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Simvastatin Stimulates Apoptosis in Cholangiocarcinoma by Inhibition of Rac1 Activity

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Abstract

Background—Simvastatin is a cholesterol-lowering drug that is widely used to prevent and treat atherosclerotic cardiovascular disease. Simvastatin exhibits numerous pleiotropic effects including anti-cancer activity. However, the effect of simvastatin on cholangiocarcinoma has not been evaluated.

Aim—The aim of our study was to determine the effect of simvastatin on cholangiocarcinoma proliferation.

Methods—The effect of simvastatin was evaluated in five human cholangiocarcinoma cell lines (Mz-ChA-1, HuH-28, TFK-1, SG231, and HuCCT1) and normal cholangiocyte cell line (HiBEpiC).

Results—We found that simvastatin stimulates a reduction in cell viability and apoptosis of cholangiocarcinoma cell lines, while in normal human cholangiocytes, HiBEpiC, simvastatin inhibits proliferation with no effect on apoptosis. Simvastatin-induced reduction of cell viability was partially blocked by pre-treatment with metabolites of the mevalonate pathway. In Mz-ChA-1 cells, pre-treatment with cholesterol alone stimulated an increase in the number of viable cells and fully restored cell viability following simvastatin treatment. Treatment with simvastatin triggered the loss of lipid raft localized Rac1 and reduction of Rac1 activity in Mz-ChA-1 cells. This effect was prevented by pre-treatment with cholesterol.

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Conflict of Interest: The authors have no conflicts to disclose.

Conclusion—Collectively, our results demonstrate that simvastatin induces cholangiocarcinoma cancer cell death by disrupting Rac1/lipid raft colocalization and depression of Rac1 activity.

Keywords

Cholangiocarcinoma; simvastatin; Rac1; apoptosis

INTRODUCTION

Cholangiocarcinoma is a deadly neoplasm that results from the malignant transformation of cholangiocytes, arising from both intrahepatic and extrahepatic origins [1]. The incidence of cholangiocarcinoma is increasing worldwide and treatment options remain limited [2]. Survival is quite limited with conventional chemotherapeutic options [3] while complete surgical resection offering the only hope for long-term survival. Thus, alternative chemotherapeutic strategies must be developed [2,4].

Simvastatin competitively inhibits, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis [5]. Originally developed for lowering serum cholesterol levels [6], increasing evidence indicates that statins have therapeutic effects in a number of diseases including cancer [7–10]. Statins trigger a number of pleiotropic effects, including inhibition inflammation [11], modulation angiogenesis [12], and inhibition growth and/or triggering cell death of various human tumor cell lines [13–21].

Inhibition of HMG-CoA reductase results in the depletion of several important cholesterol pathway intermediates, including mevalonate, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). The isoprenoids, FPP and GGPP serve as important lipid attachments for the post-translational modification of small GTP binding proteins, such as Rho, Rac and Ras, whose correct membrane localization in cholesterol lipid rafts and activity are dependent upon isoprenylation [22–24]. Inhibition of mevalonate synthesis prevents the covalent attachment of farnesyl residues on small GTP binding proteins preventing their attachment to the cell membrane and subsequently blocks cell growth [25]. The Rho family of small GTPases includes Rac1, RhoA, and Cdc42 and has been the focus of new targets for novel cancer therapies [26]. These proteins serve as molecular switches that regulate a wide range of cellular responses including cytoskeletal reorganization, cell migration, cellular transformation, and metastasis [27].

Rac1 regulates multiple signaling pathways that control cytoskeleton organization, transcription, and cell proliferation [28]. Rac1 also modulates cytoskeleton organization (such as, actin stress fibers), which is controlled by integrins that regulate the targeting of Rac1 to lipid rafts [29,30]. Disruption of the targeting of activated Rac1 (GTP-bound) to the plasma membrane results in the localization of GTP-Rac1 in the cytoplasm [31,32]. In the cytoplasm, GTP-Rac1 bound to Rho guanine nucleotide dissociation inhibitor (RhoGDI) and effectively uncoupled from its role downstream signaling mechanisms activated by growth factor receptors [33]. Direct inhibition of Rac1 activity induces cell cycle arrest and apoptosis in breast cancer cells [34].

