POTENTIAL EFFECTS OF TRITERPENOIDS IN OSTEOARTHRITIS MODEL

SYSTEMS

By

NOOPUR SNEHAL SHAH

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ABSTRACT OF THE THESIS

POTENTIAL EFFECTS OF TRITERPENOIDS IN OSTEOARTHRITIS MODEL

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Thesis Director:

DR. NANJOO SUH

Osteoarthritis, a degenerative joint disease, is one of the most common rheumatic disorders and the leading cause of chronic disability in the United States. Currently there are many pharmacologic and non-pharmacologic therapies for osteoarthritis however; these therapies do not appear to concomitantly affect disease symptoms and structure. Here, we have investigated the effects of synthetic oleanane triterpenoids, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), and its analogs CDDO-imidazolide (CDDO-Im) and CDDO-ethyl amide (CDDO-EA) in the anabolic as well as catabolic pathways of osteoarthritis using two different osteoarthritis model systems.

We found that CDDO-Im and CDDO-EA, at concentrations as low as 200 nM, induce chondrogenesis in organ cultures of new born mouse calvaria in a time and dose dependant manner. The cartilage phenotype was measured histologically with metachromatic toluidine blue staining for proteoglycans and by immunohistochemical staining for type II collagen. Real time PCR analysis using mRNA from calvaria after a 7 day treatment with CDDO-Im and CDDO-EA showed upregulation of SOX9 and collagen type 2 and well as other cartilage markers. We also found that LG100268, a

rexinoid, downregulates the expression of primary cartilage markers in mouse calvaria suggesting a possible role for rexinoids in chondrogenesis. Vitamin D (1α $25(OH)_2$ D3) did not show any significant effects at the dose tested. With respect to the catabolic pathway, we established that CDDO-EA and CDDO-Im are involved in suppression of tumor necrosis factor-α (TNF-α) and interleukin 1-β (IL-1β) induced matrix metalloproteinase (MMP) expression in SW1353 chondrosarcoma cells.

These results suggest that synthetic triterpenoids CDDO-Im and CDDO-EA can be considered as potentially useful agents for the treatment of osteoarthritis.

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INTRODUCTION

I. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common autoimmune disease that is manifested as a destructive arthritis in association with serological evidence of autoreactivity. It primarily affects the small diarthrodial joints of the hands and feet and is characterized by chronic pain and joint destruction, premature mortality and elevated risk of disability, with high cost of those suffering from this disease and for the society (Firestein, 2003). Ongoing systemic inflammation also causes extra-articular complications such as lung disease (Gabbay, 1997). It affects up to 0.5% of the world's population, with a male-to-female ratio of 3:1, and is the most common inflammatory joint disease. The onset of the disease can occur at any age; however the prevalence increases with age and the peak incidence is between the fourth and the sixth decade (Abdel-Nasser, 1997). The incidence fluctuates by a small margin across populations, with 5–50 per 100,000 adults developing RA annually (Scott, 2012).

A. Pathophysiology of RA

Rheumatoid arthritis is characterized by synovial inflammation and hyperplasia ("swelling") of the peripheral joints, autoantibody production (rheumatoid factor and anti–citrullinated protein antibody [ACPA]), cartilage and bone destruction ("deformity"), and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders (McInnes, 2011). The pathogenic basis of RA is a

sustained specific immune response against yet unknown self antigens. The two primary pathologic events leading to RA are (1) hyperplastic synovial lining cells, the layer in direct contact with the intra-articular cavity, and (2) mononuclear cell infiltration in the subintimal layer (Saxena, 2011). The synovium is normally a relatively acellular structure with a delicate intimal lining (Firestein, 1995). In RA, CD4 + T cells, B cells, dendritic cells, fibroblasts, granulocytes, macrophages and mast cells infiltrate the synovium and sometimes organize into discrete lymphoid aggregates with germinal centers (Korb-Pap, 2012). Extreme swelling of the intimal lining occurs due to a marked increase in macrophage-like and fibroblast-like synoviocytes (Korb-Pap, 2012). Locally expressed degradative enzymes, including metalloproteinases, serine proteases and aggrecanases, digest the extracellular matrix and destroy the articular structures (Korb-Pap, 2012). In addition to inflammation in the synovium, which is the joint lining, the aggressive front of tissue called pannus invades at the interface between cartilage and bone and destroys local articular structures. The formation of the pannus is a unique characteristic of RA progression which differentiates it from other arthropathic diseases (Firestein, 2003).

B. Treatment options

Recent advances in the field of immunology and rheumatology have helped in development of better understanding of the immune dysfunction in RA. Treatment has evolved from nonspecific immunosuppressive therapy to specific molecule-targeted biologics such as anti-cytokine agents, T-cell co-stimulator blocking agents, anti B-cell agents and signal kinase inhibitors (Cohen, 2002).

B.1. Traditional drugs

Early initiation and rapid dose escalation of disease-modifying anti-rheumatic drugs (DMARDs) has been shown to be crucial to optimizing disease control, protecting joints from destruction and preserving health-related quality of life. They prove to downregulate the abnormal immune response in RA, thereby preventing or suppressing structural damage to the joint. DMARDs namely methotrexate and leflunomide are folate antimetabolites, most commonly used for treatment of rheumatoid arthritis. They work effectively to inhibit purine and pyrimidine synthesis thus causing antiproliferative and anti-inflammatory effects. They can be used independently or in combination with other DMARDs for effective therapy. Sulfasalazine and hydroxychloroquine belong to second line disease modifying agents and are considered to be less potent than methorexate or leflunomide. Although they have been known to be very effective for the treatment of RA, their precise mechanism has not been elucidated yet (Saag, 2008).

Corticosteroids can be prescribed for oral use but are often given by intra-muscular or joint injection in times of flare to minimize the risks of side effects. Recent findings suggest that local administration of glucocorticoids leads to a significant decrease in synovial citrullination (Makrygiannakis, 2012). The rational use of non-steroidal anti-inflammatory drugs (NSAIDs) and analgesia remains important for symptom management in patients.

B.2. Biologics for RA

The introduction of biologics has revolutionized RA treatment. Their success has underlined the key roles of inflammatory cytokines in the pathogenesis of inflammatory arthritis, particularly tumor necrosis factor-α (TNFα) and interleukin (IL) 1 and 6. They have also refocused attention on T cells and B cells (Choy, 2001) (Feldmann, 2002) (Maini, 2010) (Silverman, 2006).

The biologics used in inflammatory arthritis are genetically engineered proteins derived from human genes. They primarily inhibit specific components of the immune system that play fundamental roles in driving or inhibiting inflammation in arthritis (Scott, 2012). The goal is to weaken or immobilize those features of the immune system that are triggering autoimmune diseases without the adverse side effects that come from broadly weakening the immune system (Feldmann, 2005), (Rayachaudhuri, 2009). Currently there are four classes of biologics namely tumor necrosis factor inhibitors, interleukin-1 receptor antagonists, B-cell inhibitiors and T-cell costimulation inhibitiors that are commercially used for the treatment of rheumatoid arthritis, each inhibiting a different component of the immune system. Tumor necrosis factor inhibitors namely etanercept, infliximab, and adalimumab are classified as first generation agents while certolizumab and golimumab are second generation agents. They are successfully approved for use in routine clinical care. The first generation agents bind to TNFα with high affinity, thus inhibiting the cytokine's interaction with its receptor. The second generation biologics binds and neutralizes membrane-bound and soluble human TNF-α (Scott, 2012).

Interleukin-6 (IL-6), an important pro-inflammatory cytokine promotes inflammation through the expansion and activation of T cells, differentiation of B cells, and induction of acute-phase reactants by hepatocytes (Kishimoto, 2010). Tocilizumab, a recombinant humanized antihuman IL-6 receptor monoclonal antibody of the IgG1 subclass is the only IL-6 inhibitor used commercially for the treatment of arthritis (Mima, 2009). The third class of biologics includes Rituximab, a genetically engineered chimeric monoclonal antibody depletes the B-cell population by targeting cells bearing the CD20 surface marker. It was introduced for the treatment of lymphomas but was subsequently found to be effective in RA (Korhonen, 2010). The fourth class of biologics including T cell modulators is less focused on compared to the other cytokine targeting agents (Raychaudhuri, 2011). Abatacept is a fusion protein constituting an immunoglobulin fused to the extracellular domain of cytotoxic T-lymphocyte antigen 4 (Scott, 2012). Cytoxic T-lymphocyte antigen 4 is a molecule that binds with a high affinity to the CD80/86 ligand on antigen-presenting cells (Scott, 2012). The abatacept molecule blocks the interaction between the antigen-presenting cell's CD80/86 ligand and the CD28 ligand on the T cell, which is necessary for T-cell activation. This results in decreases in T cell proliferation and in cytokine production (Pham, 2009).

Since the late 1990s, the success of biological agents in the treatment of RA has dramatically altered the approach for treating this disease and a variety of other inflammatory diseases.

