Published in final edited form as:

Inflamm Bowel Dis. 2014 January ; 20(1): 154–165. doi:10.1097/01.MIB.0000437615.98881.31.

Novel Rho/MRTF/SRF Inhibitors Block Matrix-stiffness and TGF- β -Induced Fibrogenesis in Human Colonic Myofibroblasts

Laura A. Johnson, BS^{*}, Eva S. Rodansky, MS^{*}, Andrew J. Haak, BS[†], Scott D. Larsen, PhD[‡], Richard R. Neubig, MD, PhD[‡], and Peter D. R. Higgins, MD, PhD, MSc^{*}

^{*}Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan

[†]Department of Pharmacology, University of Michigan, Ann Arbor, Michigan

[‡]Vahlteich Medicinal Chemistry Core, College of Pharmacy, University of Michigan, Ann Arbor, Michigan

Abstract

Background—Ras homolog gene family, member A (RhoA)/Rho-associated coiled-coil forming protein kinase signaling is a key pathway in multiple types of solid organ fibrosis, including intestinal fibrosis. However, the pleiotropic effects of RhoA/Rho-associated coiled-coil forming protein kinase signaling have frustrated targeted drug discovery efforts. Recent recognition of the role of Rho-regulated gene transcription by serum response factor (SRF) and its transcriptional cofactor myocardin-related transcription factor A (MRTF-A) suggest a novel locus for pharmacological intervention.

Methods—Because RhoA signaling is mediated by both physical and biochemical stimuli, we examined whether pharmacological inhibition of RhoA or the downstream transcription pathway of MRTF-A/SRF could block intestinal fibrogenesis in 2 in vitro models.

Results—In this study, we demonstrate that inhibition of RhoA signaling blocks both matrix-stiffness and transforming growth factor beta-induced fibrogenesis in human colonic myofibroblasts. Repression of alpha-smooth muscle actin and collagen expression was associated with the inhibition of MRTF-A nuclear localization. CCG-1423, a first-generation Rho/MRTF/SRF pathway inhibitor, repressed fibrogenesis in both models, yet has unacceptable cytotoxicity. Novel second-generation inhibitors (CCG-100602 and CCG-203971) repressed both matrix-stiffness and transforming growth factor beta-mediated fibrogenesis as determined by protein and gene expression in a dose-dependent manner.

Conclusions—Targeting the Rho/MRTF/SRF mechanism with second-generation Rho/MRTF/SRF inhibitors may represent a novel approach to antifibrotic therapeutics.

Keywords

Crohn's disease; fibrosis; matrix stiffness; Rho/ROCK; MRTF-A

Reprints: Peter D. R. Higgins, MD, PhD, MSc, Assistant Professor in Gastroenterology, Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of Michigan Medical Center, SPC 5682, Room 6510D, Medical Science Research Building One, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0682 (; Email: phiggins@med.umich.edu)

The authors have no conflicts of interest to disclose.

Fibrosis is the final common pathway to organ failure in many chronic diseases, including heart failure, kidney failure, liver cirrhosis, and lung fibrosis.¹⁻⁴ In Crohn's disease (CD), which affects more than 600,000 people in the United States, intestinal fibrosis is the critical final pathway of intestinal failure.⁵ Although intestinal fibrosis is initiated by inflammation, effective control of inflammation with potent anti-inflammatory medications has had minimal impact on the inexorable development of fibrosis.⁶⁻⁸ Indeed, recent evidence in a mouse model demonstrated that intestinal fibrosis, once initiated, may become autopropagative despite the eradication of inflammation.⁹ The lack of effective antifibrotic drugs and the autopropagative nature of fibrosis underscore the need for novel approaches to antifibrotic therapeutics.¹⁰

Pathologically activated intestinal myofibroblasts are the key effector cells of intestinal fibrosis.¹¹ Myofibroblast activation occurs through a serum response factor (SRF)-mediated gene transcription program that is induced by several stimuli including the physical environment (mechanical stress) and cytokines (transforming growth factor beta [TGF- β]).^{12,13} As we have demonstrated in both human CD ex vivo tissue and a rodent colitis model, fibrotic tissue is pathologically stiff.¹⁴ Classically, tissue stiffness has been described as an outcome, not a propagator, of tissue fibrosis.¹⁵ However, as we and others have demonstrated, pathological tissue stiffness triggers changes in fibroblast morphology and function from a quiescent, non-proliferative to an activated, fibrogenic myofibroblast phenotype.¹⁶⁻¹⁹

TGF- β is a key profibrotic cytokine in the etiology of CD in humans and in animal models of intestinal fibrosis.^{20,21} In CD strictures, tissue fibrosis is linked to alterations in TGF- β signaling and pathological activation of intestinal myofibroblasts.²⁰ In isolated intestinal myofibroblasts, profibrotic signaling occurs through a canonical TGF- β /similar to mothers against decapentaplegic-mediated pathway.²² However, noncanonical TGF- β profibrotic signaling through similar to mothers against decapentaplegic-independent pathways, including Rho/Rho-associated coiled-coil forming protein kinase (ROCK), has been described in other organ systems.²³

