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ORIGINAL ARTICLE

Synergistic anti-tumor efficacy of lovastatin and protein kinase C-beta inhibitor in hepatocellular carcinoma

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Abstract
Purpose Hepatocellular carcinoma (HCC) is characterized by hypervascularity and chemoresistance. Protein kinase C (PKC) participates in cancer progression by enhancing anti-apoptotic signals, angiogenesis, and chemoresistance. Statins have a selective anti-cancer effect due to over-expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) in cancer cells, but statins may also activate PKC. Thus, we hypothesized that simultaneous treatment with statin and PKC inhibitor might synergistically enhance their anti-tumor efficacies against HCCs.
Methods Hepatocellular carcinoma cell growth was assessed using MTS assays, apoptotic cell death by DAPI staining, and apoptotic signaling cascades were explored by immunoblotting. An in vivo model of HCC was established in C3H mice intradermally implanted with MH134 cells. Lovastatin and/or a PKC inhibitor (enzastaurin) was subsequently administered. Anti-tumor efficacies were evaluated by measuring tumor volumes and quantifying apoptotic cells and microvessel densities (MVD).
Results Co-treatment with lovastatin and enzastaurin was found to synergistically suppress HCC cell growth in vitro. Lovastatin induced HCC cellular apoptosis by activating mitochondrial apoptotic signals, and although enzastaurin alone did not induce apoptosis, its addition significantly enhanced lovastatin-induced apoptosis. This enhanced apoptosis was attributed to increased caspase-9 activation in these cells. Moreover, tumor growth was significantly suppressed in mice co-treated with lovastatin and enzastaurin, and percentages of TUNEL-positive cells were significantly increased and MVDs were significantly decreased in those mice.
Conclusion Combinatorial treatment with statin and PKC inhibitor was found to enhance anti-tumor efficacy in vivo and in vitro. Further studies are warranted to prove anti-tumor efficacy of this potential therapy in human HCCs.

Keywords Hepatocellular carcinoma · Statin · Protein kinase C · Apoptosis

Abbreviations

HCC	Hepatocellular carcinoma
PKC	Protein kinase C
HMG-CoAR	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
MVD	Microvessel densities
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
XIAP	X-linked inhibitor of apoptosis protein

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Introduction

Hepatocellular carcinomas (HCCs) are frequently derived from chronic liver diseases, such as viral hepatitis or alcoholic liver disease [1]. The incidence of HCC is increasing in Europe, the United States, and in certain areas in Asia [2]. Currently, HCC ranks as the third leading cause of cancer-related death [3]. HCC surveillance programs have led to an increase in the adoption of radical therapies, such as resection, liver transplantation, and percutaneous ablation, which are currently applicable for early or small HCCs [4].

Despite the institution of surveillance programs, HCCs often present at an advanced stage [5]. Transarterial chemoembolization (TACE) can confer survival benefit in such patients, but only for selected patients [6], and consequently, systemic chemotherapy is often the only alternative in these patients [1, 7]. However, systemic chemotherapies show minimal anti-tumor efficacy against HCC due to their innate resistance to chemotherapeutic drugs [8, 9]. Moreover, cytotoxic chemotherapeutics can cause liver injury in patients with underlying liver cirrhosis, and the resulting inadequate dosages adopted eventually attenuate anti-tumor efficacy [10]. Thus, there is an urgent need to develop efficient systemic strategies that focus on specific molecular targets without compromising liver function.

The molecular pathogenesis of HCC is complex and involves the abnormal clonal expansion of dysplastic hepatocytes, anti-apoptotic signaling, and the stimulation of angiogenesis-associated growth factors [11]. In particular, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) and its receptors are frequently over-expressed in cancer cells [12]. The statins, which inhibit HMG-CoAR, are widely used, safe lipid-lowering drugs. In accord with the selective over-expression of HMG-CoAR in cancer cells, statins have a selective anti-cancer effect, which is attributed to the induction of tumor cell-specific apoptosis in certain cancer cell lines, whereas they have little effect on human hepatocyte viability [13, 14]. In particular, lovastatin is known to potently induce the apoptosis of mammary cancer cells [15]. These observations clearly suggest that statins have therapeutic potential for the treatment of HCCs.

Protein kinase C (PKC) activation is involved in cancer progression via enhancing anti-apoptotic signals, angiogenesis, and chemoresistance [16–18]. Thus, the inhibition of PKC signaling offers a potential means of enhancing pro-apoptotic signals and chemosensitivity, and of inhibiting angiogenesis. However, statins may also activate intracellular PKC signals, which probably reduce their anti-apoptotic efficacies [19]. Recently, enzastaurin (a novel PKC inhibitor) was demonstrated to have tolerable toxicities by several clinical studies [20, 21], which supports its use as an HCC treatment, particularly in combination with statins. Therefore, we hypothesized that simultaneous treatment with statin and enzastaurin might synergistically enhance their anti-tumor efficacies against HCC.

