

Rosuvastatin Promotes Osteoblast Differentiation and Regulates SLC01A1 Transporter Gene Expression in MC3T3-E1 Cells

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Key Words

Statin • Osteoblast differentiation • Bone morphogenetic protein 2 (BMP-2) • Solute carrier (SLC) gene family transporter • *In vitro*

Abstract

Rosuvastatin (RSV) is a synthetic statin with favourable pharmacologic properties including minimal metabolism, hepatic selectivity and enhanced inhibition of HMG-CoA reductase. An induction of osteoblast differentiation has been reported *in vitro* with lipophilic statins but not with RSV, which, like pravastatin, is relatively hydrophilic compared with other statins. To mediate its action, an active transport mechanism via solute carrier (SLC) transporters from the SLC16, SLC21/SLCO and SLC22 gene family - specifically Slc16a1, Slco1a1, Slco2b1 and Slc22a8 - may be present to allow effective entry in osteoblastic cells. In this study, we demonstrate that RSV induced osteoblast differentiation, as measured by increased BMP-2 gene expression and secretion, and ALP activity in MC3T3-E1 osteoblast cells, without significantly affecting cell proliferation within the concentration range of 0.001-10 μ M. Low concentrations of RSV (0.001-0.01 μ M) were protective against cell death whereas higher concentrations (10-100 μ M) showed cytotox-

icity. Moreover, MC3T3-E1 osteoblasts expressed high levels of Slco1a1 and Slc16a1 mRNA and low levels of Slco2b1 and Slc22a8 mRNA, when compared with kidney and liver tissues from mice. Slco1a1 gene expression increased 12-fold during osteoblast differentiation and was further regulated after RSV treatment. In conclusion, as for other statins, RSV promotes osteoblast differentiation, and also, demonstrated for the first time, regulates the expression of Slco1a1, which may constitute the transport system for RSV across the cell membrane in mature osteoblasts.

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Introduction

Statins are commonly prescribed drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and decrease hepatic cholesterol biosynthesis. Consequent up-regulation of hepatic LDL receptors reduces serum cholesterol concentrations and lowers the risk of cardiovascular disease [1, 2]. Statins are also

known to elicit numerous pleiotropic effects including inhibition of proliferation and migration of smooth muscle cells, changes to extracellular matrix expression, inhibition of tumour growth, and anti-inflammatory properties [3-5]. Furthermore, statins have been demonstrated to stimulate bone formation [6, 7] and to play a role in reducing bone resorption by inhibiting osteoclast differentiation [8, 9]. These recent findings indicating statins anabolic action on bone have generated enormous interest among researchers and clinicians and their combined efforts may lead to an exciting option for the treatment of osteoporosis [10-14].

Statins act on the mevalonate pathway in osteoblasts and enhance the expression of the bone morphogenetic protein-2 (BMP-2), which is an important growth factor for osteoblast differentiation [7]. Thus, by inhibiting the mevalonate pathway and preventing the prenylation and function of small GTPases, BMP-2 expression may be stimulated, causing increased osteoblast differentiation and, consequently, enhanced bone formation [15-21]. Additionally, statins act on the same pathway upstream of the biphosphonates in osteoclasts via inhibition of HMG-CoA reductase, leading to a decrease in protein prenylation that is essential for normal osteoclast function [7-9, 22, 23].

Although all statins share a common mechanism of action, they differ in terms of their chemical structure, pharmacokinetic profile and lipid modifying efficacy [24]. Rosuvastatin (RSV) is a synthetic statin with favourable pharmacologic properties including minimal metabolism, hepatic selectivity and enhanced inhibition of HMG-CoA reductase [24]. Statins are classified into lipophilic and hydrophilic types according to their aqueous solubility [1]. In contrast to lipophilic statins that can pass through the cell membrane by passive diffusion but also be transported with specific carriers, hydrophilic statins (e.g. pravastatin and RSV) do not readily permeate the cell bilayer lipid membrane and probably depend on specific transporters for entry into the cells [25-27].

Currently, several human and rat genes of the solute carrier (SLC) gene transporter family, i.e. SLC16/MCT, SLC21/SLCO and SLC22/OAT, have been isolated and an active uptake of a number of statins has been demonstrated [28, 29]. Statins transporters are expressed in a variety of tissues including intestine, liver, kidney and brain, suggesting that they play a critical role in drug absorption, distribution and excretion [30-32]. Distribution of these transporters has also been investigated in several different mouse tissues and has shown that expression of Slc1a1 mRNA is highest in liver and kidney, Slc22a8/

Oat-3 is expressed in the liver, kidney and brain [30], while Slc2b1 [33] and Slc16a1/MCT-1 [32] appear to be ubiquitously expressed. However, it still remains unknown whether these transporters are expressed in bone and/or osteoblasts, thereby potentially facilitating the entry of hydrophilic statins into these cells.

The main aim of this study was to investigate the effect of different concentrations of RSV on cytotoxicity, proliferation, bone morphogenetic protein-2 (BMP-2) gene expression and secretion, and alkaline phosphatase (ALP) activity in MC3T3-E1 osteoblasts in an *in vitro* system. Secondly, expression of specific transporters from the SLC16, SLC21/SLCO and SLC22 family - specifically Slc16a1, Slc1a1, Slc2b1 and Slc22a8 - which are known to have different tissue distributions, were studied in MC3T3-E1 osteoblasts during osteoblast differentiation, and after RSV stimulation.

