Protein Geranylgeranylation Is Critical for the Regulation of Survival and Proliferation of Lymphoma Tumor Cells

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ABSTRACT

Purpose: Prenylation is essential for membrane localization and participation of proteins in various signaling pathways. The following study was conducted to examine the importance of protein farnesylation and geranylgeranylation for the regulation of lymphoma cell survival and proliferation.

Experimental Design: Lymphoma cells were treated with the β-hydroxy-β-methylglutaryl-CoA reductase inhibitor lovastatin, which inhibits protein farnesylation and geranylgeranylation by the depletion of intracellular pools of farnesylpyrophosphate and geranylgeranylpyrophosphate. In addition, farnesyl transferase and geranylgeranyl transferase activities were specifically inhibited by FTI-277 and GGTI-298, respectively.

Results: Only inhibition of geranylgeranylation by lovastatin led to reduction of cell viability in lymphoma cell lines and purified tumor cells from lymphoma patients in a time- and dose-dependent way. Reduction in the number of viable cells was mediated by both induction of apoptosis and inhibition of proliferation. In addition, GGTI-298 was more effective in induction of apoptosis and inhibition of proliferation than FTI-277. Apoptosis induced by inhibition of protein geranylgeranylation was associated with a reduction of Mcl-1 protein levels, collapse of the mitochondrial transmembrane potential, and caspase-3 activation. Inhibition of proliferation resulted from the induction of G1 arrest. Furthermore, lovastatin at low concentrations sensitized lymphoma cells to dexamethasone, including cells resistant to this drug.

Conclusion: These results indicate that protein geranylgeranylation is critical for the regulation of lymphoma tumor cell survival and proliferation and that pharmacological agents such as lovastatin or geranylgeranyl transferase inhibitors, alone or in combination with other drugs, may be useful in the treatment of lymphoma.

INTRODUCTION

The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate and is catalyzed by the enzyme HMG-CoA reductase (1). Mevalonate is an intermediate in the synthesis of cholesterol, which is essential for membrane integrity; of dolichol, which is required for glycoprotein synthesis; of polyisoprenoid side chains of heme A and ubiquinone, which are involved in oxidative respiration; and of isopentenyladenine, which is present in some tRNAs. Furthermore, mevalonate is also a precursor of the isoprenoids FPP and GGPP. These isoprenoids are used for the post-translational modification of proteins, including Ras (farnesylation; Refs. 2, 3) and the Rho family members Rac-1, RhoA, and Cdc42 (geranylgeranylation; Ref. 4). FTase and GGTase transfer farnesyl and geranylgeranyl moieties from FPP and GGPP, respectively, to the thiol group of conserved cysteine residues at or near the COOH terminus of target proteins. Prenylation is essential for membrane association (2) and participation of proteins in various signaling pathways regulating growth and survival (3, 5–7).

Lovastatin is a potent competitive inhibitor of HMG-CoA reductase and thereby prevents the conversion of HMG-CoA to mevalonate and the synthesis of the other products of the mevalonate pathway. We and others have previously shown that lovastatin induces apoptosis and inhibits proliferation in various cancer cell lines (8–12) and in purified tumor cells derived from patients with multiple myeloma (12) or acute myeloid leukemia (11).

NHL and B-CLL are characterized by initial sensitivity to cytotoxic drugs in the majority of the patients. However, multidrug-resistant disease will ultimately develop in many of these patients. Recent studies have shown that defects in apoptotic pathways contribute significantly to resistance of cancer cells to chemotherapeutic agents (13). Antiapoptotic signaling pathways, such as PI-3K/Akt (14–20) or nuclear factor-κB (21), and expression of antiapoptotic Bcl-2 family members, including Bcl-2 (22–27), Mcl-1 (17, 28–30), and Bcl-XL (31), are involved in the regulation of lymphoma tumor cell survival and proliferation.
Inhibition of Protein Geranylgeranylation in Lymphoma Cells

This report shows that inhibition of protein geranylgeranylation by depletion of GGPP by lovastatin or by inhibition of GGtase I activity induces apoptosis and inhibits proliferation in lymphoma cell lines and tumor cells from patients. In addition, we found that lovastatin enhanced the sensitivity of lymphoma cells to dexamethasone. Altogether our results indicate that interference with the geranylgeranylation of proteins involved in survival and proliferation may be a new treatment strategy in lymphoma.

MATERIALS AND METHODS

Reagents. Lovastatin and simvastatin were obtained from Merck & Co., Inc. (Rahway, NJ) and were chemically activated by alkaline hydrolysis before use as described previously (32). Pravastatin Sodium was purchased from Bristol-Meyers Squibb (New Brunswick, NJ) and dissolved in PBS (20 mM). Atorvastatin Calcium was obtained from Pfizer GmbH (Freiburg, Germany) and dissolved in ethanol containing 3% DMSO (Riedel-de Haen, Seelze, Germany; final concentration of atorvastatin, 10 mM). Mevalonate and FOH were purchased from Sigma (St. Louis, MO), and GGOH was obtained from ICN Biomedicals, BV (Zoetermeer, the Netherlands). FOH and GGOH are metabolized in cells to FPP and GGPP, respectively (33). FTI-277 and GGTI-298 were obtained from Calbiochem (Schwalbach, Germany).

