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Original Paper

Potentiated Antitumour Effects of Cisplatin and Lovastatin Against MmB16 Melanoma in Mice

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Lovastatin, the drug used in the treatment of hypercholesterolaemia, has previously been reported to exert synergistic antitumour activity in a melanoma model in mice when used together with some immune response modifiers. In this study, we examined the antitumour effect of cisplatin augmented by its combined application with lovastatin, both *in vitro* and *in vivo*, in a murine melanoma model. The results of this study suggest that lovastatin may enhance the therapeutic effects of cisplatin in the treatment of malignant melanomas. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: lovastatin, cisplatin, melanoma, tumour therapy
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INTRODUCTION

As the incidence of melanoma is rising and its treatment remains disappointing, new approaches to the therapy of this malignant tumour are urgently needed. Cisplatin (CDDP) is one of the most widely used chemotherapeutics but the response rate in the treatment of malignant melanoma in humans is still poor. However, combining cisplatin with other chemo- and immunotherapeutics seems to be more effective [1, 2].

Competitive inhibitors of 3-hydroxy-3methylglutaryl-coenzyme A (HMG-CoA) reductase (the statins) inhibit the synthesis of mevalonic acid, which is an essential precursor of isoprenoid compounds including cholesterol, dolichol and ubiquinone [3]. Some proteins, such as laminin B and Ras proteins, are post-translationally modified by isoprenylation [4]. The isoprenylation of Ras proteins is critical for their cellular function, and inhibition of isoprenylation abolishes the transforming activity of mutated Ras proteins [5]. Lovastatin, one of the HMG-CoA-reductase inhibitors, has been shown to exert antiproliferative activity on tumour cells *in vitro* [6–8] and to demonstrate antitumour effects in experimental tumour models [7, 9–12]. The results of a phase I study of lovastatin administered to cancer patients have already been reported [13]. Combining statins with other antitumour agents may enhance their antitumour efficacy and indicate directions to be pursued in clinical trials. The antitumour activity of lovastatin was synergistically enhanced by tumour necrosis factor-α [6, 10] and simvastatin showed synergistic antitumour effects with N,N′-bis(2-chlorethyl)-*N*-nitrosourea (BCNU, carmustine) and β-interferon [14].

It has previously been shown that lovastatin arrests various cells in the G1 phase of the cell cycle [8, 15]. According to some reports, G1-arrested cells show increased sensitivity to cisplatin [16, 17]. Therefore, lovastatin could make tumour cells more vulnerable to the action of cisplatin, enhancing its antitumour activity. In our present study, we examined the influence of lovastatin on the antitumour activity of cisplatin in the MmB16 melanoma model in mice.

MATERIALS AND METHODS

Animals

Female (C57BL/6×DBA/2)F1 mice, thereafter called $B_6D_2F_1$, were used throughout the experiment. Breeding pairs were originally obtained from the Inbred Mice Breeding Centre (Institute of Immunology and Experimental Therapy, Wrocław, Poland). All mice were kept under conventional conditions during the experimental period and were 13 weeks old at the time of inoculation of tumour cells.

Reagents

Lovastatin in the inactive lactone form was kindly provided by Dr A.W. Alberts (Merck, Sharp & Dohme Research Laboratories, Rahway, New Jersey, U.S.A.). It was converted to the active form by dissolving in ethanol, heating at 50°C in 0.1 N NaOH, neutralising with HCl and adjusting with distilled water to a final concentration of 8 mg/ml. This stock solution was stored frozen (−27°C).

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Two forms of cisplatin (CDDP) were used. In the *in vivo* experiments, Platidiam (cisplatin 10 mg, mannitol 100 mg, sodium chlorate 90 mg in 10 ml of sterile water for injection; Lachema, Brno, Czech Republic), which is a cisplatin formula applied in cancer therapy in humans, was applied. In the *in vitro* experiments, pure cisplatin (Sigma, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was used.

For *in vivo* experiments all agents were diluted with 0.9% NaCl (saline) before injection.