The Ras homolog gene family, member A (RhoA) family signaling pathway mediates numerous cellular responses including cell adhesion, migration, contraction, proliferation, and cytoskeletal remodeling.²⁴ Through RhoA binding to the effector molecules, ROCK or mDia (mammalian diaphanous), extracellular signals are transduced to the nucleus by actin polymerization, which triggers nuclear translocation of myocardin-related transcription factor (MRTF).²⁵⁻²⁷ Recent evidence has implicated this transcriptional regulation by SRF and its transcriptional cofactor myocardin-related transcription factor A (MRTF-A) in the induction of pathological myofibroblast differentiation.²⁸ This RhoA/MRTF/SRF pathway has been implicated in fibrosis in several organ systems including kidney, skin, eye, heart, and lung.²⁹⁻³²

RhoA signaling is activated by numerous extracellular mechanisms including mechanical (cell adhesion, mechanical stress) and receptor-mediated (growth factors, cytokines) stimuli.^{23,33} Several studies have linked the RhoA pathway to the cellular response to matrix stiffness.^{17,34} In addition, Rho/ ROCK signaling has been described in similar to

mothers against decapentaplegic-independent TGF- β signaling in radiation-induced fibrosis of the intestine.³⁵ Downstream of RhoA signaling, pharmacological inhibition of MRTF-A/SRF-mediated gene transcription mechanisms using CCG-1423, which was discovered by our group, inhibits TGF- β -induced fibroblast activation.³⁶ Because 2 conceptually different profibrotic stimuli (mechanical stiffness and TGF- β) potentially intersect through a common RhoA-mediated signaling pathway, we examined whether pharmacological inhibition of RhoA/MRT/SRF pathway could block intestinal fibrogenesis in matrix-stiffness and TGF- β -mediated fibrosis models.

MATERIALS AND METHODS

Reagents and Plasmids

CCG-1423, CCG-100602 (1-[3,5-bis(trifluoromethyl)benzoyl]-*N*-(4-chlorophenyl)-3-piperidinecarboxamide), and CCG-203971 [*N*-(4-chlorophenyl)-1-{[3-(furan-2-yl)phenyl]carbonyl}piperidine-3-carboxamide] were synthesized by the Vahlteich Medicinal Chemistry Core at the University of Michigan.³⁷ Recombinant human TGF- β was obtained from R&D Systems (Minneapolis, MN). All other reagents, unless otherwise noted, were obtained from Sigma (St Louis, MO).

Cell Culture

Human colonic fibroblast CCD-18co cells (CRL-1459, derived from a female donor) were obtained from ATCC (Bethesda, MD). Cells were cultured in α -MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and subcultured weekly. Human intestinal fibroblasts were derived from normal human colon samples generously provided by Claudio Fiocchi and Florian Rieder (the Cleveland Clinic, Cleveland, OH).

In Vitro Stiffness Model

Low-passage number colonic myofibroblasts, CCD-18co cells, were seeded at 1×10^5 cells/mL on 6-well plates containing acrylamide gels corresponding to the matrix stiffness of normal or fibrotic tissue. Technical methods of acrylamide stiffness gels preparation were as detailed in Ref.¹⁸. Briefly, human colonic myofibroblasts were plated on collagen-coated acrylamide substrates corresponding to normal compliant intestinal tissue (4.3 kPa) or fibrotic stiff intestine in CD (28 kPa). Cells were allowed to attach to the matrix for 2 hours before the transfer of the coverslip and hydrogel to a new 6-well plate containing minimal serum (0.5%) to avoid serum stimulation of SRF signaling and potential paracrine signaling from cells adhering to the plastic surrounding the coverslip. To determine the effect of Rho/ROCK or Rho/MRTF/SRF inhibitors, 1 to 25 μ M concentrations of inhibitors were added to cells plated on either stiff or compliant matrices and cultured for 24 to 48 hours. Cells were photographed using an Olympus (inverted) light microscope (Olympus, Center Valley, PA) and harvested for RNA, protein, or fixed and immunostained for confocal microscopy.

TGF- β Fibrogenesis Model

Low-passage colonic myofibroblasts (CCD-18co cells) were seeded at 1×10^5 cells/mL on 6-well tissue culture plates. Cells were serum-starved overnight before stimulation with 1 ng/mL of TGF- β as previously described.^{18,38} To determine the effect of Rho/ROCK or

Rho/MRTF/SRF inhibitors, cells were cotreated with 1 ng/mL TGF- β and 1, 3, 10, 17.5, or 25 μ M of inhibitors and incubated for 48 hours before harvest for molecular analysis. The media was replaced at 24 hours with fresh media containing TGF- β or TGF- β and inhibitors.

Immunoblotting

Immunoblotting was used for the detection of alpha-smooth muscle actin (α SMA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described.¹⁸

Quantitative Real-time PCR

RNA was extracted from cultured cells using the RNeasy kit (Qiagen, Valencia, CA). RNAs were treated with RNase-free DNase before cDNA synthesis using the First Strand Synthesis kit (Invitrogen) according to manufacturer's protocol. The analysis of gene expression of colonic myofibroblasts was determined by quantitative real-time PCR (qRT-PCR) of collagenIA1 (colIA1), myosin light chain kinase (MYLK), MKL1 (MRTF-A) genes, and GAPDH was performed with the TaqMan gene expression assays (ABI, Foster City, CA). qRT-PCR for SMA (ACTA2) was performed using the SYBR Green assay using the following primers (ACTA2-F 5'-AATGCAGAAGGAGATCACGC-3', ACTA2-R 5'-TCCTGTTTGCTGATCCACATC-3'). All qRT-PCR was performed using a Stratagene M $\times$ 3000P RT PCR system (Stratagene, La Jolla, CA). Cycling conditions were 95°C 10 minutes, followed by 40 cycles of 95°C 15 seconds and 62°C 60 seconds. C_t values were calculated from GAPDH expression.

Immunofluorescence Imaging

Expression of activated myofibroblast markers was analyzed by confocal immunofluorescence microscopy using the Olympus FluoViewTM FV500/IX system (Olympus America, Center Valley, PA) at the University of Michigan Microscopy and Image Analysis Laboratory as previously described.¹⁹

High Content Imaging and Analysis

CCD-18co cells were plated in a range between 5×10^3 and 2×10^4 cells per well in a 96-well plate. Cells were fixed and stained for 4',6-diamidino-2-phenylindole (DAPI) and MRTF-A as previously described.¹⁹ Images were acquired from 16 sites per individual well using the automated ImageXpress Micro Cellular Imaging (IXM) and Analysis System (Molecular Devices, Sunnyvale, CA) at the University of Michigan Center for Chemical Genomics. Nuclear localization of MRTF-A was defined by colocalization of MRTF-A staining with DAPI and quantified using the Translocation Application Module of MetaXpress (Molecular Devices), normalizing the Integrated Inner Intensity to cell number and excluding wells with an average cell number fewer than 10 cells per site to reduce sampling error effects.

Statistical Analysis

Comparisons across treatment groups were analyzed with analysis of variance, whereas pairwise comparisons between 2 groups were performed with Student's *t* test.

RESULTS

Inhibition of RhoA Signaling Blocks Matrix Stiffness–induced Fibrogenesis

Y27632, an inhibitor of p160 Rho kinase and Rho/ROCK signaling, was originally described as an antihypertensive.³⁹ However, subsequent work has demonstrated antifibrotic effects in multiple fibrosis models across different organ systems, including radiation-induced fibrosis of the intestine.³⁵ In normal human colonic myofibroblasts (CCD-18co cells), pharmacological inhibition of Rho/ROCK signaling by Y27632 repressed stiffness-induced morphological changes with a notable reduction in actin stress fiber formation characterized by diffuse and disorganized actin staining (Fig. 1A). Similarly, Y27632 inhibited formation of well-organized mature focal adhesions. Actin stress fiber contraction is regulated by MYLK, which facilitates myosin II binding to actin filaments within stress fibers.⁴⁰ In human colonic myofibroblasts, Y27632 treatment repressed MYLK mRNA levels nearly 3-fold ($P = 0.028$, Fig. 1B).

MYLK, a SRF-responsive gene, is regulated in part by SRF cofactors myocardin-related transcription factors MRTF-A and MRTF-B.⁴¹ Upon actin polymerization, MRTF-A is released and translocates to the nucleus where it acts as a transcriptional cofactor for SRF-responsive genes.^{26,42} As determined by our group in colonic myofibroblasts and others in pulmonary fibroblasts, fibrogenic activation by increased matrix stiffness is associated with increased MRTF-A transcription and MRTF-A nuclear translocation.^{19,43} In human colonic myofibroblasts, inhibition of Rho/ROCK signaling by Y27632 blocked MRTF-A nuclear translocation, as evidenced by increased cytoplasmic staining (Fig. 1C, D).

Targeted Inhibition of MRTF-A nuclear Localization Blocks Fibrogenic Activation by Matrix Stiffness

Recently, RhoA transcription pathway inhibitors that specifically disrupt MRTF-A nuclear localization have been demonstrated to inhibit Rho/MRTF/SRF signaling in carcinogenesis and cell invasion models.³⁷ One compound, CCG-1423, potentially targeted RhoA/C-activated SRE-luciferase ($IC_{50} \approx 1 \mu M$) and blocked cell invasion but showed significant toxicity in vitro and in vivo (compound 1 in Ref. ³⁷ and Fig. 2B). Using a structure–activity relationship approach, a second-generation compound, CCG-100602, with lower cellular toxicity was identified (compound 4g in Ref. ³⁷). Additional structure–activity relationship by Larsen's group to improve potency and selectivity while reducing cytotoxicity produced CCG-203971, which possesses slightly increased potency of Rho/MRTF/SRF inhibition versus CCG-100,602 and further attenuated acute cytotoxicity (Fig. 2A and compound 8a in Ref. ⁴⁴).

To determine whether Rho/MRTF/SRF inhibitors can block stiffness-induced MRTF-A nuclear shuttling, we treated normal human colonic myofibroblasts (CCD-18co cells) cultured on soft or stiff matrices with CCG-100602. CCG-100602 treatment for 24 hours at 25 μM reduced stiffness-induced actin stress fiber formation and MRTF-A nuclear translocation (Fig. 3A). Similar to Y27632, CCG-100602 decreased mRNA levels of the actin-contraction gene, MYLK by 2.3-fold ($P < 0.001$) (Fig. 3B). CCG-100602 also targeted transcription of the SRF transcriptional cofactor MRTF-A (MKL1), significantly repressing

mRNA levels of MKL1 to basal levels representative of low substrate stiffness ($P < 0.001$, Fig. 3C).

Because fibrogenic activation is characterized by increased α SMA and collagen expression and MRTF-A is a potent transcriptional coactivator of both α SMA and collagen expression,^{41,45,46} we assayed the effects of CCG-100602 on α SMA (ACTA2) and collagen I (col1A1) mRNA levels. In CCD-18co cells, treatment with 25 μ M CCG-100602 for 24 hours significantly repressed matrix stiffness-induced ACTA2 expression to levels below untreated cells ($P < 0.001$, Fig. 4A). A similar transcriptional response was observed for col1A1 (Fig. 4B). In addition to transcriptional repression, CCG-100602 dramatically repressed stiffness-induced α SMA protein expression (Fig. 4C).

