Combining Simvastatin with the Farnesyltransferase Inhibitor Tipifarnib Results in an Enhanced Cytotoxic Effect in a Subset of Primary CD34+ Acute Myeloid Leukemia Samples

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Abstract

Purpose: To show whether the inhibitory effects of the cholesterol synthesis inhibitor simvastatin on human CD34+ acute myeloid leukemia (AML) cells can be further promoted by combining it with the farnesyltransferase inhibitor tipifarnib.

Experimental Design: Normal CD34+, AML CD34+, and CD34- sorted subfractions, and AML cell lines (TF-1 and KG1A) were exposed to simvastatin and tipifarnib.

Results: Both simvastatin and tipifarnib showed a cytotoxic effect on AML cell lines, which was additive when used in combination. In primary sorted CD34+ AML cells, a heterogeneous response pattern was observed upon treatment with simvastatin when analyzing cell survival. A group of normal (n=12) and abnormal (n=10) responders were identified within the AML CD34- subfraction when compared with normal CD34+ cells. This distinction was not observed within the AML CD34+ cell fraction. When the CD34+ AML cells were exposed to simvastatin and tipifarnib, a significant enhanced inhibitory effect was shown exclusively in the normal AML responder group, whereas the AML CD34+ cell fractions all showed an enhanced inhibitory effect. The observed heterogeneity in AML responsiveness could not be explained by differences in effects on cholesterol metabolism genes or extracellular signal-regulated kinase phosphorylation in response to simvastatin and tipifarnib treatment.

Conclusion: The results suggest that combined treatment with statins and farnesyltransferase inhibitors may be beneficial for a subset of AML patients that can be defined by studying the AML CD34+ fraction.

Acute myeloid leukemia (AML) is a clonal hematopoietic disease characterized by the accumulation of immature myeloid blasts in the bone marrow. A minor tumor subpopulation with self-renewal potential, referred to as leukemic stem cells, is responsible for the sustained expansion of the leukemia (1, 2). Thus far, these leukemic stem cells are phenotypically characterized by CD34+CD38- (3, 4), but a recent study has challenged this view, suggesting that leukemic stem cells might also belong to the CD34+CD38+ cell fraction (5).

Cholesterol synthesis and the processing of low-density lipoprotein (LDL) is hyperactive in AML (6, 7), as indicated by high mRNA levels of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) and LDL receptor as well as LDL uptake studies. These findings are in line with the high expression of key genes of cholesterol metabolism in CD34+CD38+ AML cells (8). In addition, AML cells possess several mechanisms to protect them against the cytotoxic effects of chemotherapeutics, including a rapid increase in their cellular cholesterol levels after exposure to chemotherapeutic drugs (9, 10). Therefore, interfering with this protective mechanism potentially offers the opportunity to improve standard antileukemic treatment.

Statins, targeting HMG-CoA reductase, are widely used plasma cholesterol–lowering drugs. Statins inhibit cholesterol synthesis at the level of the conversion of mevalonate and, as a consequence, also inhibit the production of various byproducts of the mevalonate pathway. These byproducts include farnesyl and geranylgeranyl isoprenoids that are involved in the signaling of GTPases, including the small G protein Ras (9, 11). Ras GTPases must be transferred from the cytoplasm to the plasma membrane by isoprenylation to allow them to function as signal transducers (12–14). Inhibiting farnesylation is of interest because farnesylated proteins, particularly the protein products of the Ras gene family, are frequently activated in AML; e.g., by Ras mutations or due to the autocrine or paracrine production of growth factors (15). However, the statin concentrations necessary to inhibit specific protein isoprenylation are 100-fold to 500-fold higher than those required to inhibit cholesterol synthesis (16).

The specific inhibition of Ras farnesylation can also be realized by the use of the Ras inhibitor tipifarnib. Tipifarnib is...
Combining Simvastatin and Tipifarnib in CD34+ AML Cells

Translational Relevance

In this article, we assessed the inhibitory effects of the cholesterol synthesis inhibitor simvastatin in combination with the farnesyltransferase inhibitor tipifarnib on the CD34+ subfraction of acute myeloid leukemia (AML) cells, which is enriched for leukemic stem cells. We observed that 50% of the AML CD34+ cells had a response pattern comparable with normal CD34+ cells, whereas the remaining CD34+ samples showed a reduced responsiveness to both agents. This variability in responsiveness was exclusively observed in the CD34+ AML subfraction and not noticed in CD34- AML cells, indicating that in vitro responsiveness of patients can only be predicted by the use of selected cell fractions, which might be useful for future clinical trials.