Cell Lines. The follicular lymphoma cell line DoHH2 was obtained from the German Collection of Microorganisms and Cell Cultures, and the follicular lymphoma cell line SU-DHL-6 (34) was kindly provided by Dr. J. Jansen (University Medical Center Nijmegen, Nijmegen, the Netherlands). The Burkitt’s lymphoma cell lines Raji, Ramos, and Daudi were purchased from the American Tissue Culture Collection. Cell lines were cultured in RPMI 1640 (Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaan- lines were cultured in RPMI 1640 (Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaan- lines were cultured in RPMI 1640 (Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaan-

Patients. After receiving informed consent, we obtained mononuclear cells by Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density centrifugation of peripheral blood from 13 patients suffering from leukemic NHL, B-CLL or hairy cell leukemia; of bone marrow from one patient with follicular lymphoma; and of pleural fluid from one patient with mantle cell lymphoma. The clinical characteristics of the patients are shown in Table 1.

Tumor cells from patients 3 and 9 were purified from bone marrow mononuclear cells or from peripheral blood mononuclear cells, respectively, by MACS based on CD19 expression. To this end, mononuclear cells were subsequently labeled with anti-CD19 (BDIES, Erembodegem, Belgium) and rat antimumouse IgG1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated on a high-gradient magnetic separation column placed in a strong magnetic field (Miltenyi), according to the instructions of the manufacturer. Purity after MACS selection of these samples was >95%.

Tumor cells from patient 7 were purified from an axillary lymph node. The organ was cut into small pieces with a scalp knife and incubated with 1 mg/ml collagenase IV (Sigma) and 0.1 mg/ml DNase I (Sigma) for 1 h at 37°C in 5% CO₂. The cell suspension was subsequently filtered through an open-filter chamber (NPBI; Emmer-Capascum, the Netherlands), and mononuclear cells were obtained by ficoll-paque density centrifugation.

Table 1 Clinical characteristics

The lymphoproliferative disorders were diagnosed according to the REAL classification. The NHL and CLL patients were classified according to the Ann Arbor and Rai classification.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Stage</th>
<th>Disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FL grade I</td>
<td>IVA</td>
<td>Untreated</td>
</tr>
<tr>
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<td>FL grade I</td>
<td>IVA</td>
<td>Refractory</td>
</tr>
<tr>
<td>3</td>
<td>FL grade I</td>
<td>IVA</td>
<td>Refractory</td>
</tr>
<tr>
<td>4</td>
<td>FL grade I</td>
<td>IVA</td>
<td>Refractory</td>
</tr>
<tr>
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<td>FL grade I</td>
<td>IVA</td>
<td>Responsive disease</td>
</tr>
<tr>
<td>6</td>
<td>FL grade III</td>
<td>IIIA</td>
<td>Refractory</td>
</tr>
<tr>
<td>7</td>
<td>MCL</td>
<td>IVA</td>
<td>Refractory</td>
</tr>
<tr>
<td>8</td>
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<td>IVA</td>
<td>Refractory</td>
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<tr>
<td>9</td>
<td>MCL</td>
<td>IVB</td>
<td>Refractory</td>
</tr>
<tr>
<td>10</td>
<td>MZL</td>
<td>IVA</td>
<td>Responsive disease</td>
</tr>
<tr>
<td>11</td>
<td>PT L</td>
<td>IVA</td>
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<td>12</td>
<td>Richter</td>
<td>IVA</td>
<td>Refractory</td>
</tr>
<tr>
<td>13</td>
<td>B-CLL</td>
<td>II R</td>
<td>Untreated</td>
</tr>
<tr>
<td>14</td>
<td>B-CLL</td>
<td>III R</td>
<td>Untreated</td>
</tr>
<tr>
<td>15</td>
<td>B-CLL</td>
<td>IV R</td>
<td>Untreated</td>
</tr>
<tr>
<td>16</td>
<td>HCL</td>
<td>Untreated</td>
<td></td>
</tr>
</tbody>
</table>

FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; PT L, peripheral T-cell lymphoma; Richter, Richter’s syndrome; HCL, hairy cell leukemia.

The percentages and monoclonality of the malignant cells were established by flow cytometry (FACS Calibur; BDIS) by analysis of the light chain distribution within the surface-bound immunoglobulins on CD19-positive cells or the CD4/CD8 ratio within CD3-positive T cells in case of patient 11. Percentages of clonal cells present in the samples after density centrifugation or MACS selection (patients 3 and 9) ranged from 75 to 99.4% of the nucleated cells (see Table 3). For experiments, tumor cells were resuspended in growth medium (see above).

Cell Viability. Cell viability was examined by the MTT assay as described previously (12). In short, cells were seeded in a concentration of 0.3 × 10⁶ cells/ml for the lymphoma cell lines or 1 × 10⁶ cells/ml for the tumor cells of patients in a 96-well flat-bottomed plate (100 μl/well; Nunc, Roskilde, Denmark) and treated with different concentrations ofLovastatin (for concentrations, see figure legends) alone or in the presence of mevalonate, FOH, or GGOH. Fixed concentrations of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) were used. These concentrations proved to be optimal in rescuing myeloma cells from lovastatin-induced apoptosis (data not shown). After 2 or 4 days, 25 μl of MTT (5 mg/ml) was added to each well. After an incubation of 2 h at 37°C, the reaction was stopped by the addition of 100 μl of 20% SDS (Boehringer Mannheim, Mannheim, Germany)—0.025 M HCl—0.35 M acetic acid in a mixture of (1:1, v/v) N,N-dimethylformamide (Merck, Darmstadt, Germany) and distilled water. After an overnight incubation at 37°C, the absorbance of the samples was measured at 570 nm.
Cell Proliferation. Cells (3 x 10^5) were seeded in 96-well flat-bottomed plates (Nunc) in 100 μl of growth medium with lovastatin (for concentrations, see figure legends) alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298, respectively (for concentrations, see figure legends). After 2 or 4 days, cells were harvested, washed in ice-cold PBS, and resuspended in Annexin V-FITC (Nexins Research, Kat- tendijke, the Netherlands) and PI. After 10 min of incubation at room temperature, the cells were analyzed by flow cytometry as described previously (12). Apoptotic cells were defined as early apoptotic cells (Annexin V positive and PI negative) and late apoptotic cells (Annexin V and PI positive).