II. Osteoarthritis

Osteoarthritis (OA), a degenerative joint disease, is one of the most common rheumatic disorders, and the leading cause of chronic disability in the United States (Arden, 2006). There is evidence that a majority of individuals over the age of 65 have radiographic and/or clinical evidence of osteoarthritis (Arden, 2006). Changes that lead to the development of osteoarthritis may result from extremely slow changes over 15-20 years. The most frequently affected sites are hands, knees, hips and spine. It is a complex disease believed to be a consequence of mechanical and biological events that destabilize the normal coupling of degradation and synthesis within articular joint tissues. (Buckwalter, 1998). The disease process affects not only the articular cartilage, but also the entire joint structure including the subchondral bone, ligaments, joint capsule, synovial membrane and periarticular muscles (Martel-Pelletier, 1999). There are several systemic and biomechanical risk factors that are particularly important in weight bearing joints and modifying them may present opportunities for prevention of osteoarthritis pain and disability (Felson, 2000). Despite advances in therapies that target inflammation and tissue destruction in chronic arthritis as well as stimulation of tissue repair and restoration of joint function, the ultimate goal of treatment is far from achieved.

A. Symptoms of Osteoarthritis

Symptoms in osteoarthritis are often associated with significant functional impairment as well as signs and symptoms of inflammation including pain, morning

stiffness and loss of mobility (Goldring, 2006). On physical examination, people often have tenderness to palpation, bony enlargements and crepitus on motion (Cobb, 1957).

B. Risk factors

Osteoarthritis is a multifactorial disorder involving interplay between systemic factors (such as genetics, dietary intake, age and gender) as well as biomechanical factors (such as muscle weakness, obesity and joint injury) (Felson, 2000).

B.1. Age

Age is the risk factor most strongly correlated with the development of OA (Cooper, 2000). Prevalence of the disease increases dramatically with age with a greater incidence in subjects between 40 and 50 years of age. 50% of people over the age of 65 have arthritis in at least one joint and that increases to 80% after the age of 75 (Rodriguez-Fontenla, 2011). Cumulative exposure to various risk factors and biological changes that occur due to aging make a joint less capable to cope with stress such as cartilage thinning, weak muscle strength, poor proprioception and oxidative damage (Felson, 2000).

B.2. Gender

Overall, the prevalence as well as severity of OA is higher in females compared to males (Zhang, 2010). Men appear to have a significantly reduced risk of OA of the hip

and hands but are at a higher risk of OA of the knee and spine (Felson, 2000). Studies show a definite increase in OA in females around the time of menopause which has led investigators to hypothesize the possible relationship between hormonal imbalance and OA (Zhang. 2010). Current evidence is best suggestive of a protective effect of estrogen on osteoarthritis. Data from a women's health initiative showed that women placed on estrogen replacement therapy were 15% less likely to require total hip or knee arthroplasty compared to the control group (Cirillo, 2006).

B.3. Nutritional factors

Dietary factors are of a considerable interest in the field of OA; however the results of studies are conflicting. Vitamin D proves to be one of the most promising nutritional factors for the disease (Felson, 2000). Without enough of vitamin D, bones can become thin, brittle or misshapen. In a study, people with moderate or extremely low levels of 25-hydroxyvitamin D_3 showed a threefold increase for progressive knee OA (McAlindon, 1996). However, results from two cohort studies failed to establish a protective effect of Vitamin D on the actual worsening on the knee and concluded that it is unrelated to the risk of joint space or cartilage loss in knee OA (Felson, 2007).

Evidence indicates that continuous exposure to oxidants contributes to the development of many common age-related diseases, including osteoarthritis. Additionally, chondrocytes are potent sources of reactive oxygen species which may lead to damage of cartilage collagen as well as hyaluronate in the synovial fluid that helps maintain its viscosity (Felson, 2000). In this case, antioxidants may provide defense

against tissue injury (Felson, 2000). A cohort study showed a threefold reduction in risk for progressive radiographic osteoarthritis in people with middle and very high vitamin C intake (McAlindon, 1996).

B.4. Genetic Predisposition

Osteoarthritis in all its heterogeneous forms appears to be strongly genetically determined. Genetic factors account for at least 50% of cases of the disease in the hands and hips and a smaller percentage in the knees (Felson, 2000). These factors are either mutations or variations in genes that result in defects and variability in cartilage matrix properties and chondrocyte metabolism. For example, mutations of genes expressed in the cartilage and of the latent transforming growth factor (TGF) binding protein 3 can affect the structure and consistency of cartilage and bone respectively (Dabovic, 2002). Familial OA can result from abnormal cartilage structure and properties such as a consequence of a mutation in the type II Collagen COL2A1 gene, which causes not only cartilage dysplasia but also a severe form of OA with defective collagen (Holderbaum, 1999). In the case of matrix defects caused by mutations in the matrix genes, age dependant cartilage degeneration may occur as a result of normal mechanical stresses on a joint (Li, 2007). Twin studies have indicated a significantly higher concordance for OA between monozygotic twins compared to dizygotic twins (Zhai, 2007).

B.5. Obesity

Studies show that people who are overweight have a high prevalence of knee and hip osteoarthritis (Lemetowski, 2008). Because of an increased amount of pressure on the knee joint, there is a higher possibility for cartilage breakdown and failure of ligamentous and other structural support (Li, 2007). Recent studies implicate a role for adipokines, a product of adipocytes, in the inflammatory changes seen in OA. Leptin is an adipose derived hormone that plays a role in regulation of body weight and metabolism (Ahima, 1996). Leptin expression is increased in the cartilage and osteophytes of subjects with OA and may help explain the association between obesity and risk for onset and progression of OA (Dumond, 2003). The effect of obesity on osteoarthritis is made more portentous because obesity is a serious and growing health problem in the United States. Reduction in weight can help reduce the risk for the disease.

B.6. Joint Malalignment

Joint malalignment is one of the most important risk factors of knee OA (Sharma, 2007). Knee alignment (i.e., the hip-knee-ankle angle) is a key determinant of load distribution. Any shift from a neutral or collinear alignment of the hip, knee and ankle affects load distribution at the knee (Zhang, 2010). Therefore one would consider a relationship between malaligned knees and a higher risk of developing OA. In a cohort study of primary knee OA, it was demonstrated that angled alignment is responsible for an increase in risk of OA. Also, the burden of malalignment predicts decline in physical

function (Sharma, 2001). An interesting observation of a recent study was that new bone marrow lesions occurred at a higher frequency in malaligned limbs (Hunter, 2006).

B.7. Injury

Joint injuries are the most common risk factor for the development of OA in young adults. Severe injury to the structures of a joint, particularly a trans-articular fracture, meniscal tear requiring meniscectomy, or anterior cruciate ligament injury, can result in an increased risk of OA development and musculoskeletal symptomatology (Lohmander, 2004) (Roos, 2001). Studies have shown that majority of patients between age 35 and 44 years with OA of the knee had a previous history of knee injury (Roos, 2005). OA of the spine may follow a severe back injury.

C. Structural Pathology in OA

The joint is a specialized structure whose design allows for stability, loading and movement. The human joints do not typically wear out even after completing a million movements a year and undergoing frequent situations with extreme loading (Anandarajah, 2011). A typical synovial joint is comprised of bone, articular cartilage, joint capsule, menisci, muscles, tendons, ligaments and bursa. Traditionally OA was considered to be a primary disorder of the articular cartilage, however now it is generally appreciated that all of the joint structures are affected, including the calcified cartilage, subchondral cortical and trabecular bone, joint capsular tissues and the synovium

(Goldring, 2010). Damage to any of the joint structures can lead to an alteration in the delicate balance of joint function and consequently lead to joint damage (Goldring, 2010). Insights into the pathological and pathogenic processes associated with the initiation and progression of OA have been provided by the remarkable advances in defining the composition and structural organization of articular cartilage and in elucidating the molecular mechanisms regulating the anabolic and catabolic activities of chondrocytes (Goldring, 2010).

C.1. Articular Cartilage

All joint surfaces are covered by a thin layer of cartilage. Articular cartilage is a specialized form of hyaline cartilage that is characterized by its fibrous structure and does not have nerves, blood vessels or lymphatic flow. Approximately 75% of the cartilage is composed of water, with collagen and proteoglycans accounting for the rest (Anandarajah, 2011). The articular cartilage is extremely strong and flexible to withstand the compressive stresses, to decrease friction during movement and to help distribute the forces to other parts of the joint (Goldring, 2009). It is composed of four regions: 1) the superficial tangential zone composed of thin collagen fibril, 2) the middle zone with radial bundles of thicker collagen fibril, 3) the deep zone where the collagen bundles are the thickest and 4) the calcified cartilage located just above the subchondral bone (Goldring, 2009). Proteoglycans, especially aggrecan are responsible for the flexibility and elasticity of the cartilage. Tensile strength is due to collagen which also forms an inter-territorial matrix composed of a fibrillar collagen network that entraps chondrocytes

(cartilage cells), aggrecan molecules in addition to a number of other molecules including small proteoglycans (Goldring, 2010). The collagen fibers comprise of type II collagen fibrils with type XI collagen within the fibril and type IX collagen integrated in the fibril surface, permitting association with other matrix components and retention of proteoglycans (Goldring, 2008).

