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SM04690, a Potential Disease-Modifying Treatment for Knee Osteoarthritis, Functions Through Inhibition of CLK2 and DYRK1A, Novel Molecular Regulators of Wnt Signaling, Chondrogenesis, and Inflammation

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Introduction: In the synovial joint, upregulated Wnt signaling affects osteoarthritis (OA) pathogenesis by increasing inflammation and subchondral bone formation, as well as thinning cartilage. A novel small molecule, SM04690, was previously shown to exhibit OA disease-modifying properties through Wnt pathway inhibition *in vitro* and *in vivo*[1]. Herein, we describe the novel mechanism of action of SM04690 affecting Wnt pathway inhibition, chondrocyte differentiation, and anti-inflammatory activity.

Methods: Wnt pathway inhibition was measured using a cell-based luciferase reporter assay controlled by a β-catenin/TCF-responsive promoter in SW480 colon cancer cells, an APC mutant cell line with constitutively active Wnt signaling. A kinome screen (318 kinases) and kinase assays were performed. Effects of SM04690 on phosphorylation of proteins including serine and arginine rich splicing factor (SRSF) proteins, Sirt1, and FoxO1 in hMSCs, chondrocytes, and synovial fibroblasts were measured using Western blot. siRNA mediated knockdowns were performed in hMSCs and BEAS-2B cells. Effects of SM04690 and siRNA knockdowns on chondrogenic and Wnt pathway gene expression were measured using nCounter® gene expression panels (NanoString Technologies). Effects of SM04690 and siRNA knockdowns on LPS-induced expression of inflammatory cytokines (IL-6, IL-8, TNF-α) in BEAS-2B cells were measured by qPCR and ELISA. *In vivo,* SM04690 pharmacodynamic effects were evaluated in rat knee OA models: (1) An inflammatory monosodium iodoacetate (MIA) injection-induced knee OA model and (2) a surgical anterior cruciate ligament transection with partial medial meniscectomy (ACLT+pMMX) model, followed by single intra-articular SM04690 or vehicle injections. Knee cartilage was isolated at Days 1, 10, 28, and 35 and phosphorylation and expression of SRSF proteins, NFKB, STAT3, and Sirt1 were measured by Western blot. Animal studies were approved by the Samumed, LLC Animal Committee and performed in accordance with the U.S. Department of Agriculture guidance. Statistical analyses used one-way ANOVA for multiple group comparisons and t-tests for comparison between two groups.

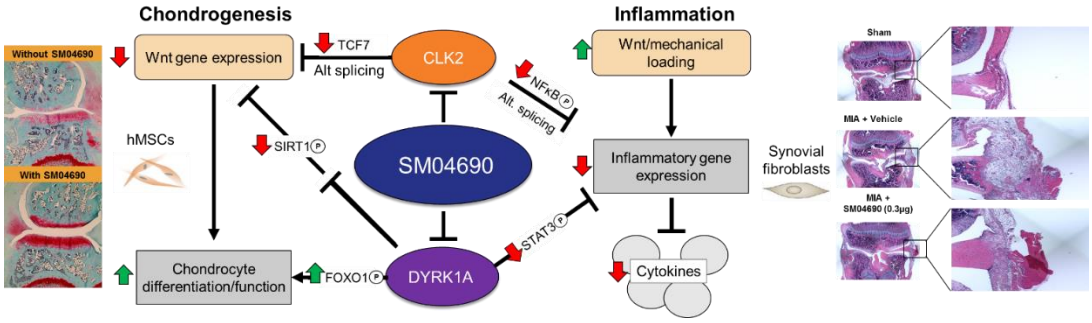
Results: SM04690 was a potent (EC_{50} = 11nM) and specific inhibitor of Wnt signaling. Biochemical assays identified cdc-like kinases (CLKs) and dual-specificity tyrosine kinase (DYRK1A) as molecular targets of SM04690. SM04690 potently inhibited CLK-mediated phosphorylation of SRSF proteins compared with DMSO controls. Separately, SM04690 inhibited DYRK1A mediated phosphorylation of Sirt1 and FoxO1 resulting in increased levels of total and nuclear localized FoxO1 compared with DMSO. While DYRK1A knockdown alone did not lead to chondrogenesis, a combined DYRK1A/CLK2 knockdown, demonstrated increased expression of several chondrocyte genes (*COL2A1, ACAN, COMP, CD44*) compared with siRNA control or CLK2 knockdown alone. Knockdowns of CLK2 and DYRK1A led to inhibition of Wnt pathway genes (*AXIN2, TCF7, TCF7L2, LRP5, FZD6, FZD7, PITX2,* etc.) with upregulation of secreted Wnt inhibitors (SFRP1, 2) and no effects on β-catenin levels compared with siRNA

controls. Furthermore, TCF7 knockdown, but not LEF1, TCF7L2 or β-catenin knockdowns led to chondrocyte differentiation. SM04690 treatment of IL-1β stimulated synovial fibroblasts resulted in decreased phosphorylation of NFkB and STAT3 compared with DMSO. Knockdown of DYRK1A was sufficient to inhibit production of inflammatory cytokines (IL-6, IL-8, TNF-α) in LPS stimulated BEAS-2B cells while combined knockdown of DYRK1A/CLK2 enhanced anti-inflammatory effects of DYRK1A knockdown, compared with siRNA control. Effects on the Wnt pathway, chondrogenesis, and anti-inflammatory activity were confirmed using CLK2 specific inhibitor (CC-671) and DYRK1A specific inhibitor (Harmine), compared with DMSO. Treatment with SM04690 inhibited SRSF proteins, Sirt1, and FoxO1 phosphorylation, as well as Wnt pathway gene expression in rat cartilage in the ACLT+pMMX and MIA models of OA compared with vehicle.

Discussion: SM04690 was a potent and selective Wnt pathway inhibitor that appeared to inhibit CLK2 and DYRK1A. Knockdown of CLKs (CLKs 1-4), compared with control siRNA identified a primary role for CLK2 in the induction of early chondrocyte differentiation from hMSCs. The effects on Wnt pathway genes highlighted the importance of modulating specific proteins (e.g. TCF7) in the Wnt pathway for chondrogenesis. Pharmacological and genomic studies demonstrated, for the first time, the role of CLK2 inhibition in chondrogenesis through effects on TCF7 and DYRK1A inhibition in maintenance of chondrocyte function through effects on Sirt1 and FoxO1, supporting the potential for OA disease modification with SM04690 **(Image 1)**. Furthermore, inhibition of STAT3 phosphorylation via DYRK1A inhibition mechanistically supports the anti-inflammatory effects of SM04690, with the effects in parallel with NFKB potentially mediated through CLK2 inhibition. These data support symptomatic improvements. The pharmacodynamic effects of SM04690 mediated through CLK2 and DYRK1A were demonstrated *in vivo* in cartilage from both surgical and inflammatory rat models of OA.

Significance: SM04690 showed potential as a single agent which may benefit symptoms and provide disease modification in OA through its effects on two distinct, novel molecular targets (CLK2 and DYRK1A) acting in a dual mechanism, modulating Wnt signaling, inducing chondrogenesis, improving chondrocyte function, and inhibiting inflammation. Human trials are ongoing.

Image 1:



References: 1. Deshmukh et al. OAC 2017