Caspase-3 Activity. The caspase-3 activity assay (Roche) was used to determine caspase-3 activity. Briefly, cells were washed in ice-cold PBS and then resuspended in lysis buffer (1 x DTT) and incubated for 1 min on ice. Supernatants were obtained after centrifugation at 14,000 rpm for 1 min at room temperature. Supernatants were added to anti-caspase-3 coated wells and incubated at 37°C for 1 h. After three washing steps, substrate solution (Ac-DEVD-AFC) was added, and the wells were incubated for 2 h at 37°C. Fluorescence was measured with a 400 nm excitation filter and a 505 nm emission filter.

Measurement of Mitochondrial Transmembrane Potential. Changes in mitochondrial transmembrane potential (ΔΨm) were evaluated by staining with 40 nM DiOC6[3] (Molecular Probes, Leiden, the Netherlands). Cells were incubated with DiOC6[3] in PBS for 15 min at 37°C, washed, and resuspended in PBS. The cells were analyzed on a flow cytometer (FACScalibur).

Western Blotting. Cells (1 x 10^6 cells in 1.5 ml) were incubated for 2 days with lovastatin (for concentrations, see figure legends) in the presence or absence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Inhibition of FTase and

Fig. 1 Lovastatin reduces cell viability in cell lines and in purified tumor cells from lymphoma patients in a time- and dose-dependent way. Cell lines DoHH2 (A) and Raji (B), purified tumor cells from patient 1 (C), and purified tumor cells from patient 7 (D) were treated for 2 or 4 days with solvent control or with different concentrations of lovastatin (1–150 μM). The percentage of viable cells relative to the solvent-control-treated cells, was measured by MTT assay. Experiments were performed three times in duplicate in case of cell lines and one time in triplicate in case of purified tumor cells from patients. Data are presented as mean ± SE (bars). In some cases the SE is smaller than the symbol. FL, follicular lymphoma; MCL, mantle cell lymphoma.
GGTase I was accomplished by FTI-277 and GGTI-298, respectively (for concentrations, see figure legends). After harvesting, whole-cell lysates were made by washing cells twice in ice-cold PBS and then resuspending them in lysis buffer [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, and a cocktail of protease inhibitors] at 4°C for 20 min. Insoluble material was removed by centrifugation at 14,000 rpm for 6 min at 4°C. Protein concentrations were determined by the BCA assay.

Protein were mixed with 2% H11003 (Pierce, Rockford, IL). Samples containing equal amounts of protein were subjected to SDS-PAGE at 4°C for 20 min. Insoluble material was removed by centrifugation at 14,000 rpm for 6 min at 4°C. Protein concentrations were determined by the BCA assay.

or by flow cytometric analysis of CD3/CD4/CD8 in case of a T-cell malignancy. GGTase I was accomplished by FTI-277 and GGTI-298, respectively.

Selection was performed by MACS based on CD19 expression.

Table 2: Effect of lovastatin on cell viability in five lymphoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MTT50 t2 (μM)</th>
<th>MTT50 t4 (μM)</th>
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<tbody>
<tr>
<td>DoHH2</td>
<td>4.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Raji</td>
<td>20.6</td>
<td>14.0</td>
</tr>
<tr>
<td>Daudi</td>
<td>29.2</td>
<td>18.5</td>
</tr>
<tr>
<td>Ramos</td>
<td>27.4</td>
<td>19.5</td>
</tr>
<tr>
<td>SU-DHL-6</td>
<td>45.7</td>
<td>29.3</td>
</tr>
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</table>

a MTT50 and MTT25 represent the concentrations of lovastatin, which after a 2- (t2) or 4- (t4) day incubation reduced cell viability by 50% and 75%, respectively. Lovastatin reduces cell viability of lymphoma cell lines. The lymphoma cell lines (DoHH2, Raji, Daudi, Ramos, and SU-DHL-6) were incubated with different concentrations of lovastatin for 2 or 4 days. Lovastatin reduced viability in a dose- and time-dependent way. Representative examples from the DoHH2 and Raji cell lines are shown in Fig. 1, A and B.

Lovastatin reduces cell viability of tumor cells from NHL patients. Studies were then conducted to determine the effect of lovastatin in purified tumor cells from lymphoma.

Table 3: Effect of lovastatin on cell viability of purified tumor cells from patients with lymphoproliferative disorders

<table>
<thead>
<tr>
<th>Patient</th>
<th>Source</th>
<th>Purity (%)</th>
<th>MTT50 t2 (μM)</th>
<th>MTT50 t4 (μM)</th>
<th>MTT25 t2 (μM)</th>
<th>MTT25 t4 (μM)</th>
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<tr>
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<td>6.4</td>
<td>116</td>
<td>3.2</td>
<td>16.3</td>
</tr>
<tr>
<td>2</td>
<td>PB</td>
<td>87.2</td>
<td>4.4</td>
<td>118</td>
<td>4.4</td>
<td>8.8</td>
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<tr>
<td>3</td>
<td>BM</td>
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<td>NR</td>
<td>NR</td>
<td>16.9</td>
<td>57.0</td>
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<td>NR</td>
<td>118</td>
<td>142</td>
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<td>146</td>
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</tr>
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<td>PB</td>
<td>93.4</td>
<td>90.4</td>
<td>18.2</td>
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</tr>
<tr>
<td>8</td>
<td>PB</td>
<td>94.5</td>
<td>101</td>
<td>17.5</td>
<td>27.6</td>
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<tr>
<td>9</td>
<td>PB</td>
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<td>14.7</td>
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<td>PB</td>
<td>96.0</td>
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<td>NR</td>
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</tr>
<tr>
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<td>PB</td>
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<td>3.1</td>
<td>0.72</td>
<td>1.8</td>
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<tr>
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<td>PB</td>
<td>75.0</td>
<td>108</td>
<td>NR</td>
<td>41.5</td>
<td>91.2</td>
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a Percentage of tumor cells in patient samples; determined by detection of CD19 and membrane immunoglobulin light chain (s) in case of a B-cell malignancy or by flow cytometric analysis of CD3/CD4/CD8 in case of a T-cell malignancy.

b MTT50 and MTT25 represent the concentrations of lovastatin that after a 2-day (t2) or 4-day (t4) incubation reduced cell viability of purified lymphoma tumor cells by 50% and 75%, respectively, compared with the solvent-control-treated cells.

c PB, peripheral blood; BM, bone marrow; PF, pleural fluid; LN, axillary lymph node.

d Selection was performed by MACS based on CD19 expression.

e NR, MTT50 or MTT25 values could not be calculated because the concentration–response curves reached a plateau.

RESULTS

Lovastatin Reduces Cell Viability of Lymphoma Cell Lines. The lymphoma cell lines (DoHH2, Raji, Daudi, Ramos, and SU-DHL-6) were incubated with different concentrations of lovastatin for 2 or 4 days. Lovastatin reduced viability in a dose- and time-dependent way. Representative examples from the DoHH2 and Raji cell lines are shown in Fig. 1, A and B. DoHH2 was the cell line most sensitive to lovastatin, whereas SU-DHL-6 was most resistant to lovastatin. The concentrations of lovastatin that reduced viability with 50% (MTT50) and 75% (MTT25) ranged from 4.7 to 45.7 and 9.7 to 11.8 μM at day 2, respectively. At day 4, MTT50 varied between 4.4 and 29.3 and MTT25 between 7.9 and 46.6 μM (Table 2).

Lovastatin Reduces Cell Viability of Tumor Cells from NHL Patients. Studies were then conducted to determine the effect of lovastatin in purified tumor cells from lymphoma.
patients (n = 16). Tumor cells were purified from peripheral blood (n = 13), bone marrow (n = 2), pleural fluid (n = 1), or an axillary lymph node (n = 1). Tumor cell percentages were >75% after purification (Table 3). The purified tumor cells were incubated with different concentrations of lovastatin for 2 or 4 days. Similar to cell lines, lovastatin reduced cell viability in a dose- and time-dependent way in tumor cells from all patients. Representative examples of dose–response data from two patients are shown in Fig. 1, C and D. Lovastatin sensitivity differed among the samples from the lymphoma patients. MTT50 and MTT25 were in the range of 1.9 to >150 and 3.1 to >150 μM at day 2, respectively. At day 4, MTT50 varied from 0.72 to 118 and MTT25 from 1.8 to 142 μM (Table 3). No obvious correlations were observed between lymphoma type or previous therapy and the effects of lovastatin on cell viability. Patients 1, 11, 13, 14, and 16 had not received treatment at the

![Image](image_url)

**Fig. 2** Lovastatin inhibits protein farnesylation and geranylgeranylation and induces apoptosis by depletion of intracellular pools of GGPP. A, DoHH2 and Raji cells were treated for 2 days with solvent control or lovastatin (30 μM) alone or in the presence of mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM). After protein isolation, processing of DnaJ and Rap1a was determined by Western blot analysis. The faster migrating band represents mature and processed protein, the slower band represents nonprenylated, unprocessed protein. The data shown are representative of at least three independent experiments. B, DoHH2 cells were treated for 4 days with solvent control or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM). The percentage of apoptotic cells was examined by the Annexin V assay. The percentages of viable plasma cells (Annexin V−/PI−), early apoptotic cells (Annexin V+/PI−) and late apoptotic cells (Annexin V+/PI+) in each dot plot are indicated in the corresponding quadrants. Results are representative of three experiments performed in triplicate. C, DoHH2 and Raji cells were treated for 4 days with solvent control or lovastatin (5, 10, 40, 100, or 150 μM) in combination with mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM), after which apoptosis was determined by Annexin V assay. Shown is the sum of the percentages of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± SE (bars). In some cases the SE is smaller than the symbol.
time that the \textit{in vitro} experiments were performed. Patients 1, 13, and 16 responded to subsequent chemotherapy, whereas patients 11 and 14 were lost to follow-up. There was no difference among patients with chemosensitive or chemoresistant disease and lovastatin sensitivity.