The organic matrix of cartilage is synthesized and maintained by chondrocytes (Goldring, 2009). These are a set of highly specialized cells that live singly or in small clusters and are capable enough to respond to mechanical forces and structural changes in the surrounding cartilage matrix as well as intrinsic and extrinsic growth factors, cytokines and other inflammatory mediators (Goldring, 2009). Chondrocytes stimulate matrix production through synthesis of growth factors such as bone morphogenetic protein 2 (BMP-2), TGF-β and IGF-1 (Anandarajah, 2011). Also, chondrocytes embedded in the superficial zone of the cartilage express lubricin that is essential for boundary lubrication while chondrocytes in the deep zone adapt to low oxygen tension by upregulating hypoxia-inducible factor-1α (HIF-1α) (Cheng, 2007),. HIF-1α regulates a number of genes associated with cartilage anabolism and chondrocyte differentiation, including vascular endothelial growth factor (VEGF) and Sox9 (Murphy, 2009). These sets of cells are also responsible for secreting matrix metalloproteinases (MMPs), nitric oxide (NO) and inflammatory cytokines that regulate the catabolic activity (Anandarajah, 2011). There is a dynamic equilibrium between the anabolic and catabolic activity of chondrocytes, which when disturbed causes joint destruction.

C.1.1 Inflammation and Cartilage Destruction in OA

The loss of articular cartilage is an important feature in the pathogenesis of OA. Under normal circumstances, if there is damage to the matrix, it would be compensated by an increase in the anabolic activity of chondrocytes to replace the lost cartilage and a subsequent decrease in the catabolic activity (Goldring, 2010). Since, this delicate balance is destroyed in OA, there is an exaggerated attempt to induce catabolic activity associated with gradual loss of proteoglycans followed by type II collagen degradation. The anabolic activity proves to be insufficient (Goldring, 2010).

Since the typical clinical signs of inflammation are not present in OA, it was designated to be a 'noninflammatory' or degenerative joint disease. However, recent research supports a role for inflammation in the pathogenesis of OA (Goldring, 2011). Also, there is increasing evidence to demonstrate that inflammation is a predecessor to cartilage destruction (Attur, 2002). Inflammatory mediators such as TNF-α and IL-1β act within the cartilage to cause degradation of cartilage in OA (Attur, 1998). The inflammatory process is mediated by the chondrocytes, which are activated by the cytokines produced by the synovium that diffuse into the cartilage from the synovial fluid. The chondrocytes, when activated along with the synovium release several pro inflammatory cytokines and chemokines as well as members of the reactive oxygen species (Nitric Oxide) in response to mechanical stimuli (Roach, 2008). These pro inflammatory mediators cause upregulation of cartilage degrading proteinases, promote apoptosis of chondrocytes and inhibit matrix synthesis (Roach, 2008).

C.1.2. The Role of Cytokines and other Inflammatory Mediators

Evidence from in-vivo and in-vitro studies indicates that synoviocytes, chondrocytes, and cells from other joint tissues can produce and/or respond to a number of cytokines and chemokines that may also be detected in osteoarthritis synovial fluid (Goldring, 2011). Interleukin (IL)-1β and tumor necrosis factor α (TNF-α), in particular, control and play major roles in cartilage degradation by evoking a cascade of events leading to both increased catabolism and chondrocyte hypertrophy (Marcu, 2010). They also stimulate the production of other cytokines such as IL-6, IL-8, leukocyte inhibitory factor, proteases and prostaglandins in addition to stimulating their own production (Van de Loo, 1995). IL-1β is extremely important to cartilage destruction while TNF-α drives the inflammatory process (Caron, 1996).

IL-1β is primarily synthesized as a 31 kD precursor and released in the active form of 17.5 kD after modification by IL-1β converting enzyme (ICE) (Black, 1988). This cytokine has been found in the active form in the articular joint tissue including the synovial membrane, synovial fluid and cartilage. Ex vivo experiments have demonstrated the ability of the OA synovial membrane to secrete this cytokine (Pelletier, 1995). The biological activation of cells by IL-1β is mediated through the association with specific cell surface receptors namely IL-1R Type I and II (Slack, 1993). Research shows a significant increase in type I IL-1 receptors in OA chondrocytes and synovial fibroblasts, thus making these cells extremely sensitive to IL-1β stimulation (Martel-Pelletier, 1992). This process increases proteolytic enzyme gene upregulation, which in turn enhances cartilage destruction.

In OA, TNF-α is an extremely important mediator of matrix degradation and a pivotal cytokine involved in the inflammation of the synovial membrane, although it is detected at very low levels in OA articular tissue. It is synthesized as a prosequence of 76 amino acids which is further cleaved by TNF-α converting enzyme (TACE) to form a mature cytokine that oligomerizes to form trimers (Black, 1997) (Aggarwal, 1985). TNF-α binds to two specific receptors namely TNF receptor 55 (TNF-R55) and TNF receptor 75 (TNF-R75) on the cell membrane (Shalaby, 1987). Experiments have been conducted in the case of TNF-α that demonstrate an increase in the expression of TNF-α-converting enzyme and TNF R-55 in OA chondrocytes and synovial fibroblasts (Amin, 1999) (Alaaeddine, 1997). This correlates with an increased amount of catabolic activity in the chondrocytes and insufficient anabolic activity.

Several other cytokines have been implicated in the pathogenesis of OA. Most of them are mediated by TNF-α and IL-1β. Interleukin 8 (IL-8), a potent chemotactic cytokine enhances the release of inflammatory cytokines in the blood vessels and lining of the synovial membrane (Yu, 1994). It is also present in the articular chondrocytes and has been shown to increase the production of oxidative products (Schroder, 1989). Leukemia inhibitory factor (LIF) is responsible for 1) enhancing IL-1β and IL-8 expression in chondrocytes and IL-1β and TNF-α expression in synovial fibroblasts (Villiger, 1993), 2) inducing the expression of collagenase and stromelysin by human articular chondrocytes (Lotz, 1992) and 3) stimulating cartilage proteoglycan resorption and nitric oxide (NO) production (Carroll, 1993). TNF-α and IL-1β also induce other proinflammatory cytokines, such as Interleukin -6 (IL-6), Interleukin-17 (IL-17) and

Interleukin-18 (IL-18). Overall, all of these factors synergize with one another in promoting chondrocyte catabolic responses.

IL-1β is synthesized by chondrocytes at levels that are capable of inducing the expression of matrix metalloproteinase (MMP) genes and aggrecanases. Several MMPs are known to play an important role in cartilage degradation. Three of the most important ones include MMP-1, MMP-9 and MMP-13 out of which the last one is specific for type II collagen (Tetlow, 2001). The crucial role of MMP-13 in OA is further substantiated by reduced OA pathology in MMP-13 deficient mice (Takaishi, 2008). Aggrecanases, which belong to a family of proteases known as disintegrin and metalloprotease with thrombospondin motifs (ADAMTS), play an important role in the pathogenesis of OA, with ADAMTS-4 and ADAMTS-5 known to be specifically involved in cartilage degradation (Tetlow, 2001). This was demonstrated by an experiment conducted with a double knockout of ADAMTS-4 and ADAMTS-5 in mice that successfully prevented the progression of OA (Majumdar, 2007)

Nitric Oxide (NO), a member of reactive oxygen species (ROS) also plays an essential role in cartilage catabolism. TNF-α and IL-1β upregulate production of NO by the cartilage via an increase in the inducible nitric oxide synthase (iNOS) (Grabowski, 1997). NO is responsible for a variety of catabolic functions such as inhibiting synthesis of cartilage matrix components such as aggrecans and collagen, enhancing the activity of MMPs, reducing the production of IL-1 receptor antagonist, increasing susceptibility to injury by other oxidants and increasing chondrocyte apoptosis (Hashimoto, 1998). In addition, NO leads to the upregulation of inducible cyclooxygenase-2 (COX-2) which

further increases the synthesis of prostaglandin E2. This prostaglandin produced by OA cartilage has been shown to decrease proteoglycan synthesis and enhance the degradation of aggrecan and type II collagen (Abramson, 2009).

The signal-transduction mechanisms of IL-1β/TNF-α have been studied extensively. IL-1β and TNF-α have different membrane-based receptors, but their signal-transduction cascades involve common pathways, e.g., the family of mitogen activated protein kinase (MAPKs), which comprises the extracellular signal-regulated kinases (ERKs), p38 and c-Jun N-terminal kinase (JNK) (Roach, 2008) (Malemud, 2004). The activities of these kinases converge on the transcription factor namely nuclear factor kappa activated B cells (NFκB) (Roman-Blas, 2006). The NF-κB pathway is a central regulator of the inflammatory cytokine-induced catabolic actions in chondrocytes. The transcription factors in the pathway can be triggered by a host of stress related stimuli including pro-inflammatory cytokines, excessive mechanical stress and ECM degradation products. Activation of canonical NF-κB (p65/p50) signaling is essential for the chondrocytes to express inflammation-related genes including MMP-1, 3 and 13, iNOS, COX2, IL-6, IL-1 and TNF-α (Marcu, 2010). In addition, NF-κB participates in the chondrocyte catabolic responses to ECM degradation products and mechanical stress. The imbalance in homeostasis in OA leads to cartilage erosion and release of ECM matrix components that are in turn recognized by special cell surface receptors. These receptors enhance the inflammatory response that leads to aggravation of cartilage erosion. NF-κB mediates chondrocyte activation caused by fibronectin fragments leading to the increased expression of inflammatory cytokines, MMPs or chemokines and other proteins (Pulai, 2005) (Marcu, 2010).