To test this hypothesis, we formulated the following questions: (1) does statin enhance PKC activity in HCC cells? (2) Does simultaneous treatment with statin and PKC inhibitor synergistically enhance HCC cell apoptosis? (3) What is the mechanism of this apoptotic enhancement? (4) Is this effect also present in an in vivo HCC model? Collectively, the results of the current study demonstrate that statin activates PKC signals, which counteracts statin-induced HCC cell apoptosis, and that PKC inhibition enhances statin-induced caspase-9 activation. Moreover, the synergistic anti-tumor efficacy was confirmed in an animal model, implicating that further efforts are needed to show anti-tumor efficacy of this potential therapy in patients with advanced HCCs.

Materials and methods

Materials and reagents

Lovastatin was obtained from the Choong Wae Pharmaceutical Co. (Seoul, Korea). The inactive lactone form of lovastatin was converted into its active dihydroxy-open sodium salt form, as described previously [22], and stock solutions (10 mM) were stored frozen at −20°C for the in vitro study. For the in vivo study, lovastatin (as the sodium salt) was dissolved in a solvent mixture composed of ethanol and propylene glycol (9:1 v/v containing 2 g/L of lovastatin) prior to injection. The PKCβ-selective inhibitor, enzastaurin (LY317615.HCl) was donated by Eli Lilly and Company (Lily Research Labs, Indianapolis, IND, USA). Enzastaurin was dissolved in DMSO at a concentration of 10 mM and stored at −20°C for the in vitro study. For the in vivo study, enzastaurin was resuspended in 5% dextrose in water at a concentration of 112.5 mg/kg and sonicated in a warm bath.

Cell culture

Huh-7 cells (a well differentiated human HCC cell line) were used for the in vitro experiments, and MH134 cells (a mouse HCC cell line) were used for the in vivo experiments [23, 24]. Huh-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100,000 U/L), and streptomycin (100 mg/L), whereas MH134 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin

(100,000 U/L), and streptomycin (100 mg/L). Cells were serum starved overnight to avoid serum-induced signaling prior to all experiments performed in this study.

Measurement of PKC activity

Huh-7 cells were cultured in 25 cm^2 culture flasks. After incubation for the indicated time period with test agents, cells were collected in ice-cold phosphate buffered saline (PBS) and centrifuged at 10,000×*g* for 10 min at 4°C. PKC activity was measured based on the phosphorylation of a PKC substrate peptide using Non-Radioactive ELISA Protein Kinase Assay Kits (Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions. Results are presented as the percentage of PKC activity under control conditions.

Cell proliferation

Cell proliferation was measured using the CellTiter 96 Aqueous One Solution cell proliferation tetrazolium salt assays (Promega, Madison, WI, USA). Following each treatment, 20 μL of dye solution was added into each well in 96-well plate and incubated for 2 h. Subsequently, absorbance was recorded at 490 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

Quantitation of apoptosis

Levels of apoptosis were determined using the nuclear binding dye 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) to quantify apoptotic cells by fluorescence microscopy (Zeiss, Germany). Briefly, DAPI was added to the treated cells for 30 min, after which they were examined by fluorescence microscopy. Apoptotic cells were identified by chromatin condensation and nuclear fragmentation. Percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted × 100. A minimum of 400 cells were counted for each treatment.

Immunoblot analysis

Cells were lysed for 20 min on ice using lysis buffer (50 mM Tris–HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium DC; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μg/mL of each of aprotinin, leupetin, pepstatin; 1 mM Na_3VO_4; 1 mM NaF) and centrifuged at 14,000×*g* for 10 min at 4°C. The same amount of total protein (35–50 μg) was loaded on the gels. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blotted with appropriate primary antibodies, and incubated with peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, USA). Bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to Kodak X-OMAT film. Rabbit anti-caspase-8, rabbit anti-caspase-9, rabbit anti-caspase-3 and mouse anti-cytochrome *c* were obtained from BD Pharmingen (San Diego, CA, USA). Goat anti-AIF and goat anti-actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and mouse anti-Smac/DIABLO was obtained from BD Transduction Laboratories (San Jose, CA, USA).

Immunoprecipitation analysis

Cell lysates were incubated with rabbit anti-sera to the X-linked inhibitor of apoptosis protein (XIAP) (Cell Signaling Technology Inc., Beverly, MA, USA) overnight at 4°C. Immune complexes were immunoprecipitated with 20 μL of protein A/G PLUS agarose beads (Santa Cruz Biotechnology Inc.) for 2 h at 4°C and then washed five times with lysis buffer. Polypeptides were then resolved by boiling with 35 μL of Laemmli sample buffer for 5 min, and immunoprecipitates obtained were immunoblotted with anti-caspase-9 (BD Pharmingen) and anti-XIAP antibody (Cell Signaling Technology Inc.).

Animals

Animal experiments were performed using 4-week old male C3H/He mice (Charles River Laboratories, Wilmington, MA, USA). Mice were housed under specific pathogen-free conditions, and treated in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

Mouse HCC model

We used a previously described established s.c. HCC mouse model [25]. Briefly, 2.5×10^5 viable MH134 cells suspended in 0.1 mL of RPMI-1640 were injected subcutaneously to produce a bleb in right flanks of C3H/He mice. When tumor volumes reached $0.5 \sim 1.0$ cm^3, lovastatin (25 mg/kg) was administered i.p. with/without enzastaurin (112.5 mg/kg), which was administered by gavage daily for 14 consecutive days. Fifteen days after administering lovastatin with/without enzastaurin, mice were killed by exsanguination via cardiac puncture under general anesthesia induced by isoflurane inhalation. Tumor masses and liver tissues were harvested, fixed in 10% formaldehyde, and cryopreserved.