Tumour cells

MmB16 melanoma cells, a metastasising subclone of B16 melanoma adapted to growth *in vitro*, was kindly provided by Dr C. Radzikowski (Institute of Immunology and Experimental Therapy, Wrocław, Poland). The original B16 melanoma was obtained from the National Cancer Institute, Bethesda, Maryland, U.S.A. Cells were maintained in RPMI-1640 (Gibco BRL, Paisley, U.K.) supplemented with antibiotics, 2-mercaptoethanol (50 μM), L-glutamine (2 mM) and 10% fetal calf serum (all from Gibco BRL) (culture medium) and passaged every 3–4 days after a short trypsinisation with trypsin/EDTA (Gibco BRL).

MTT assay

The cytostatic/cytotoxic effects of cisplatin and/or lovastatin on melanoma cells *in vitro* were tested in a standard MTT assay. 100μl aliquots containing 8×10^3 tumour cells were dispensed into 96-well microtitre plates (Corning, Bibby Sterlin Ltd, Staffordshire, U.K.). Plates were incubated overnight at 37°C in 5% CO_2 and then serial dilutions of lovastatin were added (100 μl, final concentrations 0.25–2 μM) to a final volume of 200 μl. After an incubation period of 72 h, the medium was removed and the cells were washed three times in culture medium. Then serial dilutions of cisplatin (50 μl, final concentrations 1.25–10 μg/ml) and lovastatin (50 μl, final concentrations 0.25–2 μM) were added and supplemented with culture medium to a final volume of 200 μl. After an incubation period of 24 h, a standard MTT assay was performed as described in detail previously [18]. Briefly, 25 μl 3-(45-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) (Sigma) solution was added to each well. The plates were centrifuged 4 h later (350 *g*/10 min) and 200 μl supernatant was carefully removed from the wells and replaced with 200 μl acid dimethylsulphoxide. Complete solubilisation of formazan crystals was achieved by repeated pipetting of the solution. The plates were read on an ELISA-reader (SLT-Labinstruments GmbH, Salzburg, Austria) using a 550 nm filter. The means and standard deviations were determined for quadruplicate samples. The cytostatic/cytotoxic effect of cisplatin and/or lovastatin was expressed as the relative viability (% of control) and was calculated as follows:

$$\begin{aligned}\text{Relative viability} = [&(\text{experimental absorbance} \\ &- \text{background absorbance}) \\ &\div (\text{absorbance of untreated controls} \\ &- \text{background absorbance})] \times 100\%\end{aligned}$$

Clonogenic cell survival assay

Cells in expotential growth were exposed to concentrations of cisplatin varying from 1–5 μg/ml for a period of 1 h (37°C, 5% CO_2). After removing cisplatin by washing twice with phosphate-buffered saline (PBS), the cells were removed with trypsin-EDTA, counted and replated in triplicate at density of 10^3 cells in 60 mm Petri dishes. Then serial dilutions of lovastatin were added (final concentrations 1–4 μM). After an incubation period of 72 h the medium was removed, cells were washed and grown in the absence of lovastatin in culture medium for an additional 7 days; 10 days after plating, the plates were fixed and stained with 0.5% crystal violet in 20% methanol, and colonies with 50 or more cells were scored. The means and standard deviations were determined for triplicate samples. The survival fraction was determined as the ratio of the colony number observed after treatment to the number of colonies in the control dishes.

Drug interaction analysis

To examine an interaction between lovastatin and cisplatin, isobolanalysis was used as described in detail elsewhere [6]. Briefly, inhibition of cell proliferation was determined as described above. Equi-effective concentrations (concentrations of either drug alone or in combination, which gave equivalent inhibition of cell growth as compared to untreated control cells at $P \leq 0.01$, Student's *t*-test) were analysed. The interaction index for two drug combinations was computed according to the following equation:

$$\text{Interaction index} = \frac{\text{Lov}_c}{\text{Lov}_e} + \frac{\text{CDDP}_c}{\text{CDDP}_e}$$

where Lov_e and CDDP_e are concentrations of lovastatin and cisplatin, respectively, that produce some specified effect when used alone; and Lov_c and CDDP_c are concentrations of lovastatin and cisplatin, respectively, that produce the same effect when used in combination.

