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Comparative gene expression profiling in three primary human cell lines after treatment with a novel inhibitor of Rho kinase or atorvastatin

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Abstract

Inhibitors of Rho kinase (ROCK) are a relatively new class of drugs with potential benefits in oncology, neurology, and fibrotic and cardiovascular diseases. ROCK-inhibitors modulate many cellular functions, some of which are similar to the pleiotropic effects of statins, suggesting additive or synergistic properties. Studies to date have used compounds which inhibit both isoforms of ROCK, ROCK1 and ROCK2. This study was designed to compare gene expression profiles of atorvastatin with the newly developed ROCK2-inhibitor SLx-2119 in primary cultures of normal human endothelial cells, smooth muscle cells, and fibroblasts. Cells were treated with each compound for 24 hours, after which total RNA was isolated and genome-wide gene expression profiles were obtained with Illumina arrays. Because of the known effect of statins on the actin cytoskeleton and on connective tissue growth factor (CTGF), a prominent growth factor involved in tissue fibrosis, the effects of SLx-2119 and atorvastatin on the actin cytoskeleton and CTGF mRNA were also examined in cultures of smooth muscle cells with a fibrotic phenotype, isolated from biopsies of human intestine with radiation-induced fibrosis. Although SLx-2119 and atorvastatin affected expression of genes belonging to the same biological processes, individual genes were mostly different, consistent with synergistic or additive properties. Both SLx-2119 and atorvastatin reduced CTGF mRNA and remodeled the actin cytoskeleton in fibrosis-derived smooth muscle cells,

suggesting that both compounds have anti-fibrotic properties. These results form the basis for further studies to assess the possible therapeutic benefit of combined treatments.

Keywords

Genome Expression Profiling; Endothelial Cells; Smooth Muscle Cells; Fibroblasts; rho-Kinase; Hydroxymethylglutaryl-CoA Reductase Inhibitors

Introduction

Rho proteins belong to a family of small GTP-binding proteins involved in a wide range of cellular functions, including stress fiber formation, hypertrophy, migration and production of cytokines and growth factors [1]. Rho proteins largely operate via their downstream mediators, Rho kinase (ROCK)1 and ROCK2. ROCK-inhibitors are a relatively newly developed class of drugs that affects cellular functions by inhibiting the ROCK pathway. Despite their recent development, ROCK-inhibitors have already shown substantial benefit in a wide range of diseases, including oncological disorders [2], neurological disorders [3], fibrotic disease [4] and several cardiovascular disorders [5].

The effects of ROCK-inhibitors overlap to a certain extent with the pleiotropic (non-lipid lowering) effects of hydroxy methylglutaryl-coenzyme A reductase inhibitors (statins). While the main indication for statins is hyperlipidemia disorders, statins also show benefit in trauma, vascular disease, diabetes, as well as immune, inflammatory, and fibroproliferative disorders [6,7]. Notably, there is clinical and preclinical evidence supporting the use of statins for the prevention of intestinal radiation fibrosis [8–10].

The pleiotropic effects of statins are mostly mediated via a reduced formation of intermediates of the cholesterol biosynthesis pathway, such as farnesyl pyrophosphate and geranylgeranylpyrophosphate. These intermediates serve as substrates for isoprenylation of several peptides, including Rho proteins such as Rho, Rac, and cdc42, which is critical for their function and intracellular trafficking. As a result of altered Rho protein function, statins increase the expression and activity of endothelial nitric oxide synthase (eNOS) [11]. In addition to Rho proteins, statins also alter isoprenylation of other small proteins, such as Ras proteins. Moreover, certain pleiotropic statin effects, for example up-regulation of thrombomodulin (TM), involve Rac and cdc42, but not Rho [12]. In contrast to statins, ROCK-inhibitors selectively inhibit the Rho kinase pathway. Considering these properties of statins and ROCK-inhibitors, one may expect some overlap but also important differences in the effects of the two classes of compounds. Moreover, the degree of overlap between ROCK-inhibitors and statins may vary depending on the cell type or the cell phenotype.