Materials and Methods

Normal and AML hematopoietic cells and cell lines. Normal mobilized peripheral CD34+ blood cells were collected from either healthy donors or patients awaiting autologous stem cell transplantation undergoing granulocyte colony-stimulating factor treatment, in accordance with institutional guidelines. After informed consent, bone marrow or peripheral blood cells were collected from AML patients at diagnosis. The Medical Ethical Committee of the University Medical Center Groningen (The Netherlands) approved the protocol. Patients were classified according to the French-American-British AML classification (23). Mononuclear cells were enriched by density gradient centrifugation (Lymphocyte Separation Medium LSM 1077; PAA Laboratories GmbH) and freshly used or cryopreserved in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (ICN) at 37°C and 5% CO2. Cytogenetic analysis was done by determining the DNA content of cells by staining with PI (IQ Products) in sodium citrate (1 mg/mL; Sigma-Aldrich) containing 100 μg/mL RNase A (Sigma-Aldrich), 20 μg/mL PI, and 0.1% Triton X-100 (Sigma-Aldrich) for 60 min at room temperature. PI fluorescence was analyzed by fluorescence-activated cell sorting analysis (FACSCalibur; Becton Dickinson). Cell cycle distributions were calculated with ModFit LT (Verity Software House).

Western blotting. TF-1 cells were treated in GM-CSF–free medium for 48 h with simvastatin and/or tipifarnib, after which GM-CSF (10 ng/mL) was added for 15 min. Unsorted mononuclear AML cell fractions were cultured in RPMI 1640 supplemented with 10% FCS and treated for 24 h. Whole cell extracts were obtained by lysing 5 × 10^5 cells in boiling Laemmli sample buffer for 5 min. Samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore) in Tris buffer with the use of a semidry blotting apparatus. After blocking with 5% nonfat dry milk, membranes were incubated with antibodies according to the manufacturer's protocols. The antibodies used were extracellular signal-regulated kinase (ERK)1/2 (K23; Santa Cruz Biotechnology, Inc.) and phospho-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology Inc.). Immunodetection of phospho-ERK and total ERK was done according to standard procedures, and binding of antibodies was detected by enhanced chemiluminescence. Densitometry was carried out with the use of ImageJ.

Quantitative real-time PCR. Total RNA was isolated with the use of the RNaseasy mini kit (Qiagen) and was reverse transcribed with the use of RevertAid H Minus M-MuLV reverse transcriptase (Fermentas). Quantitative PCR was done with the ABI Prism 7700 Sequence Detector (Applied Biosystems). Primers and probes for the human ATP-binding cassette (ABC) transporters and cholesterol metabolism genes were used as described before (8, 25). As endogenous control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used. The primers were obtained from Invitrogen. The probes were labeled by a 5′-carboxyfluorescein reporter and quenched by
6-carboxytetramethylrhodamine at its 3' end (Eurogentec). We used 4 
µL of diluted cDNA in each PCR reaction in a final volume of 20 
µL containing 900 nmol/L of sense and antisense primers; 200 nmol/L of 
the Taqman probe; 5 mmol/L MgCl₂, KCl, and Tris-HCl; 0.2 mmol/L 
dATP, dCTP, dGTP, and dUTP; and 0.5 U of AmpliTaq DNA 
polymerase (qPCR Core Kit; Eurogentec). The PCR program was 95°C 
for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

The expression of the genes was standardized for the expression of 
glycerinaldehyde-3-phosphate dehydrogenase. Serial cDNA dilutions 
of the AML samples were used to generate calibration curves. The 
expression of each gene in each sample was analyzed in duplicate.

Statistical analysis. Student's t test was used to calculate the 
differences between cell line samples; Friedman's and Mann-Whitney's 
nonparametric tests were done to calculate significant differences 
between AML samples. Data were expressed as mean ± SD or SE, as 
indicated. All P values are given for two-sided tests, and P ≤ 0.05 was 
considered significant. Additivity was defined as an increased effect 
of the combination exposure compared with the single exposure of 
simvastatin or tipifarnib. Normal CD34+ AML responders were defined 
as follows: at the concentration of either simvastatin or tipifarnib at 
0.2 µmol/L; 50% of the first significant effect was observed (i.e., 25 
µmol/L simvastatin and 1 µmol/L tipifarnib) with the use of the cell viability 
assay, the decrease in viability of the normal CD34+ cells ± SD was 
considered as normal. AML CD34+ cells with viabilities above this value 
were considered to give an abnormal response.