Tumor growth kinetics

An exponential model was selected to describe tumor growth kinetics and the equation used after lovastatin

administration was: $V = V0 \times \exp(k \times T)$ where $V0$ and V are the tumor volumes at baseline and T days later, respectively, and k is the growth rate constant related to tumor doubling time. Data were analyzed using a nonlinear mixed effect modeling (NONMEM) software program (version V, level 1.1, Double Precision), using the first-order conditional estimation method and the PRED routine [26]. Inter-individual variabilities for $V0$ and k were modeled using exponential random effect models. For example, the tumor volume at baseline was modeled as $V0_i = V0 \times \exp(\eta_i)$, where $V0_i$ is the tumor volume at baseline for individual i, $V0$ is the typical value for tumor volume at baseline for the population, and η_i is a normally distributed random variable with mean zero and variance ω^2_{V0}.. In addition, residual variability was modeled using a combined additive and proportional error model. The combined error model is described by $Y_{ij} = \hat{Y}_{ij} \times (1 + \varepsilon_{ija}) + \varepsilon_{ijp}$ where Y_{ij} is the jth observed tumor volume in individual i, $\hat{Y}_{ij}$ is the jth predicted tumor volume in individual i, and ε_{ija} and ε_{ijp} are normally distributed random variables for individual i and measurement j, with mean zero and variances σ^2_a and σ^2_p, respectively. The minimal objective function value (OFV) calculated by NONMEM was equal to -2 times the log likelihood of the data. The hypothesis test between nested models was performed using the likelihood ratio test, in which a change in OFV approximates the χ^2 distribution, and one model was preferred to the other when the decrease in OFV was more than 3.84 (the critical value for the χ^2 distribution at $P = 0.05$ with 1 degree of freedom). The magnitudes of inter-individual and residual variabilities were expressed as coefficients of variation (%CV), approximated by the square root of the variance estimate. Goodness-of-fit was determined using the OFV and by visual inspection of the scatter plots of data versus the population and individual predictions, and versus weighted residuals.

To evaluate treatment group effects, a categorical group variable was incorporated while assuming that each group had a unique growth rate constant value or that some groups had the same value. For the final model, the stepwise forward addition followed by backward elimination process was used. Treatment group effects were considered significant when the addition of this variable resulted in an OFV decrease of >3.84 and the elimination of this variable resulted in an OFV increase of >3.84. For convenience, tumor doubling times were calculated from the growth rate constant estimates using, $\mathrm{Td} = \ln 2/k$; where Td is the tumor doubling time, k is the growth rate constant.

Apoptosis

Apoptosis in tumor tissue was investigated by TUNEL staining using ApoTag Peroxidase In Situ Apoptosis Detection Kits (Chemicon International, Temecula, CA, USA) after fixing fresh tissue in 4% paraformaldehyde. Positive TUNEL cells were counted in six different high-power fields at ×400 and averaged. Cell numbers are expressed as percentages of total cells and these are referred to as apoptotic indexes.

Microvessel density

Immunohistochemical staining for anti-CD 31 (Vector Laboratories, Burlingame, CA, USA) on paraffin-embedded sections was performed using Vectastain Elite ABC Kits (Vector Laboratories). CD 31-positive microvessels within the most vascular areas of tumor tissues were counted in six different high-power fields at ×400 and averaged. Intratumoral mean microvessel density (MVD) was expressed as numbers of microvessel/mm^2.

Statistical analysis

All data represent at least three independent experiments and are expressed as means ± SDs. Statistical evaluations of numeric variables in each group were conducted using the Mann–Whitney U test and the Kruskal–Wallis test. All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL, USA). We considered $P < 0.05$ as statistically significant.

Results

Lovastatin enhanced PKC activity in a human HCC cell-line

Baseline PKC activities in Huh-7 cells were measured by ELISA, and relative changes in PKC activities were assessed 30 min and 1 h after 50 μM lovastatin treatment. As shown in Fig. 1, lovastatin significantly enhanced PKC activity 30 min later (124.1 ± 12.4% SD; $P < 0.05$ vs. pretreatment), confirming that it activates PKC signals in HCC cells.

