# Selective ROCK2 inhibitors for the treatment of fibrosis

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## INTRODUCTION

### ROCK2 is central to disease processes driving fibrosis pathology

- The Rho Associated Coiled-Coil Containing Protein Kinase (ROCK) serine/threonine kinases, ROCK1 and ROCK2, are central signalling proteins that regulate a range of cellular responses.
- These processes are central to the aberrant wound healing response that can progress to chronic injury and organ fibrosis.
- ROCK pathway inhibition decreases fibrosis *in vivo* and fibrotic markers *in vitro*.
- ROCK2 is upregulated in diseases associated with acute and chronic inflammation, for example where damage is caused by high glucose and high fat diets.
- ROCK2 increased in liver fibrosis models and the increased ROCK2 signalling drives HSC activation.

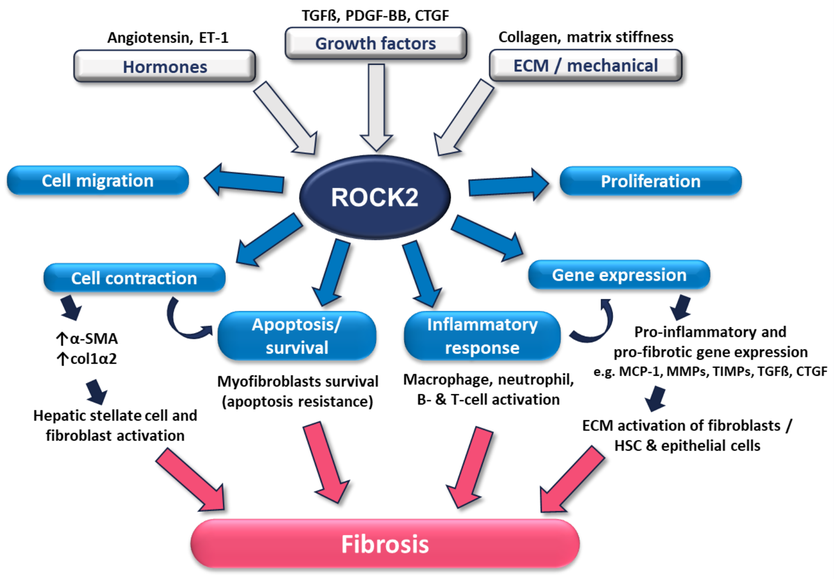



*Figure 1. ROCK2 is downstream of fibrosis mediators and drives pathways associated with fibrosis pathology.*

## RESULTS

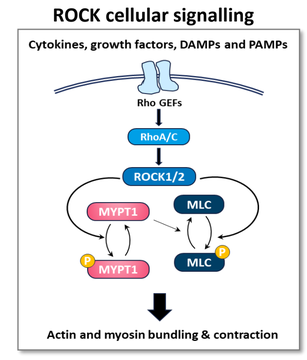
### Redx's ROCK2 inhibitors are potent and highly selective

- Redx's ROCK2 series of compounds are potent and highly selective against ROCK1 and a panel of kinases, tested in biochemical and cellular mechanistic assays.
- Cellular potency of ROCK2 selective inhibitors determined by inhibition of pMYPT1, a substrate downstream of ROCK; ROCK1 or ROCK2 selective cell lines were generated with shRNA.

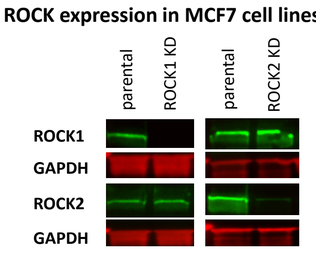
| Assay | KD025 $IC_{50}$ (µM) | REDX10178 $IC_{50}$ (µM) | REDX10616 $IC_{50}$ (µM) | REDX10843 $IC_{50}$ (µM) |
|---|---|---|---|---|
| **ROCK2 activity** | 0.16 | 0.002 | 0.004 | 0.017 |
| **ROCK1 activity** | 9.8 | 0.2 | 3.0 | 2.5 |
| **Cellular ROCK2 selective pMYPT1** | 1.0 | 0.9 | 1.0 | 2.4 |
| **Cellular parental MCF7 pMYPT1*** | 0.9 | 4.1 | 22 | 24 |
| **Cellular ROCK1 selective pMYPT1*** | > 30 | 20 | > 30 | 26 |

*Table 1. Selective ROCK2 compounds activity in biochemical and cellular assays. Comparison with KD025[22].*
**Selective ROCK2 compounds expected to be less active in parental & ROCK1 selective MCF7 assays.*

**ROCK cellular signalling**




**A: ROCK expression in MCF7 cell lines**




**B: pMYPT1 mechanistic assay**

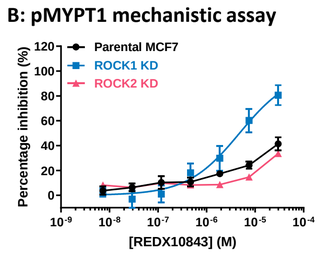


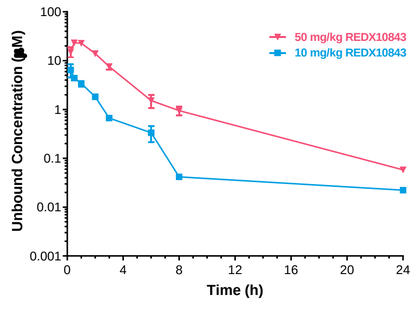

*Figure 2. MCF7 KD lines generated with shRNA express only one isoform (A). Representative inhibition of pMYPT1 in parental and ROCK1 or ROCK2 knockdown cell lines.*

## RESULTS

### REDX10843 has suitable *in vitro* ADME properties and is orally bioavailable

- REDX10843 is highly selective across a panel of 468 kinases, and in the CEREP safety panel with no significant off target activities observed.
- No activity in cardiotoxicity assays.
- No genotoxicity or mutagenicity observed in *in vitro* tests.
- Low to medium *in vitro* clearance, conserved across species (mouse, rat, dog and human) and good oral exposure in mouse, rat and dog.

**A: Mouse PK of REDX10843**




**B: Rat PK of REDX10843**

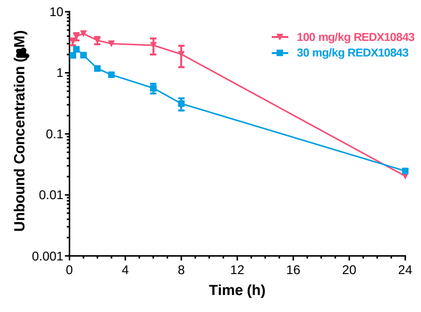



*Figure 3. Oral pharmacokinetic profiles of REDX10843 dosed in C57Bl/6 mice (A) and Han Wistar rats (B). Formulation: HPMC (0.5% w/v in water) / DMSO [90:10].*

| Assay | | REDX10843 | | | |
|---|---|---|---|---|---|
| **Selectivity** | Kinase panel (468 kinases) at 10 µM | 9 targets >90% inhibition; 32 targets >50% inhibition | | | |
| | CEREP SafetyScreen44 at 10 µM | No target > 50% inhibition; 8 targets > 25% inhibition | | | |
| **Cardiotoxicity** | hERG $IC_{50}$ | > 33 µM | | | |
| | hNav1.5 $IC_{50}$ | > 100 µM | | | |
| | hCav1.2 $IC_{50}$ | > 100 µM | | | |
| **Genotoxicity** | Mini-Ames 5 strains (± S9 plate based) | No significant increase in numbers of revertant colonies | | | |
| **Mutagenicity** | Micronucleus test using TK6 cells (± S9 metabolic activation) | No statistically significant increase in micronuclei | | | |
| | | **Mouse** | **Rat** | **Dog** | **Human** |
| **Microsome stability** | CLint (µL/min/mg) | 35 | 39 | 15 | 4.9 |
| **Hepatocyte stability** | CLint (µL/min/$10^6$ cells) | 18 | 15 | 19 | 2.3 |
| **Plasma protein binding** | Percentage free (Fu %) | 13 | 14 | 23 | 13 |

*Table 2. In vitro properties for REDX10843.*

### ROCK2 inhibitors reduce fibrosis markers in kidney models *in vitro* and *in vivo*

#### A: ROCK2 selective compounds inhibit *in vitro* expression of fibrosis markers in kidney mesangial cells

- Mesangial cells cultured in high glucose for 9 days; model diabetic environment (compounds day 4-9).
- Protein expression of CTGF, fibronectin, PDGF-BB, TIMP-1 and MCP-1 detected in the culture media.

| ASSAY | KD025 $IC_{50}$ (µM) | REDX10178 $IC_{50}$ (µM) | REDX10616 $IC_{50}$ (µM) | REDX10843 $IC_{50}$ (µM) |
|---|---|---|---|---|
| CTGF assay (WB) | Inactive | 0.4 | 0.4 | 1.5 |
| Secreted TIMP-1 | 0.9 | 0.2 | 0.9 | 2.8 |
| Secreted PDGF-BB | 2.9 | 0.2 | 0.4 | 1.4 |
| Secreted MCP-1 | 10 | 0.3 | 0.3 | 2.2 |
| Secreted MMP2 | ND | 1.2 | 2.2 | 1.1 |

*Table 3. Summary of analysis of culture supernatant from cells cultured with Redx's ROCK2 inhibitors and KD025 as a comparison. Supernatant analysed by western blot (CTGF) or ELISA. Data are from n≥3.*

**CTGF expression in culture supernatant**

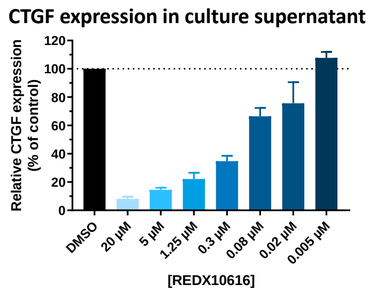
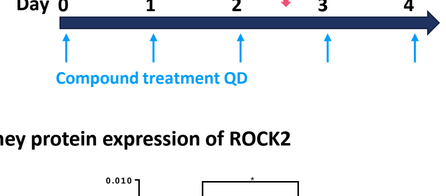



*Figure 4. Representative data for REDX10616 inhibition of CTGF.*

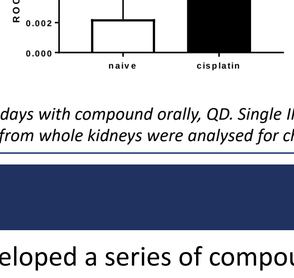
#### B: ROCK2 inhibition of inflammatory response *in vivo,* in a model of acute kidney injury

- Cisplatin induces an acute inflammatory infiltrate and response in the kidney.
- This inflammatory response induces injury in the kidney and leads to an increase in the expression of ROCK2.
- ROCK2 selective inhibitors REDX10178[†] and REDX10616[‡], reduce the expression of genes associated with inflammation and fibrosis in a dose dependent manner.

