Lovastatin Potentiates Antitumor Activity and Attenuates Cardiotoxicity of Doxorubicin in Three Tumor Models in Mice1

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ABSTRACT

Lovastatin, a drug commonly used in the clinic to treat hypercholesterolemia, has previously been reported to exert antitumor effects in rodent tumor models and to strengthen the antitumor effects of immune response modifiers (tumor necrosis factor α and IFN-γ) or chemotherapeutic drugs (cisplatin). In the present report, we show in three murine tumor cell lines (Colon-26 cells, v-Ha-ras-transformed NIH-3T3 sarcoma cells, and Lewis lung carcinoma cells) that lovastatin can also effectively potentiate the cytostatic/cytotoxic activity of doxorubicin. In three tumor models (Colon-26 cells, v-Ha-ras-transformed NIH-3T3 sarcoma cells, and Lewis lung carcinoma cells) in vivo, we have demonstrated significantly increased sensitivity to the combined treatment with both lovastatin (15 mg/kg for 10 days) and doxorubicin (3 × 2.5 mg/kg; cumulative dose, 7.5 mg/kg) as compared with either agent acting alone. Lovastatin treatment also resulted in a significant reduction of troponin T release by cardiomyocytes in doxorubicin-treated mice. This observation is particularly interesting because lovastatin is known to reduce doxorubicin-induced cardiac injury.

INTRODUCTION

Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase that has been used in the clinic to treat hypercholesterolemia (1, 2). Lovastatin has also been shown to arrest tumor and normal cells in the G1 phase of the cell cycle (3, 4) and has demonstrated antitumor effects in experimental murine models (5–11). Although clinical studies on the antitumor activity of lovastatin have been initiated (12), its effectiveness in clinical tumor therapy will probably depend on whether combination therapies are found in which lovastatin used with other drugs is shown to exert potentiated antitumor effects. As shown recently by Agarwal et al. (13), pretreatment with lovastatin increased apoptosis induced by chemotherapeutic agents in tumor cells in vitro. Lovastatin has also been shown to strengthen the antitumor activity of cisplatin (10) and tumor necrosis factor α (11) in murine tumor models.

Because lipid-lowering agents have been shown to reduce the cardiotoxic side effects of doxorubicin (14), we decided to examine how lovastatin influences the antitumor effects of doxorubicin, and whether it will reduce the cardiotoxicity of doxorubicin.

MATERIALS AND METHODS

Animals. Female 11–15-week-old BALB/c mice and (C57BL/6 × DBA/2)F1 mice (referred to hereafter as B6D2F1 mice) were used throughout the experiment. Breeding pairs were supplied by the Inbred Mice Breeding Center (Institute of Immunology and Experimental Medicine, Wroclaw, Poland). Mice were bred in a local animal facility and kept under standard conditions during the experimental period. Experiments were performed after formal approval by the Institutional Ethical Committee for Research on Animals.

Reagents. Lovastatin in the inactive lactone form was obtained from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ). It was converted to the active form by dissolving in ethanol, heating at 50°C in 0.1N NaOH, and neutralizing with HCl. Distilled water was added to a final concentration of 8 mg/ml. This stock solution was stored frozen (−27°C).

Doxorubicin hydrochloride (Adriamycin) was obtained from Farmintalia Carlo Erba (Milan, Italy). The drug was diluted in PBS for in vitro experiments and in 0.9% NaCl for in vivo experiments to obtain the appropriate concentrations.

Cell Lines. Four types of cells were used in this study: (a) Colon-26 colon adenocarcinoma cells; (b) LLC3 cells; (c)

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3 The abbreviations used are: LLC, Lewis lung carcinoma; MTT, 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cTnT, cardiac troponin T.
v-Ha-ras-transformed NIH-3T3 sarcoma cells (referred to hereafter as Ras-3T3 cells); and (d) nontransformed NIH-3T3 cells.

Murine LLC cells (syngeneic to C57BL/6) were provided by Dr. C. Radzikowski (Institute of Immunology and Experimental Medicine, Wrocław, Poland). Murine colon adenocarcinoma cells (Colon-26 cells, syngeneic to BALB/c) were purchased from the American Type Culture Collection.

