Simvastatin-Induced Apoptosis in Osteosarcoma Cells: A Key Role of RhoA-AMPK/p38 MAPK Signaling in Antitumor Activity

Walied A. Kamel,1,2,3,4 Eiji Sugiara,1 Hiroyuki Nobusue,1 Sayaka Yamaguchi-Iwai,1,5 Nobuyuki Onishi,1 Kenta Maki,3 Yumi Fukuchi,3 Koichi Matsuo,2 Akihiro Muto,3 Hideyuki Saya,1 and Takatsune Shimizu1,3

Abstract

Osteosarcoma is the most common type of primary bone tumor, novel therapeutic agents for which are urgently needed. To identify such agents, we screened a panel of approved drugs with a mouse model of osteosarcoma. The screen identified simvastatin, which inhibited the proliferation and migration of osteosarcoma cells in vitro. Simvastatin also induced apoptosis in osteosarcoma cells in a manner dependent on inhibition of the mevalonate biosynthetic pathway. It also disrupted the function of the small GTPase RhoA and induced activation of AMP-activated protein kinase (AMPK) and p38 MAPK, with AMPK functioning upstream of p38 MAPK. Inhibitors of AMPK or p38 MAPK attenuated the induction of apoptosis by simvastatin, whereas metformin enhanced this effect of simvastatin by further activation of AMPK. Although treatment with simvastatin alone did not inhibit osteosarcoma tumor growth in vivo, its combination with a fat-free diet induced a significant antitumor effect that was enhanced further by metformin administration. Our findings suggest that simvastatin induces apoptosis in osteosarcoma cells via activation of AMPK and p38 MAPK, and that, in combination with other approaches, it holds therapeutic potential for osteosarcoma. Mol Cancer Ther; 16(1): 182–92. ©2016 AACR.

Introduction

Osteosarcoma is the most common primary malignant bone tumor in childhood and adolescence (1). Individuals with osteosarcoma are currently treated with surgery, radiotherapy, and adjuvant or neoadjuvant chemotherapy, including administration of methotrexate, doxorubicin, cisplatin, or ifosphamide (1, 2). Although chemotherapy regimens have improved the survival rate of such patients by up to 60% to 70% (3), there is an urgent need for new therapeutic agents, given that many cases of osteosarcoma are initially not sensitive or acquire resistance to current chemotherapeutic drugs (4). We previously generated a mouse model of osteosarcoma on the basis of overexpression of c-MYC in bone marrow stromal cells isolated from Inkatara/Arf knockout mice (5). Injection of the resulting highly tumorigenic osteosarcoma cells (designated AXT cells) into syngeneic C57BL/6 mice results in the rapid formation of lethal tumors that mimic human osteoblastic osteosarcoma.

Drug repositioning, the identification and development of new uses for existing drugs (6), has many advantages over de novo drug development including a shortened timeline as well as reduced cost and risk. It is therefore an ideal approach to the development of new therapies for relatively rare and refractory diseases such as osteosarcoma.

Statins are 3-hydroxy-3-methylglutaryl-c-oenzyme A (HMG-CoA) reductase inhibitors that exert pleiotropic biologic effects by preventing the synthesis of mevalonic acid, the precursor of nonsteroidal isoprenoid compounds that mediate various cellular processes (7). Previous investigations have revealed that statins exhibit antineoplastic effects in a variety of cancer types, including osteosarcoma (8–14). The precise molecular mechanism by which statins induce apoptosis in osteosarcoma cells has remained unknown, however. The antitumor potential of statins for the treatment of osteosarcoma in animal models has also remained largely unexplored.

We have now screened more than 1,100 existing drugs for therapeutic potential in vitro, with statins being identified as such drug candidates. We clarified the effects of simvastatin on osteosarcoma cells in vitro and in vivo with the use of our syngeneic model, and we explored the molecular mechanisms of these effects. On the basis of the mechanistic insight, we propose that simvastatin holds potential for application to the treatment of osteosarcoma in the clinical setting by appropriate drug combination.