The potential effects of statins on cholangiocarcinoma have not been evaluated. Thus, we evaluated the effects of simvastatin on cholangiocarcinoma proliferation and apoptosis, and Rac1 signaling mechanisms in cholangiocarcinoma cells.

MATERIALS AND METHODS

Materials

Simvastatin, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), and 5-FU were purchased from Sigma-Aldrich (St. Louis, MO). General tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). FTI-277 (a farnesyltransferase inhibitor) [35], GGTI-298 (a geranylgeranyltransferase I inhibitor) [36], Rho-associated protein kinase (p160ROCK) inhibitor Y-27632 (20 μ M) [37], Rac1 Inhibitor (NSC23766) [38] and BrdU Assay Kit were purchased from EMD Biosciences, Inc. (San Diego, CA). The HMG-CoA reductase, Rac1, and β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Vibrant[®] Lipid Raft Labeling Kit, Amplex[®] Red Cholesterol Assay Kit, and Alexa-Fluor 488-phalloidin were purchased from Molecular Probes (Carlsbad, CA). CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and Caspase-Glo 3/7 Assay Systems were purchased from Promega Corporation (Madison, WI). The annexin-V-biotin kit was purchased from Roche Applied Science (Indianapolis, IN). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Cell Culture

We used five human cholangiocarcinoma cell lines (Mz-ChA-1, HuH-28, TFK-1, SG231, and HuCCT1) of different origins. Mz-ChA-1 cells, from human gallbladder, were a gift from Dr. G. Fitz (University of Texas Southwestern Medical Center, Dallas, TX) [39]. HuH-28 cells, from human intrahepatic bile ducts [40], and TFK-1 cells, from human extrahepatic bile ducts [41], were acquired from the Cancer Cell Repository, Tohoku University, Japan (provided by Dr. Gianfranco Alpini, Texas A&M Health Science Center). Mz-ChA-1, HuH-28, and TFK-1 cell lines were maintained at standard conditions [42]. HuCC-T1 and SG231 cells, originating from intrahepatic bile ducts, were obtained from Dr. A.J. Demetris (University of Pittsburgh, PA) and cultured as described [43–45]. The normal human intrahepatic biliary cell line, HIBEpiC, was purchased from ScienCell Research Laboratories (Carlsbad, CA) and cultured as recommended by the vendor. All experiments were performed when cells reached 80% confluence and conducted in serum-free medium with serum deprivation for 24 h prior to experiments.

HMG-CoA Reductase Expression

The expression of HMG-CoA reductase in Mz-ChA-1, TFK-1, SG231, HuCCT-1, HuH-28 and HIBEpiC was detected using immunofluorescence and Western blotting as described [46]. Sections were visualized using an Olympus IX-71 inverted confocal microscope. Sections were visualized using an Olympus IX-71 inverted confocal microscope (Tokyo, Japan). Western blots were performed as described [47]. Images of Western bands were obtained using a Storm 860, Amersham Biosciences (Piscataway, NJ, USA) and subsequent densitometry was carried out using Scion Imaging Software (Frederick, MD, USA).

Cell Viability and Proliferation Assays

We analyzed the effects of simvastatin on cholangiocarcinoma cell viability using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) proliferation assay from Promega [48] with the following cell lines: Mz-ChA-1, TFK-1, SG231, HuCCT-1, and HuH-28 and the normal human cholangiocyte line, HIBEpiC. Cells were stimulated with simvastatin (1 to 100 μ M) for 48 h at 37°C. Other investigators have previously utilized these dosages of simvastatin [49,50]. In addition, Mz-ChA-1 and HIBEpiC cells were stimulated with simvastatin (2 and 5 μ M) for 24, 48, and 72

h. The number of viable cells was assessed using a colorimetric cell assay (CellTiter 96Aqueous, Promega Corp., Madison, WI), and absorbance was measured at 490 nm by a microplate spectrophotometer (SpectraMax M5^e, Molecular Devices, Sunnyvale, CA). The data were expressed as the number of surviving cells (%) compared to the basal treatment.

To determine possible mechanisms of action for simvastatin, Mz-ChA-1 and HiBEpiC cells were pretreated with downstream metabolites of the mevalonate pathway, including mevalonate, FPP, GGPP, and cholesterol with dosages that were previously reported [51]. Mz-ChA-1 and HiBEpiC cells were pretreated mevalonate (1 mM), FPP (10 mM), and GGPP (10 mM) and cholesterol (1 mM) prior to stimulation with simvastatin (5 μ M) for 48 hr. Cell proliferation/viability was evaluated by MTS assay as described above [48].

