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Lorecivivint (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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Background: In the synovial joint, Wnt pathway upregulation contributes to osteoarthritis (OA) through increasing osteocyte differentiation, cartilage thinning, and inflammation. SM04690, a novel small molecule, has previously demonstrated OA disease-modifying potential through Wnt pathway inhibition *in vitro* and *in vivo*. Further studies have elucidated a novel mechanism of action for SM04690 leading to Wnt pathway inhibition, chondrocyte differentiation, and anti-inflammatory activity.

Methods: Wnt pathway activity was measured using a cell-based luciferase reporter assay controlled by a TCF/LEF promoter in SW480 colon cancer cells, a mutant cell line exhibiting active Wnt signaling. A kinome screen (318 kinases) and biochemical kinase assays were performed. SM04690 effects on protein phosphorylation of serine and arginine rich splicing factor (SRSF) proteins, Sirt1, and FoxO1 in hMSCs, chondrocytes, and synovial fibroblasts were measured using Western blot. SM04690 and siRNA knockdown effects on chondrogenic and Wnt pathway gene expression were measured using nCounter® gene expression panels (NanoString Technologies), and effects on LPS-induced inflammatory cytokines (IL-6, IL-8, TNF-α) expression in BEAS-2B cells were measured by qPCR and ELISA. *In vivo*, SM04690 (0.1 µg, 0.3 µg, 1.0 µg) 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 injection. Knee cartilage was isolated at Days 10 and 35 and phosphorylation and expression of SRSF proteins, NF-κB, STAT3, and Sirt1 were measured by Western blot. 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) 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 to DMSO control. Separately, SM04690 inhibited DYRK1A-mediated phosphorylation of Sirt1 and FoxO1 resulting in increased levels of total and nuclear FoxO1 compared to DMSO. While DYRK1A knockdown alone did not lead to chondrogenesis, combined DYRK1A/CLK2 knockdown increased expression of several chondrocyte genes (*COL2A1* (P<0.001; P<0.001), *ACAN* (P<0.05; NS), *COMP* (P<0.001; P<0.001), *CD44* (P<0.001; P<0.001)) compared to siRNA control or CLK2 knockdown alone. Compared to siRNA controls, knockdowns of CLK2 and DYRK1A led to inhibition of Wnt pathway genes (*AXIN2* (P<0.001; P<0.001), *TCF7* (P<0.05; P<0.05), *TCF4* (P<0.05, ns), *LRP5* (P<0.001; P<0.05), *FZD6, FZD7, PITX2,* etc.) with upregulation of secreted Wnt inhibitors (SFRP1, 2) and no effects on CTNNB1 levels. Furthermore, TCF7 knockdown, but not LEF1, TCF4, or β-catenin knockdowns, led to chondrocyte differentiation. SM04690 treatment of IL-1β-stimulated synovial fibroblasts decreased phosphorylation of NF-kB and STAT3 compared to DMSO. Knockdown of DYRK1A was sufficient to inhibit

inflammatory cytokine (IL-6 (P<0.001), IL-8 (P<0.05), and TNF-α (P<0.001)) production in LPS-stimulated BEAS-2B cells, while combined DYRK1A/CLK2 knockdown enhanced anti-inflammatory effects of DYRK1A knockdown compared to 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 to DMSO. In the ACLT+pMMX and MIA models of OA, compared to vehicle, treatment with SM04690 inhibited SRSF proteins, Sirt1, and FoxO1 phosphorylation as well as Wnt pathway gene expression in rat cartilage.

Conclusion: SM04690 was a potent Wnt pathway inhibitor that appeared to act via inhibition of CLK2 and DYRK1A. Pharmacological and genomic studies demonstrated, for the first time, the role of CLK2 inhibition in early chondrogenesis through effects on TCF7, and of DYRK1A inhibition in maintenance of chondrocyte function through effects on Sirt1 and FoxO1, supporting potential OA disease modification with SM04690. The specificity of effects on Wnt pathway genes highlighted the importance for chondrogenesis of modulating specific proteins (e.g., TCF7) in the Wnt pathway. Furthermore, DYRK1A and CLK2 were also confirmed as novel targets for anti-inflammatory activity, providing support for potential added symptomatic improvement with SM04690. Therefore, as a single agent, SM04690 may potentially benefit symptoms and provide disease modification in OA through effects on two distinct, novel, molecular targets (CLK2 and DYRK1A) **(Image 1)**. Human trials are ongoing.

Image 1: