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Simvastatin and atorvastatin as inhibitors of proliferation and inducers of apoptosis in human cholangiocarcinoma cells

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ABSTRACT

Aims: In this study, we investigated whether statins induce human cholangiocarcinoma (CCA) cell death and apoptosis, and examined the mechanism by which statins act on cells.
Main methods: Four CCA cell lines, KKU-100, KKU-M055, KKU-M214, and KKU-M156 CCA cell lines were examined for HMGCR mRNA expression by the RT-PCR method. Two CCA cell lines, with low and high HMGCR mRNA expression, were used to evaluate the sensitivity to two statins, simvastatin and atorvastatin. Cytotoxic activity, antiproliferative activity, and cell migratory effects of the statins on CCA cells were evaluated using sulforhodamine B (SRB) and acridine orange/ethidium bromide (AO/EB), the colony formation assay, and wound healing assay, respectively. ROS formation was measured and apoptosis-related proteins were analyzed by Western blotting.
Key findings: Both statins induced KKU-100 and KKU-M214 cell death in a time- and dose-dependent manner. Statins induced cell death more potently in the KKU-100 cells exhibiting low HMGCR expression than the KKU-M214 cells which had high HMGCR expression. Simvastatin was more potently cytotoxic than atorvastatin with lower IC_{50} values. Treatment with statins also caused a concentration-dependent decline in colony forming ability and cell migration. Both statins induced reactive oxygen species (ROS) formation in KKU-100 cells, but not in KKU-M214 cells. Simvastatin enhanced the release of cytochrome *c*, caspase 3, and increased p21 levels, especially for the KKU-100 cells.
Significance: Statins induced CCA cell death, inhibited cell migration, and induced apoptosis. Cell death was probably induced via the mitochondrial pathway. Statins could potentially be developed as novel chemotherapeutic agents for CCA.

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1. Introduction

Statins are a class of drugs that lower serum cholesterol by inhibiting HMG-CoA reductase (HMGCR), the rate-limiting enzyme of the mevalonate (MVA) pathway. They are currently widely used, relatively safe, well-tolerated, and effective agents for the treatment of dyslipidemia, particularly, hypercholesterolemia. Statin-induced decreases in serum cholesterol, triglyceride and improvement of serum lipoprotein are associated with a reduction in cardiovascular risk, such as atherosclerosis, coronary heart disease and stroke [1,2]. Statins also have a potential role in the treatment of cancer because they interact with the MVA pathway, resulting in inhibition of intermediary products including isoprenoid precursors, geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) [2]. FPP and GGPP are necessary for post-translational modification of the small G-proteins, including Rho, Rac, and Ras, which regulate cellular mechanisms such as cytoskeletal reorganization, cellular transformation, cell migration, cell invasion, and cell proliferation [3]. Statins can activate cancer cell death and apoptosis through reduction of GGPP and FPP levels.

In vitro and in vivo studies have revealed that statins inhibit growth and induce apoptosis in numerous types of cancer cell including lung, colon, breast, and prostate cancer cells [4]. It has been suggested statins induce cancer cell apoptosis through stimulation of nitric oxide (NO) and reactive oxygen species (ROS) formation [5,6], suppression of Akt and NF-κB [7,8], as well as down-regulation of Bcl-2 and up-regulation of Bax proteins [9]. Furthermore, statins in combination with gemcitabine, cisplatin and 5-fluorouracil (5-FU) showed additive antiproliferation activity [10]. Importantly, the inhibition of cancer growth and reduction of metastasis in vivo were apparent at clinically

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achievable doses [11]. Thus, the statin family of drugs could potentially be developed as molecular targeted therapeutics to battle cancers including CCA.

Cholangiocarcinoma (CCA) is a highly malignant adenocarcinoma originating from bile duct epithelial cells [12,13]. It is a rare type of cancer worldwide; nevertheless, populations residing in certain regions in Southeast and East Asian are at a very high risk [14]. The prognosis of CCA patients is poor because most patients are already in the advanced stages of the disease at diagnosis. Currently, surgical resection with a histologically tumor free margin is the only chance for cure and extends lifespan for up to 5 years [15,16]. Chemotherapy with cisplatin based regimens does not considerably improve survival in non-resectable CCA patients [17]. Therefore, a new effective treatment is urgently needed. Several strategies in sensitizing resistant CCA cells to chemotherapeutic drugs have been attempted including inhibition of NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1) and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [18–21]. Limited information is available, however, on the therapeutic potential and molecular mechanism of action of statins against CCA. In this study, therefore, we investigated the effects of two different structural classes of statin, simvastatin and atorvastatin, on induction of antiproliferation, apoptosis and inhibition of migration in CCA cells.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and other cell culture reagents were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Simvastatin, atorvastatin, protease inhibitor cocktail, dihydroethidium (DHE), RIPA lysis buffer and sulforhodamine B (SRB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies against $p21^{Cip/WAF1}$, caspase 3, cytochrome *c*, beta-actin, and anti-rabbit IgG HRP-link antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). iScript reverse transcription Supermix for RT-qPCR and SsoFast™ EvaGreen Supermix were supplied by Bio-Rad (Hercules, CA, USA).

