Identifying the Molecular Mechanisms by Which Synthetic Triterpenoids Induce Chondrogenesis

Michael J. Susienka\(^1\), Logan A. Walsh\(^2\), Olin D. Liang\(^1\), Damian Medici\(^1\).

\(^1\)Brown University, Providence, RI, USA, \(^2\)Memorial Sloan-Kettering Cancer Center, New York, NY, USA.

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Introduction: Synthetic oleanane triterpenoids are novel compounds derived from oleanolic acid, found in many medicinal plants. Although they were originally developed for their potent anti-cancer properties, they have also been shown to exhibit anti-inflammatory, antioxidant, and cytoprotective properties [1, 2]. More recently, it was shown that CDDO-Ethyl amide (CDDO-EA) and CDDO-Imidazolide (CDDO-Im) induce chondrogenesis in organ cultures of mice calvaria and human bone marrow-derived mesenchymal stem cells (BMSCs) in culture [3]. Furthermore, it was shown that these drugs induce expression of bone morphogenic protein 2 (BMP2) [3], which has been shown to stimulate chondrogenic differentiation [4]. These results suggest that synthetic triterpenoids may someday provide a novel treatment option for patients with osteoarthritis or other degenerative joint diseases. However, very little is known about which signaling pathways are activated by these compounds to cause BMP2 upregulation. Our hypothesis was that triterpenoid-induced chondrogenesis would be dependent upon BMP2 and that CDDO-EA would cause phosphorylation of many protein kinases upstream of transcription factors that bind to the BMP2 promoter region. The goal of this study was to identify the early phosphorylation events induced by triterpenoids that eventually lead to expression of BMP2 and subsequent chondrogenesis in BMSCs.

Methods: BMSCs (ScienCell, Carlsbad, CA) were cultured in Mesenchymal Stem Cell Medium (MSCM) (ScienCell, Carlsbad, CA) until 70-80% confluent, serum-starved overnight, and then cultured in DMEM-high glucose with 1% antibiotic/antimycotic (GIBCO, Carlsbad, CA) containing 100 nM CDDO-EA, CDDO-Im, or vehicle (DMSO) for 14 days in the presence of 1 μg/ml BMP2-neutralizing antibodies or non-specific IgG (LifeSpan BioSciences, Seattle, WA). Cells were lysed with RIPA buffer and western blots were performed for type II collagen and β-actin (Abcam, Cambridge, MA) as a loading control. Next, we conducted a pilot study to verify that CDDO-EA was able to induce phosphorylation of a common kinase, extracellular signal-regulated kinase 1/2 (ERK1/2), in BMSCs in culture. BMSCs were cultured in MSCM until 90% confluent, serum-starved overnight in DMEM-high glucose with 1% antibiotic/antimycotic, and then treated with varying concentrations (1, 25, 50, and 100 nM) of CDDO-EA or vehicle (DMSO) for 1 hour. Cells were lysed with RIPA buffer and western blots with 20 μg total protein/lane were performed for phospho-ERK1/2, ERK1/2, (Cell Signaling Technology, Danvers, MA) and β-actin as a loading control. For the phospho-kinase arrays, BMSCs were plated in 75 cm\(^2\) flasks in DMEM-high glucose with 10% fetal bovine serum and 1% antibiotic/antimycotic. Once 90% confluence was reached, the cells were serum-starved overnight, and then stimulated with 100 nM CDDO-EA or vehicle (DMSO) for 1 hour and lysed. Protein amounts in each cell lysate were quantified using the DC Protein Assay (Bio-Rad, Hercules, CA) and western blots for β-actin were performed to verify relative protein amounts. Next, 200 μg of each cell lysate was assayed using the Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions, allowing for the detection of relative phosphorylation levels of 43 distinct kinases. After visualizing and developing the arrays, we quantified the relative phosphorylation (activation) levels of each kinase via densitometry using ImageJ (NIH, Bethesda, MD) and normalized all values to the relative protein amounts determined above via western blot.

Results: Our results suggest that triterpenoid-induced type II collagen protein expression in BMSCs is dependent upon BMP2. The cells treated with synthetic triterpenoids in the presence of BMP2-neutralizing antibodies exhibited reduced type II collagen expression, while those treated with non-specific IgG antibodies showed robust type II collagen expression (Figure 1).
The results of the phospho-ERK1/2 pilot study show that CDDO-EA treatment caused robust phosphorylation of ERK1/2 in BMSCs at 1 hour (Figure 2).

The data from the phospho-kinase arrays suggest that CDDO-EA induced phosphorylation of many protein kinases in BMSCs (Figure 3). Quantification of the array data via densitometry indicated that GSK-3α/β, TOR, CREB, Src, Hck, and Chk-2 exhibited at least a two-fold increase in phosphorylation (relative to vehicle treatment) in BMSCs after 1-hour treatment with 100 nM CDDO-EA (Figure 3). These results indicate that treatment with CDDO-EA affects many prominent cell signaling pathways, including PI3K-Akt-mTOR and PTH-CREB.

**Figure 2:** CDDO-EA induces ERK1/2 phosphorylation in hMSCs. BMSCs were treated with varying concentrations of vehicle (DMSO) or CDDO-EA for 1 hour. Western blot for p-ERK1/2, total ERK1/2, and β-actin (loading control).

**Discussion:** Our results show that triterpenoid-induced chondrogenesis is dependent upon BMP2 and that CDDO-EA causes rapid phosphorylation of numerous protein kinases in BMSCs. These individual phosphorylation events will be confirmed via phospho-specific western blotting, which will provide a more robust signal for quantification. However, further work needs to be
done to link these kinases to transcription factors that bind to the BMP2 promoter to cause its upregulation that eventually induces chondrogenesis. Preliminary results from this promoter analysis using MatInspector software (Genomatix, Munich, Germany) with an input of 2000 base pairs upstream of the BMP2 start site (promoter region) have revealed that there are several promising transcription factor binding sites in this region, including HIF-1, CREB, LEF-1, p53, and NF-κB binding sites. We anticipate that one or more of these transcription factors will be implicated in triterpenoid-induced BMP2 expression and chondrogenesis.

**Significance:** Synthetic triterpenoids have been shown to induce stem cell differentiation to chondrocytes by increasing expression of BMP2; however, the molecular mechanisms behind triterpenoid-induced BMP2 expression and subsequent chondrogenesis are currently unknown. Identifying the signaling pathways activated by these compounds will help researchers to understand their applications for use in the treatment of orthopaedic disorders.

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