Materials and Methods

Cell culture

The mouse osteosarcoma AXT cells were established in our laboratory as described previously in 2010 (5, 15) and resuscitated and

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Division of Gene Regulation, Institute for Advanced Medical Research, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan. 2Laboratory of Cell and Tissue Biology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan. 3Department of Pathophysiology, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Shinagawa-ku, Tokyo Japan. 4Faculty of Science, Mansoura University, Mansoura, Egypt. 5Department of Orthopedic surgery, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan.

Corresponding Author: Takatsune Shimizu, Department of Pathophysiology, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. Phone: 81-3-5498-5309. Fax: 81-3-5498-5916. E-mail: t-shimizu@hoshi.ac.jp

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maintained under 5% CO₂ at 37°C in IMDM (Thermo Fischer Scientific) supplemented with 10% FBS. Saos2 and U2OS human osteosarcoma cell lines were purchased from ATCC in September 2016, resuscitated and used for assays within 2 months after delivery. Both lines were maintained under 5% CO₂ at 37°C in McCoy 5a medium (Thermo Fischer Scientific) supplemented with 15% FBS.

Reagents
Simvastatin was obtained from Combi-Blocks, metformin from LKT Laboratories, rosuvastatin and atorvastatin from Wako, fluvastatin from Tocris Bioscience, and 5-aminooimidazole-4-carboxamide ribonucleotide (AICAR) from Cell Signaling. Mevalonate, geranylgeranyl diphosphate (GGPP), or farnesyl diphosphate (FPP; Sigma Aldrich) were used at 200, 10, or 10 μmol/L, respectively, unless indicated otherwise. 5-(Tetracyclolxy)-2-furoic acid (TOFA) and a Rho inhibitor (C3 transferase) were from Santa Cruz and Cytoskeleton, respectively.

Cell proliferation assay
Cell viability was measured as previously described (15). For rescue experiments, the cells were exposed to p38 MAPK inhibitor X [BIRB796 (BIRR); Millipore] or 5-iodotubercidin (ido; Sigma) for 1.5 hours or to compound C (Millipore) for 1 hour before incubation with simvastatin or AICAR.

Tumor xenograft model
All animal care and procedures were performed in accordance with the guidelines of Hoshi University (Tokyo, Japan) and Keio University (Tokyo, Japan). For establishment of tumor xenografts, AXT cells (1 × 10⁶) suspended in PBS were injected subcutaneously and bilaterally into the flank of 8-week-old female syngeneic C57BL/6 mice (SLC). Administration of simvastatin was performed according to 3 different protocols: (i) mice were injected intraperitoneally with simvastatin at 40 mg/kg once a day for 5 days a week over 2 weeks beginning 7 days after cell injection; (ii) simvastatin suspended in double-distilled water containing 0.05% carboxymethyl cellulose was administered orally at 80 mg/kg once per day for 5 days a week over 3 weeks beginning 7 days after cell injection; and (iii) mice were fed with a fat-free diet (Research Diets, #D04112303) beginning from the day of cell injection. Two days after cell injection, mice were injected intraperitoneally with simvastatin (50 mg/kg), metformin (100 mg/kg), or simvastatin (50 mg/kg) plus metformin (50 or 100 mg/kg) once per day for 5 days a week over 3 weeks. Drugs for injection were suspended in normal saline.

Cell migration assay
AXT cells were transferred to the upper chamber in 200 μL of serum-free IMDM containing test agents, and 800 μL of IMDM supplemented with 10% FBS was placed in the lower chamber. After incubation for 12 hours under 5% CO₂ at 37°C, the cells on the lower surface of the filter were counted as described previously (16).