IL-1β and TNF-α control the degeneration of articular cartilage matrix, which makes them prime targets for therapeutic strategies. Several *in vitro* and animal studies provide support for this approach, although only a few clinical studies have investigated the efficacy of blocking these proinflammatory cytokines in the treatment of OA (Kapoor, 2011). An interesting study by Shakibaei *et al*, demonstrates that curcumin (diferuloylmethane), a pharmacologically safe phytochemical agent with potent anti-inflammatory properties, inhibited the IL-1β-induced stimulation of up-stream protein kinase B Akt. This further downregulated NF-κB targets including COX-2 and MMP-9. It also reversed the IL-1β-induced down-regulation of collagen type II and β1-integrin receptor expression (Shakibaei, 2007). A wide range of IL-1 blockers are currently being tested in clinical trials under the disease modifying OA drugs (DMOADs) program (Hunter, 2011).

C.1.3 Calcified Cartilage and Tidemark

Tidemark provides a line of separation between the hyaline articular cartilage and calcified cartilage (Goldring, 2010). In large joint and hand OA, thickening of the calcified cartilage is accompanied by advancement of the tidemark. This further leads to overall thinning of the articular cartilage (Bullough, 2004). The exact mechanisms involved in this process have not been definitely established as of yet.

C.2. Subchondral Bone

In addition to all the changes in the articular cartilage, there are several significant changes that take place in the structure and function of the periarticular bone. Studies utilizing isotope-labeled bone-seeking agents and radiographic techniques indicate that changes in the bone occur early in the course of OA and may manifest before articular changes such as increase in cartilage thickness and joint space narrowing are detected (Hutton, 1986). The periarticular bone can be separated into three different units that includes the the subchondral bone plate, the subchondral trabecular bone, and the bone at the joint margins. The subchondral bone plate consists of cortical bone, which is relatively nonporous and poorly vascularized. The calcified cartilage zone separates the plate from the articular cartilage (Goldring, 2010).

Under normal circumstances, the cell-mediated processes of remodeling and modeling contribute to the modification of the architecture and properties of the periarticular cortical and trabecular bone (Anandarajah, 2011). Bone remodeling is a process that under physiological conditions allows repair of the bone when exposed to mechanical stress (Anandarajah, 2011). This process involves bone resorption mediated by bone osteoclasts and the activation of quiescent bone surfaces. The resorptive process is followed by a phase of bone formation mediated by osteoblasts (Anandarajah, 2009). There is always a balance between the amount of bone removed and added during the resorption and formation processes so that the integrity of the bone is maintained. This cellular system allows adaptation to of the skeleton to changing mechanical influences and importantly provides a mechanism for repairing damage that occurs to the bone

tissue during mechanical loading (Martin, 2007) (Goldring, 2010). Bone modeling is responsible for growth and mechanically induced adaption of bone and requires that the processes of bone formation and bone resorption, although globally coordinated, occur independently at distinct anatomical locations (Raggatt, 2010).

During the course of OA, the changes that take place in the bone include osteophyte formation (formation of new bone at the joint margins), increase in subchondral plate thickness and development of bone marrow edema (Goldring, 2010). A significant increase in bone turnover and remodeling (bone formation and resorption) of the bone–cartilage interface occurs early in the course of the disease, especially in areas underlying damaged cartilage areas (Wieland, 2005).

Subchondral bone changes are extremely important for the pathogenesis of OA. Animal models of OA are used to observe the gradual changes in the subchondral bone. The use of microfocal computed tomography (micro-CT) in smaller animal models like mice has demonstrated the thinning of the subchondral plate early in the pathogenesis of the disease, in contrast to changes found in human end-stage OA (Mastbergen, 2011). This suggests that bone remodeling is biphasic phenomenon which involves an early decrease in subchondral plate thickness followed by a phase in which the subchondral bone becomes denser and stiffens. This biphasic theory is supported by recent research related to human OA (Bolbos, 2008). Several studies have demonstrated a significantly greater thickness of the subchondral cortical plate in patients with OA compared to subjects without arthritis (Buckland-Wright, 1992). The thickening of subchondral bone in osteoarthritis is due to the increased turnover and reactivation of the secondary center

of ossification that result from the underlying change in joint mechanics (Wieland, 2005). Reactivation of the secondary center of ossification is caused due to pathological changes in the zone of calcified cartilage leading to reduplication of the tidemark and subsequent movement of spikes of granulation tissue and fibrous tissue into the non-calcified articular cartilage. Formation of bone in this area leads to thinning of the hyaline articular cartilage (Wieland, 2005). This process causes joint destruction as thinned articular cartilage is highly prone to further damage and loss (Brandt, 2006).

The formation of osteophytes represents one of the radiographic hallmarks of OA. They are characterized by skeletal outgrowths localized to joint margins that can be a source of pain as well as loss of function (Van der Kraan, 2007). They occur as a result of an adaptive response to stabilize an already damaged joint in an attempt to maintain joint function and stability to deal with load and strain (Pottenger, 1990). However, these changes adversely affect the capacity of the joint to adapt to mechanical stress. The development of these bony outgrowths appears to be associated with but does not completely correlate with cartilage loss (Gilbertson, 1975). The formation of osteophytes begins with proliferation of periosteal cells at the joint margin. These cells further undergo differentiation into chondrocytes along with deposition of matrix molecules such as aggrecan at joint margins. This is followed by hypertrophy of chondrocytes and process of endochondral ossification to create an enlarging skeletal outgrowth at the joint margin (Anandarajah, 2011) Local growth factors such as TGF-β, IGF-1 and leptin are shown to be associated with osteophyte formation (Uchino, 2000).

C.3. Synovium

Under normal physiological conditions, the synovium is a thin tissue that consists of a pseudo epithelial lining layer with synovial fibroblasts, macrophages and loose connective tissue in the sublining zone. It is physiologically important as it both nourishes chondrocytes via the synovial fluid and joint space and removes metabolites and products of matrix degradation (Sellam, 2010). Recent studies demonstrate that synovitis in more common in OA than previously appreciated (Ayral, 2005) (Krasnokutsky, 2007). In OA, an increased presence of synovial hypertrophy and hyperplasia is prevalent along with an increase in the number of lining cells. These changes are often accompanied by infiltration of the sub-lining tissue with scattered foci of mononuclear cells (lymphocytes and macrophages). Activated B and T cells as well as overexpression of proinflammatory mediators are extremely common in established OA (Benito, 2005). The release of proteins from the cartilage and the bone triggers the non specific inflammation of the synovium. The synovium, when activated releases excess synovial fluid that results in joint swelling. It also produces proteases and cytokines such as prostaglandins, NO, IL-1β and TNF-α that accelerate cartilage breakdown (Anandarajah, 2011) (Loeser, 2006). Synovial cells and OA chondrocytes both produce large quantities of MMPs (MMP-1, MMP-3, MMP-9 and MMP-13) which lead to degradation of the cartilage (Yuan, 2004).

Synovial inflammation plays a key role in stimulating chondrocyte dysregulation (Anandarajah, 2011). Cartilage breakdown products in turn lead to release collagenase and other hydrolytic enzymes by the synovium and contribute to vascular hyperplasia.

Angiogenesis, a key component of chronic inflammation is facilitated by the cartilage breakdown products which then further potentiate inflammatory changes in the synovium and accelerate the progression of the disease. It further perpetuates the inflammatory response by forming new blood vessels to provide access to the inflammatory cells and nutrients at the sites of inflammation (Walsh, 2007) (Bonnet, 2005). Studies also show that synovial thickening is more common in people with knee OA with pain compared to people who have OA without any pain. These findings suggest an important relationship between synovitis and pain (Hill, 2001) (Hill, 2007).

III. Treatment for OA

Arthritic disorders may cause a variety of symptoms, but among the most common of these are pain and impairment of function. Functional impairment may be caused by pain, structural joint damage or both. Relief of pain and prevention of joint damage should therefore be the primary goals of arthritic therapies. In the case of osteoarthritis, joint damage and pain are ultimately the result of cartilage breakdown and abnormal joint mechanics that result from the process. Currently, various pharmacologic and non pharmacologic therapies are used for pain relief and the management of osteoarthritis. The treatment plan must be individualized and should be based on numerous factors including the presence of such comorbid conditions as hypertension, heart disease, peptic ulcer disease or kidney disease, which influence decisions about drug therapy.

A.   Insufficiency of current therapeutics

Currently clinical management of OA typically entails a combination of treatment options to reduce pain and improve tolerance for functional activity. Existing pharmacologic therapies for OA help to reduce symptoms, but are only moderately effective and leave the patients with substantial pain and a functional burden. Generally placebo effects are substantial in clinical trials for these drugs; differences in effect between the placebo and many widely-used therapies (including acetaminophen, NSAIDs, cyclo-oxygenase 2 (COX-2) inhibitors, hyaluronic acid and glucosamine) are generally quite modest (Hunter, 2008). Also, many of these drugs have side effects that have raised a number of questions about their safety for long term use. At present, these therapies do not appear to concomitantly affect disease symptoms and structure. Unlike disorders like rheumatoid arthritis and osteoporosis, where the discovery of DMARDs and biologic agents have successfully changed the course and management of the disease, none of the current pharmacologic therapies designed for OA have demonstrated a clinically relevant structure modifying effect in a well designed trial (Hunter, 2008).

Given this current situation regarding insufficiency of effective therapeutics, much of the drug development in OA nowadays focuses on modification of structural progression. However, therapeutic development in this area is challenged by several factors including the heterogeneous clinical manifestations of OA, the poor relationship generally found between structural progression and clinical endpoints, and the need for long-term follow-up to observe changes in disease structure (and the potential drug effects on structure) (Hunter, 2009)(Hunter, 2011).

