for atorvastatin versus DMSO and a two-tailed P = 0.00007 for fluvastatin versus DMSO.
Figure 3. Atorvastatin and fluvastatin induced PARP cleavage requires JNK activity. A, top, NB4 cells were incubated in the presence or absence of atorvastatin and fluvastatin (5 μM/L) for the indicated times (hrs) or exposed to DMSO as solvent control. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the cleaved form of PARP. Bottom, the same blot was stripped and reprobed with an anti-tubulin antibody to control for protein loading. B, (a), top NB4 cells were preincubated for 60 min in the absence or presence of the JNK inhibitor SP600125 (20 μM/L) and then treated with or without atorvastatin or fluvastatin (10 μM/L) for the indicated times or exposed to anisomycin (1 μg/mL) for 30 min. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the cleaved form of PARP. Bottom, the same blot was then stripped and reprobed with an anti–glyceraldehyde-3-phosphatedehydrogenase (anti-GAPDH) antibody to control for protein loading. (b), top NB4 cells were preincubated for 60 min in the absence or presence of the indicated final concentrations of the JNK inhibitor SP600125 and then treated with fluvastatin (10 μM/L) for 24 h, as indicated. Bottom, equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the cleaved form of PARP. The same blot was then stripped and reprobed with an anti–glyceraldehyde-3-phosphatedehydrogenase antibody to control for protein loading. C, top NB4 cells were preincubated for 60 min in the absence or presence of SP600125 (20 μM/L) or MEK inhibitor PD98059 (10 μM/L), or the p38 MAPK inhibitor SB203580 (10 μM/L) and treated with or without atorvastatin or fluvastatin (10 μM/L), as indicated. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the cleaved form of PARP. Bottom, the same blot was then stripped and reprobed with an anti–glyceraldehyde-3-phosphatedehydrogenase antibody to control for protein loading. D, left, NB4 cells were preincubated for 60 min in the absence or presence of SP600125 (20 μM/L) or atorvastatin (10 μM/L) or fluvastatin (10 μM/L). The induction of apoptosis was evaluated by flow cytometry using Annexin Vpropidium iodide staining. Columns, mean % early and late apoptotic cells of two independent experiments; bars, SE. Right, NB4 cells were preincubated for 60 min in the absence or presence of JNKI peptide (10 μM/L) and treated for 48 h with atorvastatin (5 μM/L), in the continuous presence or absence of the JNK inhibitor peptide, which was re-added again to the cultures at the same concentration after 24 h. The induction of apoptosis was evaluated at 48 h by flow cytometry using Annexin Vpropidium iodide staining. Columns, mean % early and late apoptotic cells of four independent experiments; bars, SE. Paired two-tailed t test analysis showed a two-tailed P = 0.0028 for atorvastatin versus JNKI + atorvastatin.
induction of expression of the CD11b marker (Fig. 4A). Moreover, combinations of either atorvastatin or fluvastatin with low concentrations of ATRA resulted in high numbers of CD11b-positive cells (Fig. 4A), consistent with a strong induction of cell differentiation by such combinations.

When similar studies were done in the retinoid-resistant NB4 variants NB4.007.6 and NB4.306, we found that either atorvastatin or fluvastatin (Fig. 4B and C) had dramatic effects on the ATRA-resistant lines and reversed the resistance that these cells exhibit to the differentiating effects of ATRA (Fig. 4B and C). Two-tailed paired t test analysis showed that the combination of ATRA + atorvastatin or ATRA + fluvastatin induced significant leukemic differentiation compared with ATRA in both NB4.007/6 and NB4.306 cells (Fig. 4B and C). We also examined the effects of
statins on leukemic blasts obtained from an ATRA-resistant case. In these primary leukemic blasts, neither ATRA nor statins induced significant cell differentiation (Fig. 4, left). However, combinations of atorvastatin or fluvastatin with ATRA resulted in induction of leukemic cell differentiation (Fig. 4, left), further establishing that statins are capable of reversing retinoid resistance in APL. The effects of either fluvastatin or atorvastatin in reversing RA resistance were dose dependent, and at higher final concentrations (5 μmol/L), the ability of statins to reverse ATRA resistance was remarkably enhanced (Fig. 4, right).

In other studies, we determined whether statins exhibit growth-inhibitory effects on RA-resistant cells. In methyl thiazolyl tetrazolium assays, we found that statins inhibited the growth of ATRA-sensitive NB4 cells in a dose-dependent manner (data not shown). Importantly, both atorvastatin and fluvastatin blocked the growth of the ATRA-resistant NB4.007/6 and NB4.306 variant cell lines, both of which were refractory to the growth-inhibitory effects of ATRA (Fig. 5). Combinations of statins with ATRA also resulted in substantial growth inhibition of the ATRA-resistant cell lines (Fig. 5). Thus, in addition to promoting ATRA-dependent cell differentiation of retinoid-resistant cells, statins exhibit growth-inhibitory effects on such cells. To examine whether statins exhibit growth-inhibitory effects on AML primary leukemic progenitors from non-APL subtypes of AML, we did clonogenic assays in methylcellulose using hematopoietic progenitors obtained from the bone marrow or the peripheral blood of patients with AML. Addition of ATRA alone to the cultures exhibited minimal growth-inhibitory effects, whereas fluvastatin suppressed leukemic CFU-GM colony formation by ~60% on average (Fig. 6A). Importantly, the combination of ATRA + fluvastatin resulted in nearly 90% suppression of leukemic progenitor cell growth (Fig. 6A). To obtain information on the potential mechanisms by which statins inhibit the growth of AML cells, we examined their effects on the expression of the p21WAF1/Cip1 protein. There is previous evidence that up-regulation of the p21WAF1 protein is involved in the generation of the growth-inhibitory effects of retinoids (38), whereas recent studies have shown that mevastatin induces its expression in colon cancer cells to regulate inhibition of cell growth (39). Both statins up-regulated p21 protein expression, and such effects were detectable after 24 or 48 h of treatment (Fig. 6B and C), suggesting the existence of a mechanism for the generation of statin-dependent growth-inhibitory responses in leukemic progenitors.