Immunoblot analysis
Cells were lysed with 2× Laemmli sample buffer (Bio-Rad) supplemented with β-mercaptoethanol. Immunoblot analysis was performed according to standard procedures (15), with primary antibodies to cleaved caspase-3, to phosphorylated or total forms of p38 MAPK, AMPK-activated protein kinase (AMPK), AKT, and acetyl coenzyme A carboxylase (ACC), to RhoA, to integrin-β1, to Rho-GDP dissociation inhibitor (Rho-GDI; all from Cell Signaling), and to actin (Santa Cruz Biotechnology).

Detection of Rho-GDI and RhoA interaction
AXT cells were lysed with a lysis buffer containing 0.5% Nonidet P-40 (17), and the resulting supernatant was incubated with rotation for 10 minutes at room temperature with antibodies to Rho-GDI (Millipore) and protein G-coupled magnetic Dynabeads (Life Technologies) as mentioned previously (17).

Detection of apoptotic cells and analysis of cell-cycle profile by flow cytometry
Cells were collected, washed with ice-cold PBS, suspended in Annexin V-binding buffer (BD Biosciences), and stained with propidium iodide (Sigma-Aldrich) and allophycocyanin-conjugated Annexin V (BD Biosciences). After incubation on ice in the dark for 15 minutes, the cells were assayed for apoptosis by flow cytometry. For cell-cycle analysis, cells were collected, fixed with 70% ethanol for 48 hours at −20°C, washed 3 times with ice-cold PBS, and stained with propidium iodide for 30 minutes on ice. Data were analyzed with the use of FlowJo software (Tree Star).

Detection of activated RhoA
Cells were lysed with a magnesium-containing buffer (Millipore) and the RhoA status was evaluated as described previously (18).

Determination of the subcellular localization of RhoA
AXT cells were fractionated with the use of a membrane fractionation kit (Abcam). Cytosolic and membrane fractions were subjected to immunoblot analysis with antibodies to RhoA to determine the relative amounts of RhoA in each compartment.

Reverse transcription and real-time PCR analysis
Total RNA extraction, reverse transcription, and real-time PCR analysis were performed as previously described (15, 19). The sequences of PCR primers (forward and reverse, respectively) are 5'-CAACCGTGAAAGATGCC-3' and 5'-TAGGACAGGG-CATACAG-3' for Actb as well as 5'-GTGCCACGTTTGGAAAC-3' and 5'-GTTGCTGATGATAAGAG-3' for Rhoa.

Statistical analysis
All assays were performed in triplicate, and quantitative data are expressed as means ± SD relative to the control value unless indicated otherwise. Data were analyzed with the use of a Student t test, and a P < 0.05 was considered statistically significant. *P < 0.05; **P < 0.005; ***P < 0.0005; NS, not significant.

Results
Simvastatin induces morphologic changes as well as inhibits the proliferation and migration of osteosarcoma cells
To discover a safe and effective therapeutic agent for osteosarcoma, we screened more than 1,100 FDA-approved drugs with the use of a novel humanoid robot (Mahoro, Robotic Biology Institute). Three statins—lovastatin, simvastatin, and fluvastatin—among the 6 statins included in the library were found to inhibit the proliferation of AXT cells (Fig. 1A and B). This antiproliferative
The effect of simvastatin was potent and concentration-dependent, and it was accompanied by the adoption of a spherical morphology by AXT cells and their irreversible detachment from the culture dish within 12 hours (Fig. 1C and D). To determine whether these effects of simvastatin were due to inhibition of the mevalonate pathway, we examined whether they might be influenced by the addition of intermediate metabolites of this pathway including mevalonate, GGPP, and FPP (20). Mevalonate and GGPP each markedly attenuated the effects of simvastatin on both cell morphology and proliferation, whereas the actions of FPP in this regard were less pronounced (Fig. 1E; Supplementary Fig. S1A). Simvastatin also inhibited the proliferation of Saos2 and U2OS human osteosarcoma cells by targeting the mevalonate pathway (Fig. 1F). In addition, simvastatin attenuated the migration of AXT cells in a concentration-dependent manner (Fig. 1G; Supplementary Fig. S1B), and this effect was largely prevented by mevalonate or GGPP and was partially attenuated by FPP (Fig. 1H and I). Together, these results suggested that simvastatin inhibits the viability and migration of osteosarcoma cells by targeting the mevalonate pathway.