Increased α SMA expression and subsequent assembly of α SMA into mature stress fibers activates a profibrotic phenotype in myofibroblasts.⁴⁷ To determine whether Rho/MRTF/SRF inhibitors inhibit the architectural characteristics of fibrogenic activation, CCD-18co cells were stimulated with TGF- β with or without the Rho/MRTF/SRF inhibitor, CCG-100602. Myofibroblast activation was assayed by staining for cellular α SMA, the quintessential marker of activated myofibroblasts. In TGF- β -stimulated cells, α SMA staining illuminated well-organized intensely stained actin stress fibers (Fig. 4D). In contrast, cells stimulated with TGF- β and treated with CCG-100602 exhibited diffuse, muted α SMA staining with a notable lack of organized stress fibers similar to the α SMA staining observed in both untreated cells and cells treated with CCG-100602 alone (Fig. 4D). Because of limitations of the stiffness model (e.g., use of collagen-coated acrylamide matrices precluded isolation of cellular collagen), the effects of CCG-100602 on collagen protein expression could not be determined.

Inhibition of Rho/MRTF-A/SRF Blocks TGF- β - mediated MRTF-A Localization

As illustrated in the matrix stiffness model, physical stimuli induce MRTF-A nuclear translocation, whereas the small molecular inhibitor CCG-100602 produces cytoplasmic retention of MRTFA. To determine whether the effects of biochemical stimuli are similarly attenuated by pharmacological inhibition of MRTF-A localization, CCD-18co cells were induced with TGF- β and treated with 25 μ M of CCG-100602. MRTF-A compartmental localization was compared with untreated cells or cells treated with CCG-100602. In human colonic myofibroblasts, increased cytoplasmic staining of MRTF-A was observed in the CCG-100602-treated cells compared with untreated cells (Fig 5A). In cells stimulated with TGF- β and treated with CCG-100602, marked MRTF-A cytoplasmic staining was observed. Surprisingly, the greatest MRTF-A cytoplasmic levels occurred in cells cotreated with TGF- β and CCG-100602. Therefore, we examined whether MKL1 (MRTF-A) transcription was affected by TGF- β and CCG-100602.

Treatment of CCD-18co cells with 1 ng/mL TGF- β significantly induced MKL1 mRNA (>3-fold, $P < 0.0001$ versus untreated). Though cotreatment with CCG-100602 did not completely abrogate MKL1 transcription to levels of untreated cells, CCG-100602 repressed TGF- β -induced MKL1 transcription by ~2-fold compared with MKL1 transcription treated with TGF- β alone ($P = 0.006$, Fig. 5B).

Because the subcellular localization of MRTF-A is critical for its function, the effects of CCG-100602 on MRTF-A nuclear localization were determined. Since, visually, differential MRTF-A nuclear localization is difficult to ascertain, MRTF-A nuclear localization was quantified by image analysis with MetaXpress software. In the matrix stiffness model, increased matrix stiffness triggers MRTF-A nuclear localization. Consistent with this observation, in the TGF- β model, where cells are plated on very stiff substrate (i.e., plastic), MRTF-A nuclear staining was not statistically different between untreated cells and TGF- β -treated cells (Fig. 5C). However, in TGF- β -stimulated cells, CCG-100602 significantly reduced MRTF-A nuclear localization 2-fold ($P = 0.006$, Fig. 6C). In unstimulated cells, CCG-100602 treatment demonstrated a reductive trend, with a modest 50% reduction in nuclear staining ($P = 0.054$, Fig. 5C).

Inhibitor Comparison: TGF- β - mediated Fibrogenesis

In other cell lineages such as pulmonary fibroblasts, a first-generation Rho/MRTF/SRF inhibitor, CCG-1423, repressed TGF- β - induced fibrogenesis and α SMA protein expression.³⁶ Because CCG-1423 demonstrated profound effects on TGF- β -induced fibrosis, yet has an unacceptable toxicity profile, we compared the efficacy of second-generation Rho/MRTF/SRF inhibitors CCG-100602 and CCG-203971 with the parent compound CCG-1423 in TGF- β -stimulated colonic fibroblasts.

In CCD-18co cells, TGF- β strongly induced α SMA protein expression. Cotreatment with TGF- β and Rho/MRTF/SRF inhibitor demonstrated a concentration-dependent relationship between inhibitor concentration and α SMA protein expression. The first-generation Rho/MRTF/SRF inhibitor, CCG-1423 repressed α SMA protein expression at 1 and 3 μ M (Fig. 6A, B). However, higher concentrations of 17.5 and 25 μ M were clearly cytotoxic (data not shown). Although higher concentrations of CCG-100602 and CCG-203971 were needed to inhibit TGF- β -induced α SMA protein expression, neither of these compounds produced cytotoxic effects, despite marked repression of α SMA protein (Fig. 6A, B). In primary human intestinal fibroblasts cells stimulated with TGF- β , CCG-100602 and CCG-203971 (25 μ M) strongly repressed α SMA protein expression (data not shown).