\textbf{Lovastatin Inhibits Protein Prenylation.} The effect of lovastatin on prenylation in DoHH2 and Raji cell lines was determined by analysis of the migratory behavior during electrophoresis of DnaJ, a protein prenylated exclusively by FTase (35, 36), and of Rap1a, a protein prenylated exclusively by

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Depletion of GGPP by lovastatin is associated with Mcl-1 protein reduction, collapse of the mitochondrial transmembrane potential, and caspase-3 activation. \textit{A}, lymphoma cell lines DoHH2 and Raji were treated for 2 days with solvent control or lovastatin (30 \(\mu\)M) alone or in combination with mevalonate (\textit{meva}; 100 \(\mu\)M), FOH (10 \(\mu\)M), or GGOH (10 \(\mu\)M). After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax levels were determined by Western blot analysis. In addition, the proapoptotic 23-kDa Bcl-2 form was detected after long exposure of the film. The data shown are representative of at least three independent experiments. \textit{B}, DoHH2 cells were treated for 4 days with solvent control or lovastatin (30 \(\mu\)M) alone or in combination with mevalonate (\textit{meva}; 10 \(\mu\)M), FOH (10 \(\mu\)M), or GGOH (10 \(\mu\)M). Collapse of the mitochondrial transmembrane potential was determined by staining the cells with DiOC\(_6\)\([3]\). Results are representative of three experiments performed in triplicate. The fraction of cells with low (DiOC\(_6\)\([3]\)\textsuperscript{low}) and the fraction of cells with intact (DiOC\(_6\)\([3]\)\textsuperscript{high}) mitochondrial transmembrane potential are indicated in gates \textit{M1} and \textit{M2}, respectively. \textit{C}, DoHH2 cells were treated for 4 days with solvent control or different concentrations of lovastatin (10, 30, 50, 100, or 150 \(\mu\)M) alone or in combination with mevalonate (\textit{meva}; 100 \(\mu\)M), FOH (10 \(\mu\)M), or GGOH (10 \(\mu\)M). Collapse of the mitochondrial transmembrane potential was determined by staining the cells with DiOC\(_6\)\([3]\). Shown is the percentage of cells with low mitochondrial transmembrane potential. Experiments were performed three times in triplicate. Data are presented as mean \(\pm\) SE (bars). In some cases the SE is smaller than the symbol. \textit{D}, DoHH2 cells were exposed to solvent control or lovastatin (30 \(\mu\)M) alone or in combination with mevalonate (\textit{meva}; 100 \(\mu\)M), FOH (10 \(\mu\)M), or GGOH (10 \(\mu\)M) for 2 days, at which time the cells were harvested and caspase-3 activation was assessed as described in “Materials and Methods.” Experiments were performed three times in duplicate. Data are presented as mean \(\pm\) SE (bars).
GGTase I (37; Fig. 2A). Inhibition of prenylation of these proteins can be monitored by immunoblotting because the non-prenylated forms of these proteins display reduced mobility in SDS-PAGE relative to their prenylated versions. In solvent-control-treated cells, DnaJ and Rap1a were in the processed, prenylated forms. Lovastatin inhibited the farnesylation of DnaJ and geranylgeranylation of Rap1a. Addition of mevalonate restored the processing of DnaJ and Rap1a. Treatment with lovastatin in the presence of GGOH (10 μM), which is metabolized to GGPP in the cells (33), restored geranylgeranylation of Rap1a but had no effect on the inhibition of farnesylation of DnaJ. In contrast, FOH (10 μM), which is metabolized to FPP (33), restored DnaJ farnesylation but not Rap1a geranylgeranylation.

Lovastatin Induces Apoptosis by Depletion of GGPP. DoHH2, Raji, Daudi, Ramos, and SU-DHL-6 cells were treated with lovastatin alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM), and apoptosis was assessed by the Annexin V assay. Lovastatin treatment resulted in an increase of the percentage of apoptotic cells in a time- and dose-dependent way for DoHH2 and Raji (Fig. 2, B and C). Treatment of cells with mevalonate or GGOH prevented lovastatin-induced apoptosis. However, addition of FOH had no effect. Similar to lovastatin, other hydrophobic HMG-CoA reductase inhibitors, including simvastatin and atorvastatin, also induced apoptosis, which could be abrogated by addition of mevalonate or GGOH but not FOH. However, the hydrophilic inhibitor pravastatin had no effect (data not shown). This indicates that inhibition of geranylgeranylation by depletion of intracellular pools of GGPP by inhibition of HMG-CoA reductase induces apoptosis in lymphoma cell lines.

Fig. 4 Lovastatin synergizes with dexamethasone in reducing cell viability. DoHH2 (A), Raji (B), Daudi (C), SU-DHL-6 (D), and Ramos cells (E) were incubated with different concentrations of dexamethasone (0–100 μM) in combination with lovastatin (lova; 2, 5, 10, 20, or 30 μM) or solvent control for 4 days. Cell viability was determined by MTT assay and compared with cells treated without dexamethasone and lovastatin. Results are representative of at least three experiments performed in triplicate and are presented as the mean ± SE (bars). In some cases the SE is smaller than the symbol. *, significantly higher reduction in cell viability than the sum of the effects observed in the corresponding lovastatin- and dexamethasone-treated groups (P < 0.05).