Synergy occurs when the interaction index is less than 1.0.

Animal experiments and dose-toxicity studies

On the day of inoculation, the cells were harvested from the cultures and washed twice in RPMI-1640 medium. 1×10^6 MmB16 melanoma cells in 20 μl medium were injected subcutaneously (s.c.) into the footpad of the right hind limb. In the preliminary experiments, in which the maximum tolerated dose (MTD) of cisplatin was established, melanoma-bearing mice were injected intraperitoneally (i.p.) on day 7 following inoculation of melanoma cells with a single dose of either cisplatin or 0.9% NaCl solution (control). Mice in each group were weighed, the tumour diameter was measured three times per week and the MTD was determined. A dose producing drug-related deaths or body weight loss of $\geq 20\%$ of the initial weight of the mice within one week after injection of cisplatin was considered as excessively toxic. In the experiments using combination therapy, antitumour activity was evaluated at the MTD of cisplatin.

To investigate the effects of the lovastatin, mice were injected intratumorally (i.t.) either for 7 consecutive days, starting from day 7 following tumour inoculation (days 7–13) (Experiment 1) or for 5 consecutive days, starting from day 7 following tumour inoculation (days 7–11 and then on days 14–18) (Experiment 2). Lovastatin was given at a dose of 100 μg per injection in 20 μl of saline. Injections of 20 μl of 0.9% NaCl (saline) were given as controls for the lovastatin treatment. Both the dose and the route of administration of

lovastatin used in this experiment were estimated on the basis of the results of our previous studies [10].

Treatment and monitoring

Treatment of melanoma started on day 7 after inoculation of tumour cells, at the moment when tumour nodules were already visible in the footpads. To investigate the effects of both drugs, melanoma-bearing mice were divided into four groups (5–6 mice) and injected in two different regimens:

(1) (a) Saline; (b) lovastatin (100 μg/day, i.t., on days 7–13); (c) cisplatin (10 mg/kg, i.p., single dose on day 7); (d) a combination of both drugs (100 μg lovastatin/day, i.t., on days 7–13 + single dose of cisplatin, i.p., on day 7).
(2) (a) Saline; (b) lovastatin (100 μg/day, i.t., on days 7–11 and on days 14–18); (c) cisplatin (10 mg/kg, i.p., on days 7 and 14); (d) a combination of both drugs (100 μg lovastatin/day, i.t., on days 7–11 and 14–18 + two doses of cisplatin, i.p., on days 7 and 14).

Local tumour growth was determined as described previously [18]. Briefly, footpad diameter was measured with calipers every second day, starting on the first day of treatment. Tumour size was calculated according to the formula:

Tumour diameter in the footpad
= {diameter of the tumour-bearing footpad}
− {diameter of the non-treated contralateral footpad}

Mice were observed daily for survival.

Statistical analysis

Differences between samples in tests *in vitro* and differences in tumour diameter in experiments *in vivo* were analysed for significance by Student's *t*-test (two-tailed). The data from *in vivo* studies were analysed additionally with the non-parametric ANOVA (Kruskal–Wallis) test followed by a Dunn's multiple comparisons test (Instat™, GraphPad Software, San Diego, California, U.S.A.). Survival time of animals after treatment was analysed for significance by log-rank survival analysis. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Analysis of the influence of cisplatin and lovastatin on tumour cell proliferation in vitro

To assess the direct influence of lovastatin and cisplatin on *in vitro* growth of melanoma cells, we performed an MTT assay. When used alone, both lovastatin and cisplatin exhibited dose-dependent cytostatic/cytotoxic activity on MmB16 melanoma cells (Figure 1). Moreover, lovastatin and cisplatin demonstrated potentiated antitumour effects *in vitro* when used in combination (Figure 1).