Similar effects of the Rho/MRTF/SRF inhibitors were observed for collagen I protein expression as 1 and 3 μ M CCG-1423 repressed TGF- β -induced collagen I protein expression (Fig. 6A). Both CCG-100602 and CCG-203971 demonstrated a concentration-response relationship for collagen I protein expression. Again, 17.5 and 25 μ M of either inhibitor reduced collagen I protein expression to untreated levels (Fig. 6A).

Inhibition of MRTF-A Activity Blocks TGF- β - mediated Expression of Profibrogenic Genes

The MRTF family of transcriptional cofactors (including MRTF-A/MLK1) is a critical mediator of TGF- β fibrogenic signaling.⁴⁸ In pulmonary fibroblasts, TGF- β stimulated MKL1 transcription, whereas a first-generation Rho/MRTF/SRF inhibitor, CCG-1423, repressed MKL1 transcription.^{28,49} Given these reports and the demonstrated efficacy of the second-generation Rho/MRTF/SRF inhibitors on MKL1 transcription in our matrix stiffness model, we determined the effect of second-generation Rho/MRTF/SRF inhibitors CCG-100602 and CCG-203971 on TGF- β -mediated fibrogenic transcription.

Treatment of CCD-18co cells with 1 ng/mL TGF- β significantly induced MKL1 mRNA levels (>3-fold, $P < 0.0001$ versus untreated). Consistent with published observations in pulmonary fibroblasts, CCG-1423 repressed MKL1 mRNA at concentrations as low as 1 μ M (Fig. 7A). However, notable cytotoxicity was observed at concentrations >3 μ M (data not shown). Both second-generation Rho/MRTF/SRF inhibitors CCG-100602 and CCG-203971 significantly repressed TGF- β -induced MKL1 expression at 17.5 and 25 μ M concentrations (Fig. 7A).

TGF- β treatment transcriptionally activates both collagen and α SMA expression whereas MRTF-A/MKL1 mediates TGF- β -induced α SMA expression.⁵⁰⁻⁵² Therefore, we examined the effects of second-generation Rho/MRTF/SRF inhibitors on TGF- β -responsive genes, including actin stress fiber contractile (MYLK) and profibrotic (ACTA2, col1A1) genes (Fig. 7B–D). As demonstrated in the matrix stiffness model above, Rho/MRTF/SRF inhibitors transcriptionally repress the actin assembly gene MYLK. In the TGF- β model, TGF- β induces MYLK mRNA >3-fold ($P < 0.0001$ versus untreated). Cotreatment of CCD-18co cells with TGF- β and increasing amounts of the first-generation Rho/MRTF/SRF inhibitor CCG-1423 strongly repressed MYLK mRNA from 1 to 25 μ M with notable cytotoxicity observed at concentrations >3 μ M (Fig. 7B, and data not shown). Second-generation Rho/MRTF/SRF inhibitors CCG-100602 and CCG-203971 repressed MYLK mRNA levels in a concentration-dependent manner, with partial, yet significant, repression observed for both CCG-100602 and CCG-203971 at 10 μ M ($P = 0.019$ and $P = 0.05$, respectively) and complete repression to basal (untreated levels) at 17.5 and 25 μ M (Fig. 7B).

Since both TGF- β and MRTF-A regulate profibrogenic gene expression, including α SMA (ACTA2) and collagen I (col1A1), the effects of first- and second-generation Rho/MRTF/SRF inhibitor on ACTA2 and col1A1 were determined. TGF- β induced a 4-fold increase ACTA2 transcription. Treatment with CCG-1423 significantly repressed ACTA2 transcription to levels below untreated cells (Fig. 7C). The second-generation Rho/MRTF/SRF inhibitors repressed ACTA2 in a dose-responsive manner, with significant transcriptional inhibition observed at 17.5 and 25 μ M ($P < 0.001$, Fig. 7C). Similar transcriptional responses were observed for collagen (col1A1), with significant inhibition observed with 17.5 and 25 μ M of either CCG-100602 or CCG-203971. However, CCG-203971 was more potent at lower concentrations. A significant reduction of nearly 2-fold was observed at 1 and 3 μ M doses ($P = 0.024$, $P = 0.049$ versus TGF- β -treated, respectively, Fig. 7D).

DISCUSSION

The pleiotropic functions of the RhoA signaling pathway, from cellular mechanosensing and motility to signal transduction and transcriptional regulation, are well established in vitro.²⁴ At the organismal level, the RhoA signaling pathway, originally identified as a master regulator in cardiovascular disease and cardiac fibrosis,⁵³ is now appreciated, through a growing body of experimental evidence, as a key pathway in multiple types of solid organ fibrosis.²⁹⁻³² Intestinal fibrosis recapitulates many key features of solid organ fibrosis.¹⁰ In addition, pathogenic activation of RhoA signaling has been described in intestinal radiation-

induced fibrosis, suggesting the RhoA signaling pathway may be a useful clinical target for intestinal fibrosis.⁵⁴

Intestinal fibrosis is mediated by key effector cells, activated intestinal myofibroblasts, which produce and maintain components of the extracellular matrix.¹¹ As we have demonstrated, both physical (matrix stiffness) and biochemical (TGF- β) stimuli activate a gene transcriptional program in intestinal myofibroblasts triggering morphological changes, including development of actin stress fibers and focal adhesions, and increased expression of profibrotic genes/proteins, notably α SMA and collagen. In this study, pharmacological inhibition of RhoA signaling blocked both matrix stiffness and TGF- β -mediated fibrogenesis. The fact that RhoA inhibition targeted 2 distinctly different profibrogenic pathways (TGF- β and matrix stiffness-induced) underscores the potential utility of RhoA inhibition in intestinal fibrosis.

Although the RhoA signaling pathway has been implicated in multiple pathological processes, including fibrosis, its pleiotropic effects have frustrated selective drug discovery efforts. To date, relatively few inhibitors have been identified.⁵⁵ Early pharmacological efforts yielded Y27632 and fasudil, which are nonselective kinase inhibitors that have biochemical efficacy in the nanomolar range but are typically used in micromolar concentrations in vivo. This lack of potency increases the risk of off-target effects.⁵⁵ Indeed, problematic cardiac side effects of Y27632 have been described.⁵⁶ Though ROCK inhibitors, including Y27632, have been used in clinical trials in humans, the potential for adverse off-target effects and weak efficacy have limited their clinical utility.

Currently, the focus of pharmacological development has shifted from inhibition at the upstream locus (i.e., ROCK) to targeting the specific transcription mechanism involved in myofibroblast activation (MRTF/SRF).⁵⁵ We have identified novel small molecular inhibitors of Rho/MRTF/SRF mechanisms with increased specificity and potency.⁵⁷ These compounds disrupt RhoA/MRTF/SRF transcription by functional inhibition of MRTF-A nuclear localization. Though the precise mechanism of action is unknown, MRTF nuclear localization is dependent on cytoskeletal changes, specifically actin polymerization.⁴¹

In intestinal myofibroblasts, CCG-1423 blocked TGF- β -mediated α SMA and collagen I protein expression. In addition, CCG-1423 repressed TGF- β induction of fibrogenic (ACTA2, col1A1), actin-contraction (MYLK), and transcriptional cofactor (MKL1/MRTF-A) genes. These results in intestine agree with observations in pulmonary myofibroblasts, including very recent work demonstrating that CCG-1423 represses both matrix-stiffness and TGF- β -induced α SMA expression.^{28,49} Although CCG-1423 demonstrated antifibrotic efficacy in vitro, this first-generation Rho/ MRTF/SRF inhibitor produced notable cytotoxicity in our intestinal fibrogenesis model, consistent with reports in other in vitro and in vivo systems.³⁷ Therefore, we assayed the antifibrotic efficacy of 2 second-generation inhibitors (CCG-100602 and CCG-203971), which have far less cytotoxicity than the parent compound CCG-1423.^{37,44}

In the matrix stiffness model, targeted inhibition of Rho/ MRTF/SRF by CCG-100602 repressed stiffness-induced morphological changes including actin stress fiber formation and

increased MYLK transcription. Mechanosensing of the extracellular environment is mediated by 2 RhoA effectors, ROCK and mDia, which alter the actin cytoskeleton and regulate downstream cellular signals.⁵⁸ ROCK directly phosphorylates MYLK, stimulating actin cross-linking and stress fiber contraction, effectively liberating bound MRTF-A, which then readily translocates to the nucleus to function as a transcriptional cofactor for a number of profibrotic genes including α SMA and collagen I (Fig. 8, Ref. ⁴²). mDia catalyzes the polymerization of globular (G-actin) to filamentous actin (F-actin), induces cytoskeletal rearrangement and the formation of actin stress fibers, and stimulates nuclear translocation of MRTF-A.⁵⁹ Thus, MRTF-A is a nexus downstream of both ROCK and mDia action. We previously showed that Rho-regulated gene transcription is more effectively blocked by CCG-1423 than by ROCK inhibitors (supplementary materials in Ref. ⁵⁷).

As demonstrated by Chaturvedi et al.,⁶⁰ the intestinal epithelial cellular response to mechanical deformation is mediated by RhoA signaling through both ROCK and mDia. We have demonstrated in intestinal myofibroblasts that repression of stiffness-induced actin stress fiber formation by CCG-100602 was associated with cytoplasmic retention of MRTF-A. In pulmonary fibroblasts, α SMA and collagen transcription is activated by MRTF-A nuclear translocation.^{41, 45} In intestinal myofibroblasts, inhibition of MRTF-A nuclear translocation by CCG-100602 dramatically repressed both α SMA and collagen I transcription, as well as stiffness-induced α SMA protein expression. In addition, CCG-100602 repressed MYLK transcription, suggesting a feedback loop between MYLK contraction of F-actin, MRTF-A release, fibrogenic transcription, and maintenance of MYLK transcription.

In the TGF- β -mediated fibrosis model, TGF- β induced morphological changes in intestinal myofibroblasts, characterized by the presence of strongly stained α SMA+actin fibers, nuclear translocation of MRTF-A, and increased MKL1 (MRTF-A) transcription. Cotreatment with CCG-100602 blocked the formation of α SMA +stress fibers and MRTF-A nuclear translocation. Surprisingly, higher cytoplasmic retention of MRTF-A was observed in the cotreated TGF- β +CCG-100602 cells compared with CCG-100602 cells alone. As we subsequently demonstrated, TGF- β induced MKL1 (MRTF-A) transcription. Though CCG-100602 cotreatment significantly repressed TGF- β -induced MKL1 transcription, MKL1 levels remained ~50% higher than untreated cells. Though not statistically significant, this observation, combined with the observed increase in MRTF-A cytoplasmic localization in the TGF- β +CCG-100602-treated cells, suggests even in the presence of increased MKL1 (MRTF-A) levels, CCG-100602 inhibition compensates for the effects of TGF- β , sequestering excess MRTF-A within the cytoplasm.