Apoptosis Induction by Lovastatin Is Associated with Mcl-1 Protein Reduction, Collapse of the Mitochondrial Transmembrane Potential, and Caspase-3 Activation. Expression of Bcl-2 family proteins was determined in DoHH2 and Raji cells treated with lovastatin (30 μM) alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) by Western blot analysis. Lovastatin treatment resulted in a significant reduction of Mcl-1 expression levels (Fig. 3A). Only in DoHH2 cells did lovastatin reduce Bcl-XL and increase Bax protein expression. Although Bcl-2 protein levels remained un-
altered, there was an increase of the proapoptotic 23-kDa Bcl-2 form, which could be visualized after long exposure of the film compared with Bcl-2. Importantly, mevalonate and GGOH prevented reduction of Mcl-1 and Bcl-XL expression and increases in Bax and the proapoptotic Bcl-2 form. In contrast, FOH had no effect.

Members of the Bcl-2 family are involved in regulation of the mitochondrial transmembrane potential, release of cytochrome c, and caspase-3 activation (38, 39). Treatment of DoHH2 and Raji cells with lovastatin resulted in loss of the mitochondrial transmembrane potential, as shown for DoHH2 cells (Fig. 3, B and C). The collapse was time (data not shown) and dose dependent (Fig. 3C). To determine whether lovastatin treatment resulted in activation of caspase-3, cell lysates were analyzed for caspase-3 activity. Treatment of DoHH2 and Raji cells with lovastatin resulted in activation of caspase-3, as shown for DoHH2 cells (Fig. 3D). Bcl-2 is a known substrate of caspase-3 (40, 41). Exposure of cell lines to lovastatin stimulates caspase-3 activity, which in turn leads to generation of the Bcl-2 cleavage product that promotes apoptosis. The increase in the proapoptotic 23-kDa Bcl-2 form was demonstrated by Western blot analysis (Fig. 3A).

Treatment of DoHH2 and Raji cells with lovastatin in the presence of mevalonate or GGOH prevented collapse of the mitochondrial transmembrane potential and activation of caspase-3. However, incubation of cells with lovastatin in combination with FOH had no effect compared with lovastatin alone (Fig. 3, B–D).

**Synergy between Lovastatin and Dexamethasone in the Induction of Lymphoma Cell Death.** Cell lines were treated with dexamethasone (0, 5, 10, 50, or 100 μM) or doxorubicin (0, 10, 25, 50, 100, 200, or 500 nM) alone or in combination with lovastatin (0–30 μM). The combination of lovastatin with doxorubicin produced a significant additive effect (data not shown). Furthermore, low concentrations of lovastatin potently sensitized both dexamethasone-sensitive (DoHH2, SU-DHL-6, Ramos) and dexamethasone-resistant (Raji, Daudi) lymphoma cells to dexamethasone in a synergistic fashion (Fig. 4).

**Depletion of GGPP Inhibits Proliferation by Induction of G1 Arrest.** DoHH2, Raji, Daudi, Ramos, and SU-DHL-6 were treated with lovastatin (0–150 μM) alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Proliferation was examined by measuring [3H]thymidine incorporation. Lovastatin inhibited proliferation in a time- (data not shown) and dose-dependent way as shown in Fig. 5A for DoHH2 and Raji at day 4. Mevalonate and GGOH, but not FOH, restored proliferation in lovastatin-treated lymphoma cell lines. Cell cycle analysis revealed that lovastatin treatment in-

![Fig. 5 Depletion of GGPP by lovastatin inhibits proliferation by the induction of a G1 block. A, DoHH2 and Raji cells were treated for 4 days with solvent control or different concentrations of lovastatin (1, 5, 10, 30, 50, 100, or 150 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Proliferation was determined by [3H]thymidine incorporation during the last 16 h of culture. Data are from three experiments performed in triplicate and are presented as mean ± SE (bars). In some cases the SE is smaller than the symbol. B, DoHH2 cells were treated with solvent control or lovastatin (lova; 10 or 30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM) for 2 days, at which time the cells were harvested and cell cycle distribution was determined by BrdUrd (BrdU) assay. The percentage of apoptotic cells (quadrant 1) and the percentages of cells in G1 phase (quadrant 2), G2-M phase (quadrant 3), and S-phase (quadrant 4) of the cell cycle are indicated in each dot plot. Results are representative of three experiments performed in triplicate.](https://cancergenome.org)
creased the number of cells in the G1 phase of the cell cycle, whereas the number of cells in the S-phase decreased (Fig. 5B). The blockade of cell cycle progression responsible for the inhibition of proliferation of lymphoma cells was abrogated by addition of mevalonate or GGOH, whereas FOH had no effect (Fig. 5B).

Depletion of GGPP Reduces Cell Viability, Down-Regulates Mcl-1 Expression, and Inhibits Proliferation in Purified Tumor Cells from Lymphoma Patients. Purified tumor cells from lymphoma patients were incubated with lovastatin alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Similar to the effects in cell lines, addition of mevalonate and GGOH to purified tumor cells from patients (n = 4) abrogated lovastatin-induced reduction of cell viability, whereas FOH had no effect. This is shown for a representative patient (patient 15) in Fig. 6A. Mcl-1 levels were determined in cells from three patients. In all cases, Mcl-1 protein levels were decreased when the cells were incubated with lovastatin. The reduction was 54.3% for patient 3, 35.1% for patient 7, and 98.4% for patient 15. Bcl-XL, Bcl-2, and Bax protein levels remained unchanged. Mcl-1 protein expression was recovered when mevalonate or GGOH was added to lovastatin-treated cells. FOH had no effect, as shown in Fig. 6B for patient 15. In addition, proliferation of purified tumor cells (n = 2) was restored by addition of mevalonate or GGOH but not FOH. A representative example from patient 7 is shown in Fig. 6C.