Until now the only pharmacological tools for studying ROCK biology have been compounds which do not differentiate between ROCK1 and ROCK2. This is far from ideal as knockout [13] and siRNA [14] studies have demonstrated that the 2 isoforms have unique and specific effects both at the cellular level and *in vivo*. This study used microarrays to perform a genome-wide comparison of the effects of the small molecule ROCK2-selective inhibitor, SLx-2119, with the effects of atorvastatin on gene expression in 3 different cell types, i.e., primary cultures of human endothelial cells (EC), smooth muscle cells (SMC), and fibroblasts. The relevance of the findings to human pathology was addressed by studying the effects of these compounds on SMC derived from clinical resection specimens of chronic intestinal radiation injury in order to model fibrosis *in vitro*. Our findings are consistent with the notion that the 2 classes of drugs may have synergistic properties and suggest that combination therapies should be explored.

Methods

Cell cultures

All cell culture media were from Cambrex (East Rutherford, NJ). Human microvascular EC (HMVEC; CC-2527, Cambrex) were cultured in EGM-2 MV BulletKit medium, containing endothelial cell basal medium-2, human EGF, hydrocortisone, human basal FGF (hFGF-B), VEGF, recombinant IGF-I, ascorbic acid, gentamicin, amphotericin-B, and 5% FBS. Human pulmonary artery smooth muscle cells (PASMC; CC-25841, Cambrex) were cultured in SmGM-2 BulletKit medium, containing smooth muscle basal medium, human EGF, hFGF-B, insulin, gentamicin, amphotericin-B, and 5% FBS. Human dermal fibroblasts (NHDF, CC-2511, Cambrex) were cultured in FGM-2 BulletKit medium, containing fibroblast basal medium, hFGF-B, insulin, gentamicin, amphotericin-B, and 2% FBS.

Smooth muscle cells were isolated from human ileal *muscularis propria* as previously described [15], either normal *muscularis propria* (N-SMC), or from *muscularis propria* biopsies of patients with delayed radiation enteropathy (RE-SMC). Intestinal tissues were collected according to the guidelines on human subject research from the French Medical Research Council. Smooth muscle cells were isolated by enzymatic digestion at 37°C (0.2% type II collagenase and 0.1% soybean trypsin inhibitor) and subcultured in SmGM-2.

All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Radiometric truncated enzyme ROCK1 and ROCK2 assays

Cell-free enzyme assays were performed to determine the selective inhibition of ROCK1 and ROCK2 by SLx-2119. Reactions were performed on non-binding surface microplates (Corning Inc., Corning, NY). Four mU of human ROCK1 and ROCK2 (Invitrogen, Carlsbad, CA) were used to phosphorylate 30 µM of the synthetic ROCK peptide substrate S6 Long (sequence: KEAKEKRQEIQAKRRRLSSLRASTSKSGGSQK), prepared at American Peptide (Sunnyvale, CA) with the addition of 10 µM ATP (Sigma), containing ³³P-ATP (Perkin Elmer, Waltham, MA) in the presence of 10 mM Mg²⁺, 50 mM Tris, pH 7.5, 0.1 mM EGTA and 1 mM DTT at room temperature. One unit is the amount of kinase needed to catalyze the transfer of 1 nmol phosphate/min to the peptide. The reactions were allowed to proceed for 45 minutes and then stopped with 3% phosphoric acid to a final concentration of 1%. The reactions were captured on phospho cellulose filtration microplates (Millipore, Billerica, MA) and washed with 75 mM phosphoric acid and methanol using a vacuum manifold. Phosphorylation was measured on a Perkin-Elmer MicroBeta 1450.

ROCK1 and ROCK2 Western Blot analysis

Western blots were used to determine whether HMVEC, NHDF and PASMC express ROCK1 and ROCK2. All cells were collected at passage 3 and lysed on ice in 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% tritonX-100, 10% glycerol, 10 mM NaF and a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined using a BCA protein assay reagent (Pierce Chemical Company, Rockford, IL). Cell lysates (35 µg) were separated on 7.5% or 12.5% SDS-PAGE polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to PVDF membrane filters. Membranes were blocked in 5% non-fat milk in TBS containing 0.1% Tween 20. Blots were probed with antibodies to ROCK1 (Bethyl Laboratories), ROCK2 (H-85, Santa Cruz Biotechnology, Santa Cruz, CA) or actin (Santa Cruz Biotechnology) and washed well before incubation with HRP-conjugated secondary antibodies and visualization with an enhanced chemiluminescence (ECL) kit (Healthcare, Piscataway, NJ).