Sequestration of MRTF-A within the cytoplasm is associated with concurrent depletion of nuclear MRTF-A. As illustrated in the matrix stiffness model, high substrate stiffness induces nuclear translocation of MRTF-A. In the TGF- β model, cells are cultured on a typical stiff tissue culture substrate. Therefore, no differences in nuclear localization were observed between unstimulated and TGF- β stimulated cells. Consistent with the matrix stiffness results, CCG-100602 repressed MRTF-A nuclear localization in both the unstimulated and TGF- β stimulated cells. Surprisingly, the most significant repression was

observed in the cotreated TGF- β +CCG-100602 cells, suggesting that CCG-100602 can overcome the combined profibrogenic stimuli of matrix stiffness and TGF- β signaling.

Activated profibrogenic myofibroblasts express α SMA and produce/secrete excess collagen.^{61, 62} Both first-generation (CCG-1423) and second-generation (CCG-100602, CCG-203971) Rho/ MRTF/SRF inhibitors repressed TGF- β -induced α SMA and collagen I transcription and protein expression in a dose-response manner. In addition, the second-generation Rho/MRTF/SRF inhibitors transcriptionally repressed key effector molecules within the RhoA/ MRTF-A pathway, including MYLK (which is involved in the release of actin-bound MRTF-A by facilitating stress fiber contraction) and MRTF-A itself.

Targeting RhoA signaling downstream of the ROCK and mDia effectors using Rho/ MRTF/SRF inhibitors may be an effective pharmacological strategy, which reduces the off-target effects and toxicity associated with current pan-RhoA inhibitors. Though higher effective doses of second-generation Rho/MRTF/ SRF inhibitors were required to achieve comparable repression (17.5, 25 μ M versus 1 μ M), minimal toxicity and biologically achievable concentrations identify these as attractive antifibrotic candidates for future therapeutic studies.

The lack of antifibrotic therapeutics is a critical unsolved problem in organ fibrosis in general, and particularly in CD. Although organ fibrosis is a multifactorial process, a rapidly advancing appreciation of the RhoA/MRTF/SRF pathway in the development of fibrosis in multiple organ systems, combined with the isolation of novel downstream inhibitors, has the potential to offer effective and specific pharmacological intervention for currently intractable diseases.

Acknowledgments

Supported by an NIH Grant, K08DK080172 (Higgins). The ImageXpress Micro screening system is supported in part by a National Functional Genomics Center Grant (W81XWH-10-2-0013) from the Department of Defense to the University of Michigan Center for Chemical Genomics.

The authors thank Steven Swaney at the University of Michigan Center for Chemical Genomics, Life Sciences Institute, for assistance with IXM imaging and analysis; Claudio Fiocchi and Florian Rieder of the Digestive Disease Institute, the Cleveland Clinic (Cleveland, OH), for the human intestinal fibroblast (HIF) cells; and Susan M. Wade and Raelene VanNoord of the University of Michigan, Department of Pharmacology, for their technical expertise.

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FIGURE 1.

Inhibition of RhoA signaling blocks matrix stiffness-induced fibrogenesis. A, Y27632 represses the formation of mature focal adhesions (red) and organization of actin stress fibers (green) normally induced by a stiff extracellular matrix. Cells were cultured on soft (4.3 kPa) or stiff (28 kPa) collagen-coated polyacrylamide gels for 24 hours and treated with 33 μ M Y27632 (DAPI = blue, vinculin = red, phalloidin = green (actin stress fibers)). B, Myosin light chain kinase (MYLK) gene expression is induced by increasing matrix stiffness (round markers). Y27632 treatment (triangular markers) inhibits matrix stiffness induction of MYLK gene expression as determined by qRT-PCR. MYLK expression was normalized to GAPDH expression. Results are from 5 experimental replicates. Statistical comparisons are made between the untreated and Y27632 treated at each stiffness point, $*P < 0.05$. C, In cells cultured on a stiff (28 kPa) matrix, MRTF-A (red) localizes

predominantly to the nucleus. In Y27632-treated cells cultured on a stiff matrix, MRTF-A remains cytoplasmic (DAPI = blue, MRTF-A = red, phalloidin = green). D, Inset of individual cells from (C) detailing MRTF-A localization within the nucleus (circled, white) or cytoplasm (denoted in yellow).

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Compound	IC ₅₀ SRE.L (μ M)	% inhib. Renilla (10 μ M)	% inhib. WST (10 μ M)
CCG-1423	1.5	60	60
CCG-100602	10	20	0
CCG-203971	6.2	0	0

FIGURE 2.

Structure and IC₅₀s of Selective RhoA/MRTF-A/SRF inhibitors. A, Structure of the first-generation (CCG-1423) and second-generation (CCG-100602, CCG-203971) RhoA/MRTF-A inhibitors. B, SRE transcription and cytotoxicity profiles of first- and second-generation RhoA/MRTF-A inhibitors. SRE.L, SRE-luciferase assay; Renilla; WST metabolism (measure of cytotoxicity).

FIGURE 3.