Materials and Methods

Chemicals

Rosuvastatin calcium (di-calcium bis (+) 7-[4-(4-fluorophenyl)-6-isopropyl-2-(N-methyl-N-methylsulfonylaminopyrimidin)-5-yl]-(3R,5S)-dihydroxy-(E)-6-heptenoate) (AstraZeneca, UK).

Cell culture

The mouse osteoblastic cell line MC3T3-E1 (DSMZ, Braunschweig, Germany) was selected as an *in vitro* model. Cells were routinely cultured at 37°C in a humidified atmosphere of 5% CO₂, and maintained in α -MEM supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU penicillin/ml and 50 μ g streptomycin/ml). Cells were subcultured 1:4 before reaching confluence using PBS and trypsin/EDTA. All experiments were performed after 6 passages of the MC3T3-E1 cells.

Cells were seeded in plates and maintained in α -MEM supplemented with 10% FCS, antibiotics and in the presence or absence of different concentrations of RSV. RSV treatment was continuous during the entire period of the experiment. The culture media were collected when harvesting the cells to test for cytotoxicity, BMP-2 secretion and ALP activity. Cells were harvested at different time points using Trizol reagent to analyze the expression of BMP-2 and SLC transporters following RSV incubation and their gene expression during osteoblast differentiation.

Determination of cell viability: LDH activity

Lactate dehydrogenase (LDH) activity in the culture media was used as an index of cell death. LDH activity was determined spectrophotometrically after 30min incubation at 25°C of 100 μ l of culture and 100 μ l of the reaction mixture, by measuring the oxidation of NADH at 490nm in the presence of pyruvate, according to the manufacturer's kit instructions (Roche

Gene	Primer sequence	GeneBank accession number	Amplicon size (bp)	T _m (°C) amplicon
Slc1a1	S 5'-ATCCAGTGTGTGGGGACAAT -3'	NM_013797	107	85.5
	A 5'-CGTCGACGTTAAAACTTTGT -3'			
Slc2b1	S 5'-TGCAGGTTCTGTGAGTCAG-3'	NM_175316	124	89.5
	A 5'-GGTGTGGAGAGTCCTACTCGT-3'			
Slc16a1	S 5'-TCCAGTAATGATCGCTGGTG -3'	NM_009196	125	84.5
	A 5'-AGTTGAAAGCAAGCCCAAGA-3'			
Slc22a8	S 5'-ATCTTCCGATTCTGTGTGG -3'	NM_031194	108	87.5
	A 5'-AGTTGTTGATGAGATGGCCC -3'			
BMP-2	S 5'-GCTCCACAAACGAGAAAAGC -3'	NM_007553	178	87.0
	A 5'-AGCAAGGGGAAAAGGACACT -3'			
ALP	S 5'-AACCCAGACACAAGCATTCC -3'	X13409	151	87.0
	A 5'-GAGAGCGAAGGGTCAGTCAG -3'			
BSP	S 5'-GAAAATGGAGACGCGATAG -3'	L20232	141	84.0
	A 5'-ACCCGAGAGTGTGGAAAGTG -3'			
Runx-2	S 5'-GCCTTCAAGGTGGTAGCCC-3'	NM_004348	67	84.0
	A 5'-CGTTACCCGCCATGACAGTA -3'			
Collagen-I	S 5'-AGAGCATGACCGATGGATTG -3'	NM_007742	177	89.0
	A 5'-CCTTCTTGAGGTTGCCAGTC -3'			
Osteocalcin	S 5'-CCGGGAGCAGTGTGAGCTTA -3'	X04142	80	85.0
	A 5'-TAGATGCGTTGTAGGCGGTC -3'			
Osterix	S 5'-ACTGGCTAGTGGTGGTCAG -3'	NM_007419	135	89.0
	A 5'-GGTAGGGAGCTGGGTAAAGG -3'			
18S rRNA	S 5'-GTAACCCGTTGAACCCCAATT -3'	X00686	151	86.0
	A 5'-CCATCCAATCGGTAGTAGCG -3'			
GAPDH	S 5'-ACCCAGAAGACTGTGGATGG -3'	XM_132897	171	90.0
	A 5'-CACATTGGGGGTAGGAACAC -3'			

Table 1. Oligonucleotide sequences of sense (S) and antisense (A) primers used in the real-time PCR of target and housekeeping genes.

Diagnostics, Mannheim, Germany). After removing the background in the absorbances of the culture media without cells, results from all the samples were presented relative to the LDH activity in the medium of cells seeded on plastic (low control, 0% of cell death) and on plastic where Triton X-100 was added at 1% (high control, 100% cell death). The percentage of LDH activity was calculated according to the manufacturer's protocol.

Cell proliferation

To test the effect of different concentrations of RSV on osteoblast proliferation, 2×10^3 cells were seeded in each well (96-well plate) and cultured for 24h. Culture medium was changed and different concentrations of RSV (0.001-100 μ M) and bromodeoxyuridine (BrdU) were added to the wells. Cells were cultured for an additional 24h for the incorporation of BrdU to the proliferating cells. Incorporated BrdU was measured as described by the manufacturer (Roche Diagnostics, Mannheim, Germany).