Lovastatin and enzastaurin synergistically suppressed human HCC cell growth

We next evaluated growth suppression by lovastatin and/or enzastaurin in Huh-7 cells using MTS assays. As shown in Fig. 2, lovastatin treatment dose-dependently suppressed Huh-7 cell growth, and resulted in significant growth suppression at a concentration of 50 μM. On the other hand, enzastaurin at 5 μM did not cause significant growth suppression, but the addition of 50 μM lovastatin significantly suppressed cell growth as compared with 50 μM

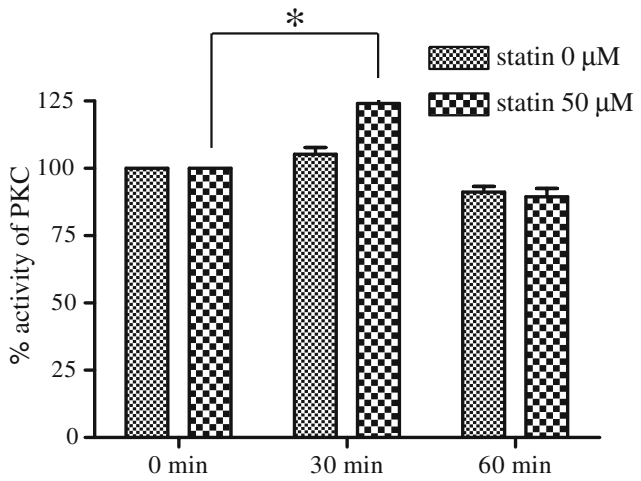

Fig. 1 Lovastatin-induced PKC activity. Huh-7 cells were treated with lovastatin (50 μM) for the indicated time period. At each time point, PKC activities, expressed as % of control, were measured by ELISA. Columns, the means of three independent experiments; *bars*, SD. * $P < 0.05$, versus pretreated control

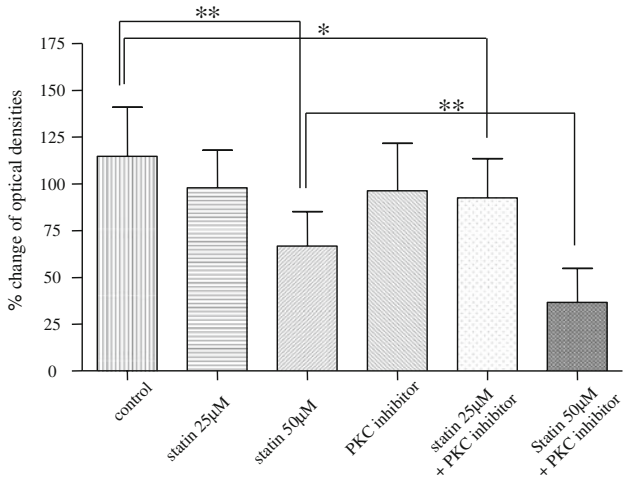

Fig. 2 Lovastatin/enzastaurin co-treatment suppressed HCC cell growth. Huh-7 cells were treated with lovastatin (0–50 μM) and/or enzastaurin (0, 5 μM) for 24 h. Cell growths were determined using MTS assays. Columns, the means of three independent experiments; *bars*, SD. * $P < 0.05$, ** $P < 0.01$ by 1-way ANOVA

lovastatin alone. These findings suggest that lovastatin and enzastaurin act synergistically to inhibit HCC cell growth.

Enzastaurin enhanced lovastatin-induced apoptosis in HCC cells

We next evaluated if enzastaurin-enhanced, lovastatin-induced growth suppression was due to increased Huh-7 cell apoptosis. Lovastatin treatment dose-dependently increased the proportion of apoptotic cells measured by DAPI staining (Fig. 3), while enzastaurin (5 μM) alone did

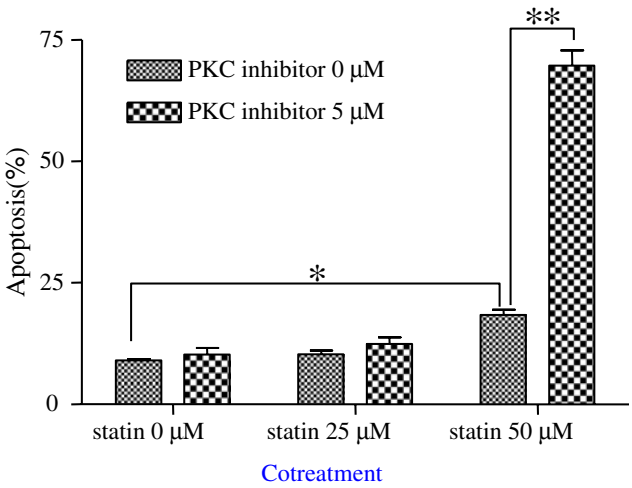

Fig. 3 Co-treatment with enzastaurin significantly enhanced lovastatin-induced apoptosis. Huh-7 cells were treated with lovastatin (0–50 μM) in the presence or absence of enzastaurin (5 μM). After 6 h, apoptotic cells were counted by DAPI staining and fluorescent microscopy. Columns, the means of three independent experiments; *bars*, SD. * $P < 0.05$, ** $P < 0.01$ by 1-way ANOVA

not induce significant cellular apoptosis. However, co-treatment with enzastaurin (5 μM) and lovastatin (50 μM) significantly enhanced Huh-7 cell apoptosis as compared with 50 μM lovastatin alone. These observations indicate that enzastaurin enhances lovastatin-induced HCC cell apoptosis.