Simvastatin induces apoptosis in AXT cells

To examine whether the suppression of osteosarcoma cell proliferation by simvastatin was due to the induction of apoptotic cell death, we determined the proportion of apoptotic cells after exposure of AXT cells to simvastatin for 24 hours. Flow cytometric analysis of cells stained with Annexin V and propidium iodide revealed that the numbers of both early (Annexin V+, propidium iodide−) and late (Annexin V+, propidium iodide+) apoptotic cells were increased by simvastatin treatment. Furthermore, consistent with their effects on the antiproliferative action of simvastatin, the addition of mevalonate or GGPP essentially prevented simvastatin-induced apoptosis whereas that of FPP had a more modest inhibitory effect (Fig. 2A–D). The cleaved form of caspase-3, another indicator of apoptosis (21), was also detected in AXT cells treated with simvastatin for 24 hours (Fig. 2E), and the simvastatin-induced cleavage of caspase-3 was blocked by mevalonate or GGPP (Fig. 2F). Finally, the induction of AXT cell apoptosis by simvastatin was confirmed by cell-cycle analysis as an increase in the size of the sub-G1 cell fraction (Supplementary Fig. S1C and S1D).
Simvastatin changes the expression and localization of RhoA in AXT cells

FPP and GGPP are required for posttranslational prenylation of various proteins including small GTPases of the Ras and Rho (Rac, Rho, and Cdc42) families (22, 23). The covalent attachment of FPP or GGPP to Ras or Rho family members, respectively, results in the translocation of these proteins from the cytosol to the plasma membrane, where they participate in the regulation of various cellular processes (22, 23). Whereas RhoA is detected in the membrane fraction of cells under normal conditions (24), we found that most RhoA was present in the cytosolic fraction of AXT cells after treatment with simvastatin (Fig. 3A). In addition, simvastatin increased the expression of RhoA in AXT cells and the active, GTP-bound form of RhoA (RhoA-GTP) in a manner sensitive to inhibition by mevalonate or GGPP but to a lesser extent by FPP (Fig. 3B–D). Rho-GDI negatively regulates the activity of Rho GTPases in the cytosol by preventing the release of GDP (25). An immunoprecipitation assay revealed that simvastatin attenuated the formation of a complex between RhoA and Rho-GDI (Fig. 3E), which is supposed to be a main cause of the accumulation of RhoA-GTP in cytosol. Together, these results suggested that simvastatin induces the accumulation of RhoA in the cytosol as well as disrupts the interaction between RhoA-GDP and Rho-GDI in AXT cells, indicating that, despite its increased amount, the function of RhoA-GTP is likely impaired.

Simvastatin induces apoptosis via activation of AMPK/p38 MAPK signaling

Statins have been found to induce apoptosis in cancer cells via activation of AMPK (26) or p38 MAPK (27). Furthermore, activation of AMPK and p38 MAPK shows antitumor potential in human osteosarcoma (28). We therefore examined the effects of simvastatin on AMPK and p38 MAPK activation status in AXT cells. Simvastatin induced activation of both AMPKα (phosphorylation at Thr172) and p38 MAPK (phosphorylation at Thr180 and Tyr182) in a concentration- and time-dependent manner (Fig. 4A and B). Phosphorylation of AMPKα at Ser485 was not affected by simvastatin in AXT cells (Supplementary Fig. S2A).

Activation of AMPK and p38 MAPK by simvastatin was prevented by mevalonate or GGPP as well as inhibited to a lesser extent by FPP (Fig. 4C), indicating that the activation of both kinases is mediated via inhibition of the mevalonate pathway.
Exposure of AXT cells to the AMPK inhibitor 5-iodotubercidin or to the p38 MAPK inhibitor BIRB796 before treatment with simvastatin attenuated both the statin-induced change in cell morphology (Supplementary Fig. S2B) and its inhibition of cell viability (Fig. 4D). Such treatment with 5-iodotubercidin or BIRB796 also markedly inhibited the induction of apoptosis by simvastatin in AXT cells as revealed by flow cytometric analysis of Annexin V and propidium iodide staining (Fig. 4E and F) as well as by immunoblot analysis of cleaved caspase-3 (Supplementary Fig. S2C and S2D). These findings thus indicated that activation of AMPK and p38 MAPK mediates simvastatin-induced apoptosis in AXT cells.

The activation of p38 MAPK by simvastatin was inhibited by 5-iodotubercidin (Fig. 4G) whereas that of AMPK was not inhibited by BIRB796 (Fig. 4H), indicating that AMPK functions as an upstream regulator of p38 MAPK in simvastatin-induced AXT cell death. Consistent with these findings, the AMPK activator AICAR induced the phosphorylation of both p38 MAPK and ACC (Supplementary Fig. S2E), the latter of which is a downstream substrate of AMPK (29). In addition, AICAR markedly attenuated the proliferation of AXT cells in a manner sensitive to prior exposure of the cells to the AMPK inhibitor compound C (Supplementary Fig. S2F), indicating that activation of AMPK inhibits cell proliferation. Notably, inhibition of Rho activity by C3 transferase triggered the activation of AMPK and p38 MAPK as well as morphologic changes mimicking those caused by simvastatin treatment (Fig. 4I; Supplementary Fig. S2G). Collectively, these data indicated that simvastatin induces apoptosis in AXT cells via dysregulation of Rho GTPases accompanied by activation of the AMPK/p38 MAPK pathway in a manner dependent on inhibition of the mevalonate pathway.

Combined antiproliferative effect of simvastatin and metformin in AXT cells

Given that simvastatin activates AMPK in AXT cells, we examined whether the combination of simvastatin and metformin, a well-known inducer of AMPK activation (30), could further enhance the cytotoxicity of AXT cells. The combination of simvastatin and metformin manifested synergistic antitumor activity in prostate cancer previously (31). The combination of both drugs inhibited AXT cell proliferation to an extent greater than that

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**Figure 3.**

Simvastatin disturbs the activation and localization of RhoA in AXT cells. **A,** Immunoblot analysis of RhoA in membrane and cytosolic fractions of AXT cells treated with 10 μmol/L simvastatin for 12 hours. Integrin-β1 and actin were examined as loading controls for membrane and cytosolic fractions, respectively. **B,** RT and real-time PCR analysis of Rhoa mRNA in AXT cells treated with 5 μmol/L simvastatin for 13 hours. Data are normalized by the amount of Actb mRNA and are means ± SD of triplicates from a representative experiment. **C,** Immunoblot analysis of RhoA in AXT cells treated with simvastatin for 12 hours. **D,** Immunoblot analysis of RhoA and RhoA-GTP in AXT cells treated with 10 μmol/L simvastatin for 12 hours in the absence or presence of mevalonate, GGPP, or FPP. RhoA-GTP was isolated from cell lysates by precipitation with beads conjugated with a GST fusion protein containing the Rho binding domain of Rhotekin. **E,** AXT cells treated with 10 μmol/L simvastatin for 12 hours in the absence or presence of mevalonate were subjected to immunoprecipitation (IP) with antibodies to Rho-GDI, and the resulting precipitates as well as the input lysates were subjected to immunoblot analysis with the indicated antibodies.
apparent with either agent alone (Fig. 5A). This combined effect was attenuated only, in part, by mevalonate (Fig. 5B), suggesting that the antiproliferative effect of metformin is independent of inhibition of the mevalonate pathway. The combination of both drugs also induced the accumulation of RhoA-GTP to a greater extent than did either agent alone, with this combined effect being abolished in the presence of mevalonate and attenuated in part by that of GGPP or FPP (Fig. 5C). In addition, the combination treatment induced a shift in the localization of RhoA from the membrane fraction to the cytosol even at lower concentration of simvastatin (Fig. 3A, 5D). Furthermore, simvastatin and metformin together induced AMPK and p38 MAPK activation to a greater extent than did either drug alone (Fig. 5E and F). Together, these in vitro observations indicated that the combination of simvastatin and metformin might possess antitumor activity against osteosarcoma.