The Ras-3T3 and parental nontransformed NIH-3T3 cell lines were provided by Dr. H. Maruta (Ludwig Institute for Cancer Research, Victoria, Australia). The NIH-3T3 cell line is a nonmalignant murine fibroblast cell line derived from NIH Swiss mouse embryo culture. The Ras-3T3 cells were obtained as described previously (15). Briefly, normal NIH-3T3 fibroblasts were transfected with the v-Ha-ras oncogene inserted into the mammalian retroviral vector pMV7, leading to tumorigenic Ras-3T3 cell line. Tumorigenicity of transformed cells was assessed in the BALB/c mouse model, which indirectly confirmed stability of the transfection.

Cells were maintained in either DMEM or RPMI 1640 (Life Technologies, Inc.; Paisley, Scotland) supplemented with antibiotics, 2-mercaptoethanol (50 μM), L-glutamine (2 mM), and 10% FCS (all from Life Technologies, Inc.; culture medium) and passaged every 3–4 days.

**MTT Assay.** The cytostatic/cytotoxic effects of lovastatin and/or doxorubicin on Ras-3T3, NIH-3T3, Colon-26, and LLC cells in vitro were tested in a standard MTT assay. Portions (100 μl) of Ras-3T3 (4 × 10⁴) and NIH-3T3 (9 × 10⁴) cells as well as Colon-26 (6 × 10⁴) and LLC (5 × 10⁴) cells were dispensed into 96-well microtiter plates (Corning, Bibby Sterlin, Ltd., Staffordshire, England). The plates were incubated overnight at 37°C in 5% CO₂, and then serial dilutions of doxorubicin (50 μl; final concentration, 0.025–1 μg/ml) and/or lovastatin (50 μl; final concentration, 0.1–4 μM) were added in quadruplicate to a final volume of 200 μl. After an incubation period of 24 h, the medium was removed, and the cells were washed three times in culture medium. Serial dilutions of lovastatin (100 μl; final concentration, 0.1–4 μM) were added and supplemented with culture medium to a final volume of 200 μl. Different incubation periods for doxorubicin and lovastatin were used to obtain comparable inhibition of cell proliferation in all experiments, and these concentrations correspond to those obtained with untreated control cells at 100% (1). Briefly, inhibition of cell proliferation was determined as described above. Equieffective concentrations [concentrations of either drug, alone or in combination, that gave equivalent inhibition of cell growth as compared with untreated control cells at P ≤ 0.05 (Student’s t-test)] were analyzed. The interaction index for combinations of the two drugs was computed according to the following equation:

\[
\text{Interaction index} = \frac{\text{LOV}_c + \text{DOX}_c}{\text{LOV}_e + \text{DOX}_e}
\]  

where \(\text{LOV}_c\) and \(\text{DOX}_c\) are concentrations of lovastatin and doxorubicin, respectively, that produce some specified effect when used alone, and \(\text{LOV}_e\) and \(\text{DOX}_e\) are concentrations of lovastatin and doxorubicin, respectively, that produce the same effect when used in combination.

Synergy occurs when the interaction index is less than 1.0.

**Animal Experiments.** On the inoculation day, the tumor cells were harvested from the cultures and washed, and then 5 × 10⁵ Ras-3T3 or LLC cells or 1 × 10⁵ Colon-26 cells in 20 μl of medium were injected s.c. into the footpad of the right hind limb.

Mice were injected with lovastatin and/or doxorubicin in two different regimens: (a) starting at day 5 after inoculation of tumor cells (for Colon-26 tumor); and (b) starting at day 7 after inoculation of tumor cells (for Ras-3T3 and LLC tumors), after the tumor nodules became visible in the footpads.

Local tumor growth was determined by measuring footpad diameter with calipers every other day, starting with the first day of treatment (day 5 or 7 after inoculation of tumor cells – initial tumor volume). Tumor volume was estimated by the formula below.

\[
\text{Tumor volume (mm}^3\text{)} = \text{(longer diameter)} \times \text{(shorter diameter)}^2
\]
Fig. 2 Effects of lovastatin and/or doxorubicin on (A) Ras-3T3, (B) Colon-26, and (C) LLC cells in vitro. One-day-old monolayers of tumor cells were exposed to various concentrations of lovastatin (0.1–4 μM). After a 72-h incubation period, the medium was supplemented with doxorubicin (0.025–1 μM), and cells were incubated for an additional 24 h. Cytostatic/cytotoxic effects, expressed as the relative viability (percentage of untreated control), were tested in an MTT assay. Bars, mean ± SE.
Relative tumor volume was calculated as follows:

\[ \% \text{Vol}_X = \left( \frac{\text{Vol}_X}{\text{Vol}_{\text{ini}}} \right) \times 100\% \]  

where \( \% \text{Vol}_X \) represents mean relative tumor volume on day \( X \), \( \text{Vol}_X \) represents mean tumor volume on day \( X \), and \( \text{Vol}_{\text{ini}} \) represents mean initial tumor volume.