Atorvastatin, mevalonate, and SLx-2119 treatment

Atorvastatin (Pfizer, New York, NY) was dissolved in methanol to obtain a stock solution of 10 mM. Mevalonate (Sigma, St Louis, MO) was dissolved in 0.1 M NaOH, to obtain a stock solution of 100 mM, pH 7.4. The ROCK2-inhibitor, SLx-2119, was synthesized at Surface Logix (Brighton, MA) and dissolved in DMSO to obtain a stock solution of 20 mM.

Human microvascular endothelial cells, PASMC, and NHDF at passage 7, and N-SMC and RE-SMC at passage 4, were plated in 6 cm culture dishes with 3 ml culture medium, at a density of 1×10^6 cells/dish. After 2 days (confluence 90%) the cells were incubated for 24 hours in 3 ml culture media containing vehicle (10 μ l sterile PBS), 10 μ M atorvastatin, a combination of 10 μ M atorvastatin and 500 μ M mevalonate, 10 μ M SLx-2119, or 40 μ M SLx-2119. Three independent experiments were performed, with 3 culture dishes in each treatment group.

RNA isolation

Twenty-four hours after treatment of HMVEC, PASMC and NHDF with vehicle, SLx-2119, atorvastatin, or atorvastatin combined with mevalonate, total RNA was isolated using Ultraspec RNA isolation reagent (Biotecx Laboratories, Houston, TX), according to the manufacturer's instructions. Two μ g of RNA was kept for microarray analysis (including quality control analysis) and 2 μ g was used for real-time PCR.

Twenty-four hours after treatment of N-SMC and RE-SMC with vehicle, SLx-2119, atorvastatin, or atorvastatin combined with mevalonate, total RNA was isolated as described before [15]. This RNA was used for real-time PCR.

Real-time PCR

For real-time PCR, 2 μ g of RNA from HMVEC, PASMC and NHDF was treated with RQ-DNase I (Promega, Madison, WI) at 37 °C for 30 min, after which cDNA was synthesized using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Steady-state mRNA levels were measured with real-time quantitative PCR (TaqMan™) using the ABI Prism 7700 Sequence Detection System, TaqMan mastermix and TaqMan polymerase, and the following pre-designed TaqMan Gene Expression Assays™ (Applied Biosystems): human connective tissue growth factor (CTGF, Hs00170014_m1), human TM (Hs00264920_s1), and human thrombospondin-1 (Tsp-1, Hs00170236_m1). mRNA levels were normalized to eukaryotic 18S rRNA (Hs99999901_s1) and calculated relative to vehicle-treated cells, using the standard $\Delta\Delta C_t$ method. Modulation of CTGF gene expression in N-SMC and RE-SMC was monitored by real-time PCR as previously described [15].

Real-time PCR data were evaluated by analysis of variance followed by a Tukey-Kramer *post-hoc* test, using software packages NCSS 2000 (NCSS, Kaysville, UT) and SPSS 14.0 (SPSS, Inc., Chicago, IL). The criterion for significance was a *P* value less than 0.05. Data are reported as means $\pm$ standard error of the mean (SEM).

Illumina arrays

Microarrays were used to determine gene expression profiles in HMVEC, NHDF and PASMC after treatment with atorvastatin or with SLx-2119. Both generation of aRNA and microarray hybridization were performed by the Microarray Core Laboratory of the University of Texas Health Science Center (Houston, TX). All RNA samples were tested for integrity and minimal degradation using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Two hundred ng of total RNA was reverse transcribed and amplified overnight with T7 RNA polymerase and labeled with biotin following the manufacturer's protocol, of which 1.5 μ g of biotin-labeled aRNA was hybridized to microarrays at 58 °C overnight. Illumina Genome-Wide Expression BeadChips (Human Ref-6 v1, Illumina, San Diego, CA), representing

~43,000 human transcripts [16], were used. Arrays were incubated with Cy3 streptavidin and washed according to the manufacturer's protocol. Initial analysis of the microarray data was done using Illumina's Beadstudio V1. After background subtraction, arrays were normalized to each other by rank-invariant normalization. Changes in gene expression were tested using a modified t-test that employs estimates of variation that include sequence specific biological variation (σ_{bio}), nonspecific biological variation (σ_{neg}) and technical error (σ_{tech}), according to the Illumina User Guide (2005), rev B. Genes were considered differentially regulated at $p < 0.01$ and a fold change of at least 2.

Reversing the effects of statins by the addition of mevalonate provides an opportunity to subtract non-specific effects of statins *in vitro* [17,18]. This approach was also used in the current study, as follows: genes were considered up- or down-regulated by atorvastatin if they were up- or down-regulated in atorvastatin-treated cells versus vehicle-treated cells and when the addition of mevalonate significantly reversed the change in expression induced by atorvastatin [18].

Genes that were up- or down-regulated by SLx-2119 or atorvastatin were classified according to Gene Ontology (GO) biological processes [19]. Microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE8686.

Actin staining of N-SMC and RE-SMC

Phalloidin-FITC was used to detect stress fiber formation in N-SMC and in RE-SMC. Twenty-four hours after treatment of N-SMC and RE-SMC with vehicle, SLx-2119, atorvastatin, or atorvastatin combined with mevalonate, cells were fixed in 0.5% paraformaldehyde and permeabilized in 0.1% triton X-100. The actin cytoskeleton was visualized with phalloidin-FITC (Sigma). Staining was examined by laser scanning confocal microscopy (Zeiss LSM510).

Results

SLx-2119 selectivity

Radiometric enzyme assays confirmed that SLx-2119 selectively inhibits activity of human ROCK2 ($IC_{50} = 105$ nM), while effects on human ROCK1 in this cell-free system were minimal ($IC_{50} = 24$ μ M, Fig. 1).

Microarray reproducibility and characterization of normal primary cells

Western Blot analysis was used to confirm that primary cultures of HMVEC, PASMC and NHDF express both isoforms of ROCK (Fig. 2).

No rounded cell morphology was observed in any of the cell treatments, confirming that the drugs were not cytotoxic at the concentration used. In total, 36 microarrays were hybridized with aRNA from HMVEC, PASMC, or NHDF at 24 hours after any of the treatments. One of the arrays, which was hybridized with aRNA from HMVEC treated with SLx-2119, showed a 5-times higher background than the other arrays. This array was omitted and the remaining 35 arrays were used for further analysis. In vehicle treated NHDF 10,536 genes showed a signal above background. Similarly, 10,776 genes were detected in vehicle-treated HMVEC and 9,188 genes were detected in vehicle-treated PASMC. Reproducibility among biological duplicates was high, with a mean R^2 of 0.985 in HMVEC, 0.99 in NHDF and 0.985 in PASMC.

Real-time PCR analysis of up-regulated and down-regulated genes

The effects of atorvastatin and SLx-2119 on selected genes were examined by realtime PCR in PASMC (Fig. 3). Atorvastatin at 10 μ M induced an up-regulation of TM mRNA in PASMC

that was reversed by mevalonate, confirming that this effect was due to an inhibition of the cholesterol biosynthesis pathway by atorvastatin. SLx-2119 did not induce an up-regulation of TM mRNA in either concentration tested. On the other hand, both atorvastatin at 10 μ M and SLx-2119 at 40 μ M induced significant down-regulations of Tsp-1 and CTGF mRNA levels. Based on these real-time PCR data, concentrations of atorvastatin and SLx-2119 of 10 μ M and 40 μ M respectively, were chosen for microarray analysis.

Microarray analysis of up-regulated and down-regulated genes

To select only those genes that were up- or down-regulated by atorvastatin via inhibition of the mevalonate pathway, genes were selected only when they were up- or down-regulated in atorvastatin-treated cells versus vehicle-treated cells and when the addition of mevalonate significantly reversed the change in expression. Table 1 shows numbers of genes that were up- and down-regulated by atorvastatin or SLx-2119 in HMVEC, PASM, and NHDF. Among the genes that were up-regulated by atorvastatin in HMVEC, were well-known effectors of statins, including genes coding for eNOS, TM, heat shock protein 27, and tissue plasminogen activator (*NOS3*, *THBD*, *HSPB1*, *PLAT*, respectively). In addition, accordance with the real-time PCR data, genes coding for Tsp-1 and CTGF (*THBD* and *CTGF*) were down-regulated by atorvastatin in all 3 cell types. SLx-2119, on the other hand, induced a significant down-regulation of Tsp-1 transcripts in HMVEC and PASM only and induced a down-regulation of CTGF transcripts, although non-significant, in all 3 cell types. Interestingly, the overlap in genes up- or down-regulated by both drugs was relatively small in all cell types and even absent in NHDF. Moreover, the 3 cell types differed greatly in their response to the 2 drugs, as illustrated both in table 1 and Fig. 4.

Of all up- or down-regulated genes, 60–70% could be classified according to GO biological process (Table 2). Although the 2 drugs exhibited little overlap in terms of the individual genes that were regulated, the genes could be classified in the same GO biological processes. Examples are given in Table 3, which lists all altered genes involved in cell adhesion (GO biological process ID 7155) and coagulation (GO biological process ID 7596), in PASM. Analogously, in HMVEC and NHDF, both atorvastatin and SLx-2119 affected genes involved in the same biological processes although the specific genes were mostly different for the two compounds.

Phenotype-dependent effects of atorvastatin and SLx-2119

Fibrosis is not included as a separate GO biological process. Nevertheless, atorvastatin and especially SLx-2119 were shown to alter many genes that may play a role in fibrosis. As mentioned above, atorvastatin down-regulated the gene *CTGF* in HMVEC, PASM, and NHDF. SLx-2119 induced a down-regulation of the genes *COL1A1*, *COL1A2*, *COL3A1*, and *COL5A2* in PASM, an up-regulation of the gene *MMP1* in PASM, a down-regulation of the gene *COL3A1* in NHDF, and an up-regulation of the genes *COL7A1*, *COL4A2*, *MMP1*, *MMP3*, *MMP14*, and *MMP17* in NHDF.

To address the possibility of phenotype-dependent effects of atorvastatin and SLx-2119, SMC were isolated from biopsies of normal human intestine (N-SMC) and from human intestine with radiation-induced fibrosis (RE-SMC). Because CTGF over-expression is known to be associated with fibrotic diseases, the effects of atorvastatin and SLx-2119 on CTGF mRNA were examined by real-time PCR in N-SMC and RE-SMC (Fig. 5a). Atorvastatin and SLx-2119 did not significantly alter CTGF mRNA level in N-SMC. In contrast, both drugs reduced CTGF mRNA level in RE-SMC in which CTGF base-line expression is elevated. Inhibition of CTGF mRNA by SLx-2119 occurred at 1 μ M with no further inhibition with increasing concentrations. Atorvastatin inhibitory action on CTGF was reversed by mevalonate, confirming that this effect was due to an inhibition of the mevalonate pathway.

Modulation of CTGF induced by compounds that interfere in the Rho/ROCK pathway (such as statins and the ROCK-inhibitor Y-27632) is thought to be associated with alteration of the actin cytoskeleton, which was also suggested by the stress fiber network modifications in the current study. Atorvastatin and SLx-2119 reduced and remodeled F-actin staining, both at a concentration of 10 μ M, in RE-SMC and N-SMC (Fig. 5b). The stress fibers became sparse in the central cell body, but the cytoplasmic processes remained. Mevalonate reversed the effects of atorvastatin.