Combined antitumor effect of simvastatin and a fat-free diet with or without metformin in vivo

To determine whether simvastatin might possess antitumor activity against AXT cells in vivo, we injected syngeneic C57BL/6 mice with these cells and then treated the animals with simvastatin by intraperitoneal injection or oral gavage. Although each treatment protocol with high dosage of simvastatin induced a slight decrease in tumor weight, these effects were not statistically significant (Supplementary Fig. S3A and S3B). All mice were alive, fine, and not exhausted during the treatment.

Statins inactivate ACC, an enzyme that carboxylates Acetyl CoA and mediates de novo fatty acid biosynthesis and lipogenesis (32). ACC is also a major downstream target of AMPK, and inactivation of ACC by phosphorylation is thought to trigger apoptosis in cancer cells (33). Simvastatin induced phosphorylation of ACC (on Ser79) in a concentration-dependent manner in AXT cells, and this effect was prevented by mevalonate or GGPP but not by FPP (Fig. 6A and B). These findings suggested that simvastatin inactivates ACC via inhibition of the mevalonate pathway. The simvastatin-induced inactivation of ACC was also inhibited by 5-iodotubercidin (Fig. 6C), suggesting that this effect of the statin is also mediated by AMPK activation. Treatment of AXT cells with the ACC inhibitor TOFA attenuated cell proliferation in a concentration-dependent manner (Supplementary Fig. S3C). Furthermore, the combination of simvastatin

Figure 4.

Simvastatin-induced apoptosis mediated by activation of AMPK and p38 MAPK in a manner dependent on mevalonate pathway inhibition. A and B, Immunoblot analysis of phosphorylated (p-) and total forms of AMPK (A) and p38 MAPK (B) in AXT cells treated with the indicated concentrations of simvastatin. C, Immunoblot analysis of AMPK and p38 MAPK phosphorylation in AXT cells treated with 10 μmol/L simvastatin for 20 hours in the absence or presence of mevalonate, GGPP, or FPP. D, Viability of AXT cells exposed to 10 μmol/L BIRB796 (BIRB) or 3 μmol/L 5-iodotubercidin (Iodo) in the additional presence of simvastatin for 1 day. E and F, Flow cytometric analysis of AXT cells exposed to 2 μmol/L 5-iodotubercidin or 20 μmol/L BIRB796 in the additional presence of 5 μmol/L simvastatin for 20 hours. Representative profiles (E) and the percentages of Annexin V+ cells (F) are shown. G and H, Immunoblot analysis of AMPK and p38 MAPK phosphorylation in AXT cells exposed to the indicated concentrations of 5-iodotubercidin (G) or BIRB796 (H) in the additional presence of 5 μmol/L simvastatin for 14 hours. I, Immunoblot analysis of AMPK and p38 MAPK phosphorylation in AXT cells exposed to C3 transferase for 24 hours.
and TOFA inhibited cell proliferation to a greater extent than did either agent alone (Fig. 6D). Together, these data suggested that simvastatin targets fatty acid synthesis via inactivation of ACC.

Then, on the basis of the mechanistic insight above, to enhance the antitumor effect of simvastatin in vivo by further activation of AMPK and its downstream effect, we examined combination of metformin and a fat-free diet. We applied 50 mg/kg of simvastatin which was assumed to be safely administrable by the former single treatment (Supplementary Fig. S3A and S3B).