**A: Schematic of dosing regime**




**B: Kidney protein expression of ROCK2**




**C: Gene expression in the kidney**

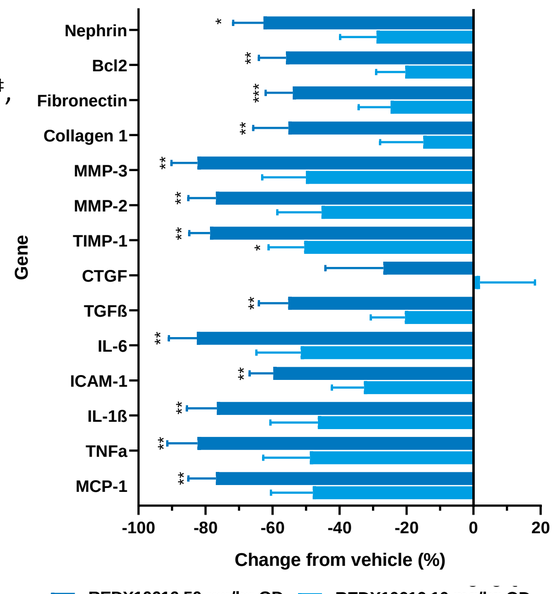


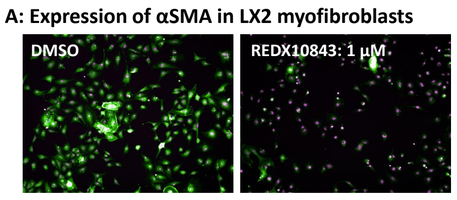

*Figure 5. Mice treated for 5 days with compound orally, QD. Single IP injection of cisplatin on day 3 (A). Protein expression of ROCK2 detected by western blot normalised to GAPDH (B). RNA isolated from whole kidneys were analysed for changes in gene expression by qPCR, plotted as change from vehicle treated animals (C).*

### ROCK2 inhibitors reduce fibrosis markers in liver models *in vitro* and *in vivo*

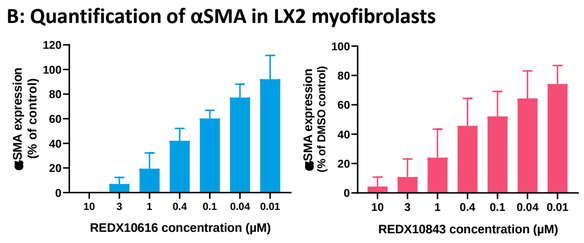
#### A: ROCK2 selective compounds reverse myofibroblast phenotype in HSC derived myofibroblasts i*n vitro*

- Hepatic stellate cell line differentiated on stiff plastic for 2 weeks demonstrate myofibroblast morphology and phenotype by induction of αSMA protein expression.
- Reduction of αSMA with ROCK2 inhibitors indicates suppression of the myofibroblast phenotype.

**A: Expression of αSMA in LX2 myofibroblasts**




**B: Quantification of αSMA in LX2 myofibrolasts**




**C: Mean $IC_{50}$ of percentage inhibition of αSMA**

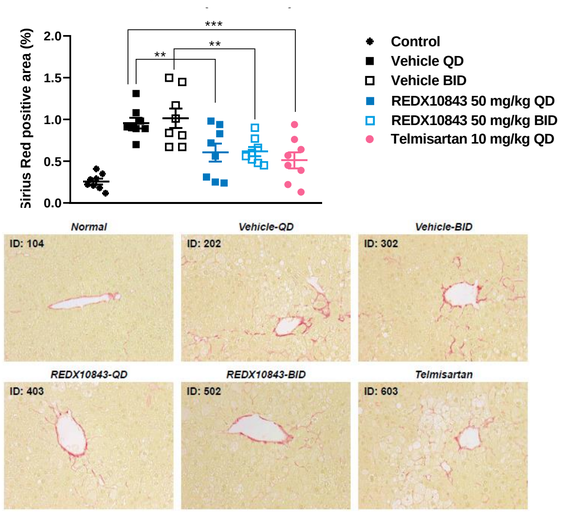
| Assay | KD025 ($IC_{50}$) | REDX10178 ($IC_{50}$) | REDX10616 ($IC_{50}$) | REDX10843 ($IC_{50}$) |
|---|---|---|---|---|
| **αSMA expression** | 0.3 µM | 0.7 µM | 0.3 µM | 0.5 µM |