RNA isolation and RT-PCR analysis

Total RNA was isolated using a monophasic solution of phenol and guanidine isothiocyanate (Invitrogen, Carlsbad, CA, USA), following the instructions of the manufacturer. RNA was quantified using a spectrophotometer set at 260 nm.

1 μ g of total RNA (from each sample) was reverse transcribed to cDNA at 42°C for 60 min in a final volume of 40 μ l,

using an iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA) containing both oligo(dT) and random hexamers. Each cDNA was diluted 1/5 and aliquots were frozen (-20°C) until the PCR reactions were carried out.

Real-time PCR was performed in the iCycler (BioRad, Hercules, CA, USA) using SYBR green detection. Real-time PCR analysis was performed for housekeeping genes (18S and GAPDH) and target genes. Primer sequences are shown in Table 1. Each reaction contained 5 μ l of cDNA, 500 nM of the sense and antisense specific primers, and 12.5 μ l of 2X IQTM SYBR® Green Supermix (BioRad, Hercules, CA, USA) in a final volume of 25 μ l. The amplification program consisted of a preincubation step for denaturation of the template cDNA (3 min 95 °C), followed by 40 cycles consisting of a denaturation step (15s 95°C), an annealing step (15s 60°C) and an extension step (30s 72°C). After each cycle, fluorescence was measured at 72°C. A negative control without a cDNA template was run in each assay. Samples were run in duplicate.

Real-time efficiencies were calculated from the given slopes in iCycler software using serial dilutions, showing all the investigated transcripts high real-time PCR efficiency rates, and high linearity when different concentrations were used. PCR products were subjected to a melting curve analysis on iCycler and subsequently 3% agarose/TAE gel electrophoresis to confirm amplification specificity, T_m and amplicon size, respectively.

To allow relative quantification after PCR, standard curves

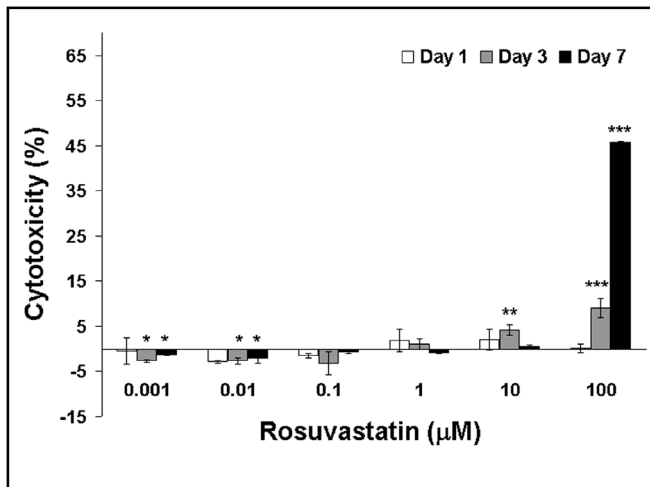


Fig. 1. Effect of rosuvastatin on lactate dehydrogenase (LDH) activity of MC3T3-E1 cells. Results were calculated relative to untreated control cells (0% cytotoxicity) and cells treated with Triton X-100 (100% cytotoxicity). Values represent the mean \pm SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the untreated control cells at each time point.

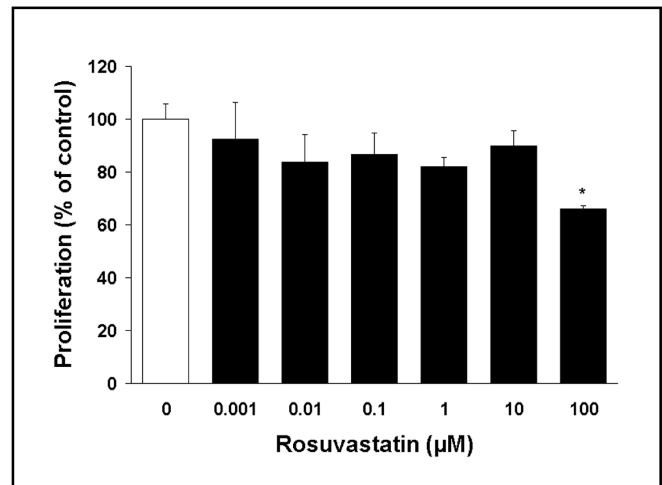


Fig. 2. Proliferation of MC3T3-E1 cells incubated with increasing concentrations of rosuvastatin. Cells were treated with different concentrations of rosuvastatin (0.001–100 μM) for 24 hours and BrdU incorporation measured. Values represent the mean \pm SEM. *, $p < 0.05$ compared with the untreated control cells.

were constructed from the standard reactions for each target and housekeeping genes by plotting Ct values (cycle threshold), i.e. the cycle number at which the fluorescence signal exceeds background, versus log cDNA dilution. The Ct readings for each of the unknown samples were then used to calculate the amount of either the target or housekeeping gene relative to the standard. Relative mRNA levels were calculated as the ratio of relative concentration for the target genes to that for the mean between the two housekeeping genes (18S rRNA, GAPDH) to correct for RNA.

Release of BMP-2 into the cell culture media

BMP-2, secreted to the culture medium after day 1 and day 7 of cell culture, was analysed by enzyme-linked immunosorbent assay (ELISA). Aliquots from the culture media were centrifuged at 1800 rpm for 5 minutes at 4°C and supernatants were used for BMP-2 determination following instructions described by the manufacturer (Quantikine Immunoassay, R&D Systems, Minneapolis). Total protein in the culture media for each sample was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and used for correction of BMP-2 secretion, expressed in pg BMP-2/mg protein.