To determine the relative role of geranylgeranylation, farnesylation, RhoA and Rac-dependent signaling mechanisms, cell viability was evaluated in the absence/presence of geranylgeranyltransferase (GGTase) inhibitor (GGTI-298) [36] and farnesyltransferase (FTase) inhibitor (FTI-277) using previously reported dosages [35,36]. GGTase and FTase convert GGPP and FPP into families of prenylated proteins including Ras, Rho, and Rac which are extremely important in various cell functions including cell proliferation, differentiation, and migration [52]. Cells were stimulated with FTI-277 (10 μ M) and GGTI-298 (10 μ M) for 48 h. The effects of the inhibition of Rho-kinase (effector kinase downstream of RhoA, p160ROCK) and Rac1 were evaluated in Mz-ChA-1 cells treated with the p160ROCK inhibitor, Y-27632 (20 μ M) [37] and Rac1 inhibitor (NSC23766; 100 μ M) [38] for 48 h. Cell viability was evaluated by MTS assay as described above [48].

Evaluation of Apoptosis

Apoptosis was evaluated using annexin V labeling of cells as described [48,53]. Cells were treated with simvastatin (5 μ M) with and without pretreatment with cholesterol (1 mM) for 24 h. The number of annexin V-positive cells was counted and expressed as a percentage of total cells in five random fields for each treatment group (a minimum of 500 cells was observed for each treatment group).

The activation of caspase 3/7 was examined Mz-ChA-1 and HiBEpiC cells treated with simvastatin. Caspase 3/7 activity was determined with the Caspase-Glo 3–7 Assay kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions. Cells were treated with simvastatin (5 μ M) with and without pretreatment with cholesterol (1 mM), incubated at 37°C for 6 h, and 100 μ l of Caspase-Glo 3/7 Reagent was added to each well. Luminescence was measured with Fluoroskan (ThermoLabsystems, Waltham, MA). Data were expressed as the fold-change of treated cells, as compared with respective basal controls.

Rac1 Activity

The G-LISATM Rac1 Activation Assay Biochem KitTM from Cytoskeleton (Denver, CO) to evaluate the effects of simvastatin on Rac1 activity in Mz-ChA-1 and HiBEpiC cells. Cells were then treated with vehicle or simvastatin (5 μ M) with and without cholesterol (1 mM) for 30 min at 37°C. Rac1 activity was then assessed according to the manufacturer's instructions, at an absorbance of 490 nm using a microplate spectrophotometer (SpectraMax M5^e, Molecular Devices, Sunnyvale, CA). Data were expressed as the fold-change of treated cells as compared with respective basal controls.

Lipid Raft Labeling

Lipid rafts were visualized using the Vibrant[®] Lipid Raft Labeling Kit (Molecular Probes) [48]. Mz-ChA-1 cells were seeded onto sterile coverslips placed in the bottom of 6-well

culture dishes and allowed to adhere overnight. Cells were treated with simvastatin (5 μ M), cholesterol (1 mM), or simvastatin plus cholesterol for 4 hours at 37°C. Coverslips were then moved into a new 6-well plate for staining. Cells were incubated in the presence of 1 μ g/ml (diluted in growth media) of fluorescent cholera toxin subunit B labeled with Alexa Fluor® 488 for 10 minutes at 4°C. Cells were then gently washed with 1x PBS, then fixed in 4% paraformaldehyde for 15 minutes at 4°C. In the lipid raft/Rac1 co-localization studies, the lipid rafts were stained as described above. Cells were permeabilized by incubating in 1x PBS containing 0.1% Triton X-100 at room temperature for 10 minutes. The cells were counterstained with an antibody against Rac1 overnight at 4°C. Immunofluorescent staining was carried out using Cy2-labeled anti-rabbit secondary antibody. Finally, the coverslips were mounted onto microscope slides with Prolong® Gold antifade reagent with DAPI, and visualized using an Olympus IX-71 inverted confocal microscope. Quantitative analysis of colocalization was performed with ImageJ. Six images were analyzed per treatment group. The data is reported as % colocalized pixels per region of interest (ROI).

Cholesterol Assays

Cholesterol levels in total cell lysates from Mz-ChA-1 and HiBEpiC cells (basal and simvastatin (5 μ M)) were measured using Amplex® Red Cholesterol Assay Kit following the manufacturer's instructions (Invitrogen).

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Differences between groups were analyzed by the Student unpaired t test when two groups were analyzed and by Mann-Whitney U test and Kruskal-Wallis H test when more than two groups were analyzed, followed by an appropriate post hoc test. A value of $p < 0.05$ was considered significant.

RESULTS

Cholangiocarcinoma and Normal Cholangiocyte Cell Lines Express HMG-CoA Reductase

All of the cholangiocarcinoma cell lines evaluated and the normal human cholangiocyte cell line, HiBEpiC, express HMG-CoA reductase by immunofluorescence (Figure 1a) and Western blotting (Figure 1b). There were no significant differences in expression levels of HMG-CoA reductase among the cell lines.

Simvastatin Reduces Cholangiocarcinoma Viability

MTS assays revealed that simvastatin treatment (1 – 100 μ M) dose-dependently reduces the number of viable (i.e., % of surviving cells) in all of the cholangiocarcinoma cell lines and to a lesser extent in HiBEpiC (Figure 2a). The time-dependent effects of simvastatin were less obvious, although the reduction in the numbers of viable cells were significant (Figure 2b).

Effect of Mevalonate Pathway Metabolites

Mz-ChA-1 and HiBEpiC cells were cultured with simvastatin in combination with mevalonate, FPP, GGPP, and cholesterol. Mevalonate, FPP, GGPP, and cholesterol are all downstream metabolites in the cholesterol synthesis pathway, and have shown to reverse the effects of statins in various studies [49,54]. However, in our study, only cholesterol completely reversed the effects of simvastatin in Mz-ChA-1 cells (Figure 3, top). Mevalonate, FPP, and GGPP all partially reversed the effect simvastatin (Figure 3, top). Cholesterol 1 mM alone increased the number of viable Mz-ChA-1 cells by MTS assay compared to the basal treatment group. In comparison with HiBEpiC, the addition of the pathway metabolite, mevalonate, fully restored the number of viable cells to that of the basal

treatment group (Figure 3, bottom). FPP, GGPP, and cholesterol all partially restored cell viability, suggesting the involvement of multiple mechanisms in simvastatin-induced reduction in cell proliferation in the normal cholangiocyte cell line, HiBEpiC (Figure 3, bottom). Interestingly, cholesterol did not stimulate the proliferation of the normal cholangiocyte cell line, HiBEpiC, which suggests that cholesterol may regulate cholangiocarcinoma proliferation.

Simvastatin Activates Apoptosis in Cholangiocarcinoma Cells

Simvastatin significantly increased the number of annexin-V positive Mz-ChA-1 cells (Figure 4a). The percentage annexin-V positive Mz-ChA-1 cells observed are presented in the graph on the right (Figure 4a). Simvastatin did not alter the number of annexin-V positive HiBEpiC cells (Figure 4a). The effect of simvastatin on the number of annexin-V positive Mz-ChA-1 cells was prevented by preincubation with cholesterol. To confirm that simvastatin stimulates apoptosis in Mz-ChA-1 cells, caspase 3/7 activity was determined. Similar to the annexin-V staining, simvastatin stimulated a significant increase in caspase activity in Mz-ChA-1 cells, which was prevented by preincubation with cholesterol (Figure 4b). Simvastatin had no effect on caspase activity in HiBEpiC (Figure 4b).

Inhibition of Isoprenylation and Rac Activity Suppresses Cell Viability in Cholangiocarcinoma Cells

The enzymes GGTase and FTase catalyze the isoprenylation of small GTPases, such as Rac1 and RhoA. We treated CCA cells with inhibitors of GGTase and FTase, FTI-277 (10 μ M) and GGTI-298 (10 μ M), respectively. Both inhibitors produced a significant decrease in Mz-ChA-1 cell viability based on MTS assays (Figure 5a). We next evaluated the potential roles of Rac1 and RhoA in the effects of simvastatin on Mz-ChA-1 cells. The inhibition of Rho-kinase (effector kinase downstream of RhoA) and Rac1 were evaluated in Mz-ChA-1 cells treated with the p160ROCK inhibitor, Y-27632 and Rac1 inhibitor (NSC23766). Inhibition of Rac1, but not p160ROCK, resulted in a significant decrease in Mz-ChA-1 cell viability (Figure 5a).