B.   Pharmacologic therapy

Current pharmacologic therapies used for the management of osteoarthritis focus on pain relief and prevention of further joint damage. The form of therapy usually depends on the location of the arthritic joint. Also, treatment of patients with OA are individualized and tailored to the severity of the symptoms. In individuals with mild symptomatic OA, treatment may be limited to patient education, physical and occupation therapy and other non pharmacologic modalities, and pharmacologic therapy including non-opioid oral and topical analgesics. Patients with a moderately severe case of OA are usually prescribed a more rigorous regimen of pharmacologic and non pharmacologic forms of treatment while severe cases of OA might require surgical treatment. A discussion of the different forms of commercially available treatment is followed by recently discovered nutraceuticals and structure-altering drugs currently undergoing research and clinical trials.

B.1. Acetaminophen

Acetaminophen also commonly referred to as 'Paracetamol', is often an effective analgesic in arthritis patients, especially in those with mild to moderate pain from OA. It has both analgesic and antipyretic actions but a comparatively weak anti-inflammatory activity (Botting, 2000). The drug is well tolerated and generally safer compared to NSAIDs in the elderly, especially in those with cardiovascular disease, renal

insufficiency or a history of acid-peptic disorders. It is usually the initial medicine of choice for systemic treatment of symptomatic OA (Clissold, 1986).

Acetaminophen produces analgesia through a combination of inhibition of prostaglandin synthesis in the central nervous system and peripheral blockade of pain impulse generation (Aronoff, 2006). However, it exhibits a highly variable capacity to inhibit PG synthesis by different cell and tissue types. For example, the analgesic and antipyretic effects of acetaminophen follow its inhibition of prostaglandin E2 (PGE2) generation whereas the failure of acetaminophen to inhibit platelet derived thromboxane A2 synthesis and inflammatory PGE2 synthesis accords with its weak antiplatelet and anti-inflammatory effects (Seppala, 1990) (Green, 1989) (Muth-Selbach, 1999) (Feldberg 1973). A study examining the combined effect of acetaminophen and NSAIDs showed that combination treatment for short courses provides more relief of pain in osteoarthritis without an increase in side effects (Buescher, 2004). Over the long term, however, this combination may increase the risk of upper gastrointestinal (GI) bleeding more than that conferred by the NSAID alone (Buescher, 2004). There has been speculation on the existence of a third isoform, COX-3, which would explain the mechanism of action of acetaminophen as it is a poor inhibitor of COX-1 and COX-2. Splice variants of COX-1 and COX-2 have emerged that have been referred to as COX-3 but were shown to have little relevance in humans (Davies, 2004).

Adverse effects are uncommon with appropriate dosing of acetaminophen. However, hepatotoxicity may occur at proper doses but typically only occurs in patients who consume excessive amounts of alcohol (Seeff, 1986).

B.2. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are one of the most commonly prescribed medicines worldwide. In the United States, it is estimated that more than 70 million prescriptions and more than 30 million over-the-counter tablets of these drugs are sold annually (Wolfe, 1999). Since arthritis is classified as an inflammatory disease; these drugs are commonly prescribed for osteoarthritis as well as rheumatoid arthritis. They are also prescribed for non arthritic conditions like headache and dysmenorrhea and for certain non painful conditions like hereditary polyposis coli and Alzheimer's disease (Schmajuk, 2011). At lower over-the-counter doses these drugs are used to treat pain. When given at very high doses, these medicines are very useful in treating inflammation. The principle of NSAID therapy dates back to the use of willow bark more than 5,000 years ago for musculoskeletal pain. The active ingredient of willow bark, salicilin had been used to treat inflammation for centuries (Brune, 2004) (Jones, 2001). Aspirin, the first anti-inflammatory drug, was synthesized more than 100 years ago. Over the next century, several compounds were formulated that share the ability of aspirin to block prostaglandin and they were classified as NSAIDs (Vane, 1998). Currently, there are more than 20 different NSAID preparations and there are important differences in the chemical structure, dosing, adverse effects and pharmacokinetics between the various agents. Drugs such as salicylic acid, naproxen and ibuprofen are commonly found at pharmacies across the United States in the form of over-the-counter medications.

Traditionally, NSAIDs have been thought to work by inhibiting the synthesis of prostaglandins, although they may also work by other mechanisms including the

inhibition of leukotriene synthesis, superoxide scavenging and control of cytokine production (Hochberg, 2003). Inhibition of prostaglandin synthesis is the primary mechanism of action used by all the currently available NSAIDs. E series prostanoic acids are pro-inflammatory, increase sensitivity to the release of bradykinins and increase vascular permeability. Thus the decrease in prostaglandin E levels result in anti inflammatory and analgesic effects. NSAIDs also inhibit the formation of prostacyclin and thromboxane, with resultant complex effects on vascular permeability and platelet aggregation. Ultimately, NSAIDs cause a significant reduction in the prostaglandin levels via the inhibition of cyclooxygenase (COX), an important enzyme required for the conversion of arachidonic acid to prostaglandin. The constitutive isoform of COX, COX-1, has clear physiologic functions. Its activation leads to the production of prostacyclin, which, when released by the gastric mucosa, is cytoprotective (Whittle, 1980). COX-2 is an inducible enzyme, levels of which are not detectable in many body tissues, but which increase in response to inflammation. As COX-2 is induced by inflammatory stimuli and cytokines in migratory and other cells, it is attractive to suggest that the anti-inflammatory actions of NSAIDs are due to the inhibition of COX-2, whereas the unwanted side-effects, such as irritation of the stomach lining and toxic effects on the kidney, are due to inhibition of COX-1 (Vane, 1996) (Vane, 1998). All of the currently available NSAIDs that inhibit COX-1 also inhibit COX-2, with the exception of low dose aspirin which is COX-1 specific (Meade, 1993).

There are several adverse effects caused by constant use of NSAIDs that make acetaminophen a wiser alternative in the long run. Most NSAID toxicity is related to the inhibition of the normal functions of prostaglandins. The toxicity potential of the drug

differs in some organ systems, depending on the degree of COX-2 selectivity. Upper gastrointestinal (GI) injury is the most important toxicity associated with frequent use of NSAIDs. They are implicated in the development of complicated peptic ulcer disease and injury to the small bowel and colon (Schmajuk, 2011). NSAIDs interfere with prostaglandin-mediated epithelial defense mechanisms and also cause direct epithelial toxicity (Scheiman, 1996). Research shows that around 20% of patients taking these agents develop gastric or duodenal ulcer, and about 3% of this group goes on to experience hemorrhage or perforation (Lanza, 1993). Age, use of glucocorticoids and anticoagulants, prior history of ulcer disease and RA, all further the risk of GI toxicity (Singh, 1996) (Straus, 2001). The GI risk can be reduced with the use of concomitant proton pump inhibitors, misoprostol or by using NSAIDs with higher COX-2 selectivity (Rostom, 2002). Management of gastrointestinal complications and dyspepsia significantly adds to the economic burden of arthritis (Spiegel, 2006).

NSAIDs may produce either acute, reversible or permanent renal toxicity and a variety of effects on electrolyte and water homeostasis. Research shows that COX-2 specific NSAIDs have similar deleterious effects on renal function compared to the non-specific NSAIDs (Murray, 1993). There is a potential risk of cardiovascular toxicity associated with NSAIDs. An increase in risk related to myocardial infarction, heart failure, and hypertension, appears to be dependent on duration of exposure (Antman, 2007). The American Heart Association recommends that all NSAIDs should be used at their lowest effective dose. These and other guidelines, including those from the American College of Rheumatology, recommend that all NSAIDs, and particularly COX-2-selective agents, should be avoided where possible in patients with cardiovascular risk

factors (such as hypertension, hypercholesterolemia, angina, edema, recent bypass surgery, and a history of myocardial infarction or other cardiovascular events), and should be used only when sufficient pain relief is not achieved with other therapies and the benefit outweighs the increased cardiovascular risk (Conaghan, 2011)(Chan, 2008) (Lanza, 2009) (Scheiman, 2007).

Overall, NSAIDs prove to be better in treatment of inflammatory symptoms of osteoarthritis compared to acetaminophen. However, due to the high prevalence of risk factors, specific strategies are designed to incorporate NSAIDs along with gastroprotective agents as a form of treatment. For example, Arthrotecis a combination product containing diclofenac sodium 50–75 mg plus misoprostol 200 μg that is approved for the treatment of osteoarthritis or rheumatoid arthritis in patients at high risk of developing NSAID-associated ulcers and their complications (Bocanegra, 1998). While strategies exist to prevent complications in patients at risk of NSAID-associated gastrointestinal and cardiovascular injury, they are often underutilized or difficult to apply, particularly in patients with both types of risk factor. Despite all the recent debates concerning the safety of their long term use, NSAIDs have still to be considered as one of the most important drugs to allow the osteoarthritic patients to maintain an acceptable quality of life.

B.3. COX-2 Inhibitors (Coxibs)

Traditional NSAIDs act by inhibiting both COX-1 and COX-2, thereby locking the synthesis of PGs. The GI adverse events of NSAIDs are predominantly due to the

decrease in synthesis of the gastroprotective prostaglandins PGE2 and PGI2, which are mainly produced by COX-1 (Bertolini, 2002) (Hinz, 2002). To significantly reduce the GI toxicity of NSAIDs associated with acute and chronic use and to obtain similar or better efficacy, pharmaceutical companies conducted intensive international research which led to the development of COX-2 inhibitors. Due to the great expectation, these drugs were rapidly introduced in the market and gained a remarkable commercial and therapeutic success (Grosser, 2006) (Mathew, 2011).