Determination of ALP activity

Alkaline phosphatase (ALP) activity was calculated by measuring the cleavage of p-Nitrophenyl Phosphate (pNPP) (Sigma, Saint Louis, Missouri, USA) in a soluble yellow end product which absorbs at 405nm. In parallel to the samples, a standard curve with calf intestinal alkaline phosphatase (CIAP) (Promega, Madison, USA) was constructed by mixing 1 μl from the stock CIAP with 5 ml of alkaline phosphatase (1:5000 dilution), and then making 1:5 serial dilutions. Total protein was determined using a bicinchoninic acid (BCA) protein assay kit,

following the manufacturer's kit instructions (Pierce, Rockford, USA).

Animal tissue

Adult female mice (FVB) from the animal unit (The Norwegian School of Veterinary Science, Oslo, Norway) were euthanised by cervical dislocation and the following tissues rapidly removed and frozen in liquid N₂: liver and kidneys. The tissues were stored at -70°C until RNA isolation.

Statistical analyses

The data were presented as mean values \pm SEM of three separate experiments performed in duplicate. Differences between groups were assessed by one-way ANOVA and least-significant difference for post hoc comparisons using a statistical software package (SPSS, Chicago, IL). Results were considered statistically significant at the p -values < 0.05 .

Results

Effect of rosuvastatin on cytotoxicity of MC3T3-E1 osteoblasts

To assess the cytotoxicity of different concentrations of RSV on MC3T3-E1 cells, we measured the LDH activity from the culture medium (Fig. 1). At lower concentrations (0.001 and 0.01 μM), RSV showed a protective effect on osteoblasts against cell damage compared with untreated cells. LDH activity showed no significant changes in treated compared with untreated cells for 0.1 and 1 μM of RSV. However, a significant increase in

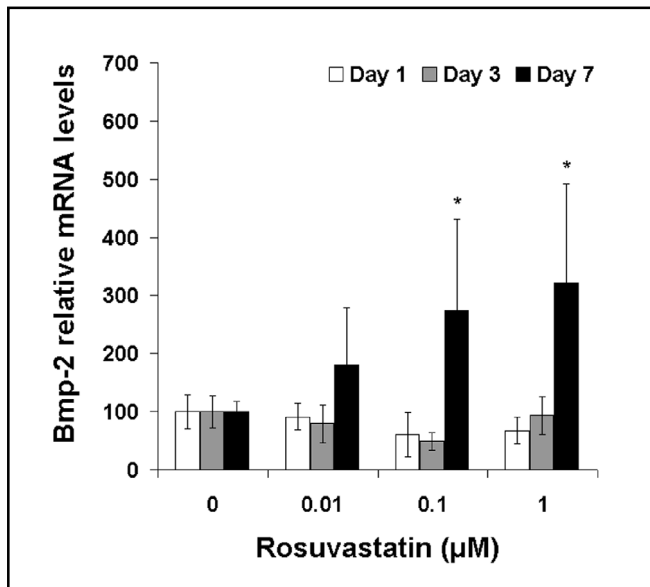


Fig. 3. Effect of rosuvastatin on BMP-2 mRNA levels in MC3T3-E1 cells. Cells were treated with rosuvastatin (0.01-1 μ M) for the time periods indicated. Results are expressed in % of control cell samples at each time point. Values represent the mean \pm SEM. *, $p < 0.05$ compared with the untreated control cells.

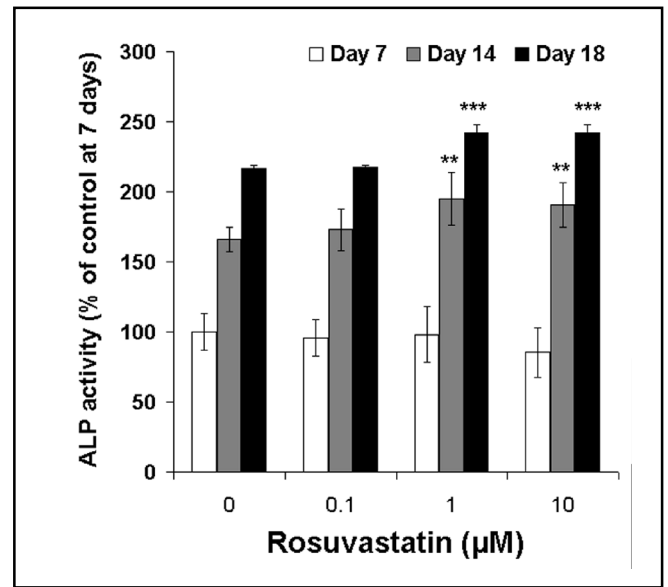


Fig. 4. ALP activity in the culture media of MC3T3-E1 cells after incubation with rosuvastatin. Cells were treated with rosuvastatin (0.1-10 μ M) for the time periods indicated. Results are expressed in % of control at 7 days. Values represent the mean \pm SEM. **, $p < 0.01$; ***, $p < 0.001$ compared with the untreated control cells at each time point.

LDH activity was found after incubation for 3 and 7 days at the highest concentrations of RSV (10 and 100 μ M). After 7 days incubation with 100 μ M of RSV, 45% of damaged cells had released LDH into the culture medium. However, these high concentrations are supra-pharmacological exposures not seen in clinical situations.

