ROCK2 inhibitors for the treatment of fibrosis

Emily P. Offer, Nicolas E.S. Guisot, Stuart A. Best, Philip MacFaul, Sara Ceccarelli, Matthew R. Box, Neil Hawkins, Rosie Knowles, Rebecca Holland, Peter R. Bunyard, Clifford D. Jones, Richard Armer.

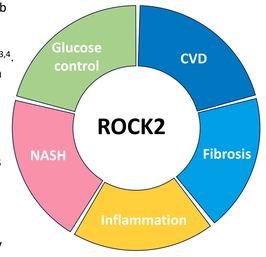
Redx Pharma Plc, Alderley Park, Macclesfield, United Kingdom.

KEYSTONE SYMPOSIA on Molecular and Cellular Biology — Accelerating Life Science Discovery

INTRODUCTION

ROCK2 is central to disease processes driving fibrosis pathology

- ROCK2+/- on HFD protected against loss of insulin sensitivity & protected from heart abnormalities[1].
- ↑ROCK activity in vascular endothelium of db/db mice[2].
- ↑ROCK2 in vascular endothelium diabetic animals, ↑ hypertension & vascular dysfunction[3,4].
- ROCK2+/- haplotype mouse showed attenuation of diabetes induced hypertension[6].



- ↑ROCK2 in liver of db/db mice[9] and liver fibrosis models[10,11].
- ↑ROCK2 signalling drives HSC activation and portal hypertension[9,10,12] and blocking this pathway reduces fibrosis[11].
- ROCK2 implicated in non-canonical Wnt pathway induced steatohepatitis[13].

- ↑ROCK2 arterial smooth muscle cells, cultured from patients with PAH[5].
- ROCK2 conditional KO protected against hypoxia induced hypertension[5].
- Cardiomyocyte specific ROCK2 KO mice protected against angiotensin induced hypertrophy[7].
- ROCK2-/- bone marrow reduced atherosclerosis on high cholesterol diet[8].

- ROCK inhibitors reduce fibrosis severity in lung[18], heart[19], and liver fibrosis models[10,11].
- ROCK inhibition reduces fibrosis parameters and albuminuria in kidney fibrosis models[14,15,16].
- ROCK2+/- protected from bleomycin induced lung fibrosis[20].
- ROCK2 inhibitor reduces fibrosis in human cGVHD.

- ↑ROCK2 in acute inflammation in the kidney[17].
- ROCK inhibition reduces pro-inflammatory cytokine release and leukocyte recruitment[14,16,17].

NOTE: *ROCK1 and ROCK2 expression in the vascular endothelium is responsible for the control of vascular tone and systemically active pan-ROCK inhibitors have been shown to induce a drop in arterial blood pressure leading to a corresponding increase in heart rate[21]. We hypothesise that targeting only ROCK2 would not induce hypotension.*

RESULTS

Redx's ROCK2 inhibitors are potent and highly selective

- REDX10178 and REDX10616 are potent and highly selective ROCK2 inhibitors.
- Cellular potency of ROCK2 selective inhibitors determined by measuring inhibition of pMYPT1, a substrate downstream of ROCK in MCF7 cell lines; ROCK1 or ROCK2 selective cell lines were generated with shRNA.

ASSAY	KD025 IC_{50}	REDX10178 IC_{50}	REDX10616 IC_{50}
ROCK2 activity	0.07 µM	0.001 µM	0.004 µM
ROCK1 activity	5.1 µM	0.1 µM	2.4 µM
Cellular ROCK2 selective pMYPT1	1 µM	0.8 µM	0.9 µM
Cellular parental MCF7 pMYPT1*	0.9 µM	3.9 µM	> 30 µM
Cellular ROCK1 selective pMYPT1*	0.8 µM	8.8 µM	> 30 µM

*Table 1. ROCK2 selective tool compounds are active in biochemical and cellular assays and selective in kinase panel. Comparison with KD025 – Kadmon's ROCK2 selective compound[22]. *Note: Selective ROCK2 compounds expected to be less active in parental and ROCK1 selective MCF7 assays.*

REDX10616 inhibition of pMYPT1

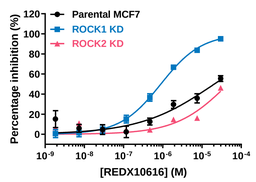
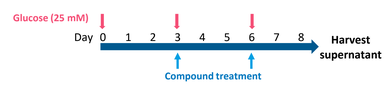


Figure 1. Inhibition of pMYPT1 in parental and ROCK1 or ROCK2 knockdown cell lines. ROCK2 selective compounds are more potent in ROCK1 knockout lines due to compensation of ROCK1 signalling in the wildtype parental lines. Data are from n>3.

ROCK2 inhibitors prevent the release of pro-inflammatory and pro-fibrotic factors in kidney mesangial cells grown in high glucose

- Protein expression of CTGF, fibronectin, PDGF-BB, TIMP-1 and MCP-1 detected in the culture media.