Discussion

In this study, microarrays were used to perform a genome-wide comparison of the effects of ROCK2 selective inhibitor SLx-2119 with the effects of atorvastatin in primary cultures of human EC, SMC, and fibroblasts. The study showed that, although SLx-2119 and atorvastatin induced altered expression of genes belonging to the same biological processes, individual genes affected by these drugs were mostly different. These results suggest that there may be synergistic effects between statins and ROCK2-inhibitors, and that a possible therapeutic benefit may be achieved by combining the 2 classes of drugs.

When interpreting effects of drugs on cells in culture, it is important to consider to what extent *in vitro* concentrations are attainable *in vivo*. In our laboratory, the investigation of pleiotropic effects of statins such as TM up-regulation [20] and CTGF down-regulation [21] are a major focus. Hence, we have shown previously that atorvastatin at a concentration of 10 μ M consistently up-regulates TM gene and protein expression in cell cultures [20]. Moreover, administration *in vivo* of another HMG-CoA reductase inhibitor to mice at a nontoxic dose also caused increased TM gene expression levels in lung tissue (unpublished observation, 2008). In human subjects, peak plasma concentrations after oral administration of clinically relevant doses of atorvastatin, administered for its lipid-lowering effects (10 – 40 mg), are 0.5 – 10 nM [22,23]. Peripheral tissue concentrations and intracellular concentrations of atorvastatin have, to our knowledge, not been described. In the current study SLx-2119 down-regulated Tsp-1 and CTGF transcripts but only at a concentration of 40 μ M. Hence, the *in vitro* microarray studies reported here were performed with concentrations of atorvastatin and SLx-2119 of 10 μ M and 40 μ M, respectively. These concentrations are likely higher than tissue and/or intracellular concentrations that can be achieved *in vivo*. On the other hand, cells *in vivo* are generally exposed to a drug for much longer periods of time than cells in culture. Because of these and other considerations, studies in animals and/or humans will ultimately be required to examine whether the synergistic effects of SLx-2119 and atorvastatin suggested by the present study apply to the *in vivo* situation.

In radiometric assays, The IC₅₀ value of SLx-2119 for inhibition of ROCK1 was 24 μ M. Partial inhibition of ROCK1 may thus be expected at the concentration of SLx-2119 used in the microarray studies (40 μ M). However, SLx-2119 is an ATP-competitive inhibitor of ROCK. Therefore, in cell based assays where intracellular levels of ATP are 10–100 fold higher than the concentration of ATP used in the radiometric assay, the potency of SLx-2119 for ROCK2 may be expected to be in the 1–10 μ M range and for ROCK1 $\gg$ 200 μ M. Hence, at the concentrations used in this study, SLx-2119 selectively inhibits ROCK2.

An example of how ROCK2-inhibitors and statins may be complimentary to each other can be found in the effects of SLx-2119 and atorvastatin on genes involved in blood coagulation. Statins have known anti-fibrinolytic effects on vascular cells [24]. Moreover, the current and former studies have shown that statins up-regulate expression and function of TM [12,18,20]. TM is located on the luminal surface of EC in most normal blood vessels, where it forms a complex with thrombin. When complexed to TM, thrombin no longer cleaves fibrinogen to form fibrin and no longer activates cellular thrombin receptors, but instead activates protein

C. Activated protein C counteracts the pro-coagulant, inflammatory, and fibroproliferative effects of thrombin. Recent studies have demonstrated that increased levels of TM attenuate inflammatory responses in a variety of settings, including endotoxin-induced tissue damage and atherosclerosis [25,26]. Statins may be therapeutically beneficial in various disorders by up-regulating expression of TM. Interestingly, SLx-2119 did not affect expression of TM in any of the investigated cell types. However, this ROCK2-inhibitor could possibly enhance the therapeutic benefit of statins, by affecting the expression of other anti-fibroproliferative or anti-coagulant factors that are not affected by statins, such as urokinase plasminogen activator (PLAU) and urokinase plasminogen activator receptor (PLAUR). SLx-2119 does not alter prothrombin time (J.L. Ellis, unpublished observation).