Effect of rosuvastatin on MC3T3-E1 cell proliferation

The resulting effects of the RSV-induced osteoblast proliferation are shown in Figure 2. BrdU incorporation showed that RSV treatment had no significant effects on cellular proliferation within the concentration range of 0.001-10 μ M in MC3T3-E1 osteoblasts. However, cell proliferation was suppressed to 66 % of control levels with 100 μ M of RSV.

Effect of rosuvastatin on BMP-2 gene expression and secretion in MC3T3-E1 osteoblasts

It has been shown in several studies that statins induce bone formation through the induction of bone morphogenetic protein-2 (BMP-2) both *in vitro* and *in vivo* [7, 15, 17, 18, 20]. However, no studies have been carried out to assess the effect of RSV on BMP-2 gene expression and secretion in osteoblasts. Therefore, we

investigated the effect of different concentrations of RSV (0.01, 0.1 and 1 μ M) on BMP-2 mRNA levels (Fig. 3). These concentrations were chosen based on previous LDH activity analysis. RSV significantly enhanced expression of BMP-2 after 7 days with concentrations of 0.1 and 1 μ M. Based on the gene expression data, the release to the culture media was determined using a BMP-2 immunoassay after 1 and 7 days in control and in RSV-treated (1 μ M) samples. RSV-treated cells showed higher BMP-2 secretion after 1 day (647.2 ± 272.9 pg BMP-2/mg protein) and 7 days (565.6 ± 350.4 pg BMP-2/mg protein) compared to control cells after day 1 (221.0 ± 56.8 pg BMP-2/mg protein) and 7 days (280.9 ± 64.6 pg BMP-2/mg protein).

Effect of rosuvastatin on ALP activity in MC3T3-E1 osteoblasts

To examine whether MC3T3-E1 cells began a differentiation program after the induction of BMP-2 with RSV, ALP activity was quantified in the culture medium after longer incubation periods of 7, 14 and 18 days with RSV (Fig. 4). ALP activity in the culture medium significantly increased after 14 and 18 days in the RSV-treated cells (1 μ M and 10 μ M) compared with untreated control cells.

Fig. 5. Tissue specificity of *Slco1a1*, *Slco2b1*, *Slc16a1*, *Slc22a8* gene expression in MC3T3-E1 osteoblasts and mouse tissues, liver and kidney. A. Confirmation of amplification specificity of the PCR using 2% agarose/TAE gel electrophoresis (L: liver, K: kidney, OB: osteoblast). B. Transporters *Slco1a1*, *Slco2b1*, *Slc16a1*, *Slc22a8* displayed different C_t values depending on the starting amount of mRNA in MC3T3-E1 osteoblasts *in vitro* and mouse tissues (liver, kidney) *in vivo*. This relative expression was normalized by 18S ribosomal RNA and GAPDH. The expression of transporter genes studied is calculated relative to the osteoblast samples, which were assigned a value of 1.

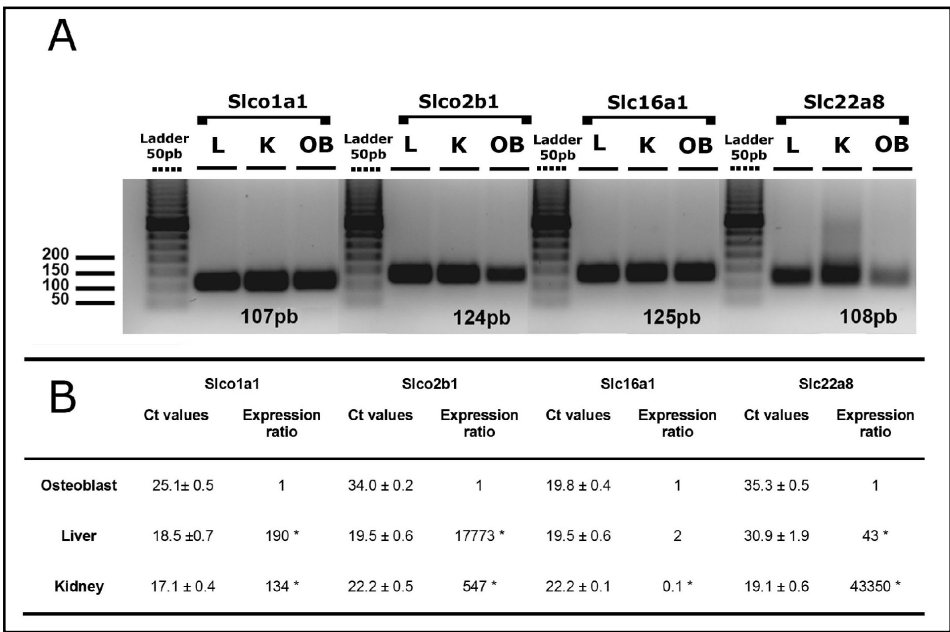


Fig. 6. Temporal expression of *Slco1a1*, *Slco2b1*, *Slc16a1*, *Slc22a8* and several osteoblast specific marker genes (collagen-I, bone sialoprotein, osterix, runx2, ALP and osteocalcin) during differentiation. MC3T3-E1 cells were cultured from 1 to 42 days after reaching confluence. Expression ratios of target genes relative to housekeeping genes (18S and GAPDH) were expressed as a percentage of cell samples at day 1, which were assigned as 100%. Values represent the mean ± SEM.