Several clinical trials have been conducted in the past few years to evaluate the efficacy of the COX-2 inhibitors. Celecoxib Long-term Arthritis Safety Study (CLASS) and Vioxx Gastrointestinal Outcomes Research Study (VIGOR), two long term trials were conducted specifically for patients with osteoarthritis and rheumatoid arthritis. (Shi, 2008). The studies effectively showed that two of the most important coxibs namely, celecoxib and valdecoxob significantly reduced the risk of GI toxicity compared to traditional NSAIDs (Silverstein, 2000). However, research also shows that COX-2 inhibitors have adverse cardiovascular and renal effects. Patients taking refecoxib, a frequently prescribed coxib on a daily basis showed a fourfold increase in myocardial infarction compared to the naproxen group (Bombardier, 2000). Several randomized trials involving patients with osteoarthritis have successfully demonstrated that COX-2 inhibitors are similar in efficacy when compared to NSAIDs (Deeks, 2002) (Garner, 2005).

Any patient requiring chronic NSAID treatment for the management of arthritis may benefit from the COX-2 therapy. Patients who are at a high risk of GI bleeding, have

a history of intolerance to traditional NSAIDs, or are not responding to traditional NSAIDs may be appropriate candidates for treatment with COX-2 selective inhibitors (Mathew, 2011). Overall, a meticulous assessment of the patient's medical condition and complete evaluation during therapy may help reduce the amount of toxicity caused by generic NSAIDs and COX-2 inhibitors.

B.4. Opioid Analgesics

The term opiate and opioid refer to a group of analgesics that have properties of morphine. These drugs are specifically used to reduce pain in arthritic patients under the following circumstances 1) contraindication to NSAIDs, acetaminophen or adjuvant analgesics and 2) failure to respond adequately to non narcotic analgesics (Pascoe, 2000). Opioids bind to and activate opioid receptors located in the brain, spinal cord and peripheral sensory nerves. The activation of opioid receptors results in a diversity of psychological effects including analgesia as well as alterations in respiratory, cardiovascular, GI and neuroendocrine functions (Ballantyne, 2008).

There are multiple adverse effects that arise due to constant intake of opioid analgesics although these symptoms typically wane with continued use. The most severe mishaps with opioids are related to their respiratory depressant effect, which is widely influenced by factors such as pain, previous opioid experience and awareness (Schmajuk, 2011). Other relevant central nervous system effects of opioids include cough suppression, nausea and vomiting, rigidity, pruritus and miosis (Porreca, 2009). The cardiovascular adverse effects of opioids are mainly related to histamine release and

differ widely between agonists and agonist-antagonists. GI effects such as constipation and spasms of the bile duct are extremely common especially in the elderly (Schug, 1992). The main concern regarding constant use of opioids for pain reduction is that the analgesic efficacy of these set of drugs is not always maintained over prolonged courses of treatment despite dose escalation and stable pain. Possible loss of analgesic efficacy is very concerning, considering the fact that many people develop psychological dependence making it extremely hard to withdraw therapy (Ballantyne, 2008) (Nicholson, 2003). Overall, opioids are prescribed by physicians in case of severe arthritis related pain which is unresponsive to other less rigorous forms of treatment.

B.5. Intraarticular corticosteroid injections

Steroid injections are one of the most common forms of treatment in OA of the knee that results in reduction of swelling and pain. In patients who have an effusion and local signs of inflammation, judicious use of intraarticular corticosteroid injections is considered to be appropriate (Neustadt, 1992). When joints are painful and swollen, aspiration of fluid, followed by intraarticular injection of a corticosteroid preparation is an effective short term method of reducing pain (Dieppe, 1980) and increasing quadriceps strength (Fahrer, 1988). Commonly used corticosteroids such as triamcinolone, acetonide, hexacetonide and prednisone are usually injected at a dose of 40 mg. Joints are aspirated/injected using aseptic technique and the fluid is sent for cell counts and gram stains if an infection is suspected (Gatter, 1995). Injection can be used as monotherapy in selected patients or as an adjunct to systemic therapy. Evidence

supports short term (up to two weeks) improvement in symptoms of osteoarthritis of the knee after corticosteroid injections. For a longer term of benefit (16-24 weeks) in some cases, higher doses of prednisone (50 mg) can be injected (Arroll, 2004). However, studies have been conducted to show that intra-articular corticosteroids are effective for short term relief of pain but predicting responders is not possible (Jones, 1996). Ultrasound experiments show that consistent long term advantages have not been established which does not prove to be very beneficial for the disease on a whole (Chao, 2010). Also, sometimes patients may experience a mild flare of synovitis related to a reaction to the crystalline suspensions; however, these post injection flares are short lived. The risk of introducing infection into an osteoarthritic joint using standard aseptic technique is exceedingly small. It is generally recommended that injection of corticosteroids in a given joint should not be performed more than 3-4 times in a given year because of concern about the possible development of progressive cartilage damage because of repeated injection in the weight bearing joints (Neustadt, 1992). Most individuals who require more than 3-4 intraarticular injections per year to control symptoms are probably candidates for joint lavage or surgical intervention.

B.6. Hyaluronic Acid

Hyaluronic acid (HA), also known as hyaluronan or hyaluronate, is a high-molecular-weight glycosaminoglycan composed of continuously repeating molecular sequences of glucuronic acid and N-acetyl-glucosamine. In addition to providing joint lubrication and shock absorbancy, HA acts as the backbone for the proteoglycans of the

extracellular matrix, creating a hydrated pathway through which cells can migrate (Brockmeier, 2006) (Strauss, 2009). Recent studies have also suggested that HA promotes chondrocyte proliferation and differentiation, which has increased interest in its use as a scaffold component in tissue-engineering techniques (Kujawa, 1986) (Yagishita, 2005).

In the case of osteoarthritis, HA is a sterile, viscoelastic, non-pyogenic solution that is indicated as a medical device for the treatment of pain in patients with osteoarthritis of the knee who have failed to respond adequately to conservative non-pharmacological therapy and simple analgesics (Curran, 2010). In an arthritic joint, the concentration of HA is decreased by almost 50% limiting its role to effectively lubricate the joint surface and distribute the stresses associated with weightbearing (Strauss, 2009). The primary goals of viscosupplementation (injection) are to replace the lost HA and potentially stimulate the endogenous production of HA in the joint (Bagga, 2006). Research shows that hyaluronan injections are very effective for supplying required lubrication to the joint as well as providing significant anti-inflammatory effects within the joint space, affecting leukocyte function and reducing the concentration of inflammatory mediators such as prostaglandins and fibronectin (Ghosh, 1994) (Watterson, 2000). In cases where patients undergo arthroscopic knee procedures, HA injections post treatment proves to maintain the pain-relieving and functional benefits of the surgical procedure to a great extent (Hempfling, 2007).

The injections are very well tolerated on a whole with a mild chance of some side effects. The most common complication with HA injection is an inflammatory reaction at

the injection site, characterized by localized injection site pain and a knee effusion. These reactions are generally very mild and resolve by themselves within 1-3 days. Other non serious side effects include post injection itching, calf pain and headaches (Goldberg, 2004). Overall, HA injections are extremely effective in providing pain relief and reduction in inflammation by maintaining normal biomechanics of the joint.

B.7. Disease Modifying OA Drugs (DMOADs)

Currently available therapeutics in the field of OA are extremely effective in providing short term pain relief however they fail to target the actual cause of the disease. Patients are completely reliant on daily use of painkillers and injections to improve their quality of life which in the long run can cause adverse effects in addition to already bothersome OA related pain. Current drug development focuses on a new strategy to modify the progression of structural change in OA in order to improve the effect of increasing OA prevalence (Hunter, 2011).

Articular cartilage is one of the main tissues that is affected in the course of rheumatic disorders. Many investigations conducted previously have looked at the characteristics of one single cell present in this tissue – the chondrocyte. Substances that protected the articular cartilage during the course of destructive joint diseases were termed chondroprotective agents as they targeted the chondrocyte and the surrounding matrix. When the same effect occurred in vivo in the case of osteoarthritis, these agents were termed as disease-modifying osteoarthritis drugs (Verbruggen, 2006). With respect to disease outcomes, the use of DMOADs can cause slowing of the rate of disease

progression, a complete stop in disease progression, a reverse in disease progression and even the prevention of disease development (Hunter, 2011).

At present, there is a modest collection of agents for OA disease modification that are in various phases of development. The promising ones are in phase II clinical trials and beyond. One of the best examples includes calcitonin, a hormone involved in the regulation of calcium in the body that has shown to have effects on osteoclasts. *Ex vivo* studies with cartilage explants demonstrated that the hormone exerts direct anabolic effects on articular chondrocytes, resulting in increased proteoglycan synthesis. Also, the anti-catabolic effects of calcitonin might involve induction of cAMP, resulting in attenuation of MMP-mediated cartilage degradation (Karsdal, 2007). Clinical trials with daily ingestion of oral salmon calcitonin (sCT) resulted in reductions in markers of bone resorption and cartilage degradation in patients with OA (Karsdal, 2010). These preclinical and early clinical findings are currently the focus of ongoing clinical trials of sCT in knee OA.

Piascledine, an avocado-soybean unsaponifiable substance, is composed of one third avocado and two thirds soybean unsaponifiables, the oily fractions that do not produce soap after hydrolysis. They have been reported to repress chondrocyte catabolic activities and to increase the accumulation of proteoglycan by OA chondrocytes in culture (Henrotin, 2003). A randomized clinical trial reported that ASU significantly reduced the progression of joint space loss as compared with placebo in the subgroup of patients with advanced joint space narrowing (Lequesne, 2002). Further clinical trials are been conducted to evaluate the effects.

Vitamin D3 primarily known as cholecalciferol is extremely important for normal bone and cartilage metabolism. Research shows that dietary intake of vitamin D in patients with OA is 80% less compared to normal consumption levels (O'Connor, 1986). Low intake and low serum levels of vitamin D each appear to be associated with an increased risk for progression of osteoarthritis of the knee. Studies conducted suggest that an adequate amount of vitamin D might slow disease progression and even help to prevent the development of OA (McAlindon, 1996).

An important example includes fibroblast growth factor 18 (FGF-18), a well-known anabolic growth factor involved in chondrogenesis and articular cartilage repair (Moore, 2005). The growth factor exerts anabolic effects in human articular chondrocytes by activating FGFR3, increasing matrix formation and cell differentiation while inhibiting cell proliferation, leading to dispersed cells surrounded by abundant matrix (Ellman, 2008). These pre clinical findings form the basis for current clinical trials in the field.

Other DMOADs include inhibitors of inducible nitric oxide synthase, bone morphogenetic protein-7 and IL-1 inhibitors might work against progression of OA however they are weak at this stage and under constant research and clinical trials. The discovery and development of DMOADs is not a straightforward task and involves many challenges. These include the slow progression of OA, the multitude of disease risk factors, the regulatory hurdles, connecting the structural changes to a clinically meaningful endpoint, the complex etiopathogenesis and the heterogeneity of clinical presentations—creating a complex environment for therapeutic development aimed at

modifying disease structure (Hunter, 2011). None of the existing pharmacological modalities have shown promise in the disease modification field. However, future agents still under research are trying to work around these results to deliver promising results.

C. Non-pharmacologic therapy

Non-pharmacological therapies are extremely important in osteoarthritis to improve the quality of life. Once a patient has been diagnosed with OA, appropriate non-pharmacologic methods are employed as first-line treatments in mild disease or in adjunct with pharmacologic management in progressive disease. The most common physician recommendations include therapeutic exercises (physical and occupational therapy), weight loss, acupuncture and self management programs. Each form of this treatment is individually devised, taking into account the anatomical distribution, the phase and the progression rate of the disease. Indications, contraindications, dosage and precautions are as important in non-pharmacological therapy as they are in drug treatment (Géza Bálint, 1997). Multidisciplinary collaboration allows the patient to access the broad range of non-pharmacological interventions used in OA treatment. These can be provided by healthcare providers such as physiotherapists, occupational therapists, massage and manual therapists, personal trainers, exercise physiologists, dieticians and nurses. These kinds of therapies are the basis for management with the goal of decreasing pain and increasing function (McKenzie, 2010).

C.1. Exercise

Therapeutic exercises also known as physiotherapy decrease pain, increase muscle strength and range of joint motion, restore normal neuromuscular function as well as improve endurance and aerobic capacity for the knee and hip joints (Hunter, 2009). Strengthening and functional therapy, as the name suggests, focuses on altering lower limb alignment and improving muscle strength. Also, strengthening exercises that focus on the quadriceps, adductor, and abductor muscles have been demonstrated to improve both knee and hip stabilization (Page, 2011). Low-impact aerobic exercises such as walking, swimming, or bicycling help with overall blood circulation and improving the flexibility of the muscles. At this stage, there is no proof to suggest that one form of exercise is better compared to the other, although a combination of strengthening, aerobic and functional exercise is recommended (Page, 2011). All exercise regimens are initiated by a trained physiotherapist and continued at home, with intensity and duration increasing slowly as tolerated (Bennell, 2005).

Patients with knee OA tend to have reduced muscle strength as a consequence of reductions in physical activity and pain inhibition (Messier, 1992). The quadriceps are the largest group of muscles crossing the knee joint and have the greatest potential to generate and absorb forces at the knee (Page, 2011). Clinical trials in patients with OA of the knee showed that strengthening of quadriceps musculature with either isometric or resistive exercises was associated with significant improvement in quadriceps strength, knee pain and function when compared with the control group (Jan, 1993) (Feinberg, 1992). Results from recent studies effectively demonstrate that improvement in knee

function and pain can be gained by increasing hip muscle strength (Bennell, 2010). This highlights the importance of individual assessment and management strategies for patients with knee OA.

C.2. Diet and Lifestyle

Obesity is the most important and modifiable risk factor for the development of OA (Seed, 2009). A 4 year cohort trial conducted by Wang *et al* determined that that there was a 3- to 4-fold increased risk of primary joint replacement associated with increased body weight, BMI, fat mass, and percentage fat (Wang, 2009). Increased waist circumference and waist-to-hip ratio were also associated with an increased risk, suggesting that both biomechanical and metabolic mechanisms associated with adiposity contribute to the risk of joint replacement (Wang, 2009). Experiments conducted to examine the effects of dietary fat on OA progression demonstrated that an increase in dietary fat was associated with changes in cartilage thus leading to worsening of OA in both normal and malaligned joints (Brunner, 2012). Therefore, exercise and weight reduction are recommended by physicians to all patients especially with OA of the hip and knee.

C.3. Assistive Devices

The American College of Rheumatology (ACR) recommends using a cane contralaterally to the affected knee joint to manage OA symptoms and provide stability

(Recommendations). Taping the knee, in particular the patella, is a physiotherapy treatment strategy recommended in the management of knee OA by some clinical guidelines (Page, 2011). It involves the application of an adhesive rigid strapping tape to the patella and surrounding tissue structures to realign the region and ultimately reduce knee pain. Short term reductions in pain have been successfully demonstrated in several randomized controlled trials in patients with knee OA (Hinman, 2004). In addition, patients benefit from shoe inserts to correct abnormal mechanics due to angular deformities of the knees (Sasaki, 1987). Biomechanical studies have demonstrated that lateral wedges reduce the adduction moment during walking by 4-12% compared to walking barefoot or with normal shoes (Butler, 2007) (Hinman, 2008) (Hinman, 2008) (Kuroyanagi, 2007). Finally, the use of light weight braces made of a single sleeve of neoprene has proven to be helpful in reducing pain for patients with knee OA. The design of the brace is such that it changes the way the force is distributed at the knee by forcing load away from the painful side (Lindenfeld, 1997). Overall, assistive devices help reduce the pain from inflammation in osteoarthritis thus improving the quality of life. In moderate to severe cases they alone are not enough and are usually combined with pharmacological measures to provide relief from the symptoms.

D.   Surgical treatment

Osteoarthritis is a progressive disease which ultimately damages the entire joint. At first, the disease is treated conservatively using non pharmacological and pharmacological modalities however if symptoms persist, surgical procedures are used

for treatment. Determination of an appropriate surgical procedure depends on several factors including the location and severity of OA damage, patient characteristics and risk factors (Lützner, 2009). Arthroscopic procedures, osteotomy and joint replacement surgery are widely known surgical forms of treatment currently available for OA.

D.1. Arthroscopic Lavage and Debridement

In osteoarthritis of the knee, arthroscopic lavage with or without debridement is useful in certain patients who have concomitant meniscal disease or symptoms of locking of the knee and have failed to respond to common pharmacologic treatment. The procedure is performed using a 2.5 mm wrist arthroscope that removes debris such as microscopic or macroscopic fragments of cartilage involved in the induction of synovitis. It also removes calcium phosphate crystals that are detectable in most severely osteoarthritic knees and may be associated with synovitis. Debridement consists of smoothing rough, fibrillated articular and meniscal surfaces, shaving tibial-spine osteophytes that interfere with the motion of the joint, and removing inflamed synovium to improve the mechanical condition of the joint (Felson, 2002). These steps are expected to help lessen knee pain and disability as well as decrease the mechanical stress on the cartilage, thus causing less cartilage loss.

The Osteoarthritis Research Society International (OARSI) views arthroscopic debridement for OA as controversial (Zhang, 2008). Earlier controlled trials have demonstrated that people who underwent debridement and lavage showed a significant improvement in knee pain as compared to people who received no treatment (Merchan,

1993) (Ike, 1992). However, most published studies of arthroscopic procedures are of limited quality, owing to lack of randomization, lack of a control group, short-term follow-up or inconsistent assessment methods (Siparsky, 2007). Also, randomized controlled trials over the last decade have failed to demonstrate a substantial improvement in pain relief or improvement of function when compared to placebo, no arthroscopy or joint injection of corticosteroid alone (Choong, 2011) (Kirkley, 2008). Therefore, effectively evaluated studies regarding the advantages of arthroscopic surgery are required in the field of OA.