Results

Patient characteristics. The 22 AML patients studied, with a 
median age of 52 years (range, 19-79 years), were classified as 
the French-American-British classification groups M0 (n = 1), 
M1 (n = 6), M2 (n = 5), and M4/5 (n = 10). The clinical 
characteristics of the patients are shown in Table 1. The median 
peripheral blast cell count at diagnosis was 62 × 10⁹/L (range, 
2-220). The majority of patients were treated according to 
going HOVON protocols; i.e., for patients <60 years, the 
HOVON SAKK AML-42 study (26), and for patients >60 years, 
the HOVON 43 study (27). Palliative treatment consisting of 
treatment with 6-mercaptopurine (28) was given to patients 
who were ineligible for intensive chemotherapy (n = 3).

Tipifarnib and simvastatin decrease cell viability by inducing 
apoptosis and cell cycle arrest. First, the effects of tipifarnib 
and simvastatin on the hematopoietic cell lines KG1A and TF-1 
were assessed. With simvastatin alone, a dose-dependent 
decrease in cell survival of up to 30% was shown (Fig. 1) in 
both cell lines. Similar results were obtained with tipifarnib (up 
to 60% decrease of survival), whereas the combined use 
showed an even more efficient decrease of up to 75% in cell 
viability, which was additive compared with either treatment 
alone. To define whether this decline in cell survival was due to 
cell cycle arrest or apoptosis, cell cycle status and cell survival 
were defined by PI staining and an Annexin V/PI assay, 
respectively. Treatment of TF-1 cells with tipifarnib increased 
the number of cells in the G₂-M cell cycle phase (9% versus 27% 
(0.2 µmol/L; P = 0.002) and lowered the number of cells in the 
G₀-G₁ phases (51% versus 36% at 0.2 µmol/L; P = 0.049; 
Fig. 2A). Cells in S phase remained the same. Treatment with 50 
µmol/L simvastatin resulted in fewer cells in the S phase (40% 
versus 24%) and more in G₀-G₁ (51% versus 69%; Fig. 2A), but 
these differences did not reach significance (P = 0.2). When 
combining both treatments, we observed an inhibitory effect 
on the cells in the S phase (P = 0.02), and there were more cells 
in the G₀-G₁ phases (P = 0.09) or G₂-M phases (P = 0.05) when 
using tipifarnib and simvastatin at a dose of 5 and 50 µmol/L 
(Fig. 2A).

In KG1A cells, we observed similar effects: the combination 
of both treatments resulted in an increase of cells in the G₀-G₁ 
phases (Fig. 2B; 47% versus 64% at 50 µmol/L simvastatin and 
5 µmol/L tipifarnib; P = 0.002) and a decrease of cells in the S 
phase (41% versus 25%; P < 0.001). Thus, treatment of TF-1 
and KG1A cells with simvastatin and tipifarnib results in a

Table 1. Clinical and cellular characteristics of AML patients

<table>
<thead>
<tr>
<th>AMLs FAB Classification</th>
<th>Leukocytes at presentation (×10⁹/L)</th>
<th>% CD34**</th>
<th>Cytogenetics</th>
<th>Normal responder to simva in CD34+ cells</th>
<th>Normal responder to simva + tipi in CD34+ cells</th>
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<tr>
<td>1 M2</td>
<td>56</td>
<td>29</td>
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<td>2 M1</td>
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<td>1</td>
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<td>25</td>
<td>28</td>
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<td>15</td>
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<td>60</td>
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<td>5q-, trisomy 6</td>
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<td>11</td>
<td>46N,xy +11q23</td>
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*Percentage of CD34+ cells in the AML mononuclear cell fraction.

1 Normal responders (Yes) and abnormal responders (No) to simvastatin and tipifarnib were defined as described in Materials and Methods.
G₀-G₁ cell cycle arrest. It seemed that this cell cycle arrest was associated with an increased number of cells in apoptosis, which was especially noticed for the TF-1 cell line (Fig. 2C and D). Also, here we found additive effects when combining both treatments.