Simvastatin Suppresses Rac1 Activity in Cholangiocarcinoma Cells

Evaluation of Rac1 activity revealed that simvastatin significantly inhibits Rac1 activity in Mz-ChA-1 cells but not HiBEpiC cells (Figure 5b). The inhibition of Rac1 activity in Mz-ChA-1 cells was prevented by preincubation with cholesterol (Figure 5b). Cholesterol restored Rac1 activity to levels significantly higher than basal Rac1 activity levels (Figure 5b). Simvastatin had no effect on Rac1 activity in HiBEpiC cells (Figure 5b).

Simvastatin Disrupts Rac1 Co-localization in Lipid Rafts

Since simvastatin is known to reduce cellular cholesterol levels, we evaluated the effects on simvastatin on Mz-ChA-1 and HiBEpiC total cellular cholesterol levels [55]. We found that simvastatin induces a significant reduction in total cellular cholesterol levels in both Mz-ChA-1 and HiBEpiC cell lines (Table 1). Mz-ChA-1 cells had higher basal total cholesterol levels compared to the normal cell line, HiBEpiC (Table 1). To characterize the effect of simvastatin on lipid raft/Rac1 colocalization, staining for lipid rafts and Rac1 was performed. In the basal treatment, lipid rafts Rac1 co-localization is present in the cell membrane (Figure 5c and 5d). Mz-ChA-1 cells treated with simvastatin exhibited a lack of lipid raft localization of Rac1. In Mz-ChA-1 cells treated with simvastatin, Rac1 staining appeared as punctate structures in the cytoplasm (Figure 5c and 5d). However, cells treated with cholesterol displayed increased Rac1 co-localization in the lipid rafts (Figure 5c and 5d). The lipid raft and Rac1 co-localization disrupting effects of simvastatin were prevented by pre-incubation with cholesterol (Figure 5c and 5d).

DISCUSSION

Statins have been extensively studied due to their serum cholesterol lowering actions in humans. However, statins are pleiotropic agents, which have been shown to have antitumor effects. In the present study, we demonstrate that simvastatin stimulates cholangiocarcinoma apoptosis and that this activity was closely associated with (i) decreased total cellular cholesterol, (ii) disruption of Rac1 co-localization in lipid rafts, (iii) the down regulation of Rac1 activity, and (iv) along with increased apoptosis characterized by increased numbers of annexin V-positive cholangiocytes and increased caspase activity.

In our study, there was no detectable difference in the expression levels of HMG-CoA reductase expression levels in the normal compared to the cholangiocarcinoma cell lines. However, several investigators have reported that there is either deficient feedback control of HMG-CoA reductase or increased expression and activity in a number of tumor types including hepatocellular carcinoma and colorectal cancer [56–60]. Two alternatively spliced isoforms have been identified: full-length HMG-CoA reductase and a version that lacks exon 13 [61]. Overall risk of developing colorectal cancer was associated with expression of HMG-CoA reductase lacking exon 13, which suggested that patients expressing this isoform would not benefit as greatly from the protective benefits of statin utilization [62]. We evaluated HMG-CoA reductase expression only in cells lines cultured *in vitro*, which may not be completely indicative of expression levels and activity in human cholangiocarcinoma tissue samples. However, our evaluation of total cellular cholesterol levels does indicate that there is increased cholesterol levels in cholangiocarcinoma cells compared to the normal cholangiocytes, which would indicate a dysregulation of HMG-CoA reductase activity or feedback control. Further investigation of HMG-CoA expression in human cholangiocarcinoma tissue samples are required to understand if there is a dysregulation of the mevalonate pathway and over or under expression of HMG-CoA reductase or its alternatively spliced isoforms.