**Figure 5.**
Additive effects of simvastatin and metformin on AXT cell proliferation as well as on RhoA, AMPK, and p38 MAPK signaling. A and B, Viability of AXT cells treated with the indicated concentrations of simvastatin and metformin (Met) or with simvastatin, metformin, and mevalonate (B) for 2 days. C, Immunoblot analysis of RhoA and RhoA-GTP in AXT cells treated with the indicated reagents for 12 hours. D, Immunoblot analysis of RhoA in membrane and cytosolic fractions of AXT cells treated with simvastatin and metformin for 12 hours. E and F, Immunoblot analysis of AMPK and p38 MAPK phosphorylation as well as of caspase-3 cleavage in AXT cells treated with 0.5 μmol/L simvastatin and the indicated concentrations of metformin for 12 hours (E) or 24 hours (F). The arrow indicates p-p38 MAPK.
Whereas a fat-free diet alone did not affect the growth of AXT cell tumors in mice (Supplementary Fig. S3D), intraperitoneal injection of simvastatin in mice fed such a diet resulted in a significant inhibition of tumor growth (Fig. 6E and F). Furthermore, whereas metformin alone did not significantly affect tumor growth in mice fed a fat-free diet, it enhanced the antitumor effect of simvastatin in such animals (Fig. 6E and F). In fact, toxicity appeared to be a problem. In total, 5 of 6 mice in each group

Figure 6.
Antitumor effect of simvastatin in vivo. A and B, Immunoblot analysis of ACC phosphorylation in AXT cells treated with the indicated concentrations of simvastatin for 24 hours (A) or with 10 μmol/L simvastatin in the absence or presence of mevalonate, GGPP, or FPP for 20 hours (B). C, Immunoblot analysis of AMPK and ACC phosphorylation in AXT cells treated with 5 μmol/L simvastatin either alone or together with the indicated concentrations of 5-iodotubercidin for 20 hours. D, Viability of AXT cells treated with simvastatin or TOFA as indicated. E and F, Weight of AXT cell–derived tumors formed in mice fed a fat-free diet and injected intraperitoneally with saline (control), simvastatin (50 mg/kg), metformin (100 mg/kg), or both simvastatin (50 mg/kg) and metformin (50 mg/kg). Data are means ± SD (n = 10), with the P values being for comparison with control (Student t test). The excised tumors are also shown (F). G, Immunoblot analysis of AMPK and p38 MAPK phosphorylation in 5 randomly selected tumors of each group in E.
died; 1 mouse treated with metformin at 100 mg/kg, 1 with simvastatin at 50 mg/kg plus metformin at 50 mg/kg, and 3 with simvastatin at 50 mg/kg plus metformin at 50 mg/kg, respectively, albeit without any apparent organ damage (data not shown). In addition, mice treated with simvastatin (50 mg/kg) alone or with simvastatin (50 mg/kg) plus metformin (50 mg/kg) showed a body weight loss of 6.2% and 9.7%, respectively, compared with control mice. Finally, we found that the phosphorylation of AMPK was enhanced in tumors from the mice treated with simvastatin either alone or together with metformin compared with that apparent in control tumors, whereas such changes in the phosphorylation of p38 MAPK were less obvious (Fig. 6G).

**Discussion**

To identify new and effective therapeutic options for osteosarcoma, we performed a multidrug screen, which revealed statins as candidate agents. We further characterized the antitumor activity of simvastatin in vitro, finding that such activity was essentially abolished in the presence of mevalonate or GGPP and attenuated to a lesser extent by FPP. These results thus indicate that the antitumor activity of simvastatin is mediated via inhibition of the mevalonate pathway for biosynthesis of isoprenoids. Previous studies have also shown that statins exert antitumor effects by inhibiting the production of mevalonate and GGPP (34). Our results thus suggest that inhibition of protein geranylgeranylation has a greater impact on the viability of osteosarcoma cells compared with that of farnesylation.