We then explored which apoptotic signaling pathway is more activated in lovastatin/enzastaurin-treated Huh-7 cells. Whereas lovastatin did not affect caspase-8 activation, it caused cytochrome *c*, Smac/DIABLO and AIF release into cytosol and subsequent caspase-9 activation (Fig. 4a), which suggests that lovastatin activates mitochondrial apoptotic signals in HCC cells. When lovastatin and enzastaurin were co-supplemented, cytochrome *c*, Smac/DIABLO and AIF release occurred as was observed in lovastatin-treated cells alone. However, caspase-9 activation was more enhanced after co-treatment than in cells treated with lovastatin alone (Fig. 4a). Since the mitochondrial release of pro-apoptotic molecules was not affected by enzastaurin, we hypothesized that the expression level of XIAP, an inhibitor of apoptosis proteins (IAPs) and a regulator of caspase-9 activation, and/or binding between XIAP and caspase-9 may have been altered by enzastaurin. To investigate this possibility, XIAP was immunoprecipitated from cell lysates, and XIAP levels and XIAP to caspase-9 binding affinity were evaluated by immunoblotting these precipitates with antibodies for XIAP and caspase-9. As shown in Fig. 4b, lovastatin increased both the XIAP expression and the association of XIAP with caspase-9, either pro- or active forms. When enzastaurin was added, this lovastatin-induced increase in

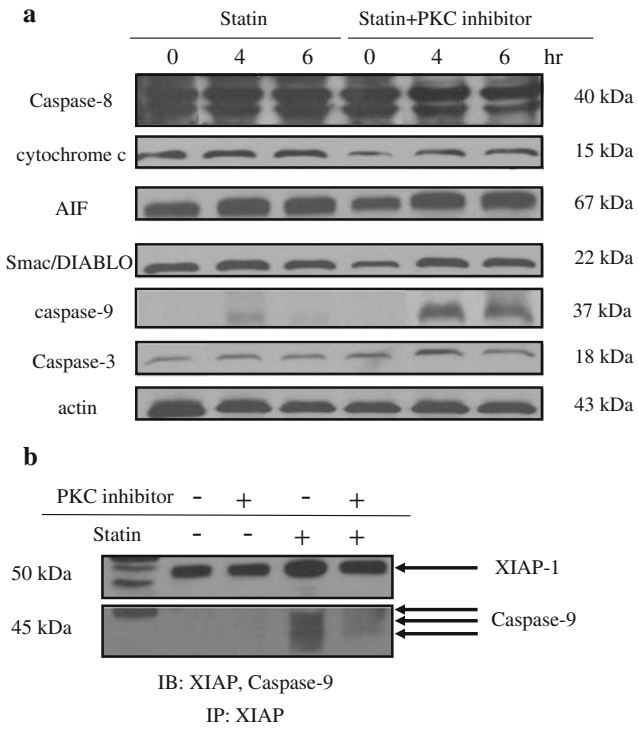

Fig. 4 Increased lovastatin-induced apoptosis by enzastaurin was due to increased caspase-9 activation resulting from reduced caspase-9/XIAP binding. **a** Huh-7 cells were treated with lovastatin (50 μM) and/or enzastaurin (5 μM) for the indicated times. At each time point, cells were lysed and immunoblot analysis was performed for caspase-8, 3, 9, cytochrome *c*, AIF, Smac/DIABLO, and *β*-actin. **b** Huh-7 cells were cultured in the presence or absence of lovastatin (50 μM), and simultaneously treated with enzastaurin (0, 5 μM) for 2 h. Cells were then lysed and XIAP was immunoprecipitated. Immunoblotting was performed for XIAP-1 and caspase-9

XIAP expression and XIAP to caspase-9 binding affinity was diminished (Fig. 4b). These observations collectively indicate that lovastatin-induced PKC activation is an anti-apoptotic signal, which increases XIAP levels and enhances XIAP to caspase-9 binding, thus preventing caspase-9 activation after lovastatin treatment.

In vivo anti-tumor efficacy of lovastatin and enzastaurin

Mean tumor volumes were significantly reduced in mice treated with lovastatin + enzastaurin as compared with control, lovastatin, or enzastaurin-treated mice (Fig. 5). We then evaluated whether lovastatin and enzastaurin had a synergistic anti-tumor effect using NONMEM assuming an exponential tumor growth kinetic curve. Growth rate constants were no different for untreated controls and enzastaurin-treated animals (Table 1). However, tumor doubling times calculated from growth rate constants were significantly longer for lovastatin + enzastaurin treated than lovastatin-treated animals, demonstrating that

