Fluvastatin, a lipophilic statin, induces apoptosis in human hepatocellular carcinoma cells through mitochondria-operated pathway

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Fluvastatin, a lipophilic statin, was known to inhibit proliferation and induce apoptosis in many cancer cells. Its potential anticancer was evaluated in three hepatocellular carcinoma (HCC) cell lines (HepG2, SMMC-7721 and MHCC-97H). Cells were treated with fluvastatin in vitro and its effect on cell proliferation, cell cycle, invasion and apoptosis was determined. Mechanism of apoptosis induced by fluvastatin on HCC cell lines was also investigated through western blotting and mitochondrial membrane potential (MMP) analysis. It was observed that fluvastatin inhibited proliferation of HCC cells by inducing apoptosis and G2/M phase arrest in a dose-dependent manner. The results of cell invasion assay revealed that fluvastatin significantly decreased the invasion potency of HCC cells. A mitochondria-operated mechanism for fluvastatin induced apoptosis might be involved and was supported by Western blotting and MMP analysis. After fluvastatin treatment, expression of Bcl-2 and procaspase-9 were downregulated, cytochrome c (cytosolic extract), Bax and cleaved-caspase-3 protein expression were increased. Furthermore, a breakdown of MMP in HCC cells was observed. To conclude, these results have provided a rationale for clinical investigations of fluvastatin in future as a potential anticancer reagent for growth control of HCC.

Keywords: Fluvastatin, Hepatocellular carcinoma, Mitochondria-operated pathway

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancy which accounts for more than 626,000 new cases per year worldwide. Despite the advances in surgical and nonsurgical therapies for HCC, the overall survival rate of patients remains unsatisfactory. A majority of patients are diagnosed at an advanced stage and this makes curative surgery impossible. Resistance to chemotherapeutic drugs is yet another reason responsible for failure of treatments. Therefore, new therapeutic strategies for more effective treatment remain to be obtained.

Statins are among the most widely prescribed drugs for the treatment of patients with hypercholesteremia and block de novo synthesis of cholesterol by inhibiting its key enzyme, 3-hydroxy-3-methyl-glutaryl coenzyme-A reductase (HMG-CoAR). Inhibition of this enzyme can reduce the synthesis of downstream products of the mevalonate pathway such as cholesterol, dolichol, ubiquinone and isoprenoid derivatives (farnesyl pyrophosphate and geranylgeranyl pyrophosphate). Besides their lipid-lowering effect, increasing evidences suggest that statins can induce in vitro apoptosis and inhibit in vivo metastasis of many solid tumor cell lines as well as hematological malignancies. Additionally, the results of clinical trials indicate that statins can prolong the survival of patients with advanced HCC. Meta-analyses suggest that statins may decrease the risk of developing certain malignancies; though some studies provide data conflicting with this hypothesis. Therefore, the present study was undertaken to investigate the effects of fluvastatin on HCC cell lines in vitro.

Materials and Methods

Cells and chemicals — The human HCC cell lines HepG2, SMMC-7721 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and MHCC-97H was obtained from Liver Cancer Institute, Fudan University (Shanghai, China). Cells were grown in Dulbecco’s modified Eagle’s medium (HyClone, Logan, Utah, USA) supplemented with 10% fetal...
bovine serum (JRH Biosciences, Lenexa, Kansas, USA), penicillin G (100 units/ml), and streptomycin (100 µg/ml). A 100 mM of fluvastatin (Merck, Germany) stock solution was made using dimethylsulfoxide and stored at -20°C. Dilutions with medium were performed at the time of each experiment. Culture medium containing 0.02% dimethylsulfoxide served as a control.

**Cell viability test (MTT assay)** — Viability of HCC cells was tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay according to the manufacturer’s instructions (Sigma, St. Louis, MO, USA). All three cell lines were treated with fluvastatin with various concentrations from 0 to 30 µM for 24 h. Viable cells were detected by measuring absorbance at 570 nm (reference 630 nm) using MRX II absorbance reader (DYNEX Technologies, Chantilly, Virginia, USA). Evaluation was based on means from at least three independent experiments, each of which included six replicates per concentration level.