Fig. 6  Treatment of purified tumor cells from lymphoma patients with lovastatin reduces cell viability, down-regulates Mcl-1 protein expression, and inhibits proliferation by reduction of intracellular pools of GGPP. A, tumor cells derived from patient 15 were treated for 4 days with solvent control or different concentrations of lovastatin (5, 10, 30, 50, 100, or 150 μM) alone or in the presence of mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). The percentage of viable cells relative to the solvent-control-treated cells was measured by MTT assay. Experiments were performed once in triplicate. Data are presented as mean ± SE (bars). In some cases the SE is smaller than the symbol. B, tumor cells derived from patient 15 were treated for 4 days with solvent control or lovastatin (30 μM) alone or in the presence of mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax were determined by Western blot analysis. C, tumor cells derived from patient 7 were treated for 4 days with solvent control or different concentrations of lovastatin (5, 10, 30, 50, 100, or 150 μM) alone or in the presence of mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Proliferation was determined by [3H]thymidine incorporation during the last 16 h of culture. Experiments were performed once in triplicate. Data are presented as mean ± SE (bars). In some cases the SE is smaller than the symbol.
Inhibition of GGTase I Reduces Mcl-1 Protein Expression and Induces Apoptosis. The importance of geranylgeranylation for the regulation of apoptosis and proliferation was further evaluated by use of specific inhibitors of FTase (FTI-277) and GGTase I (GGTI-298). FTI-277 (20 μM) inhibited the farnesylation of DnaJ, and GGTI-298 (20 μM) inhibited the geranylgeranylation of Rap1a in DoHH2 and Raji cells. The specificity of these prenylation inhibitors is illustrated by the lack of inhibition of Rap1a prenylation by FTI-277 and, likewise, the lack of inhibition of DnaJ prenylation by GGTI-298 (Fig. 7A). Treatment of DoHH2 and Raji cells with GGTI-298 (20 μM) resulted in induction of apoptosis in a time-dependent way (Fig. 7B). The effect of FTI-277 (20 μM) on induction of apoptosis was significantly less pronounced compared with GGTI-298. Apoptosis induced by GGTI-298 treatment was associated with reduction of Mcl-1 protein expression (Fig. 7C). Furthermore, in DoHH2 cells, Bcl-XL expression was reduced. Bax and Bcl-2 levels remained unchanged; however, GGTI-298 treatment led to an increase of the 23-kDa proapoptotic Bcl-2 form. In contrast, inhibition of FTase activity by FTI-277 had no effect on expression of Mcl-1, Bcl-XL, Bax, Bcl-2, and the proapoptotic Bcl-2 form (Fig. 7C).

Inhibition of GGTase I Inhibits Proliferation. The effect of FTI-277 (0–30 μM) and GGTI-298 (0–30 μM) on proliferation of DoHH2 and Raji cells was examined by measuring [3H]thymidine incorporation. Inhibition of GGTase I activity by GGTI-298 reduced proliferation in a time- and dose-dependent way (Fig. 8, A and B). Inhibition of FTase activity by FTI-277 did not inhibit proliferation or inhibited proliferation only to a small extent compared with GGTI-298 (Fig. 8, A and B).

DISCUSSION

Treatment of cell lines (n = 6) and purified tumor cells from lymphoma patients (n = 16) with the HMG-CoA reductase inhibitor lovastatin reduced the number of viable cells. The reduction in the number of viable cells was mediated by both induction of apoptosis and inhibition of proliferation. Addition of mevalonate to lovastatin-treated cells prevented induction of apoptosis and restored proliferation. This indicates that the effects of lovastatin resulted from the inhibition of mevalonate formation and not from nonspecific cell toxicity. There was no significant difference in lovastatin sensitivity between tumor cells from pretreated and untreated patients or from patients with chemosensitive or chemoresistant disease.

Mevalonate is the precursor of various molecules, including the isoprenoids FPP and GGPP (1). We found that geranylgeranylated protein(s) are important regulators of survival...
and proliferation in lymphoma tumor cells. Although lovastatin depletes intracellular pools of both GGPP and FPP, resulting in inhibition of geranylgeranylation of Rap1a and farnesylation of DnaJ, only addition of GGOH, which is converted to GGPP in cells (33), abrogated the inhibition of geranylgeranylation by lovastatin and restored both cell viability and proliferation. In contrast, although addition of FOH, which is converted to FPP (33), completely restored farnesylation of DnaJ, it had no effect on viability or proliferation. However, in some cases GGOH was less effective than mevalonate in rescuing lymphoma tumor cells from apoptosis and growth arrest. This suggests that depletion of other metabolites downstream of mevalonate may also partly contribute to the effects of lovastatin. The importance of geranylgeranylation for the regulation of growth and survival of lymphoma cells was further confirmed by specific inhibition of FTase by FTI-277 and GGTase I by GGTI-298. GGTI-298 inhibited protein geranylgeranylation and resulted in the induction of apoptosis and inhibition of proliferation, whereas inhibition of farnesylation by FTI-277 had no or only a small effect.