Statins are also well-known to down-regulate genes coding for Tsp-1 and CTGF [18,26–28]. These factors are important mediators in the transforming growth factor (TGF) pathway, as Tsp-1 is an activator of TGF and CTGF is one of the main downstream mediators of TGF. The TGF pathway plays a central role in many conditions that lead to fibrosis [29–31]. Current microarray data show that SLx-2119 alters the expression of a plethora of genes that may be related to fibrosis and tissue remodeling, including genes coding for several collagen types and MMPs, in PASMC and NHDF. These genes were not altered by atorvastatin, again suggesting that the two classes of drugs may have synergistic effects. By altering CTGF and other fibroproliferative mediators, both statins and ROCK-inhibitors have indeed been shown to alter the fibrotic phenotype of SMC and fibroblasts [15,21,32]. In this study, we compared the effects of SLx-2119 and atorvastatin on SMC isolated from radiation enteropathy biopsies. As shown previously [15,21], these cells exhibit a profibrogenic phenotype characterized by increased formation of actin stress fibers and an increased expression of CTGF when compared to SMC isolated from normal intestine. Both atorvastatin and SLx-2119 reduced stress fiber formation and CTGF expression specifically in the RE-SMC. Similar results were found in EC in previous studies, in which ROCK-inhibitors and statins reduced stress fiber formation and endothelial permeability in response to irradiation or thrombin [33,34].

Interestingly, N-SMC from intestine and normal PASMC differed in their response with regard to CTGF. Although this difference may be explained by differences in culture conditions and differences in origin between the two SMC types, different responses of different cells may also be caused by the many targets and diverse effects of Rho proteins. This was also found in a previous study, in which microarrays were used to investigate the effects of ROCK-inhibitor Y-27632 on three different human fibroblast phenotypes [35]. Similar to the current study, the ROCK-inhibitor affected many genes involved in cell cycle regulation and cytokinesis. Furthermore, it was concluded that the ROCK-inhibitor had phenotype-specific effects on these cells. A similar difference was found on effects of Rho/ROCK inhibition on normal and fibrotic phenotypes of intestinal SMC in the current study.

As reported before for statins [36], the different cell types in the current study differed greatly in their gene expression response to atorvastatin or SLx-2119, including the total number of genes affected. For example, atorvastatin had little effect on gene expression in fibroblasts whereas SLx-2119 had a major effect. This predominant response of SLx-2119 on fibroblasts likely parallels the role of ROCK2 in the fibrotic response [10] and the potential for ROCK2-inhibitors to be beneficial in fibrotic disease.

In conclusion, the results of the current gene expression profiling study show that atorvastatin and the ROCK2-inhibitor SLx-2119 exhibit little overlap, but instead are mainly complimentary in their effects on gene expression in several primary human cell cultures. These data are consistent with a potential synergistic effect between statins and ROCK-inhibitors. It should also be noted that the ROCK pathway affects the function of several target proteins by posttranslation modification. Hence, more *in vitro* and *in vivo* studies are needed to further

explore the possible therapeutic benefit of treatments in which ROCK-inhibitors and statins are combined.

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Figure 1.
Radiometric truncated enzyme assay analysis of the effects of SLx-2119 on human ROCK1 and ROCK2.

Figure 2.

Both ROCK1 and ROCK2 are expressed in HMVEC, PASMC and NHDF, as analyzed with Western-Blot.

Figure 3.

Relative mRNA levels of (a) TM, (b) CTGF, and (c) Tsp-1 in PASMC, treated with atorvastatin (10 μ M), a combination of atorvastatin and mevalonate, or SLx-2119, as measured with real-time PCR. Means $\pm$ SEM, n=3. Atorvastatin induced an up-regulation of TM (p<0.001) and a down-regulation of CTGF (p=0.03) and Tsp-1 (p=0.02). Similar to atorvastatin, SLx-2119 induced a down-regulation of CTGF (p=0.01) and Tsp-1 (p=0.01). Expression of TM, on the other hand, was not altered by SLx-2119 (p=1.0). *Significantly different from vehicle (p<0.05).

Figure 4.