The effect of simvastatin and tipifarnib on primitive CD34⁺ normal and AML cells. To show whether, in patient AML cells, comparable effects can be noticed, AML mononuclear cells (n = 22) were sorted into CD34⁺ and CD34⁻ subfractions and exposed to the two agents. The results were compared with the effects seen in normal CD34⁺ (n = 8). When exposed to simvastatin (Fig. 3A) or tipifarnib (data not shown), normal CD34⁺ cells showed a concentration-dependent decrease in cell survival, which was significant at a concentration of 25 µmol/L simvastatin (P < 0.001) and 1 µmol/L tipifarnib (P = 0.01). Treatment of normal CD34⁺ cells with 25 µmol/L simvastatin in combination with tipifarnib showed an additive inhibitory effect on cell survival compared with the effects of the separate compounds (Fig. 3B). However, within the CD34⁺ AML cell fraction, a marked variability in responsiveness could be observed. Based on the response pattern of normal CD34⁺ cells, two AML subgroups could be distinguished (see Materials and Methods) when the CD34⁺ AML cells were exposed to simvastatin. Fifty-five percent (n = 12) of the AML-CD34⁺ cells had a response pattern comparable with normal CD34⁺ (e.g., P = 0.6 at 25 µmol/L), whereas 45% (n = 10) of the AML-CD34⁺ cells showed reduced simvastatin sensitivity (Fig. 3A; e.g., P = 0.003 at 25 µmol/L). In contrast, the CD34⁻ AML subfraction showed a response pattern comparable with normal CD34⁺ cells, and no difference was observed between normal and AML CD34⁻ cells.
Next we studied whether the suppressive effects of simvastatin on AML cells can further be promoted by cotreatment with tipifarnib as shown for normal CD34+ cells. The results show that 50% (n = 11) of the AML CD34+ cells had a response pattern comparable with normal CD34+ cells, whereas the other half of the AML CD34+ cells showed reduced sensitivity for the combined treatment of simvastatin (25 μmol/L) and tipifarnib (1 μmol/L; Fig. 3B; P < 0.001). It seemed that 8 of 10 of the AMLs that were not responsive to simvastatin were also not affected when both compounds were used. When the abnormal responder group was exposed to 1 μmol/L tipifarnib and 25 μmol/L simvastatin, the decrease in cell survival was not enhanced compared with the effect of simvastatin alone (P = 0.5). In contrast, in the AML-CD34+ responder group, a significant enhancement in cell death was observed when both compounds were used at low dose (1 μmol/L; P = 0.01). At higher concentrations, a strong reduction in cell survival was observed in normal CD34+ cells, as well as in the AML abnormal and normal responder group, but these concentrations are physiologically not relevant. The AML CD34+ subpopulation showed a comparable response pattern with normal CD34+ cells, and no clear distinction could be made between responders and abnormal responders when both compounds were used (P = 0.03 and P = 0.05 for 0.2 μmol/L and 1 μmol/L tipifarnib, respectively; Fig. 3D).

Heterogeneity in AML response pattern is not related to differences in modulation of cholesterol metabolism genes and ERK phosphorylation by simvastatin and tipifarnib. To investigate whether the functionality of either simvastatin or tipifarnib was different in the normal versus the abnormal responder group, we studied important downstream targets of simvastatin and tipifarnib. The functionality of simvastatin was tested based on reported up-regulation of HMG-CoA reductase and LDL receptor, and a down-regulation of ABCA1 and ABCG1 at the mRNA level (29, 30), whereas the downstream effects of tipifarnib were analyzed by studying the change in ERK1/2 phosphorylation, a downstream target of Ras. In the 6 AML samples (3 normal responders and 3 abnormal responders to simvastatin, tipifarnib, and both drugs) studied, the expression of HMG-CoA reductase and LDL receptor was almost 2.5-fold higher after treatment with 25 μmol/L simvastatin (Fig. 4A; P = 0.001 and P = 0.003, respectively), and ABCA1 and ABCG1 expression was 2-fold decreased (P < 0.001). Treatment with tipifarnib did not alter mRNA expression, and the combination of tipifarnib and simvastatin had no additive effect compared with treatment with simvastatin alone (data not shown).

Tipifarnib (0.5 and 1 μmol/L) did not affect ERK phosphorylation in any of the studied AMLs of the normal responder or abnormal responder group (Fig. 4B). Also, simvastatin did not lead to inhibition of ERK phosphorylation. However, combining simvastatin and tipifarnib resulted, in 69% of the 16 tested AML samples, in a decrease of phospho-ERK expression (88% ± 10% for 1 μmol/L tipifarnib and 25 μmol/L simvastatin; n = 11) irrespective of the AML used. Similar results were observed when TF-1 cells were cultured with GM-CSF and incubated with simvastatin and tipifarnib (Fig. 4C).

In vitro response pattern and patient characteristics. In view of the observed in vitro difference for simvastatin and tipifarnib sensitivity, we questioned whether these findings might correspond to patient characteristics. In the group responding in vitro to both tipifarnib and simvastatin, the median percentage of CD34+ cells in the AML mononuclear cell
fraction at presentation was 28% (mean, 28; range, 1-87) and, in the abnormal responder group, 50% (mean, 36; range, 11-92; $P = 0.08$; Table 1). No correlation was found between AML responder groups, and cytogenetics and peripheral blast count. The French-American-British classifications were distributed equally over the normal and abnormal responder groups.