A: Schematic of the experiment



ASSAY	KD025 IC_{50}	REDX10178 IC_{50}	REDX10616 IC_{50}
CTGF assay – WB	Inactive	0.1 µM	0.1 µM
Fibronectin ELISA	Induction	0.4 µM	0.2 µM
Secreted TIMP-1 – ELISA	0.9 µM	0.2 µM	0.5 µM
Secreted MCP-1 – ELISA	2.9 µM	0.3 µM	0.4 µM
Secreted PDGF-BB – ELISA	10 µM	0.2 µM	0.3 µM

Table 2. Summary of analysis of culture supernatant from cells cultured with Redx's ROCK2 inhibitors and KD025 as a comparison. Data are from n≥3.

B: CTGF expression in culture supernatant

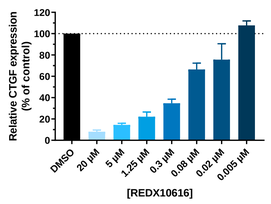


Figure 2. Mouse mesangial cells cultured for 8 days in high glucose with media refresh and compound addition on day 3 and media and compound refreshed on day 6 (A). Supernatant harvested for analysis, representative data in (B).

RESULTS

REDX10616 has suitable *in vitro* ADME properties and is orally bioavailable

- REDX10616 is a highly selective ROCK2 inhibitor across a panel of 422 kinases, and in the CEREP safety panel with no off target activities observed.
- Low to medium *in vitro* clearance and conserved across species (mouse, rat, dog and human).

Assay		REDX10616			
Selectivity	Kinase panel (422 kinases) at 10 µM	2 targets >80% inhibition; 20 targets >50% inhibition*			
	CEREP SafetyScreen44 at 10 µM	1 target > 50% inhibition			
Cardiotoxicity	hERG IC_{50}	25 µM			
Thermodynamic solubility	pH 7.4	4.2 µM			
	FaSSIF	131 µM			
	FeSSIF	375 µM			
	FaSSGF	341 µM			
		Mouse	Rat	Dog	Human
Microsome stability	Clint (µL/min/mg)	24	39	ND	7
Hepatocyte stability	Clint (µL/min/10^6 cells)	19	3.7	5.2	3.2
Plasma protein binding	Percentage free	9	7	18	7

*Table 3. In vitro properties for REDX10616. *Excluding ROCK isoforms.*

REDX10616 kinase panel at 10 µM

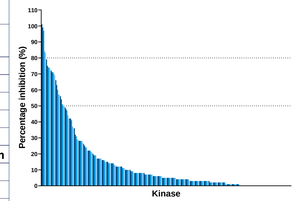


Figure 3. Waterfall plot analysis of kinase panel of 422 kinases for REDX10616 at 10 µM.

- REDX10616 has good oral exposure *in vivo* in rat and mouse.

Species	Dose (mg/kg)	C_{max}/C_0 (µM)	T_{max} (h)	$t_{1/2}$ (h)	Vdss (L/kg)	CL (mL/min/kg)	AUC_{inf} (h/µM)	AUC_{0-t} (h/µM)	% F
C57Bl/6 mouse	2 (IV)	6.5	-	1.0	1.0	17	4.3	4.3	-
	10 (PO)	7.6	2.0	1.2	-	-	30	29	> 100
	50 (PO)	56	0.5	2.2	-	-	397	397	> 100
Han Wistar rat	2 (IV)	5.2	-	3.6	1.2	14	5.1	5.1	-
	10 (PO)	3.9	0.5	1.7	-	-	19	19	73
	50 (PO)	22	3.0	3.2	-	-	245	243	> 100

Table 4. Pharmacokinetic parameters of REDX10616 in C57Bl/6 mice and Han Wistar rats.

Mouse C57Bl/6 oral pharmacokinetics

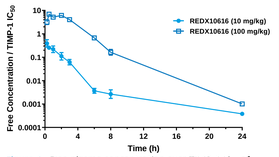


Figure 4. Free plasma concentration over TIMP-1 IC_{50} of REDX10616 in C57Bl/6 mice.

ROCK2 inhibitors reduce markers of fibrosis in human liver *in vitro* models

- Redx ROCK2 inhibitors reduce primary human hepatic stellate cell activation and and fibrotic gene expression *in vitro*.
- Redx ROCK2 inhibitors reduce lipid accumulation in primary human hepatocytes *in vitro*.

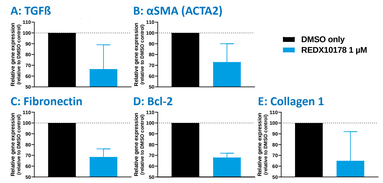


Figure 8. Primary HSC activated with TGFβ for 5 days then treated with REDX10178 for 1h. Gene expression relative to RPS18 normalised to DMSO controls.

Hepatocyte lipid accumulation

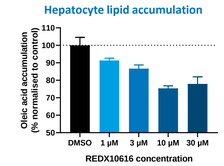
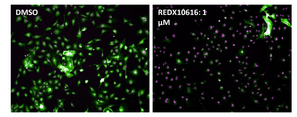


Figure 9. Primary human hepatocytes dosed with REDX10616 plus oleic acid at 1 mM. After 48 h cells labelled with Nile red probe to stain lipids then analysed by flow cytometry. Accumulation normalised to DMSO controls.