**Treatment and Monitoring.** To investigate the effects of both drugs, tumor-bearing mice were divided into four groups (6–7 mice/group) and injected with either: (a) saline; (b) lovastatin (15 mg/kg/day, i.p.; 10 consecutive days); (c) doxorubicin (2.5 mg/kg, i.p., in three doses (on the first, fifth, and ninth day of therapy)); or (d) a combination of both drugs (15 mg/kg lovastatin i.p. for 10 consecutive days + 2.5 mg/kg doxorubicin i.p. on the first, fifth, and ninth day of therapy).

Tumor growth delay was determined according to the method of Corbett et al. (17) and calculated as follows:

\[ \text{Tumor growth delay} = T - C \]  

where \( T \) represents the median time (in days) required for the treatment group tumors to reach a certain volume (in mm\(^3\)); different for various types of tumors), and \( C \) represents the median time (in days) required for the control group tumors to reach the same size.

Mice were observed daily for survival.

**Serum cTnT Levels.** B6D2F1 mice were randomized and divided into four groups (5–6 mice/group), and the therapy protocol was introduced as presented in Fig. 1. Blood was drawn from all mice on days 1, 15 (before the initial dose of doxorubicin), 21 (after two doses of doxorubicin; cumulative dose, 5 mg/kg), and 35 (after six doses of doxorubicin; cumulative dose, 15 mg/kg) of the experimental cycle. Serum levels of cTnT were measured using a commercially available cTnT ELISA kit (Enzymun-Test Troponin-T; Boehringer Mannheim GmbH, Mannheim, Germany) according to manufacturer’s instructions.

The means and SEs were determined for triplicate samples.

**Statistical Analysis.** Differences between the results of the in vitro tests and between tumor diameters in the in vivo experiments were examined and analyzed primarily by Student’s \( t \) test and Mann-Whitney test (two-tailed). The resulting data from our in vivo studies were also analyzed using the nonparametric ANOVA (Kruskal-Wallis) test, followed by a Dunn’s multiple comparisons test. In addition, the data from cTnT studies were analyzed with the nonparametric repeated measures ANOVA (Friedman) test. (all computed using Instat 2.0; GraphPad Software Inc., San Diego, CA). \( P < 0.05 \) was considered to be statistically significant.

**RESULTS**

Lovastatin and Doxorubicin Demonstrate Potentiated Cytostatic/Cytotoxic Effects When Used in Combination in Colon-26, LLC, and Ras-3T3 Tumor Cells in Vitro. To assess the direct influence of doxorubicin and lovastatin on in vitro growth of tumor cells, an MTT assay was performed as described in “Materials and Methods.” When used alone, both lovastatin and doxorubicin exhibited dose-dependent cytostatic/cytotoxic activity on Ras-3T3, Colon-26, and LLC cells (Fig. 2, A–C). Moreover, in all presented cell lines, lovastatin and doxorubicin demonstrated potentiated cytostatic/cytotoxic effects in vitro when used in combination (Fig. 2, A–C). Ha-ras-transformed Ras-3T3 cells were more sensitive to lovastatin than other tumor cells.

To determine whether lovastatin and doxorubicin exert synergistic cytostatic/cytotoxic effects in the treated cells, the isobologram analysis described by Berenbaum (16) was used. According to this analysis, synergy occurs when the interaction index is less than 1.0. The interaction index for lovastatin and doxorubicin used in combination in LLC and Ras-3T3 cells was 1.0, which indicates an additive mode of action (data not shown). However, in Colon-26 cells, the interaction indices for these two drugs were 0.5 and 0.6 (Fig. 3). Thus, the results of our in vitro experiments suggest that potentiated cytostatic/cytotoxic effects of combination therapy with lovastatin and doxorubicin may be due, at least in part, to the synergistic cytostatic/cytotoxic interaction of these two drugs acting in Colon-26 tumor cells.