To determine whether lovastatin and cisplatin exert synergistic cytostatic/cytotoxic effects on the treated melanoma cells, isobolanalysis as described by Berenbaum was used [19]. According to this analysis, synergy occurs when the interaction index is less than 1.0. The interaction index for lovastatin and cisplatin used in combination in MmB16 cells was 0.625 (Figure 2). Thus the results of our *in vitro* experiments suggest that potentiated antitumour effects of combination therapy with lovastatin and cisplatin may result from the synergistic cytostatic/cytotoxic interaction of these two drugs acting directly on melanoma cells.

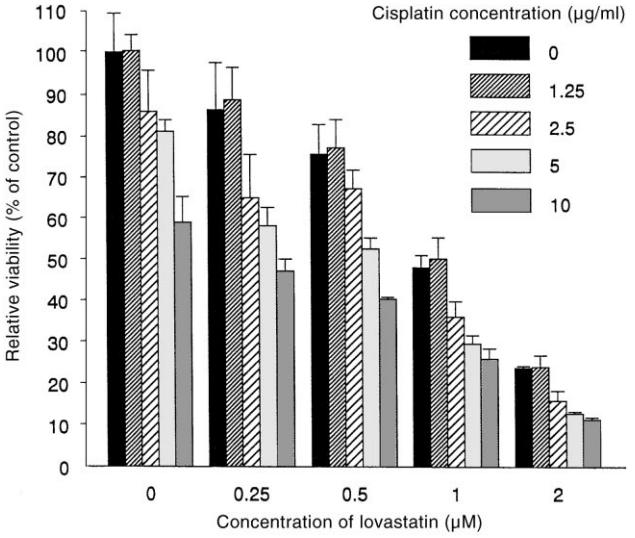

Figure 1. Effects of cisplatin and lovastatin on MmB16 cells *in vitro*. Cytostatic/cytotoxic effects, expressed as relative viability (% of untreated control), were tested in an MTT assay. Bars (mean ± S.D.).

To be able to distinguish between inhibition of cell growth and drug-induced cell killing, a clonogenic cell survival assay was performed (Figure 3). The survival curve of melanoma cells obtained by the colony formation assay treated with cisplatin alone differs significantly from those treated both with

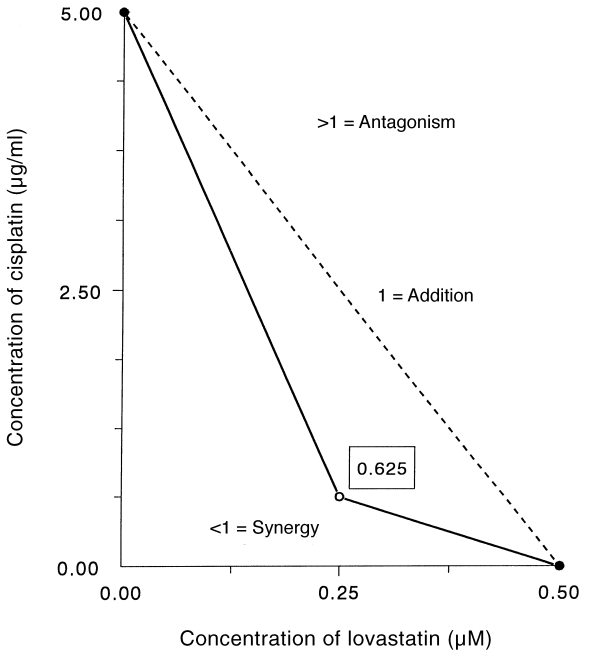

Figure 2. An isobologram analysis depicting interaction between lovastatin and cisplatin in inhibiting growth of MmB16 cells. The solid line represents concentrations of both drugs which cause a 30% inhibition of cell proliferation (IC_{30}). The broken line represents the hypothetical amounts of both drugs required to cause the same decrease in the cell proliferation as if the interactions were additive. The number in the box indicates the interaction index computed as described in Materials and Methods.