Overlap in the genes up-regulated in NHDF, HMVEC, or PASM, by (a) atorvastatin, or (b) SLx-2119. The 3 genes up-regulated by atorvastatin in all three cell types were *KLF2*, *DSIPI*, and *COPEB*. The 12 genes up-regulated by SLx-2119 in all three cell types were *MTIX*, *CXCL3*, *STC1*, *RIS1*, *SQSTM1*, *MMP1*, *CSF3*, *SPHK1*, *DUSP5*, *ESM1*, *IL8*, and the transcript of hypothetical protein LOC134285.

Figure 5.

Effects of atorvastatin, a combination of atorvastatin and mevalonate, or SLx-2119 on N-SMC and RE-SMC. Effects on (a) relative mRNA levels of CTGF as measured with real-time PCR and (b) F-actin cytoskeleton as visualized by FITC-phalloidin. Atorvastatin and SLx-2119 induced a down-regulation of CTGF mRNA in RE-SMC only ($p < 0.05$ and $p < 0.01$ respectively, means $\pm$ SEM, $n=3$). Atorvastatin and SLx-2119 reduced and remodeled F-actin staining, both at a concentration of 10 μ M, in RE-SMC and N-SMC (A: atorvastatin; A+M: atorvastatin + mevalonate).

Table 1

Numbers of genes up-regulated [and down-regulated] by atorvastatin or SLx-2119 in normal primary human cell cultures. The last column lists numbers of genes that were altered both in atorvastatin-treated cells and in cell treated with SLx-2119. The overlap between atorvastatin and SLx-2119 was small and even absent in NHDF.

Cell line	Atorvastatin	SLx-2119	Overlap between atorvastatin and SLx-2119
NHDF	13 [6]	195 [38]	0 [0]
HMVEC	146 [267]	34 [319]	13 [110]
PASMC	76 [81]	291 [264]	17 [33]

Table 2

Numbers of altered genes in all GO biological processes in which gene expression was altered (up- or down-regulated) by atorvastatin or SLx-2119 in PASM. The last column lists numbers of genes that were found altered both after atorvastatin treatment and after SLx-2119 treatment. Note that some genes belong to more than one biological process and are therefore counted multiple times.

Biological process	Number of genes altered		
	Atorvastatin	SLx-2119	Overlap
Cell adhesion / cell motility / cell shape	23	54	9
Cell cycle / cell growth	37	133	12
Cell death	6	24	3
Blood coagulation	4	8	2
Development / pathogenesis	34	63	13
DNA repair	9	3	0
Inflammatory response	13	42	7
Ion transport / ion homeostasis	4	13	3
(Molecular) metabolism	20	122	6
Protein modification	9	78	3
Signal transduction	40	127	16
Small molecule transport	11	46	7
Transcription / translation	15	77	4
Other	23	55	9
Biological process unknown	5	19	2

Table 3

Genes involved in cell adhesion (GO biological process ID 7155) and blood coagulation (GO biological process ID 7596) that were altered by atorvastatin or SLx-2119 in PASMC.

Gene symbol	Accession number	Up- or down-regulated	
		Atorvastatin	SLx-2119
<u>Cell adhesion</u>			
CLDN1	NM_021101.3	↑	
PCDH1	NM_002587.3	↑	
MTSS1	NM_014751.2	↑	
CD58	NM_001779.1	↑	
EMILIN2	NM_032048.1	↑	
CTGF	NM_001901.1	↓	
CCL2	NM_002982.2	↓	
THBS1	NM_003246.2	↓	↑
ALCAM	NM_001627.1		↑
CDH6	NM_004932.2		↑
CDH15	NM_004933.2		↑
ITGA11	NM_012211.2		↑
EDG1	NM_001400.2		↓
ITGA2	NM_002203.2		↓
LPXN	NM_004811.1		↓
<u>Blood Coagulation</u>			
THBD	NM_000361.2	↑	
PLAT	NM_000931.2	↑	
THBS1	NM_003246.2	↓	↓
F3	NM_001993.2	↓	↓
SERPIND1	NM_000185.2		↑
ITGA2	NM_002203.2		↑
PLAU	NM_002658.1		↑
PLAUR	NM_002659.1		↑
F2RL1	NM_005242.3		↑
PLA2G4A	NM_024420.1		↑

↑: up-regulated, ↓: down-regulated.