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Lorecivivint (SM04690), a Potential Disease-Modifying Osteoarthritis Drug, Inhibits CLK2 and DYRK1A, Novel Molecular Regulators of Wnt Signaling, Chondrogenesis, and Inflammation

Vishal Deshmukh, Alyssa O'Green, Carine Bossard, Tim Seo, Lisa Lamangan, Maureen Ibanez, Abdullah Ghias, Carolyn Lai, Long Do, Shawn Cho, Joseph Cahiwat, Kevin Chiu, Melinda Pedraza, Yusuf Yazici

Samumed, LLC, San Diego, CA

Background: 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, lorecivivint (LOR; 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 LOR affecting Wnt pathway inhibition, chondrocyte differentiation, and anti-inflammatory activity.

Methods: Wnt pathway inhibition was measured using a cell-based luciferase reporter assay in SW480 cells. A kinome screen (318 kinases) was performed. Effects of LOR on phosphorylation of proteins including serine/arginine-rich splicing factor (SRSF) proteins, Sirt1, and FoxO1 in hMSCs, chondrocytes, 293T cells, and synovial fibroblasts were measured using western blot. siRNA-mediated knockdowns were performed in hMSCs and BEAS-2B cells. Effects of LOR and siRNA knockdowns on chondrogenic and Wnt pathway gene expression were measured using nCounter® gene expression panels and RNA sequencing. Effects of LOR 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*, the pharmacodynamic profiles of a single intra-articular injection of LOR (or vehicle) 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. Knee cartilage was isolated and phosphorylation and expression of SRSF proteins, NF- κ B, STAT3, and Sirt1 were measured by western blot, and Wnt pathway gene expression was measured by nCounter® gene expression panels. Statistical analyses used one-way ANOVA for multiple-group comparisons and t-tests for comparison between two groups.

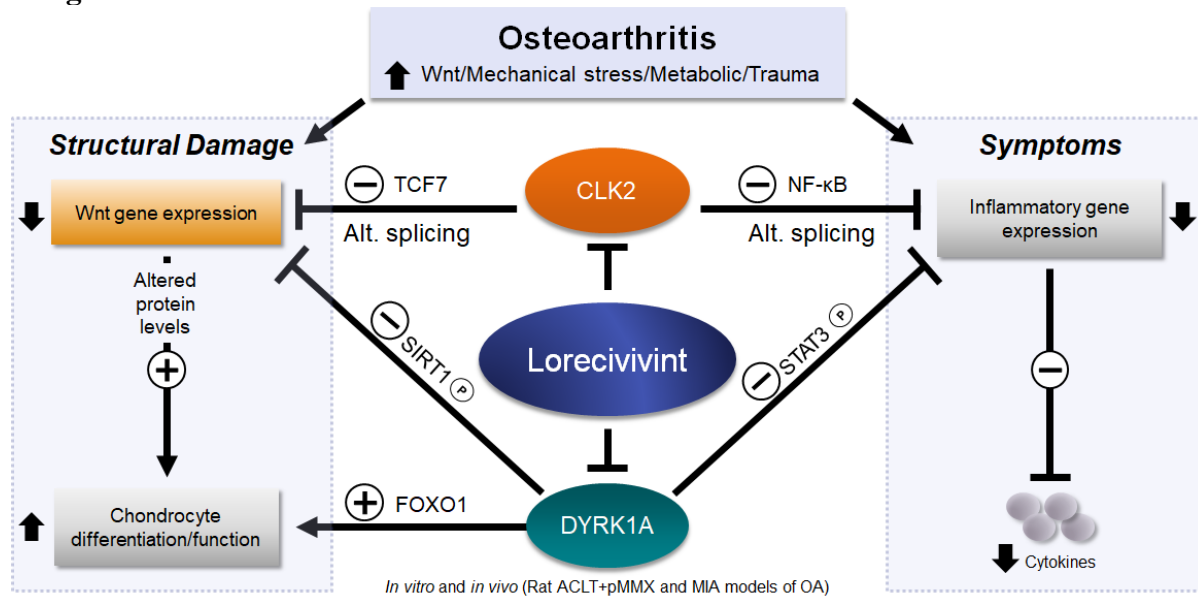
Results: LOR was a potent ($EC_{50}=11$ nM) inhibitor of the Wnt signaling pathway. Biochemical assays identified CDC-like kinases (CLKs 1-4) and dual-specificity tyrosine kinase (DYRK1A) as molecular targets of LOR. LOR potently inhibited CLK-mediated phosphorylation of SRSF proteins compared with DMSO controls. LOR inhibited DYRK1A-mediated phosphorylation of Sirt1 and FoxO1, resulting in increased levels of total and nuclear localized FoxO1 compared with DMSO. LOR decreased levels of TCF7 and AXIN2 with no effects on active and total β -catenin levels in CHIR- and Wnt3a-stimulated hMSCs, chondrocytes, and 293T cells compared with DMSO. 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, knockdown of *TCF7*, but not *LEF1*, *TCF7L2*, or β -catenin, led to chondrocyte differentiation. 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. LOR induced alternative splicing of several Wnt pathway genes (*TCF7, ERBB2, AXIN2, DVL2, LRP5*, etc.), identified by RNA sequencing and confirmed by PCR. LOR treatment of IL-1 β -stimulated synovial fibroblasts resulted in decreased Wnt pathway and NF- κ B gene expression and phosphorylation of STAT3 compared with DMSO. Compared with siRNA control, knockdown of *DYRK1A* alone was sufficient to inhibit production of inflammatory cytokines (IL-6, IL-8, TNF- α) in LPS-stimulated BEAS-2B cells; however, the combined knockdown of *DYRK1A* and *CLK2* enhanced the anti-inflammatory effects of *DYRK1A* knockdown. Effects on the Wnt pathway, chondrogenesis, and anti-inflammatory activity were confirmed using a *CLK2*-specific inhibitor (CC-671) and a *DYRK1A*-specific inhibitor (harmine) compared with DMSO. Treatment with LOR inhibited phosphorylation of SRSF proteins, Sirt1, and FoxO1, as well as Wnt pathway gene expression, in rat cartilage when compared with vehicle in the MIA and ACLT+pMMX models of OA.