Simvastatin decreased cell viability of all cholangiocarcinoma cells lines and normal cholangiocytes, HiBEpiC. Simvastatin treatment significantly increased the number of annexin V-positive Mz-ChA-1 cells and caspase 3/7 activity. However, simvastatin did not stimulate apoptosis in HiBEpiC suggesting that simvastatin triggers a cell death mechanism in cholangiocarcinoma cells while simply inhibiting the proliferation of HiBEpiC. Simvastatin stimulates apoptosis in a number of cancer cell types via a variety of intracellular signaling mechanisms [17,63]. Similar to our observation in HiBEpiC, other normal cell types have been shown to be more resistant to the antiproliferative effects of statins when compared to faster proliferating tumor cells [64,65]. It has been reported that statins can trigger apoptosis in a tumor specific manner, while non-malignant cells of the sample tissue type do not undergo apoptosis [66,67].

Due to its inhibitory effect on HMG-CoA reductase, simvastatin triggers the depletion of several important cholesterol pathway intermediates, including mevalonate, FPP, GGPP, and lastly cholesterol. Evidence exists that several cancer cell types have higher membrane cholesterol levels are more sensitive to apoptosis induced by cholesterol-depleting agents [55,63]. Mz-ChA-1 cells have higher total cellular cholesterol levels compared to HiBEpiC. Treatment with simvastatin significantly lowers total cellular cholesterol levels Mz-ChA-1 cells and slightly in the normal cells during treatment. It is important to note that the HMG-CoA reductase protein expression levels were not significantly different by Western blotting between the cholangiocarcinoma cell lines and HiBEpiC, which does not reconcile with increased cholesterol levels in Mz-ChA-1 cells and might suggest that HMG-CoA reductase activity may be higher in cholangiocarcinoma cells.

The mevalonate pathway metabolites, mevalonate, FPP and GGPP, partially prevented the simvastatin-induced reduction in cell viability in Mz-ChA-1 cells, suggests the involvement of several signaling downstream mechanisms that involve Rho family members [68]. In support of this concept, we found that inhibitors of GGTase (GGTI-298) and FTase (FTI-277) also reduce the number of viable Mz-ChA-1 cells. Pre-treatment cholesterol completely reversed the effects of simvastatin on cell viability. Interestingly, cholesterol alone stimulated a significant increase in number of Mz-ChA-1 viable cells (but not HiBEpiC), which suggests that cholesterol may play a role in regulating cholangiocarcinoma proliferation. Cholesterol stimulates prostate cancer cell proliferation *in vitro* and hypercholesteremic diet promoted xenograft growth [63,69]. In the normal HiBEpiC, mevalonate fully reversed the antiproliferative effects of simvastatin while FPP, GGPP and cholesterol partially reversed the effects of simvastatin. Cholesterol reversed the effects of simvastatin on the number of annexin V-positive cells and simvastatin-stimulated caspase activity in Mz-ChA-1 cells, which suggests that cholesterol plays a key role in the survival of cholangiocarcinoma. Using inhibitors for Rac1 and p160ROCK, inhibition of Rac1, but not RhoA (i.e., the downstream kinase p160ROCK), results in decreased Mz-ChA-1 cell viability. In support of the role of Rac1 in the regulation of Mz-ChA-1 cell viability, treatment with simvastatin significantly depresses Rac1 activity, which was blocked by pre-treatment with cholesterol.

Rac1 activity is dependent upon its localization in lipid rafts [29]. Under basal conditions, Rac1 co-localizes to lipid rafts in Mz-ChA-1 cells. Treatment with simvastatin triggers disruption of the colocalization of Rac1 in lipid raft structures in Mz-ChA-1 cells. This effect was prevented by pre-treatment with cholesterol, which potentially augments cellular cholesterol levels aiding in the stabilization of the colocalization Rac1 with lipid raft structures in Mz-ChA-1 cells.

We demonstrated that simvastatin-induced apoptosis in cholangiocarcinoma cells was dependent upon dysregulation of the cholesterol biosynthetic pathway resulting in disruption of Rac1 activity. Two potential mechanisms regulate depression of Rac1 activity: (i) suppression of isoprenoid biosynthesis inhibits the placement of Rac1 in lipid rafts; and (ii) disruption of lipid raft-Rac1 co-localization by alteration of cellular cholesterol levels. Our data indicate that targeting of Rac1 either through modulation of the cholesterol pathway or by direct inhibition for the treatment of cholangiocarcinoma deserves careful consideration. A recent study has implicated dysregulation of the mevalonate pathway in the promotion of transformation and suggests that HMG Co-A reductase may have oncogenic potential [70] and suggests that studies are needed to provide knowledge of HMG-CoA reductase expression *in situ* in cholangiocarcinoma tumors and in disease states such as primary sclerosing cholangitis, which is a risk factor for the development of cholangiocarcinoma. Considering the extensive experience on the safety of statins in humans, investigation of the utilization of statins as therapy alone or in combination with traditional chemotherapeutics for cholangiocarcinomas may be warranted.