Discussion

In this study, we addressed the question of whether the combined use of a cholesterol synthesis inhibitor and a farnesyltransferase inhibitor might have an augmented cytotoxic effect on CD34+ AML cells. The results show that, in human CD34+ AML leukemic cell lines, simvastatin and tipifarnib have an enhanced suppressive effect on cell survival compared with either compound alone, which was characterized by an inhibition of cell cycle progression and enhanced apoptosis. Several studies have shown that geranylgeranylated proteins, not farnesylated proteins, are required for the G1 to S phase transition (31) and that statins are able to block cell cycle in G1 due to geranylgeranylation rather than farnesylation (32). Apparently, this mechanism varies among different cell types. We observed G1 cell cycle arrest after treatment with both simvastatin and tipifarnib, whereas only a limited effect was observed with either compound alone. This suggests that farnesylated proteins also are responsible for cell cycle progression in AML cells. Likewise, ERK phosphorylation was strongly inhibited only when both simvastatin and tipifarnib were used. It may be that geranylgeranylation of Ras can overcome the inhibitory effects of tipifarnib on Ras farnesylation, and both geranylgeranylation and farnesylation must be blocked to decrease Ras activation. This is consistent with the idea that K-Ras and N-Ras can be both farnesylated and geranylgeranylated (22).

In contrast with AML cell lines, the observed inhibitory effect of simvastatin and tipifarnib was not found to be a general phenomenon for all primary AML samples. Most studies on statins have been focused on the total AML mononuclear cell fraction (33–35). In the total mononuclear AML fraction, a heterogeneous response to lovastatin has been observed (33). Our data show that a heterogeneous response also exists within the more homogeneous primitive AML CD34+ subfraction. Two subgroups could be defined based on sensitivity to simvastatin alone. About 50% of the AML-CD34+ showed a reduced sensitivity to simvastatin, tipifarnib, or to both compared with normal CD34+ cells, whereas the remaining AML samples showed a response pattern comparable with normal CD34+ cells.

A remarkable finding was the fact that, within the AML CD34+ subpopulation, no distinction could be made between normal and abnormal responders. These findings show that immature AML cells are intrinsically different from the CD34+...
Several clinical studies have already been done with either statins or with tipifarnib (17–21). In AML, statins have been combined with intensive chemotherapy, whereas tipifarnib has been used as single agent. Phase I and II studies have shown that only a minority of the patients were responsive to treatment. Likewise, the results of the present study suggest that, in vitro, not all AML patients are responsive to clinically relevant concentrations of simvastatin and tipifarnib (17, 19) and that this distinction can be made by using exclusively the CD34+ fraction. Therefore, it might be useful to determine whether the in vitro response is predictable for clinical response to be able to select patients who are eligible for treatment.

Importantly, to be able to predict response, the rationale behind the difference in susceptibility of AML cells to simvastatin and tipifarnib should be elucidated. We observed a considerable overlap between the simvastatin and tipifarnib responders, and no differences on studied downstream targets of simvastatin and tipifarnib were observed, suggesting that there might be a common mechanism of resistance.

It is conceivable that a specific set of antiapoptotic proteins are responsible for the differences in response. It has been shown that sorted CD34+ AML cells are more resistant to (spontaneous) apoptosis than the corresponding CD34+ fractions, which is paralleled by higher Bcl-2, Bcl-xl, Mcl-1, and Pgp, and low Bax expression levels (40). In addition, it has been shown that overexpression of Bcl-xl and Bcl-2 protects against statin-induced apoptosis (41, 42). It is unlikely that simvastatin and tipifarnib responsiveness correlates with the Ras activity status of the AML cells because both normal and abnormal responders show a basal phosphorylation level of ERK and a decrease of ERK activity upon combination treatment, which is in line with the results of Stirewalt et al. (11), who suggested that neither Ras mutations nor high Ras protein expression are found to be consistently associated with increased statin sensitivity. Likewise, in clinical studies using tipifarnib in AML, the Ras mutational status and inhibition of phosphorylated ERK did not correlate with clinical responsiveness to tipifarnib (19, 20).

Thus far, the clinical data on the use of either agent are encouraging but not convincing. Because we observed in vitro significant cytotoxic effects in an AML subgroup with clinically achievable concentrations, we predict that combining both agents in vivo will be advantageous in a subset of AML patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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