**Cell cycle analysis** — Cell cycle analysis was measured by DNA Prep Reagent Kit according to the manufacturer’s instructions (Beckman Coulter, Fullerton, CA, USA). The concentrations of fluvastatin applied in HepG2, SMMC-7721, MHCC-97H cells were 20, 10 and 5 µM, respectively. After 24 h of incubation, detached cells in the medium were discarded and the remaining cells were harvested and resuspended at a density of 3-5x10^6 cells/ml in cold PBS/2% FBS. Then, 100 µl of single cell suspension was added to 100 µl lysis and permeabilizing reagent, vortexed and incubated with 2 ml of DNA Prep Stain for 30 min. Finally, the samples were analyzed by Beckman Coulter FC500 Flow Cytometry System with CXP Software (Beckman Coulter, Fullerton, CA, USA) within 1 h.

**Cell invasion analysis** — Invasion assay was performed using Transwell chambers (8 µm pore size; Millipore, Bedford, MA, USA) as described previously. Cells were divided into three groups: DMSO control group; fluvastatin low concentration group; and high concentration group. After 24 h of incubation at 37°C, the cells in the chambers were removed by wiping gently with a cotton swab, and cells on the reverse side of the filter were fixed and stained. The number of cells that had spread through the pores of filter in five representative fields was counted using a phase-contrast microscope (Leica DMLB2, Leica Microsystems AG, Wetzlar, Germany). Each experiment was carried out in triplicate wells and repeated at least twice.

**Cell apoptosis analysis** — Cell apoptosis was examined using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) that measures phosphatidylserine membrane externalization. Cells were seeded in six-well plate at a density of 5x10^5 cells/ml and incubated overnight. Then, fluvastatin at different concentrations (0-20 µM) was added into each well. After 24 h, detached cells in the medium were collected, and the remaining adherent cells were harvested by trypsinization. The cells (1x10^6) were washed with PBS and resuspended in 100 µl of 1xbinding buffer followed by adding 5 µl of Annexin V-FITC and 10 µl of propidium iodide, and then, it was incubated at 25°C in the dark for 15 min. Finally, 400 µl of 1xbinding buffer was added to each tube, and the cells were analyzed by Beckman Coulter FC500 Flow Cytometry System with CXP Software (Beckman Coulter, Fullerton, CA, USA) within 1 h.

**Western blotting** — Cells (0.5-2x10^7 cells) were lysed with pre-chilled lysis buffer. Protein concentration was measured using a BCA assay kit (Sigma) according to the manufacturer’s instructions. After denaturation, samples (40 µg protein) were separated by 10-15% SDS-PAGE gel electrophoresis and electroblotted onto polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was then blocked with Tris buffered saline/Tween-20 (TBST) containing 5% w/v nonfat dry milk. Then, the membrane was exposed to the specific primary antibody Bcl-2, Bax, cytochrome c (Santa Cruz, California, USA), pro-caspase-9, caspase-3 (Cell Signaling, Danvers, Massachusetts, USA) at appropriate dilution in TBST at 4°C overnight. Washed with TBST thrice, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature with rotation. After washing, protein bands specific for antibody were visualized using enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

**Analysis of mitochondrial membrane potential (MMP)** — Changes in MMP during the fluvastatin-induced apoptosis in HCC cells were quantified by flow cytometry with Rhodamine-123 staining (Molecular Probes), based on depolarization of MMP resulting in the loss of Rhodamine-123 from mitochondria and a decrease in intracellular fluorescence. After 24 h treatment with fluvastatin, cells were harvested, washed twice with PBS, and
exposed to rhodamine-123 (10 µg/ml, Sigma-Aldrich) for 30 min at 37°C. The fluorescence intensity of the cells was analyzed by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Statistical analysis — Data was subjected to statistical analysis using SPSS for Windows (version 15.0, Chicago, IL, USA). Data were presented as mean ± SD. Student’s t test was used to analyze the difference between the means of the treatment and the control groups. In all analyses, \( P < 0.05 \) was considered statistically significant.

Results

Effects of fluvastatin on the growth of HCC cells — Fluvastatin was found to induce growth inhibition of HCC cells in a dose-dependent manner. The mean IC50 values of fluvastatin in the HepG2, SMMC-7721, MHCC-97H cells were 19.73, 9.87 and 4.83 µM, respectively (Fig. 1). In the following experiments, various concentrations of fluvastatin were used in HepG2 cells (10 and 20 µM), SMMC-7721 cells (5 and 10 µM) and MHCC-97H cells (2 and 5 µM) according to the results of MTT assay.

Effects of fluvastatin on the cell cycle of HCC cells — To observe the changes in cell cycle distribution induced by fluvastatin treatment, HCC cells were cultured in absence or presence of different concentrations of fluvastatin for 24 h. PI-stained cells were analyzed using flow cytometry to quantify cells in certain cell cycle stages. Flow cytometric analysis revealed that the proportion of fluvastatin treated HCC cells decreased at S-phase of the cell cycle, with a concomitant increase in the proportion of those in the G2/M phase, compared with untreated cells (Fig. 2; Table 1).

| Table 1 — Assessment of fluvastatin effect on three cell lines using flow cytometry (PI-stained cells) [Values are mean±SD of 3 replications] |
|----------------------|----------------------|----------------------|
|                      | Percent of cells in phase |          |
|                      | G0/G1                  | S                  |
| HepG2               | Control                 | 54.5±4.4            | 35.8±4.5  |
|                     | Fluvastatin (20µM)     | 55.5±6.6            | 15.7±3.8* |
| SMMC-7721          | Control                 | 54.1±6.5            | 31.2±2.4  |
|                     | Fluvastatin (10 µM)    | 63.4±5.6            | 12.5±2.6* |
| MHCC-97H           | Control                 | 60.9±4.4            | 25.1±5.2  |
|                     | Fluvastatin (5µM)      | 57.8±6.4            | 10.2±2.4* |

\( P<0.05 \) compared to control.

Fig. 1 — Antiproliferative effects of fluvastatin. Cell viability was expressed as a ratio of the absorbance between treated cells and untreated controls.

Fig. 2 — Effects of fluvastatin on cell cycle profile. Representative flow cytometry cell cycle profiles for three cell lines are presented (see Table 1 also).
Fluvastatin decreased the invasion potency of HCC cells — Impacts of fluvastatin on invasion potency of HCC cells were assessed using Transwell chambers, in which the Matrigel matrix served as a reconstituted basement membrane in vitro. As shown in Fig. 3, HepG2 cell numbers of 10 and 20 µM fluvastatin treated groups were 67 ± 9 and 17 ± 5, respectively, which were significantly less than control group (152 ± 7). SMMC-7721 cell numbers of 5 and 10 µM fluvastatin treated groups were 63 ± 7 and 37 ± 8, respectively, which were significantly less than control group (146 ± 10). MHCC-97H cell numbers of 2 and 5 µM fluvastatin treated groups were 65 ± 10 and 35 ± 4, respectively, which were still significantly less than 185 ± 8 in control group. The results suggested that treatment with fluvastatin could significantly decrease the invasion potency of HCC cells.

Fluvastatin induced significant apoptosis of HCC cells — Inhibition in cell proliferation and survival after fluvastatin treatment might be due to apoptosis, necrosis, or both. Flow cytometry was applied to identify the mode of cell death. Compared with the untreated cells, all three HCC cell lines showed an increase in apoptosis, including both early and late apoptotic cell death (Fig. 4). Data revealed that fluvastatin was able to induce apoptosis in approximate 40% of cancer cells at a concentration of 5 µM in MHCC-97H within 24 h, and more than 30% of the cancer cells have undergone apoptosis within 24 h after fluvastatin treatment (10 µM in SMCC-7721, 20 µM in HepG2). These results indicated that fluvastatin induced apoptosis, instead of necrosis, in all three HCC cell lines.

Fluvastatin induced the activation of a mitochondria-operated pathway of apoptosis in HCC cells — The present study showed that Bax, Bcl-2, cytochrome c, procaspase-9 and caspase-3 were involved in the regulation of apoptosis. Their expression levels were detected by Western blotting after 24 h of fluvastatin treatment. Figure 5 showed representative Western blots demonstrating the effects of fluvastatin on the protein expression. The treatment with fluvastatin caused dose-dependent increase in Bax expression and decrease in Bcl-2 expression. A remarkable increase of cytochrome c level was observed after fluvastatin treatment in all three HCC cells. Immunoblot analysis of the pro-caspase-9 and active forms of caspase-3 was also undertaken and observed that both could be activated through cytochrome c release. As shown in Fig. 5c, pro-caspase-9 was significantly downregulated accompanied with a subsequent increase of cleaved-caspase-3 in all three cancer cells. Due to cytochrome c release linked to the loss of MMP, we also examined the effect of fluvastatin on the MMP by using the Rhodamine-123, a fluorescent dye that accumulates rapidly and selectively within mitochondria depending on the membrane potential. Fluvastatin caused disruption of MMP as evidenced by a decrease in the proportion of cells with higher fluorescence intensity (Fig. 6). In summary, these findings suggested that fluvastatin-induced apoptosis...
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Discussion