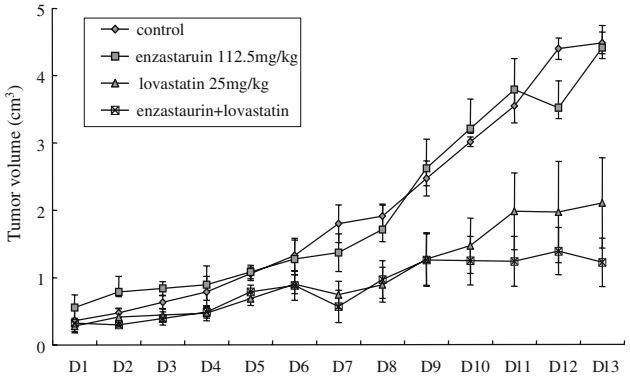

Fig. 5 An in vivo model of HCC was established in C3H mice by implantating MH134 cells subcutaneously. Lovastatin (0, 25 mg/kg, i.p.) and/or enzastaurin (0, 112.5 mg/kg, gavage) were administered to mice for 15 days after tumor volumes had reached $0.5 \sim 1.0$ cm^3. Tumor volumes were calculated using $0.5 \times L$ (cm) $\times W^2$ (cm^2), where L and W represent maximum length and width, respectively. Six mice were randomly allocated to each group. Points, mean tumor volumes; bars, SE

lovastatin and enzastaurin acted synergistically to inhibit tumor growth (Table 2).

Finally, we examined if apoptotic levels are higher in mice treated with lovastatin + enzastaurin as compared with control, lovastatin, or enzastaurin-treated mice using TUNEL staining. In addition, since PKC activity is closely related with angiogenesis, we also evaluated the anti-angiogenic effects of these treatments by comparing MVDs. As shown in Fig. 6a–c, percentages of TUNEL-positive cells were significantly increased and mean MVDs were significantly decreased in mice treated with lovastatin + enzastaurin versus the other three treatments ($P < 0.05$). These findings collectively suggest that lovastatin and enzastaurin act synergistically in vivo, and that their anti-tumor effects involve enhancing apoptosis and reducing angiogenesis.

Discussion

The key findings of the present study relate to the disruptive role of PKC activity on lovastatin-induced anti-tumor efficacy in HCC. In particular, this study demonstrates that lovastatin activates PKC signals, which counteracts lovastatin-induced HCC cell apoptosis, and that PKC inhibition enhances lovastatin-induced caspase-9 activation. Moreover, the synergistic anti-tumor efficacy of lovastatin and enzastaurin was confirmed in mice bearing HCCs.

Statins inhibit cell proliferation and induce apoptosis to a greater degree in cancer cells [27, 28] because they overexpress HMG-CoAR [12]. Impairment in the negative feedback control of cholesterol synthesis has been observed in HCC, which implies that a loss of cholesterol synthesis

Table 1 Development of the tumor growth kinetics model by forward addition to the base model and backward elimination from the full model

Hypothesis	OFV	ΔOFV	*P* value*	Conclusion
Base model	−42.908			
Was *k* different according to treatment group?	−70.755	−27.847	<0.001	Yes
Full model	−70.755			
Was *k* different between control group and enzastaurin-treated group?	−70.754	+0.001	0.975	No
Was *k* different between control group and lovastatin-treated group?	−65.037	+5.718	0.017	Yes
Was *k* different between control group and combination-treated group?	−51.111	+19.644	<0.001	Yes
Was *k* different between enzastaurin-treated group and combination-treated group?	−48.421	+22.334	<0.001	Yes
Was *k* different between lovastatin-treated group and combination-treated group?	−66.596	+4.159	0.041	Yes
Final model	−70.754			

* *P* value calculated using the likelihood ratio test indicating the OFV difference between the indicated model and the base or full model. *k* Growth rate constant

Table 2 Population parameter estimates for tumor growth kinetics

Parameter	Base model		Final model	
	Estimate (SE)	%CV	Estimate (SE)	%CV
$V0$ (cm^3)	0.378 (0.0532)		0.412 (0.0611)	
k (day^{-1})	0.153 (0.00797)			
k_I/k_{II}			0.169 (0.0128)	
k_{III}			0.135 (0.0164)	
k_{IV}			0.107 (0.0116)	
ω^2_{V0}	0.255 (0.100)	50.5	0.168 (0.0763)	41.0
ω^2_k	0.0128 (0.00634)	11.3	0.0132 (0.0115)	11.5
σ^2_a	0.168 (0.0285)	41.0	0.188 (0.0233)	43.4
σ^2_p	0.253 (0.0229)	50.3	0.221 (0.0187)	47.0
Td[a] (day)	4.53			
Td_I/Td_{II}			4.10	
Td_{III}			5.13	
Td_{IV}			6.48	

V0, tumor volume at the beginning; *k*, growth rate constant in the whole population; k_I, k_{II}, k_{III}, and k_{IV}, growth rate constants in the control, enzastaurin-treated, lovastatin-treated, and lovastatin + enzastaurin treated groups, respectively;ω^2_{V0}, variance of inter-individual variability for tumor volume at baseline**;** ω^2_k, variance of inter-individual variability for the growth rate constant; σ^2_a, variance of residual variability for the additive error; σ^2_p, variance of residual variability for the proportional error; SE, standard error; CV, coefficient of variation

[a] Tumor doubling time (Td) calculated from the growth rate constant estimate

control and carcinogenesis are related [29]. Furthermore, cancer cells require higher levels of cholesterol and cholesterol precursors like mevalonate-derived isoprenoids [30]. Statins induce apoptosis by regulating the Raf-mitogen activated protein kinase kinase (MAP2K, MEK)-extracellular signal-regulated kinase (ERK) pathway [28], and by activating mitochondrial apoptotic signaling and disrupting mitochondrial membrane potential in a variety of malignant cells [15, 31]. In the present study, we also observed that lovastatin induced HCC cell apoptosis by activating mitochondrial apoptotic signals, as was demonstrated by the release of pro-apoptotic molecules and caspase-9 cleavage without caspase-8 activation. In preclinical studies, the downregulation of serum cholesterol levels by cholesterol-free diet or fluvastatin reduced tumor growth and improved survival in animals [32, 33]. Indeed, the findings of a randomized controlled trial of pravastatin monotherapy in patients with advanced HCC implicated a potential role of statin as an anti-cancer therapy against HCC [34]. However, statin-based monotherapies in cancer patients do not result in persistent, clinically relevant anti-cancer responses [35, 36], perhaps because they activate PKC, an upstream regulator of Ras and an activator of the ERK-MAPK cascade [19, 37]. The present study confirms this statin-induced PKC activation.

Protein kinase C isoenzymes are distributed in a tissue-specific manner and differentially involved in the regulation of cell proliferation, differentiation, and apoptosis [16]. The over-expressions of PKC isoenzymes have been reported in breast cancer, melanoma and HCC [38–40], and this has also been implicated in the tumor angiogenesis mediated by vascular endothelial growth factor/basic fibroblast growth factor (VEGF/bFGF) [17, 41, 42] as well as multi-drug resistance (MDR) mediated by MDR-1/P-glycoprotein [18]. Moreover, PKC activation has been associated with both pro-apoptotic and anti-apoptotic signaling [43], wherein PKCδ and θ appear to be pro-apoptotic and PKCα and β have anti-apoptotic properties in most cells [44, 45]. Thus, enzastaurin (the PKCβ-selective inhibitor used in this study) may exhibit an apoptogenic effect in some cancer cells. However, when examined in this study, enzastaurin did not induce significant cellular apoptosis in vitro or in vivo. On the other hand, enzastaurin significantly enhanced lovastatin-induced HCC cell apoptosis, as was demonstrated by greater caspase-9 activation in Huh-7 cells treated with lovastatin + enzastaurin than in