Abbreviations

5-FU	5-fluorouracil
BrdU	Bromodeoxyuridine
DAPI	4',6-diamidino-2-phenylindole
FPP	farnesyl pyrophosphate
FTase	farnesyltransferase

GGPP	geranylgeranyl pyrophosphate
GGTase	geranylgeranyltransferase
HMG-CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MBCD	methyl- β -cyclodextrin
Mev	mevalonate
PBS	phosphate buffered saline

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Figure 1. Cholangiocarcinoma and normal cholangiocytes express HMG-CoA reductase
[a] Cholangiocarcinoma (Mz-ChA-1, HuCC-T1, SG231, HuH-28, and TFK-1) and HIBEpiC cell lines express HMG-CoA reductase by immunofluorescence. HMG-CoA reductase expression is shown in *red* with nuclei counterstained with DAPI (blue). Original magnification, $\times 60$. **[b]** Cholangiocarcinoma cell lines and HIBEpiC express HMG-CoA reductase by immunoblotting. No significant differences in the protein expression levels of HMG-CoA reductase were observed.

Figure 2. Simvastatin decreases the number of viable cholangiocarcinoma and normal cells in a dose and time-dependent manner

[a] Cholangiocarcinoma (Mz-ChA-1, HuCC-T1, SG231, HuH-28, and TFK-1) and HIBEpIC cell lines were treated with escalating doses of simvastatin (1–100 μ M) for 48 h and cell viability was determined by MTS proliferation assay. Simvastatin dose-dependently decreased the number of viable cells in the cholangiocarcinoma and normal cholangiocyte cell lines. Data are presented as mean $\pm$ SEM ($n=6-12$). *denotes significance ($p<0.05$) when compared with the respective basal treatment (Mann Whitney U). **[b]** Mz-ChA-1 and HIBEpIC cell lines were treated with 2.5 and 5 μ M simvastatin for 24, 48, and 72 h and cell viability was determined by MTS proliferation assay. Simvastatin decreased the number of viable cells in both cholangiocarcinoma and normal cholangiocyte cell lines. Data are presented as mean $\pm$ SEM ($n=6-12$). *denotes significance ($p<0.05$) when compared with the respective basal treatment (Mann Whitney U).

Figure 3. Metabolites of the mevalonate pathway prevent reduced cell viability induced by simvastatin

Mz-ChA-1 [top] and HiBEpiC [bottom] cells were pretreated with metabolites of the mevalonate pathway, mevalonate (Mev; 1 mM), farnesyl pyrophosphate (FPP; 10 μ M), and geranylgeranyl pyrophosphate (GGPP; 10 μ M) and cholesterol (Chol, 1 mM) prior to stimulation with simvastatin (Sim, 5 μ M) for 48 h. Pretreatment with Mev, FPP, and GGPP partially prevented the effects of simvastatin on cell viability in Mz-ChA-1 and HiBEpiC cells. Interestingly, cholesterol stimulated an increase in the number of viable Mz-ChA-1 cells in addition to fully preventing the effects of simvastatin. Cholesterol had no effect on the number of viable HiBEpiC cells, and partially prevented simvastatin-induced reduced cell viability. Data are presented as mean $\pm$ SEM (n=12). *denotes significance ($p<0.05$) when compared with the respective basal treatment (Kruskal-Wallis). ‡denotes significance ($p<0.05$) when compared with the simvastatin treatment (Kruskal-Wallis). §denotes significance ($p<0.05$) for cholesterol compared to basal treatment (Kruskal-Wallis).