Characterization of *Slco1a1*, *Slco2b1*, *Slc16a1* and *Slc22a8* gene expression in MC3T3-E1 osteoblasts

Although the presence of different family members has been characterized in several mouse tissues [33], no studies have been carried out to identify the expression of different SLC family transporters in osteoblasts. Thus, gene expression of *Slco1a1*, *Slco2b1*, *Slc16a1* and *Slc22a8* was evaluated in undifferentiated osteoblasts (in absence of RSV) and compared with their expression levels in mouse liver and kidney (Fig. 5). We confirmed that all transporters studied were expressed in MC3T3-E1 osteoblasts (Fig. 5A). Further, taking into account that *Slco1a1*, *Slco2b1*, *Slc16a1* and *Slc22a8* primers showed similar PCR efficiency, and that higher amounts of mRNA correlate with fewer C_t values, gene expression levels of *Slco1a1* and *Slc16a1* were higher than *Slco2b1* and *Slc22a8* in osteoblastic cells. (Fig. 5B). Thus, *Slco2b1* and *Slc22a8* could have a minor role in osteoblasts since their expression was found to be much lower (but still detectable) compared with *Slco1a1* and *Slc16a1*. Liver and kidney showed high mRNA levels for all the transporters tested, except for *Slc22a8* in the liver.

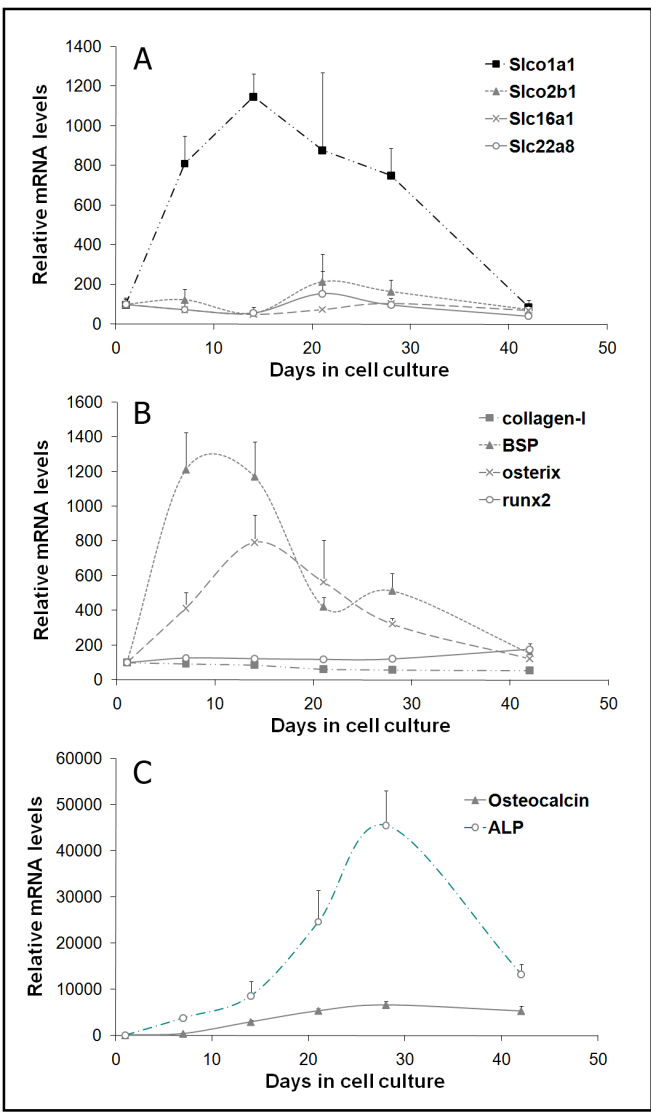
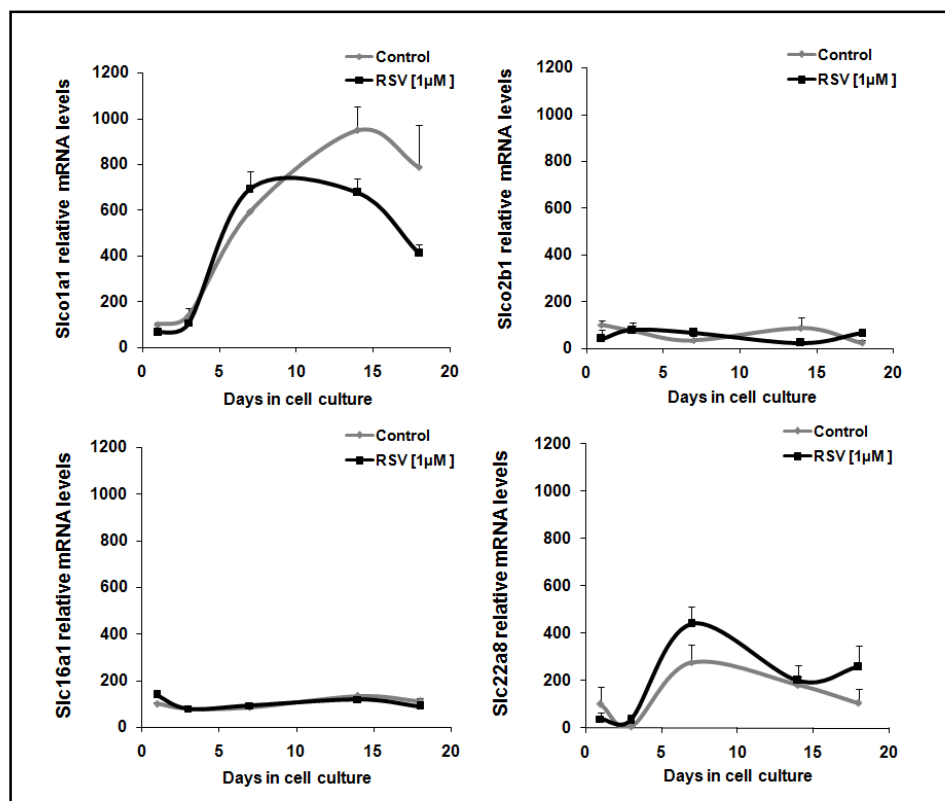