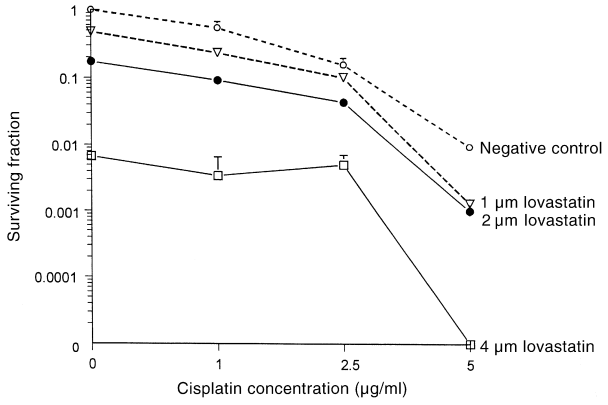

Figure 3. Clonogenic cell survival of the MmB16 cell line treated with different concentrations of cisplatin for 1 h at 37°C in 5% CO_2 and/or lovastatin for 72 h. The surviving fraction of cells plated was plotted against the concentration of cisplatin and represents triplicate samples (mean ± S.D.).

cisplatin and various concentrations of lovastatin. The MmB 16 cells were significantly more sensitive to treatment with cisplatin combined with lovastatin as compared to the treatment with either agent alone ($P < 0.05$, Student's *t*-test) (Figure 3).

Antitumour effects of combination therapy with cisplatin and lovastatin on the MmB16 melanoma in vivo

To examine whether our *in vitro* observations could have potential therapeutic significance, animal studies were performed. In the preliminary dose-toxicity study, an MTD of cisplatin was established at 10 mg/kg body weight (Table 1). This dose was used in experiments where the combined effects of lovastatin and cisplatin were tested (100 μg lovastatin/day on days 7–13 and 10 mg/kg cisplatin on day 7). Although cisplatin when used alone at this dose did not inhibit tumour growth, a significant antitumour effect came to light when lovastatin was included in the therapeutic regimen (Figure 4a). Beginning from day 15, a significant retardation of tumour growth was observed in mice treated with lovastatin in combination with cisplatin compared with each of the other groups ($P < 0.05$) (Figure 4a). Similar effects were observed with a slightly different therapeutic regimen (100 μg of lovastatin/day for 9 days and 10 mg/kg cisplatin on days 7 and 14) was used (Figure 4b).

By day 13, after inoculation of tumour cells, the footpads of all mice in the group in which lovastatin was injected had an increased diameter compared with those of the other groups (Figure 4a). This was caused by oedema developing as a result of local lovastatin injections.