Discussion

The introduction of statins in clinical medicine has been a major breakthrough that has changed the natural history of coronary artery disease in humans. A large body of evidence has also suggested that statins are inducers of apoptosis and growth inhibition of different types of malignant cells, whereas they also exhibit anti-invasive and radiosensitizing activities (11, 40). Early-generation statins, such as lovastatin, have been shown to induce apoptosis and/or growth inhibition of malignant cells, including cells of leukemic origin (11–22, 41–43). There is also evidence for statin-dependent differentiation of osteoblasts and human mesenchymal stem cells (23, 24, 44, 45), whereas lovastatin has been shown to induce differentiation of some leukemic cell lines in vitro, including NB4 cells (46). The ability of statins to suppress growth and induce apoptosis of leukemia cells in vitro has raised the prospect that these agents may exhibit antileukemic properties.
in vitro when used at doses higher than the standard cholesterol-reducing doses. In fact, when used at a high dose, lovastatin was found to suppress blast cell growth in vitro in an elderly patient with AML (47). In addition, a recent phase I study in which pravastatin was combined with idarubicin and high-dose 1-β-D-arabinofuranosylcytosine showed tolerable toxicity at very high doses of pravastatin (1,280 mg/d), whereas the maximum tolerated doses had not been reached at the time of the report (48). Importantly, this preliminary report showed higher response rates for both newly diagnosed patients with poor prognosis and for salvage patients, compared with historical controls, providing the basis for future phase II studies (48).

Although there is accumulating evidence that different statins exhibit antileukemic properties in vitro and in vivo, very little is known on the antileukemic effects of statins in APL. APL is a distinct subtype of AML, also known as AML-M3 (49), with different clinical behavior than other acute leukemias. The hallmark of APL is the presence of the t(15;17) chromosomal translocation and the ability of ATRA to induce granulocytic differentiation of the leukemic blasts (49). The t(15;17) translocation results in the abnormal fusion of the RA receptor α (RARα) gene with the promyelocytic leukemia (PML) gene, whose product is the abnormal PML-RARα fusion protein (49). In contrast to other AML-subtypes, APL blasts are highly sensitive to the differentiating effects of retinoids (ATRA) in vitro and in vivo, and the introduction of ATRA in the treatment of APL has dramatically improved the outcome of such patients (36). It is of particular interest that when ATRA is given as a single agent, the vast majority of APL patients obtain complete responses (36). However, most of these patients eventually relapse, consistent with the development of ATRA resistance by the leukemic cells (21).

In the present study, we examined the effects of new-generation statins on cells of APL origin. Our data show that atorvastatin, fluvastatin, and/or rosuvastatin inhibit the MEK/Erk pathway and activate the Rac1/Cdc42/JNK kinase cascade in NB4 cells. Different statins were capable of inducing leukemic cell differentiation of ATRA-sensitive NB4 cells, whereas combinations of statins with low doses of ATRA resulted in enhanced cell differentiation. Remarkably, atorvastatin and fluvastatin were found to induce apoptosis and cell differentiation of two NB4 variant cell lines (NB4.007/6 or NB4.306) that are refractory to the antileukemic properties of ATRA. In addition, combinations of ATRA with atorvastatin or fluvastatin were more effective in inducing differentiation of either NB4.007/6 or NB4.306 cells, indicating that statins reverse the ATRA resistance that these cells exhibit. This was further established in experiments showing reversal of resistance of primary leukemic blasts to ATRA-induced differentiation and enhancement of sensitivity of primary AML bone marrow progenitors to the inhibitory effects of ATRA. Thus, statins are capable of inducing both apoptosis and cell differentiation of leukemic blasts, as in the case of arsenic trioxide, a heavy metal derivative with significant clinical activity in APL (50). The precise contributions of apoptosis versus cell differentiation in the antileukemic effects of statins and their ability to reverse ATRA resistance remains to be established, but it is likely that both processes contribute to such effects. It should be also noted that as ATRA is not a useful agent in the treatment of non-APL cases of AML, the ability of statins to promote its antileukemic effects in such cells is of particular interest. Combinations of statins with ATRA may be an approach to restore ATRA sensitivity in vitro and possibly in vivo in non-APL cases of AML, and this remains to be determined in future studies.

Our studies also establish that the JNK/MAPK pathway is activated during treatment of APL cells with statins, and that inhibition of such JNK kinase activation reverses statin-dependent apoptosis. In addition, the upstream regulators of the JNK pathway, small GTPases Rac1 and Cdc42, are activated in a statin-inducible manner, suggesting a potential mechanism by which statins regulate downstream activation of JNK. Although the precise sequence of events that result in activation of JNK in leukemia cells remain to be defined, it seems that this MAPK cascade plays a key and specific role in the generation of the effects of statins, as the two other major MAPK cascades are either not activated (p38; current report) and/or are inhibited (Mek/Erk; ref. 14 and current report) by statins. Independently of the precise cellular mechanisms involved, our data show that APL cells are particularly sensitive to...
the differentiating and proapoptotic effects of atorvastatin and fluvastatin, and that they maintain such a response even after the development of retinoid resistance. Combinations of statins with ATRA may prove to be an effective approach for the treatment of APL patients that develop ATRA resistance. Incorporating a statin in the treatment schema may prevent development of such resistance, and, based on our data, future clinical translational efforts to address this issue are warranted.

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