The Bcl-2 family consists of proapoptotic proteins, such as Bax, Bad, and Bak, and antiapoptotic family members, including Mcl-1, Bcl-XL, and Bcl-2. The balance of anti- and proapoptotic Bcl-2 family proteins determines the survival or death of cells (38, 39). The antiapoptotic Bcl-2 family members inhibit apoptosis by forming inactivating heterodimers with proapoptotic Bcl-2 family proteins and by preventing the collapse of the mitochondrial transmembrane potential and cytochrome c release from mitochondria into the cytosol (38, 39). In the cytosol, cytochrome c and Apaf-1 can activate procaspase-9, which subsequently activates caspase-3 (38, 39). Mcl-1 is expressed in various types of human leukemias and lymphomas, including CLL (28), follicular lymphoma (31, 42), and anaplastic large cell lymphoma (43). There is increasing evidence that Mcl-1 plays a prominent role in lymphomagenesis (44) and in the survival (17, 30, 45) and chemoresistance (28, 29) of lymphoma tumor cells. We showed that apoptosis induced by inhibition of geranylgeranylation was associated with a reduction in Mcl-1 protein expression in lymphoma cell lines and patient cells, which in turn resulted in collapse of the mitochondrial transmembrane potential and caspase-3 activation.

Lovastatin treatment also led to an increase in the 23-kDa proapoptotic Bcl-2 form. Bcl-2 is a known substrate of caspase-3 (40, 41); it is therefore likely that lovastatin-mediated stimulation of caspase-3 activity led to the increase in the proapoptotic Bcl-2 form. The proapoptotic 23-kDa Bcl-2 form is able to localize to mitochondria and stimulate the release of cytochrome c into the cytosol (41). Therefore, caspase-3-dependent cleavage of Bcl-2 appears to promote further caspase activation as part of a positive feedback loop. Only in DoHH2 cells was expression of the antiapoptotic protein Bcl-XL decreased and the proapoptotic protein Bax increased. Although the alterations in expression levels of these proteins were relatively small compared with the change in Mcl-1 expression, they may contribute partly to the induction of apoptosis induced by inhibition of geranylgeranylation in this cell line.

The identities of the geranylgeranylated target protein(s) of lovastatin and GGTI-298 that mediate protection against cell death and regulate the proliferation of lymphoma tumor cells remain unknown. Candidates, however, include RhoA, Rac-1, R-Ras, and Cdc42, which are involved in important cellular functions, including the regulation of apoptosis (4, 46–50). Several pathways, including PI-3K, JAK/STAT3, and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase, can stimulate Mcl-1 transcription (51). Because RhoA (52, 53), Rac-1 (54), R-Ras (49, 55), and Cdc42 (56) have been shown to activate the PI-3K pathway, inhibition of these pro-
Inhibition of Protein Geranylgeranylation in Lymphoma Cells

Proteins by lovastatin or GGTTI-298 may be involved in McI-1 down-regulation and induction of apoptosis. Furthermore, RhoA, Rac-1, R-Ras, and Cdc42 have also been implicated as regulators of proliferation (4, 47, 48, 50, 57). We showed that inhibition of geranylgeranylation led to a reduction of proliferation attributable to arrest in G1 phase of the cell cycle. This indicates that geranylgeranylated proteins are critical for G1-S transition in lymphoma tumor cells. Also in lung adenocarcinoma (58) and in mouse fibroblasts (37), inhibition of geranylgeranylation arrested cells in the G1 phase of their cycle. Rac-1 (59, 60), Cdc42 (60–62), and RhoA (59, 60) play an essential role in G1-S-phase transition, and inhibition of processing of these proteins by lovastatin or GGTTI-298 may, therefore, be responsible for the G1 block. Ongoing studies are directed at identifying the geranylgeranylated target proteins.

Lovastatin augmented the sensitivity of both drug-sensitive and drug-resistant lymphoma cells to dexamethasone in a synergistic way. Moreover, additive cytotoxicity was observed between lovastatin and doxorubicin. Our findings are consistent with the ability of lovastatin to sensitize tumor cells to cytotoxic agents in other models (10, 12, 63–65). Because McI-1 is an important regulator of response to cytotoxic agents (28, 29, 66), the synergism between lovastatin and dexamethasone may be attributable, at least in part, to the lovastatin-mediated McI-1 down-regulation. Importantly, cell death and chemosensitization induced by lovastatin were observed at concentrations that could be achieved in vivo without significant tissue toxicity (67).

In summary, we have shown that geranylgeranylation of proteins is critical for the survival and proliferation of lymphoma tumor cells. Inhibition of geranylgeranylation, either by depletion of intracellular pools of GGPP through the inhibition of HMG-CoA reductase by lovastatin or by the specific inhibition of GGTTase I activity by GGTTI-298, resulted in the induction of apoptosis and reduction of proliferation. Apoptosis induced by inhibition of geranylgeranylation was probably attributable to a reduction of the antiapoptotic Bcl-2 family member McI-1, which in turn resulted in collapse of the mitochondrial membrane potential and activation of caspase-3. The McI-1 down-regulation may also explain the chemosensitization activity of lovastatin. Furthermore, inhibition of proliferation was associated with a G1 block. These data suggest that inhibition of protein geranylgeranylation either alone or in combination with chemotherapy warrants further investigation as a new therapeutic strategy in lymphoma and CLL.

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Protein Geranylgeranylation Is Critical for the Regulation of Survival and Proliferation of Lymphoma Tumor Cells


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