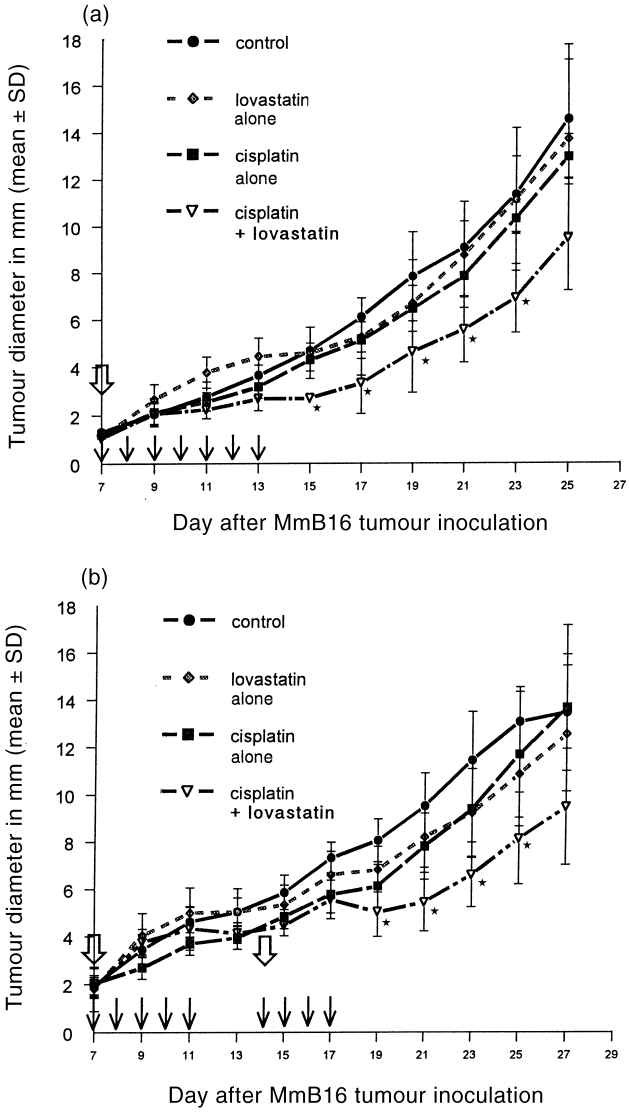

Figure 4. Effects of treatment with cisplatin and lovastatin on MmB16 tumour growth. Measurements of tumour diameter started on day 7 after inoculation of tumour cells. (a) Experiment 1. Mice were injected with (↓) lovastatin (100 μg/day, i.t., on days 7–13) and/or (⇓) cisplatin (10 mg/kg, i.p., single dose on day 7). (b) Experiment 2. Mice were injected with (↓) lovastatin (100 μg/day, i.t., on days 7–11 and on days 14–18) and/or (⇓) cisplatin (10 mg/kg, i.p., two doses on days 7 and 14). *$P < 0.05$ (Kruskal–Wallis test followed by a Dunn's multiple comparisons test) in comparison to each of the remaining groups.

Table 1. *Toxicity study to evaluate an MTD of cisplatin in MB*16 *melanoma-bearing B6D2F*1 *mice*

Dose (mg/kg)	Schedule (d)	Average body weight loss in grams/ mouse on day 11	Mortality by day 21 (No. died/No. injected)	Mean survival time (d ± S.D.)	Comments
5	7	−0.04	0/5	29.4 ± 3.9	No activity
10	7	−2.7	0/5	30.2 ± 3.7	MTD, marginal antitumour activity, 12.4% BWL
20	7	−5.2	6/6	12.33 ± 0.8	Excessively toxic, all mice died by day 13, 25% BWL

Seven days after inoculation of MmB16 cells, melanoma-bearing mice were divided into three groups and injected intraperitoneally (i.p.) with a single dose of cisplatin. Mice in each group were weighed and observed daily for survival; MTD, maximum tolerated dose; BWL, body weight loss.

All mice in our experiments died from developing tumours between days 26 and 59 following inoculation of tumour cells due to multiple metastases in the lungs and liver. Although there was no significant prolongation of the survival time in mice treated with both drugs in Experiment 1, there was a significant increase in the survival time in this group in Experiment 2 (Figure 5). Mice from the control group had a mean survival time of 41.4 ± 10.2 with a median survival time of 43 days. Combined treatment with lovastatin and cisplatin resulted in significant prolongation of the mean survival time (54.4 ± 4.2 days) and the median survival time (56 days) ($P < 0.05$ by log-rank survival analysis compared with each of the other three groups of mice).

DISCUSSION

Tumour chemotherapy is limited by the toxicity of antitumour drugs to normal tissues and by the development of resistance to these drugs. Combination therapy using two or more chemotherapeutic drugs is one of the most common strategies used in current oncology to overcome these obstacles. Therefore, we decided to test the combined effects of lovastatin and cisplatin in the murine melanoma model.