- Selective ROCK2 inhibitors reverse myofibroblast phenotype of activated human HSC.
  - Hepatic stellate cell line differentiated on stiff plastic for 2 weeks demonstrate myofibroblast morphology and phenotype by induction of αSMA protein expression.
  - Redx ROCK2 inhibitors reduce the expression of αSMA in these cells, indicating suppression of the myofibroblast phenotype.

A: αSMA expression in HSC myofibroblasts



Green: αSMA; purple: nuclei (DAPI)

B: Quantification of αSMA expression

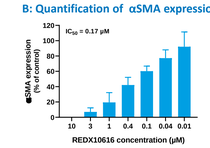


Figure 10. REDX10616 reduces αSMA expression in LX-2 differentiated myofibroblasts. Myofibroblasts expressing αSMA are dosed with compound for 48 h and αSMA expression detected by IHC. (A) Representative images shown where green indicates αSMA and purple nuclei stain (DAPI). In (B) mean quantification of αSMA expression in cells dosed with REDX10616 (n=4).

REDX10616 supresses inflammatory, fibrosis and kidney injury pathways in a model of acute kidney injury

- Mice treated for 5 days with compound orally, QD. Single IP injection of cisplatin on day 3 induces an acute inflammatory infiltrate.
- This inflammatory response induces injury in the kidney and leads to an increase in the expression of ROCK2.
- REDX10616 modifies the expression of genes associated with inflammation and fibrosis in a dose dependent manner.

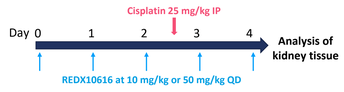


Figure 5. Schematic of the dosing regime in the acute kidney injury model.

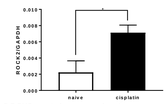


Figure 6. ROCK2 protein expression from whole kidneys.

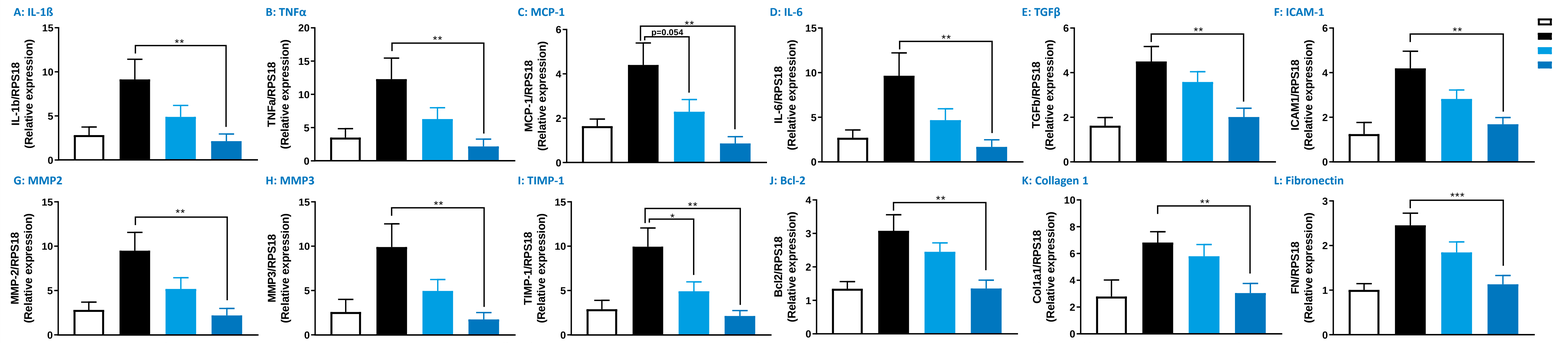


*Figure 7. Gene expression analysis from whole kidneys. Data are plotted mean±SEM n=8-10 animals. Statistical effect of treatment analysed by one way ANOVA with Fisher's LSD post test, compared to vehicle treated animals, *p<0.05, **p<0.01, ***p<0.001, naive animals excluded from analysis.*

SUMMARY

- Redx have developed a series of compounds that are potent ROCK2 inhibitors in biochemical & cellular *in vitro* assays.
- These compounds are highly selective against ROCK1 and a panel of kinases.
- Targeting ROCK2 selectively allows a safe cardiovascular profile, as previously demonstrated in telemetered rats.
- Demonstration that physiologically relevant markers of fibrosis pathways can be modulated *in vivo* with a selective ROCK2 inhibitor.
- No safety concerns highlighted from early *in vitro* assessment (hERG, CEREP).
- This encouraging profile of tool compound REDX10616 is representative of the potential of the chemical series which are currently in lead optimisation.
- *In vivo* studies with ROCK2 selective inhibitors in NASH STAM, UUO kidney and IPF animal models of fibrosis are ongoing.

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.*