We found that simvastatin upregulated the amount of RhoA-GTP, induced a shift in the localization of RhoA from membrane to cytosol, and inhibited the interaction between RhoA and Rho-GDI, with these effects also appearing to be mediated via inhibition of the mevalonate pathway. Isoprenoids are lipid moieties that are essential for posttranslational modification, membrane anchoring, and transforming activity of Ras and Rho proteins (23, 35). Statins were previously shown to induce apoptosis in osteosarcoma cells via inactivation of RhoA (12, 13). Indeed, a lack of isoprenylation of RhoA-GTP is likely responsible for the changes in cell morphology and inhibition of migration induced by simvastatin in the present study.

Simvastatin induced the activation of AMPK and p38 MAPK in AXT cells, consistent with previous findings in other cancer cell type (36). Again, these effects of simvastatin were found to be mediated through inhibition of the mevalonate pathway. Moreover, we found that this activation of both AMPK and p38 MAPK directly contributes to the induction of apoptosis by simvastatin in AXT cells. Intriguingly, as the activation of AMPK by simvastatin treatment was fully reversed by supplement of mevalonate or GGPP in AXT cells, activation of AMPK can be regulated depending on the amount of intermediate metabolites of the mevalonate pathway. Conversely, activation of AMPK was previously shown to result in inhibition of the mevalonate pathway through inactivation of HMG-CoA reductase, the rate-limiting enzyme of this pathway (37–39). Phosphorylation of p38 MAPK was previously shown to be related to the induction of cell death by various stimuli including statins, with the generation of reactive oxygen species also having been found to mediate this process (27, 40, 41). However, we found that simvastatin did not increase the level of reactive oxygen species (data not shown). Notably, the disruption of Rho activity alone can induce activation of AMPK (Fig. 4I) and a similar finding was shown in other organ system (42). Collectively, we proposed the mode of action of simvastatin, treatment of which inactivates Rho kinases accompanied by the sequential activation of AMPK and p38 MAPK that leads to induction of apoptosis in osteosarcoma cells (Supplementary Fig. S3E).

Although simvastatin alone manifested potent cytotoxic effects in osteosarcoma cells in vitro, it did not exhibit a significant antitumor effect in vivo. We therefore attempted to increase such in vivo activity of simvastatin by enhancing AMPK activation independently in the mevalonate pathway. Then, we chose combination with metformin which previously exhibited further antitumor effect in other cancers (31). Mechanistically, simvastatin and metformin showed additive effects on the accumulation of RhoA-GTP and the phosphorylation of AMPK and p38 MAPK. In addition, given that AMPK regulates fatty acid synthesis through phosphorylation of ACC (29), we examined the antitumor effect of simvastatin in animals fed a fat-free diet to further enhance this downstream effect of AMPK signaling. Simvastatin alone inhibited the growth of osteosarcoma tumors in mice fed a fat-free diet and that this effect was enhanced by metformin. Accumulating evidence suggests that statins sensitize malignant cells to various chemotherapeutic agents and strengthen the antitumor activity (43, 44). Therefore, simvastatin also might become a promising agent that improves chemosensitivity in osteosarcoma.

Collectively, our results have demonstrated the potential of simvastatin as a new therapeutic agent for osteosarcoma. The adoption of simvastatin for such treatment, however, will require further refinement of the optimal conditions for it to exert its antitumor action and to reduce the toxicity.

**Disclosure of Potential Conflicts of Interest**

H. Saya reports receiving commercial research grant from Daiichi Sankyo Inc., Eisai Co Ltd and Nihon Nohyaku Co Ltd. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: W.A. Kamel, T. Shimizu

Development of methodology: W.A. Kamel, S. Yamaguchi-Iwai, T. Shimizu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.A. Kamel, E. Sugihara, K. Maki, Y. Fukuchi, T. Shimizu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W.A. Kamel, S. Yamaguchi-Iwai, K. Maki, Y. Fukuchi, T. Shimizu

Writing, review, and/or revision of the manuscript: W.A. Kamel, K. Matsuo, T. Shimizu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.A. Kamel, H. Nobusue, N. Onishi, T. Shimizu


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