Fig. 7. Effect of rosuvastatin on Slco1a1, Slco2b1, Slc16a1, Slc22a8 mRNA levels in MC3T3-E1 cells. Cells were treated with rosuvastatin (1 μ M) for the time periods indicated. Ratios of transporter mRNA levels relative to housekeeping mRNA levels (18S and GADPH) were expressed as a percentage of untreated control cells at day 1, which were assigned as 100%. Values represent the mean \pm SEM.



Characterization of Slco1a1, Slco2b1, Slc16a1 and Slc22a8 gene expression during differentiation of MC3T3-E1 osteoblasts

We next investigated the temporal expression of these transporters in relation to the sequential expression of genes associated with osteoblast differentiation (Fig. 6). MC3T3-E1 cells were cultured after reaching confluence for different time points (from 1 to 42 days) and in the absence of RSV. There was a significant increase in Slco1a1 mRNA levels during the culture period from day 7 until day 28, with maximal expression at day 14 (12-fold increase, $p=0.003$) together with other markers related to the mature osteoblast phenotype, such as osterix and BSP (the latter with maximal expression at days 7 and 14). In contrast, Slc16a1 mRNA levels were significantly down-regulated after 7 days (0.7-fold decrease, $p=0.004$), 14 days (0.5-fold decrease, $p=0.000$), 21 days (0.7-fold decrease, $p=0.027$) and 42 days of cultivation (0.7-fold, 0.002). No changes were observed for Slco2b1 and Slc22a8 mRNA levels during osteoblast differentiation. The expression of type I collagen did not change throughout the culture period, as this early marker is associated with the proliferative stage of preosteoblasts. Nor did the expression of the transcriptional factor Runx2 change, since MC3T3-E1 cells are preosteoblasts already committed to the osteoblast lineage. Late mark-

ers, such as ALP and osteocalcin, associated with the mineralization stage of osteoblasts, showed a large increase in their mRNA levels after 4 weeks, with a reduction after 6 weeks in ALP gene expression, which is known to decrease when mineralization is well progressed.

Effect of rosuvastatin on Slco1a1, Slco2b1, Slc16a1 and Slc22a8 gene expression in MC3T3-E1 osteoblasts

To address specific regulation of transporter gene expression by RSV, MC3T3-E1 cells were treated with 1 μ M of RSV for 1, 3, 7, 14 and 18 days (Fig. 7). Slco1a1 mRNA levels decreased after incubation with RSV for 1, 14 and 18 days by 35% ($p=0.041$), 29% ($p=0.049$) and 48% ($p=0.077$) compared with control, respectively. Minor changes were observed in the mRNA levels of Slco2b1, Slc16a1 and Slc22a8 after incubation with RSV.

Discussion

The results confirm that RSV enhances BMP-2 gene expression and osteoblast differentiation *in vitro*, as previously reported for other statins [7, 15, 17, 18, 20]. The main limitation for the use of statins as systemic bone-

activation agents is their poor tissue distribution beyond the liver to where they could be active, i.e. in bone. Statins are rapidly absorbed after oral administration and their bioavailability is low due to their high liver specificity. However, in appropriate doses, statins may have therapeutic applications for the treatment of osteoporosis. In fact, several studies have demonstrated positive effects on bone when statins are administered orally [7, 22, 34], subcutaneously [7, 9, 35, 36] or locally to bone sites [37-44]. Nevertheless, the route of administration and dosage form of statins still has to be optimized to maximize their efficacy in bone.

In general, statins are safe and well tolerated [24]. The only well documented adverse reactions associated with statins are muscle toxicity (including myopathy and rhabdomyolysis) and effects on liver enzymes, but these are uncommon at standard doses [45]. In clinical studies, RSV has shown a safety profile comparable to other statins [46]. In cell studies with myoblasts, hydrophilic statins have been shown to be less toxic than lipophilic statins [27]. In the present study, we showed that RSV (0.001-1 μM) was not toxic to MC3T3-E1 cells following a 7-day incubation. Moreover, the results revealed a significant decrease in cell death at lower RSV concentrations, although this reduction was no more than 3%. RSV, similarly to our results after 24h incubation, showed absence of cytotoxicity in kidney cells in the concentration range of 0.001-100 μM [47]. In other studies in primary human osteoblasts and osteosarcoma cells, a cell viability above 85% was observed using simvastatin and atorvastatin with concentrations ranging from 0.001-1 μM [19]. In a human bladder carcinoma T24 cell line, lovastatin was found to be cytotoxic at a concentration of 50 μM [48]. From these studies it can be concluded that statin concentrations ranging between 10^{-6} and 10^{-9} M, which correspond well to clinical serum concentrations (i.e. 10^{-7} - 10^{-8} M), preserve cell viability.