Conclusion: LOR was a potent Wnt pathway inhibitor that appeared to inhibit *DYRK1A* and the *CLK* (all subtypes) intranuclear kinases. Knockdown of the individual *CLK* variants identified a primary role for *CLK2* in the regulation of the Wnt pathway and induction of early chondrocyte differentiation from hMSCs. LOR utilized a novel mechanism of action, effectively reducing Wnt pathway gene expression by modulating alternative splicing independently and downstream of β -catenin. These findings also demonstrate the previously unknown roles of *CLK2* and *DYRK1A* in Wnt signaling. In addition, the data identify an important feature of chondrogenesis: Wnt pathway regulation not by β -catenin inhibition but by modulation of specific proteins (e.g., *TCF7*) within the pathway. Overall, the results suggest a model of LOR activity (**Image 1**) in which *CLK2* inhibition downregulates Wnt pathway activity and where concomitant *DYRK1A* inhibition both enhances chondrocyte development and function while suppressing Wnt pathway activity. The data supports the potential for OA disease modification by LOR. Furthermore, reducing *DYRK1A* and *CLK2* activities suppresses STAT3 phosphorylation and NF- κ B expression (respectively), thus inducing a strong anti-inflammatory effect. The data supports the observed symptomatic improvements with LOR. The pharmacodynamic effects of LOR mediated through *CLK2* and *DYRK1A* were demonstrated *in vivo* in cartilage from both surgical and inflammatory rat models of OA.

Significance: *In vitro* and *in vivo*, LOR showed potential as a single agent that may benefit symptoms of and provide disease modification in OA through its effects on two distinct and novel molecular targets (*CLK2* and *DYRK1A*). LOR acted through a dual mechanism, modulating Wnt signaling to induce chondrogenesis, improve chondrocyte function, and inhibit inflammation. Phase 3 clinical trials are ongoing.

Image 1:



Reference: 1. Deshmukh, et al. *OAC*. 2017