2.2. Cell lines and cell culture conditions

The human cholangiocarcinoma cell lines used in this study, KKU-100, KKU-M156, KKU-M214, and KKU-M055, were kindly provided by Dr. Banchob Sripa of the Department of Pathology, Faculty of Medicine, Khon Kaen University. Four CCA cells were maintained at 37 °C and 5% CO_2 in DMEM medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and 100 μg/m$_l$L streptomycin. The DMEM media was renewed every 3 days, trypsinized with 0.25% trypsin-EDTA and subcultured in the same media.

2.3. Cell viability assay

Briefly, cells were plated in a 96-well plate for 24 h. After exposure to test compounds at various concentrations for 24 or 48 h, the cultured cells were fixed with ice-cold 10% trichloroacetic acid (TCA) for 1 h at 4 °C and stained with 0.4% SRB for 30 min at room temperature. Excess dye was removed by washing several times with 1% acetic acid, and protein-bound dye was dissolved with 200 μL 10 mM Tris base solution for determination of absorbance with a microplate reader with a filter wavelength of 540 nm.

2.4. Clonogenic assay

Five hundred viable cells were seeded onto 6-well plates and allowed to grow for 24 h. The cells were then treated with different concentrations of statin for 24 h. The cells were washed with phosphate buffered saline (PBS), and fresh medium was added. The cells were then cultured for another 10 days. After this, the cultured cells were stained with 0.5% crystal violet in 25% methanol, and the number of colonies was counted.

2.5. Wound healing assay

Cell migration was assessed using a wound healing assay described previously [22]. Briefly, KKU-100 cells were seeded into a 24-well plate and allowed to grow for 24 h. A scratch wound was made with a sterile 0.2 mL pipette tip. After washing with PBS to remove detached cells, cells were pretreated with various concentrations of statins and images of the scratched wound were taken from 0 to 48 h. The closing of the scratched wound, representing the migration process, was determined by capture of the denuded area along the scratch using Image-Pro Plus software (Media Cybernetics, LP, USA). The wound distance was calculated by dividing the area by the length of the scratch.

2.6. Reactive oxygen species (ROS) production assay

Intracellular ROS generation was measured using the cell-permeable fluorescent probe, dihydroethidium (DHE). CCA cells were seeded and cultured in black 96-well plates for 24 h. The medium was discarded and the cells were washed with PBS buffer. The cells were then treated with various concentrations of the test compounds and 25 μM DHE in serum-free medium, and kept in a 5% CO_2 atmosphere at 37 °C for 90 min in the dark. Fluorescence intensity was measured at 518 nm (excitation) and 605 nm (emission) on a fluorescent microplate reader. The data were expressed as the percentage of ROS relative to untreated controls.

2.7. Acridine orange/ethidium bromide (AO/EB) staining

AO/EB staining was used to quantify apoptotic and necrotic cells as previously described [23]. Briefly, at the end of the 24 h treatment with statins, cultured cells were rinsed with PBS and stained with AO and EB (each 1 μg/ml). The fluorescent images were captured using a Nikon Eclipse TS100 inverted microscope with excitation and long-pass emission filters of 480 and 535 nm. The number of viable, apoptotic, and necrotic cells were enumerated and calculated as a percentage by dividing by the total number of cells in the same area.

2.8. Reverse transcription real-time polymerase chain reaction (RT-PCR)

Four CCA cell lines were seeded in 6 well-plates and allowed to growth for 24 h. The total RNA was collected using TriZol® reagent, according to the manufacturer's instructions. Total RNA (1 μg) was then reverse transcribed to single-stranded cDNA by the iScript reverse transcription Supermix at 42 °C for 60 min. The reverse transcription products served as a template for real-time PCR. The primer sequences were as follows: HMGCR: forward, 5′-TTC-TTG-CCA-ACT-ACT-TCG-TGT-T-3′ and HMGCR reverse: 5′-GCT-GCC-AAA-TTG-GAC-GAC-C-3′, beta-actin: forward 5′-GCA-CAG-AGC-CTC-GCC-TT-3′ and beta-actin: reverse, 5′-GTT-GTC-GAC-GAC-GAG-CG-3′. The PCR was performed in a final volume of 20 μL containing cDNA template, 5 μM of each HMGCR primer or 2.5 μM of each beta-actin primer in SsoFast™ EvaGreen Supermix with low Rox (Bio-Rad, CA, USA). To determine the relative expression of genes, the relative quantification using standard curve method was used. The amount of HMGCR mRNA was expressed as a ratio to beta-actin mRNA.

2.9. Protein extraction and Western blot analysis

Western blot analysis was used to determine the protein expression levels of p21, cytochrome *c*, and caspase 3, with beta-actin used as an

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