Figure 4. Simvastatin stimulates apoptosis in Mz-ChA-1, but not HiBEpiC cells

[a] Mz-ChA-1 and HiBEpiC cells were treated with simvastatin (Sim; 5 μ M) and evaluated for annexin V (an early marker of apoptosis) expression by immunofluorescence. The neoplastic Mz-ChA-1 cells expressed annexin V when treated with simvastatin. Simvastatin-induced Annexin V expression was prevented by pretreatment with cholesterol (Chol; 1 mM). The normal HiBEpiC cells did not express annexin V after treatment with simvastatin. Annexin V expression is shown in *green* with nuclei counterstained with DAPI (blue). Original magnification, $\times 60$. The number of annexin V-positive cells was counted and expressed as a percentage of total cells in five random fields for each treatment group. Data are presented as mean $\pm$ SEM (n=12). *denotes significance ($p < 0.05$) when compared with the respective basal treatment (Kruskal-Wallis). **[b]** Mz-ChA-1 and HiBEpiC cells were treated with simvastatin (Sim; 5 μ M) and analyzed caspase 3/7 activity for 6 h. Simvastatin stimulated an increase in caspase 3/7 activity in Mz-ChA-1, but not HiBEpiC cells. In Mz-ChA-1 cells, simvastatin-stimulated caspase 3/7 activity was prevented by pretreatment with cholesterol (Chol; 1 mM). Data are presented as mean $\pm$ SEM (n=12). *denotes significance ($p < 0.05$) when compared with the respective basal treatment (Kruskal-Wallis). #denotes significance ($p < 0.05$) when compared with the simvastatin treatment (Kruskal-Wallis).

Figure 5. Simvastatin-induced reduction in Mz-ChA-1 cell viability is associated with inhibition of Rac1 activity and lipid raft co-localization

[a] Treatment of Mz-ChA-1 cells with geranylgeranyltransferase inhibitor (GGTI-298; 10 μ M) and farnesyltransferase inhibitor (FTI-277; 10 μ M) significantly reduced cell viability at 48 h by MTS assay. Mz-ChA-1 cell viability was also significantly decreased by a Rac1 inhibitor (NSC23766; 100 μ M), but not by an inhibitor of Rho-kinase (ROCK, Y-27632; 20 μ M). Data are presented as mean $\pm$ SEM (n=12). *denotes significance ($p < 0.05$) when compared with the respective basal treatment (Kruskal-Wallis). **[b]** Mz-ChA-1 and HiBEpiC cells were treated with simvastatin (Sim; 5 μ M) for both 30 min and the Rac1 activity was analyzed. Rac1 activity was significantly diminished by approximately 50% by simvastatin in Mz-ChA-1, but not HiBEpiC cells. In Mz-ChA-1 cells, pretreatment with cholesterol (Chol; 1 mM) reversed simvastatin-induced inhibition of Rac1 activity above basal Rac1 activity. Cholesterol pretreatment alone stimulate a significant increase in Rac1 activity in Mz-ChA-1, but not HiBEpiC cells. Data are presented as mean $\pm$ SEM (n=6). *denotes significance ($p < 0.05$) when compared with the respective basal treatment (Kruskal-Wallis). [‡]denotes significance ($p < 0.05$) compared with the simvastatin treatment (Kruskal-Wallis). [§]denotes significance ($p < 0.05$) for cholesterol compared to basal treatment (Kruskal-Wallis). **[c]** Mz-ChA-1 cells were treated with simvastatin (Sim; 5 μ M), cholesterol (Chol; 1 mM), and a combination of the two, and stained for lipid rafts and Rac1. Yellow represents the co-localization between the green lipid raft and the red Rac1 staining. Red arrows indicate example areas of colocalization. In the simvastatin treatment group, the light blue arrow indicates the intracellular expression of Rac1. **[d]** Quantitative assessment

of Rac1 lipid raft colocalization. Data is presented as mean $\pm$ SEM (n=6). *denotes significance ($p<0.05$) when compared with the respective basal treatment (Kruskal-Wallis). ‡denotes significance ($p<0.05$) compared with the simvastatin treatment (Kruskal-Wallis). §denotes significance ($p<0.05$) for cholesterol compared to basal treatment (Kruskal-Wallis).

Table 1

The effects of simvastatin on total cellular cholesterol levels.

	Basal (ng/μg protein)	Simvastatin (ng/μg protein)
Mz-ChA-1	20.5 ± 1.2 ^a	13.2 ± 1.2 ^b
HiBEpiC	13.2 ± 1.2	11.2 ± 1.0

Data are presented as a mean ± SEM (n=6).

^a p<0.05 vs HiBEpiC basal

^b p<0.05 vs Mz-ChA-1 basal