We also investigated whether RSV could have an effect on the proliferation of MC3T3-E1 cells. The treatment with RSV had no effects on cell proliferation within the concentration range of 0.001-10 μM , and significantly reduced proliferation with 100 μM of RSV, which could be related to the higher cytotoxicity found with this dose. The antiproliferative effect of statins has been shown to be more evident in cultures of human bone marrow stromal cells [15] and primary human osteoblasts [19], while it was negligible with the MG-63 osteosarcoma cell line [19]. Therefore, the high growth rate of transformed osteoblastic cell lines could account for the lack of antiproliferative effects in these in vitro models. Future

studies are necessary to verify the effect of RSV in primary osteoblastic cells in comparison with other statins. Statins have been reported to have an antiproliferative effect through their inhibition of the mevalonate pathway, as this pathway is the source of isoprenoid precursors necessary for the post-translational lipid modification (prenylation), which enhance the function of Ras and other GTPases involved in the regulation of the proliferation processes [49]. Statins have beneficial effects on the differentiation process of osteoblasts, however, if the suppressive effects on cell growth and proliferation are considerable, the net effect on bone formation may be less evident [15].

Regarding the osteoblast-differentiating effect of statins, it has been well documented that lipophilic statins increase synthesis and secretion of a wide spectrum of factors and matrix proteins related to osteoblast differentiation, and ultimately stimulate mineralization via inhibition of the cholesterol synthesis pathway [15, 17-21, 50]. Moreover, it is believed that BMP-2 has a central role in mediating the effects of statins on bone [13]. In our study, low concentrations of RSV (10^{-7} - 10^{-6} M) enhanced expression and secretion of BMP-2 at an early stage of differentiation, which is in agreement with previous studies using cells treated with simvastatin and cerivastatin [7, 17, 18, 20]. Moreover, RSV enhanced not only BMP-2 expression/secretion but also ALP activity in later osteoblast differentiation stages, which is consistent with earlier observations [15, 17, 18].

As commented previously, most statins are very lipid soluble (atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin and simvastatin) and enter cells easily by passive diffusion and by the action of specific carrier. However, two other synthetic statins, pravastatin and RSV, are relatively more hydrophilic and depend on active transporters with a high affinity for statins. Thus, whether different transporters from SLC16, SLC21/SLCO and SLC22 gene family members were expressed in the osteoblast cell membrane was investigated. To the best of our knowledge, expression of Slco1a1, Slco2b1, Slc16a1 and Slc22a8 in bone cells has not yet been reported. The present study confirms that Slc16a1 is strongly expressed in osteoblasts compared with liver and kidney tissues, which are the most important organs for chemical disposition. Slco1a1 showed moderate expression levels after its upregulation in mature osteoblasts compared to liver and kidney tissues (16- and 11-fold lower respectively), while Slco2b1 and Slc22a8 may have a minor role in this cell line due to its much lower expression. We monitored their gene expression during osteoblast differentiation in

the absence of RSV and observed a significant increase of Slc1a1 mRNA levels in parallel with the expression of specific osteoblast differentiation marker genes, while Slc16a1 mRNA levels were down-regulated. We demonstrated that maximum Slc1a1 expression levels correlate with the middle stages of osteoblast differentiation *in vitro* and not with the final stage when extracellular matrix undergoes mineralization. Slc1a1 expression was spontaneously enhanced about 12-fold during MC3T3-E1 cell differentiation, in the absence of RSV, suggesting that the gene increase during osteoblast differentiation may have important physiological functions other than importing RSV or other xenobiotics into the cells. Whether changes in Slc1a1 gene expression levels could be correlated with reduced RSV uptake in mature osteoblasts is presently not known. Further studies should investigate if a depletion or overexpression of Slc1a1 mRNA levels correlates with a change in RSV transport across the cell membrane and whether this is a requirement in mediating RSV action in MC3T3-E1 cells. Active uptake by SLC21/SLCO into the liver of many statins, such as cerivastatin, fluvastatin, pravastatin, pitavastatin and RSV, has been demonstrated in recent investigations. Thus, Slc1b1, Slc1b3, and Slc2b1 have been shown to be involved in the transport of these statins [28, 51]. In the present study, RSV reduced the steady state concentration of Slc1a1 mRNA levels in MC3T3-E1 cells after short and long term incubation, and this occurred with concentrations of RSV that were able to stimulate dif-

ferentiation of osteoblasts, which is similar to other statins [15, 17, 18, 20]. In this way, RSV may act as a regulator of Slc1a1 gene expression, equal to other xenobiotics [52], and these alterations in the expression levels and/or functional characteristics of Slc1a1 could in turn regulate intracellular accumulation of RSV in mature osteoblasts. It is well possible that statins simply enhance the rate of differentiation of osteoblasts, and that changes in transporters (and other genes) reflect the maturity status of osteoblasts. Future functional studies are needed to confirm a role for Slc1a1 in osteoblasts.

In conclusion, as with other statins, rosuvastatin promotes osteoblast differentiation and also regulates the expression of Slc1a1, which may constitute the transport system for RSV across the cell membrane in mature osteoblasts.

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