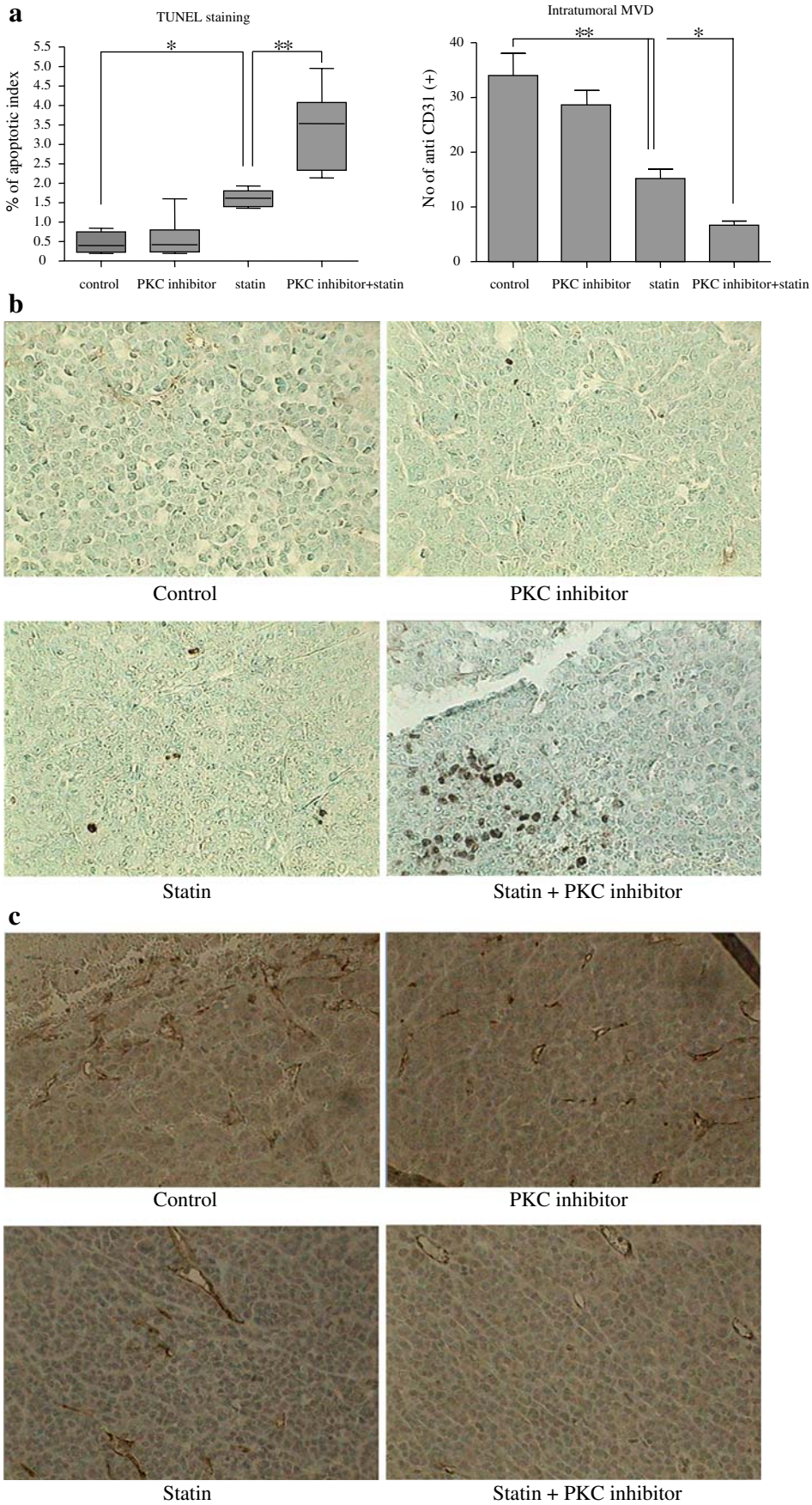

Fig. 6 TUNEL-positive cell proportions were significantly increased and mean MVDs were significantly decreased in mice treated with lovastatin + enzastaurin. **a** Apoptotic indexes and intratumoral microvessel densities (MVDs) in the mice treated with lovastatin and/or enzastaurin and in untreated mice were compared by TUNEL and immunohistochemical staining. Columns, mean values of each group; *bars*, SD. * $P < 0.05$, ** $P < 0.01$, versus untreated control. **b** Apoptosis in tumor tissues were assessed by TUNEL staining after fixing fresh tissues in 4% paraformaldehyde. Positive stained cells were counted in six different high-power fields (×400) and averaged. Cell numbers are expressed as percentages of total cells, and are referred to as apoptotic indexes. **c** Mean MVDs were determined by counting CD31 + vessels in six different high-power fields (×400) and averaged

cells treated with lovastatin, and by more TUNEL-positive cells in lovastatin + enzastaurin treated mice than in lovastatin-treated mice. These findings indicate that PKC inhibition augments the apoptotic effect of lovastatin in HCC. In addition, we observed that lovastatin increased the expression level of XIAP and XIAP/caspase-9 binding affinity, and that these effects were prevented by enzastaurin co-treatment. These findings, collectively suggest that lovastatin-induced PKC activation leads to an anti-apoptotic signal, which involves caspase-9 to XIAP binding, and that this signal can be effectively inhibited by a PKC inhibitor. Indeed, during this study lovastatin and enzastaurin were found to act synergistically in vivo.

The mechanism underlying statin/PKC-mediated XIAP induction and its association with caspase-9 is unclear. PKC activation has been previously reported to stabilize XIAP, and thus prevent apoptosis via suppressing XIAP ubiquitination and proteasomal degradation [46]. Therefore, PKC-mediated XIAP stabilization might be responsible for the increased XIAP expression observed in the present study after lovastatin treatment. PKC activation has also been demonstrated to phosphorylate caspase-9 at serine 144 in human, and thereby, seems to directly inhibit the Apaf-1-mediated activation of caspase-9 [47]. It is possible that PKC-induced caspase-9 phosphorylation may enhance caspase-9 binding to XIAP, and thereby, prevent its activation, but since active caspase-9 fragments were also observed in XIAP immunoprecipitates after lovastatin treatment in the present study, it appears unlikely that caspase-9 phosphorylation enhances its complexation with XIAP. Another plausible explanation for statin/PKC-mediated caspase-9 binding to XIAP is that XIAP phosphorylation is lovastatin-induced and PKC-mediated, and that this enhances XIAP/caspase-9 binding, though this remains to be evaluated.

HCCs are characteristically hypervascular tumors, and therefore, anti-angiogenic treatment strategies may be effective. Moreover, statins are known to have pro-angiogenic effects at low therapeutic concentrations, which are associated with the activation of the Akt/eNOS signaling pathway [48, 49], whereas at higher doses, statins can inhibit endothelial cell migration and angiogenesis [48]. In a clinical trial of lovastatin in patients with various solid tumors, doses of from 2 to 25 mg/kg daily resulted in plasma concentrations ranging between 0.1 and 3.9 μM [50]. In the present study, the lovastatin dose was 25 mg/kg daily, and was found to have an anti-angiogenic effect in mice. In addition, PKCβ, which is a component of the VEGF signaling pathway, induces endothelial proliferation [17], and PKCβ inhibitors play a role in anti-angiogenesis in human tumor xenograft-bearing mice [41]. During the present study, we also observed that enzastaurin had an anti-angiogenic effect in HCC-bearing mice. Moreover, this observed anti-angiogenic effect suggests an additional mechanistic basis underlying the effectiveness of lovastatin + enzastaurin.

In summary, the present study demonstrates that lovastatin activates PKC signals and that PKC inhibition enhances lovastatin-induced apoptosis in HCC cells. Moreover, lovastatin and enzastaurin were also found to act synergistically in mice bearing HCCs, which suggests that combinatorial statin/PKC inhibitor-based treatments may provide targeted strategies for the treatment of HCC.

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