*Figure 6. Redx ROCK2 inhibitors reduces αSMA expression in LX-2 differentiated myofibroblasts. Myofibroblasts expressing αSMA are dosed with compound for 48 h and αSMA expression detected by IHC. Representative images shown where green indicates αSMA and purple nuclei stain (DRAQ5) (A). Representative data of cells dosed with REDX10616 or REDX10843 (n=4) (B). Table of mean quantification of αSMA expression $IC_{50}$ of inhibition response (C).*

#### B: REDX10843 significantly reduces fibrosis parameters in the STAM NASH model

- Selective ROCK2 inhibitor REDX10843 dosed therapeutically in the murine STAM NASH model significantly reduces fibrosis in the liver when dosed BID or QD at 50 mg/kg.

**A: Sirius Red expression in the liver**




**B: Reticular fibroblasts (ER-TR7)**

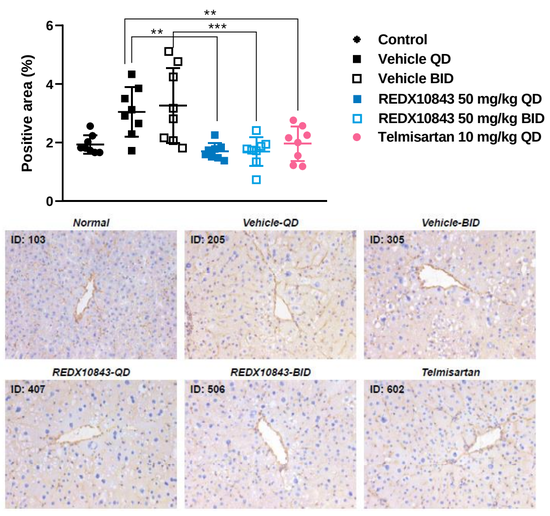



*Figure 7. In the STAM NASH model mice are treated with streptozotocin (STZ) at day 2, and fed HFD from week 4. Compounds dosed therapeutically from week 6-9. Sirius Red expression in the liver shows collagen expression (A). Quantification of reticular fibroblasts (B). Representative images are at 200x original magnification.*

## SUMMARY

- Redx have developed a series of compounds that are potent ROCK2 inhibitors in biochemical & cellular *in vitro* assays and highly selective against ROCK1 and a panel of kinases.
- Demonstration that physiologically relevant markers of fibrosis pathways can be modulated *in vitro* in disease relevant phenotypic assays.
- No safety concerns highlighted from early *in vitro* assessment (hERG, CEREP, AMES, micronucleus).
- Targeting ROCK2 selectively allows a safe cardiovascular profile, as previously demonstrated with REDX10178 in telemetered rats[†].
- The encouraging profile of series compounds is representative of the potential of the chemical series which are currently in lead optimisation.
- ROCK2 inhibition modulates fibrotic parameters *in vivo* with REDX10843, selective ROCK2 inhibitor.

***References:*** *1. Soliman et al, 2016; 2. Xie et al, 2006; 3. Waddingham et al, 2015; 4. Cicek et al, 2013; 5. Shimizu et al, 2013; 6. Yao et al, 2013; 7. Okamoto et al, 2013; 8. Zhou et al 2012; 9. Hu et al. 2018; 10. Luo et al, 2012; 11 Zhang et al 2016; 12. Trebicka et al, 2007; 13. Wang et al, 2018; 14. Kolavennu et al. 2008; 15. Baba et al. 2014; 16. Sun et al. 2006; 17. Nozaki et al 2015; 18. Zhou et al 2013; 19. Ho et al. 2012; 20. Knipe et al 2015; 21.* Kast *et al, 2017; 22. Flynn et al, 2016. Note: [†]Data presented at ASN Oct 2018, [‡]Data presented at Keystone NAFLD and NASH conference, Jan 2019.*