Tumorigenic Ras-3T3 Cells Are Less Sensitive to Doxorubicin than Control NIH-3T3 Cells in Vitro, but Lovastatin Reverses this Resistance. To study whether lovastatin could reverse the resistance of Ras-3T3 cells to doxorubicin, additional experiments were performed. In these experiments we compared the drug sensitivity of Ras-3T3 cells with that of their nontransformed counterparts (NIH-3T3) using an MTT assay. Although doxorubicin exhibited dose-dependent cytostatic/cytotoxic activity on both Ras-3T3 and control cells (Fig. 4), Ras-3T3 cells were significantly less sensitive (\( P < 0.05 \) Mann-Whitney test) to its action as compared with the nontransformed NIH-3T3 cells.

Nevertheless, adding lovastatin to the culture medium resulted in an increase in vulnerability of Ras-3T3 cells to doxor-
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Lovastatin at a concentration of 0.25 μM significantly potentiated the cytostatic/cytotoxic effects of doxorubicin (P < 0.05; Mann-Whitney test), making both Ras-3T3 cells and non-transformed NIH-3T3 cells identically sensitive to doxorubicin (Fig. 4).

Lovastatin and Doxorubicin Demonstrate Potentiated Antitumor Effects When Used in Combination. To examine whether our in vitro observations could have potential therapeutic significance, animal studies were performed. A sub-threshold dose of lovastatin in these experiments was established at 15 mg/kg body weight (data not shown). This dose of lovastatin, given i.p., did not exert any significant antitumor effects against either of the examined tumors. In the preliminary dose-toxicity study, the maximum tolerated dose of doxorubicin was established as a total of 7.5 mg/kg body weight (three injections of 2.5 mg/kg doxorubicin; data not shown). This dose was used in experiments where the combined effects of lovastatin and doxorubicin were tested. Although doxorubicin when used alone at this dose did not inhibit tumor growth in two of the three tumors studied (Colon-26 and LLC tumors), a significant antitumor effect came to light when lovastatin was included in the therapeutic regimen (Fig. 5, B and C). The resulting significant retardation of Colon-26 adenocarcinoma growth in mice treated with lovastatin combined with doxorubicin, as compared with each of the remaining groups, was observed on days 15 (P ≤ 0.005, ANOVA), 17 (P < 0.01, ANOVA), and 19, 21, and 25 (P ≤ 0.05, ANOVA; Fig. 5B). Similar effects were observed in LLC carcinoma tumors, and beginning on day 11, a significant retardation of tumor growth was observed in mice treated with lovastatin in combination with doxorubicin as compared with each of the remaining groups (days 11–23, P ≤ 0.005, ANOVA; day 25, P ≤ 0.05, ANOVA; Fig. 5C).

In the Ras-3T3 sarcoma, doxorubicin, when used alone, produced slight retardation of tumor growth as compared with the control group (P ≤ 0.05), lovastatin-treated cells versus nontreated cells (Mann-Whitney test).

To further evaluate the observed antitumor effects, we also assessed the tumor growth delay. The median tumor growth delay for mice treated with lovastatin was between 0 and 2 days, and the median tumor growth delay for mice treated with doxorubicin was between 0 and 4 days (Table 1). However, in all of our experiments, combined treatment with lovastatin and doxorubicin resulted in significant retardation of tumor growth, and the tumor growth delay was 6 days in three tumor models (Table 1). This delay in tumor growth was higher than a simple addition of the delays produced by any of the agents used alone, suggesting a synergistic mode of action for this therapeutic combination.

All mice in our experiments died with developing tumors between day 21 and day 63 after inoculation of tumor cells. However, no prolongation of the survival time was observed in either of the experimental groups.

Serum cTnT Indicates Protective Activity of Lovastatin on Doxorubicin-induced Cardiac Injury. Serum levels of cTnT become elevated in patients with minimal myocardial cell damage (18), making this enzyme an attractive, noninvasive marker of anthracycline-related cardiotoxicity both in humans (19) and in murine experimental models (20). Therefore, in our experiments, we monitored serum levels of cTnT during combination therapy with lovastatin and doxorubicin. Pretreatment cTnT level (number of animals, 21) was 0.29 ± 0.03 ng/ml (range, 0.0–0.67 ng/ml). This level remained relatively unchanged in animals receiving lovastatin or saline solution for 2 weeks (0.53 ± 0.05 and 0.28 ± 0.06 ng/ml, respectively; Fig. 6). As shown in Fig. 6, a statistically nonsignificant increase in cTnT level (3.26 ± 2.74 ng/ml; range, 0.67–14.87 ng/ml) was observed in mice treated for 1 week with a cumulative dose of 5 mg/kg doxorubicin (Friedman test, nonparametric repeated
Fig. 5 Effects of treatment with lovastatin and/or doxorubicin on (A) Ras-3T3, (B) Colon-26, and (C) LLC tumor growth. Measurements of tumor diameter started on day 5 or 7 after inoculation of tumor cells. Mice were injected with (↓) lovastatin (15 mg/kg/day, i.p.) and/or (open arrow) doxorubicin (2.5 mg/kg/dose, i.p.). Logarithmic (log10) scaling. Data represent the mean relative tumor volume ± SE. *, $P \leq 0.05$ versus each of the remaining groups; **, $P < 0.01$ versus each of the remaining groups; ***, $P < 0.005$ versus each of the remaining groups; †, $P \leq 0.05$ versus control group (Kruskal-Wallis test, followed by Dunn’s multiple comparisons test).
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Discussion

Lovastatin is a drug that is widely used to treat hypercholesterolemia (2), and there is also a growing interest in its potential antitumor effects. The mechanisms responsible for the cytostatic/cytotoxic effects of lovastatin have not been definitively elucidated. Inhibition of isoprenylation of oncogene product \( p21^{ras} \) and loss of its signal capacity (3), inhibition of phosphatidylinositol 3-kinase (21), suppression of mitogen-activated protein kinase activity (22), and inhibition of the proteasome (4) have all been suggested to participate in these effects. Recently, cyclin-dependent kinase inhibitor p27\(^{Kip1}\) has been proposed to be an important mediator of the growth inhibition effects of lovastatin (4).

In our \textit{in vitro} studies, lovastatin was used in combination with doxorubicin. This combination was found to be more effective in decreasing the viability of murine Ras-3T3,Colon-26, and LLC tumor cells than each agent alone. Furthermore, in the case of Colon-26 cells, a synergy of these two drugs was revealed. As suggested recently (23), lovastatin may increase the vulnerability of tumor cells to the action of other chemotherapeutic agents by specifically targeting drug-resistant P-glycoprotein-expressing tumor cells. This observation may be considered as a possible explanation for the increase in cytotoxicity of doxorubicin in combination with lovastatin in our study, although an augmentation of apoptosis induced by both agents cannot be ruled out (13).

In this study, Ras-3T3 cells were significantly less sensitive to the action of doxorubicin compared with nontransformed NIH-3T3 cells. These results are in accordance with previous reports showing resistance to doxorubicin in tumor cells transformed by \textit{ras} oncogene (24, 25). Lovastatin was able to reverse the resistance of Ras-3T3 cells to doxorubicin. Restoration of doxorubicin sensitivity in \textit{ras}-transformed tumor cells by lovastatin has been observed previously in osteosarcoma cells (26). The significance of these observations may be underscored by the high frequency of \textit{ras} mutations present in human tumors (27).

We then examined whether the strengthened antitumor effects of combination treatment with doxorubicin and lovastatin observed \textit{in vitro} could have any relevance for \textit{in vivo} tumor therapy. In all three tumor models, lovastatin potentiated the antitumor activity of doxorubicin, resulting in retardation of tumor growth as compared with both agents used alone and suggesting synergistic augmentation of antitumor effectiveness in mice treated with a combination of both drugs. It should be mentioned that lovastatin has already been shown to increase the antitumor activity of cisplatin (10) and tumor necrosis factor \( \alpha \)

Table 1

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>LLC (35 mm(^3))</th>
<th>C26 (45 mm(^3))</th>
<th>Ras-3T3 (60 mm(^3))</th>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lovastatin + doxorubicin</td>
<td>6</td>
<td>6</td>
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</tr>
</tbody>
</table>

Fig. 6 Effects of doxorubicin and/or lovastatin on serum cTnT concentrations in mice. Mice were treated i.p. for either 5 weeks (lovastatin) or 3 weeks (doxorubicin) as described in “Materials and Methods.” Blood was drawn on days 1, 15 (before the initial injections of doxorubicin), 21 (after two doses of doxorubicin), and 35 (after six doses of doxorubicin), and serum levels of cTnT were measured. Data are expressed as the mean ± SE of the serum level of cTnT in a group of five to six mice receiving the same therapy. **, \( P < 0.01 \) versus initial cTnT levels (Friedman test, followed by Dunn’s multiple comparisons test); \textit{ns}, nonsignificant.


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