Targeted inhibition of MRTF-A nuclear localization blocks fibrogenic signaling by matrix stiffness. Comparison of CCD-18co cells were cultured on soft (4.3 kPa) or stiff (28 kPa) collagen-coated polyacrylamide gels compared with CCD-18co cells plated on stiff gels and treated with 25 μ M CCG-100602 for 24 hours. A, In CCG-100602-treated cells cultured on a stiff matrix, MRTF-A is retained in the cytoplasm (DAPI = blue, MRTF-A = red, phalloidin = green). CCG-100602 treatment inhibits matrix stiffness induction of MYLK (B) and MKL1 (MRTF-A) (C) gene expression as determined by qRT-PCR. Gene expression was normalized to GAPDH expression. Results are from 5 experimental replicates. Statistical comparisons are made between the untreated 4.3 kPa stiffness. Pairwise comparisons are denoted with a horizontal line between groups. * $P < 0.05$, *** $P < 0.001$.

FIGURE 4.

Targeted inhibition of Rho/MRTF/SRF represses transcription of fibrogenic genes and α SMA protein expression. Expression of fibrogenic genes ACTA2 (A) and col1A1 (B) in CCD-18co cells at low (4.3 kPa) and pathologically high (28 kPa) matrix stiffness with and without 25 μ M CCG-100602 for 24 hours. C, A representative Western blot illustrating α SMA protein expression in response to low versus high matrix stiffness in Ccd18-co cells treated with 25 μ M CCG-100602 compared with untreated cells. D, α SMA staining (red) in CCD-18co cells treated with TGF- β , CCG-100602, or TGF- β +CCG-100602, compared with untreated cells. Cell nuclei in the merged images were visualized with DAPI (blue).

Statistical comparisons are made with respect to the untreated low stiffness (4.3 kPa) group. Pairwise statistical comparisons are denoted with horizontal lines, $*P < 0.05$, $***P < 0.001$. Results are from 3 independent experiments, performed in duplicate ($n = 6$).

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FIGURE 5.

Inhibition of Rho/MRTF/SRF signaling causes cytoplasmic retention of MRTF-A in TGF-β-mediated fibrogenesis. **A**, Compartmental localization of MRTF-A (red) in CCD-18co cells stimulated for 48 hours with TGF-β (1 ng/mL) or 1 ng/mL TGF-β + 25 μM CCG-100602 compared with untreated or CCG-100602 (25 μM) treated alone. Nuclei are visualized with DAPI (blue). Merged images illustrating MRTF-A localization are shown. Detailed MRTF-A localization (**A**, insets) within the nucleus (circled, white) or cytoplasm (outlined in yellow) of individual cells are shown. **B**, MKL1 gene expression in TGF-β or TGF-β + CCG-100602-treated cells compared with untreated cells. **C**, Quantitation of MRTF-A nuclear localization. Integrated inner intensity of nuclei was determined by image analysis with MetaXpress software as normalized to cell density. Statistical comparisons are between the treated groups and untreated cells. Pairwise comparisons are denoted by horizontal lines (*** $P < 0.001$, P values for nonstatistically significant trends are shown).

FIGURE 6.

Dose-response inhibition of TGF- β -mediated fibrogenesis by Rho/MRTF-A/SRF inhibitors. Expression of fibrogenic proteins in CCD-18co cells. A, Representative α SMA and collagen I Western blots of CCD-18co cells treated with 1 ng/mL TGF- β (TGF) or TGF- β + MRTF-A inhibitors CCG-100602, CCG-203971, or CCG-1423 at 1, 3, 10, 17.5, and 25 μM concentrations compared with untreated cells (un). Because of cytotoxicity, concentrations of CCG-1423 > 3 μM are not shown. GAPDH was used as a loading control. B, Quantitation of α SMA protein expression as normalized to GAPDH. Results are from 3 independent experiments. ** P < 0.01, *** P < 0.001 compared with TGF- β treated.

FIGURE 7.

Inhibition of MRTF-A blocks TGF-β-mediated gene expression. Gene expression of transcriptional cofactor MKL1 (A) actin-contraction (MYLK, B), and fibrogenic genes ACTA2 (C) and col1A1 (D) in CCD-18co cells treated with 1 ng/mL TGF-β and 1, 3, 10, 17.5, and 25 μM of MRTF-A inhibitors CCG-1423, CCG-100602, or CCG-203971. Gene expression was normalized to GAPDH expression. Results are from 3 independent experiments performed in duplicate (total n = 6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with TGFβ-treated cells.

FIGURE 8.

Activation of fibrogenesis genes through RhoA/MRTF-A/SRF signaling. Alterations in matrix stiffness are sensed through integrin interaction with extracellular focal adhesions while TGF- β function occurs through ligand binding to a TGF- β receptor complex. These physical (matrix stiffness) and biochemical (TGF- β) stimuli converge through activation of Rho GTPases its effectors including ROCK, which stimulates actin cross-linking and stress fiber contraction, ultimately releasing actin-bound MRTF-A and mDia, which triggers actin polymerization from globular (G-actin) to filamentous (F-actin). Incorporation of G-actin into F-actin during actin polymerization releases MRTF-A, which translocates to the nucleus. On nuclear translocation, MRTF-A interacts with the transcription factor SRF, inducing the transcriptional of SRF-responsive profibrogenic genes including α SMA, col1A1, and MYLK. In the presence of nuclear G-actin, MRTF-A is complexed with G-actin and exported from the nucleus, inhibiting transcription of SRF-responsive genes. Pharmacological inhibitors of RhoA (i.e., Y27632) block RhoA signaling upstream of actin polymerization. Targeted inhibitors (CCG-1423, CCG-100602, and CCG-203971) block MRTF-A-mediated processes downstream of actin polymerization and the RhoA effectors ROCK and mDia. Dotted arrows represent indirect interactions.