Recently, statins have been studied as potential anticancer therapeutics. Increasing evidence suggests that statins may be useful for cancer therapy either via effects on cell cycle arrest and induction of proliferation suppression6,7, or via induction of apoptosis of malignant cells16,17. Lovastatin suppresses EGF-induced anaplastic thyroid cancer cell proliferation and invasiveness via reduction of Rho geranylgeranylation7. Simvastatin inhibits proliferation of human prostate cancer PC-3 cells via down-regulation of insulin-like growth factors 1 receptor18. It seems that statins can suppress growth and induce apoptosis more evident in malignant cells than in normal cells19,20, probably due to increased expression of HMG-CoAR and a greater requirement for mevalonate-derived isoprenoids in cancer cells comparing with normal cells21. Mechanisms for all of those effects; however, remain unclear. It seems that statins have a complicated interaction with cancer cells in cell signaling pathways.

However, less attention has been paid to study whether fluvastatin can induce apoptosis in HCC cell lines. Moreover, even if it does work, a mechanism involved mitochondria-operated pathway in this process is still unclear. Therefore, the present study was undertaken to observe effect of fluvastatin in HepG2, SMMC-7721 and MHCC-97H cells. It was observed that fluvastatin could inhibit the proliferation of HCC cells and induce a cell cycle arrest at the G2/M phase. The invasive and metastatic potentials of tumor cells are major features of
malignancy. Metastasis of HCC is often responsible for the high mortality rate. The present study revealed that the invasion rate of three HCC cell lines was significantly decreased after incubation with fluvastatin (Fig. 3) and induce apoptosis (Fig. 4).

In the mitochondria-operated pathway of apoptosis, caspase activation is closely associated with mitochondrial outer membrane permeabilization and the mitochondrial permeability transition plays a key role in the process. Previous research has demonstrated that cholesterol may stabilize mitochondrial membranes against Bax permeabilizing action by induction of negative curvature, consistent with the accumulation of cholesterol in high-curvature regions of membranes. Cholesterol metabolism is abnormal in many malignancies with loss of cholesterol feedback and HMG-CoAR up-regulation even in cases of increased cholesterol levels. Previous research has proved that the mitochondrial cholesterol depletion by statins can increase the sensitivity of inducing apoptosis in HCC.

The present study showed that there were an increase of Bax expression and a decrease of Bcl-2 expression, together with a remarkable increase of cytochrome c level after fluvastatin treatment in all cell lines.

Fig. 5 — Mitochondria-operated pathway of apoptosis in HCC cells treated with fluvastatin. The expression of Bax, Bcl-2, pro-caspase-9, cleaved-caspase-3 in whole cell lysate and cytochrome c in cytosolic extract were examined by Western blotting. β-actin was also stained to serve as an internal control.

Fig. 6 — Effects of fluvastatin at different concentration upon MMP in human HepG2, SMCC-7721, MHCC-97H cell lines. MMP was evaluated by the uptake of a membrane potential-sensitive fluorescence dye Rhodamine-123. The fluorescence intensity was analyzed with a flow cytometer. Results were expressed as mean Rhodamine-123 fluorescence [means ± SD of 3 independent experiments, *P < 0.05 vs control].
three types of HCC cells (Fig. 5). Depletion mitochondrial cholesterol by fluvastatin make it easy for Bax to penetrate into the mitochondrial membrane and, hence, results in susceptibility to cell death. The released cytochrome c from mitochondria promotes activation of caspase-9 by forming a complex with apoptosis protease activating factor-1 in the presence of ATP. Afterwards, caspase-3 is also activated, and results in apoptosis. In the present study, pro-caspase-9 was down-regulated and subsequently there was significant increase of cleaved-caspase-3 in all three HCC cell lines (Fig. 5). On the other hand, decrease of MMP was detected (Fig. 6). These results suggested that the mitochondrial pathway could play a major role in apoptosis induction by fluvastatin in the three HCC cell lines.

Statins are generally thought to exert their effects in cancer by inhibiting the prenylation of small G-proteins, such as Ras and Rho, which in turn regulate the signal transduction of several membrane receptors proteins, such as Ras and Rho, which in turn regulate the signal transduction of several membrane receptors that are crucial for the transcription of genes involved in cell growth, differentiation, and apoptosis. In the present study, we found an alternative mechanism for fluvastatin induced cell apoptosis via activating a mitochondria-operated pathway. Although further clinic trials are necessary, fluvastatin may be a potential useful agent in the therapy of HCC in the future.

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References