We demonstrated that cisplatin and lovastatin used in combination exerted potentiated cytostatic/cytotoxic effects on MmB16 cells (Figure 1). Based on the isobolanalysis, this drug interaction could be defined as synergistic (Figure 2). Both of these agents also exhibited potentiated cell killing as assessed by the colony-forming assay (Figure 3). The data obtained in our *in vitro* experiments were confirmed in the *in vivo* study. Although neither cisplatin nor lovastatin produced any notable antitumour effects when used alone, significant retardation of tumour growth was observed as a result of their combined application (Figure 4). The data obtained from our *in vivo* study indicate that combined application of both agents may prolong the survival time of the tumour-bearing mice (Figure 5).

Locoregional administration is proposed as another approach to limit toxicity and to reduce effective doses of drugs used in the treatment of malignant melanoma [20]. In our previous experiments, lovastatin applied at the MTD (1 mg per mouse for 12 consecutive days) did not exert any significant antitumour effects against MmB16 melanoma when given intraperitoneally (data not shown). In contrast, locoregional administration of lovastatin at the dose of 200 μg for 5 consecutive days resulted in retardation of tumour growth [10]. Therefore, in our present experiment, we applied lovastatin intratumorally, though at a dose of 100 μg. Lovastatin did not produce any antitumour effects when given alone at this dose (Figure 4).

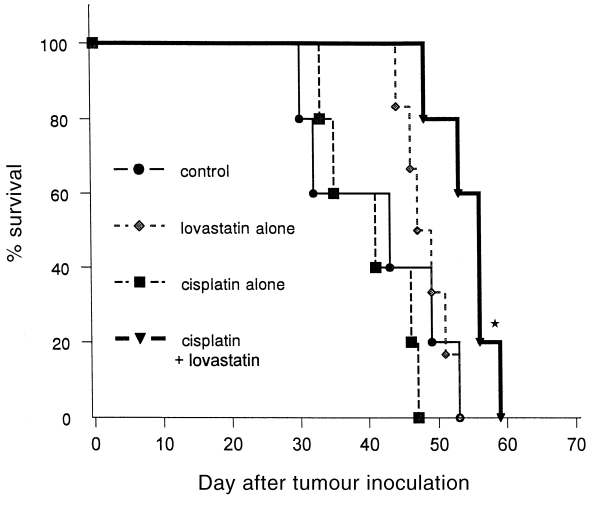

Figure 5. Effects of treatment with cisplatin and lovastatin on the survival time of MmB16 tumour-bearing mice. *$P < 0.05$ (log-rank survival analysis) in comparison to each of the other groups.

As described above, the footpads of all mice in the group in which lovastatin was injected had an increased diameter compared with those of the other groups due to oedema developing as a result of local lovastatin injections. However, unexpectedly this effect was not observed in mice treated with both lovastatin and cisplatin. Similar toxic effects were also observed in our previous studies [10] and are consistent with the results of clinical phase I trials demonstrating certain level of toxicities in tumour patients receiving lovastatin at doses much higher than those used to treat hypercholesterolaemia [13].

The precise mechanism underlying the potentiated antitumour effects of cisplatin and lovastatin remains to be elucidated. As lovastatin arrests various cells in the G1 phase of the cell cycle [8, 15] and G1-arrested cells seem to show increased sensitivity to cisplatin [16, 17], it could be suggested that lovastatin makes tumour cells more vulnerable to the action of cisplatin, enhancing its antitumour activity.

With their ability to lower blood cholesterol levels, statins are now the most commonly prescribed drugs at many medical institutions [21]. A certain number of patients develop tumours while receiving statin therapy, although no difference in the incidence of cancer between treated and placebo groups has been reported [22]. With the growing application of statins, the number of statin-treated patients developing tumours is likely to increase. Therefore, the interaction between statins and chemotherapeutics used for tumour treatment should be examined. One of the interesting and promising aspects of the interaction between statins and chemotherapeutics in tumour therapy, which has already been revealed, is the ability of statins to target specifically drug-resistant P-glycoprotein-expressing tumour cells [23]. In our present study, we demonstrated that in mice receiving lovastatin, the antitumour activity of cisplatin was significantly potentiated.

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