D.2. Bone Marrow Stimulation

Bone marrow stimulation is an extremely controlled procedure where bleeding is induced from the subchondral bone. This is followed by the formation of a fibrin clot and the migration of undifferentiated mesechymal stem cells that leads to formation of fibrocartilaginous tissue covering the full thickness of chondral lesions. Different techniques such as drilling (Pridie, 1959), microfracturing (Steadman, 2001) and abrasion arthroplasty (Johnson, 1986) are used to penetrate the bone. In these cases, healing takes place with replacement of the defect by cartilage like material causing temporary relief of symptoms (Miller, 2004). However, the fibrocartilaginous tissue produced in place of articular cartilage has biomechanical properties inferior to the latter, thus raising a number of questions regarding reliability on such measures for a long term.

D.3. Osteotomy

High tibial osteotomy is an effective procedure that involves cutting through the bone and realigning it in another position to redirect the weight bearing joints through cartilage that is unaffected by osteoarthritis. This procedure aims to relieve symptoms as well delay the need for joint replacement surgery. A meta-analysis of high tibial osteotomy demonstrated an overall 10-year failure rate of 25%, and an average of 72 months between high tibial osteotomy and conversion to total knee arthroplasty (Virolainen, 2004). The risk factors for failure include obesity, female gender and severity of osteoarthritis (Coventry, 1993). Also, computer navigated surgery has greatly improved the accuracy of osteotomy thus leading to improved outcomes (Goleski, 2008). The success of the procedure has highlighted the fact that minimally invasive surgical processes can be very effective in relieving OA related pain.

D.4. Total Joint Arthroplasty (TJA)

Currently, total joint arthroplasty (TJA) is the predominant surgery and can significantly decrease pain and improve function in patients with hip and knee OA. In the past few years over 220,000 primary total hip arthroplasties (THAs) and 430,000 primary total knee arthroplasties (TKAs) were carried out in the US. Approximately 60% were performed on patients over 65 years old, and 36% were performed on patients between 45 and 65 years old (Katz, 2006). Currently, predictable and sustainable pain relief and functional relief are obtainable after TJA in more than 90% of patients for 10-15 years postoperatively (Scott, 2006).

This surgical treatment is considered as a last resort when all the conservative treatments have been exhausted. The pain caused should be significant and disabling for the physician to recommend such an aggressive form of therapy. Also radiographic findings must correlate with clinical symptoms for affirmation of an advanced stage of OA (Matsuda, 2011).

In total knee arthroplasty, damaged articular cartilage and subchondral bone are removed and the joint surface is resurfaced with an implant. The femoral and tibial components are designed specifically for the patient in order to achieve maximum knee stability (Matsuda, 2011). Total hip arthroplasty is an orthopedic procedure that involves the surgical excision of the head and proximal neck of the femur and removal of the acetabular cartilage and subchondral bone. An artificial canal is created in the proximal medullary region of the femur and a metal prosthesis is inserted in the canal (Siopack, 1995). This procedure has improved the management of OA of the hip joint that has not responded to conventional medical therapy. This type of replacement procedure is also extremely common for OA of the hand and shoulders. Most patients who undergo TJA achieve marked pain relief and improvement of function however; there are always some complications that persist in a small segment of people. This includes pain, stiffness or instability. The patients might need revision surgery if there is a mechanical failure in the implant. Infection is another very serious complication in TJA. The prevalence of deep infection after surgery is about 1%-2% (Wilson, 1990).

Total joint arthroplasty is continuously evolving in terms of materials, prosthetic design, surgical technique, prevention of complications, and postoperative management.

Current surgical procedures are extremely successful in providing a normal standard of living for patients with OA.

IV. Triterpenoids and Rexinoids

A. Triterpenoids

Triterpenoids are the most ubiquitous non-steroidal secondary metabolites in terrestrial and marine flora and fauna. Their presence, even in non-photosynthetic bacteria, has created interest from both evolutionary and functional aspects (Mahato, 1992). They are biosynthesized in plants by the cyclization of squalene and are used for medicinal purposes in many asian countries. More than 20,000 triterpenoids are known to occur in nature; two of these, oleanolic acid (OA) and ursolic acid (UA), are anti-inflammatory and anti-tumorigenic in vivo (Huang, 1994) (Nishino, 1988). However, the biological activities of these naturally occurring molecules are relatively weak and therefore new analogues have been synthesized to enhance their potency (Honda, 1998). New synthetic oleanane triterpenoids (SO) are found to be more potent than the naturally occurring parent structure, oleanolic acid, as anti-inflammatory, antiproliferative, cell-differentiating and apoptosis-inducing agents (Suh, 1999). They are non-cytotoxic and highly multifunctional drugs that have applications for the prevention and treatment for not only cancer, but also of many other diseases with an inflammatory component. These molecules inhibit the expression of genes for inflammatory mediators, such as iNOS and inducible cyclooxygenase (COX-2), inhibit proliferation of many cancer cells, and induce monocytic differentiation of leukemia cells and adipogenic differentiation of fibroblasts

(Suh, 1999) (Wang, 2000). CDDO-Me is in phase III clinical trials for advanced kidney disease (Pergola, 2011). Further derivatization of CDDO to yield imidazolides (CDDO-Im), amides (methyl amide, CDDO-MA; ethyl amide, CDDO-EA), or a dinitrile (Di-CDDO), significantly increases biological activity (Liby, 2007).

A.1. 2-Cyano-3,12-Dioxoolean-1,9-Dien-28-oic Acid (CDDO)

CDDO is a potent multifunctional molecule that has three important properties: (a) it is a potent inducer of differentiation in both malignant and nonmalignant cells; (b) it is active at nanomolar levels as an inhibitor of proliferation of many malignant or premalignant cells; and (c) it is 100 –500-fold more potent than any previous triterpenoid in suppressing the de novo synthesis of the inflammatory enzymes iNOS and COX-2 (Suh, 1999). Because of the extremely high efficacy of this triterpenoid in regulation of primary hallmarks of cancer, it has been termed as a chemopreventive agent. In the case of cartilage destruction, CDDO has been shown to inhibit matrix metalloproteinase (MMP-1 and MMP-13) gene expression mediated by inflammatory cytokines in human chondrosarcoma cell lines and primary human chondrocytes (Mix, 2001) (Elliott, 2003). Thus, this triterpenoid has shown great therapeutic potential for the inhibition of joint degradation in OA.

A.2. CDDO-Ethyl Amide (CDDO-EA) and CDDO-Imidazolide (CDDO-Im)

CDDO-EA and CDDO-Im (Fig 1.1) are newer derivates of CDDO and are highly active in relevant cell culture assays at concentrations that can be obtained *in vivo*. They

are more potent compared to CDDO both *in vitro* and *in vivo* (Place, 2003). They are highly efficient in suppressing inflammation, activation of cytoprotective pathways, inducing differentiation, inhibiting proliferation and inducing apoptosis in cancer cells.

Liby *et al* showed that CDDO-EA successfully prevents lung cancer induced by a mutagenic carcinogen, vinyl carbamate in mice (Liby, 2007). This triterpenoid has also shown preventive effects in transgenic mouse models of pancreatic cancer (Liby, 2010). CDDO-Im has also shown similar effects in cancer cells. It successfully suppresses STAT phosphorylation and induces apoptosis in myeloma and lung cancer cells (Liby, 2006).

A.3. Triterpenoids and Nrf2 Pathway

Nuclear factor-erythroid 2 (NF-E2) - related factor 2 (Nrf2) is a redox sensitive transcription factor, which belongs to the cap 'n' collar subfamily containing the basic leucine zipper region (Kim, 2010). Nrf2 binds to antioxidant response elements (ARE) that are located in the promoter region encoding many phase II detoxifying or antioxidant enzymes that are related stress-responsive proteins (Lee, 2004). Induction of cytoprotective enzymes via Nrf2-ARE signaling provides a very effective way for achieving cellular protection against a variety of electrophilic carcinogens, reactive toxicants as well as ROS. Thus Nrf2 is considered to be a significant target in chemoprevention (Sporn, 2005). In addition to protection against oxidative stress, recent studies have exemplified the effects of Nrf2 in response to pro-inflammatory stimuli and saving cells/tissues from inflammatory injuries (Cho, 2004) (Braun, 2002) (Arisawa, 2007).

Many forms of stress increase the formation of reactive oxygen and reactive nitrogen species (ROS and RNS, respectively) (Nguyen, 2004). At low doses, these cellular stressors can induce a protective response by activating Nrf2, which in turn induces the formation of cytoprotective substances; however, high doses of these stressors can cause severe cellular damage and death (Liby, 2007). Synthetic triterpenoids are significant cytoprotective agents because at extremely low doses, they they can induce the synthesis of phase 2 enzymes that protect against the potential damaging and killing effects of ROS, RNS and electrophiles (Liby, 2007).

A.4. Triterpenoids and Smad Signaling

Suh *et al* studied the effects of CDDO and CDDO-Im on transforming growth factor (TGF)-β/Smad signaling. They showed that these agents, at nanomolar concentrations, increase the expression of TGF-β-dependent genes, such as those for plasminogen activator inhibitor 1 and the type II TGF-β receptor, and they synergize with TGF-β in this regard. They prolong the activation of Smad2 induced by TGF-β and markedly enhance the ability of Smad3 to activate a Smad binding element, CAGA-luciferase (Suh, 2003). They also showed that these triterpenoids enhance Smad signaling in the pathways of two other members of the TGF-β superfamily, namely, activin and bone morphogenetic protein (BMP). Further experiments by Ji *et al* demonstrated that CDDO-Im induces monocytic differentiation by activating the Smad and ERK signaling pathways in HL60 leukemia cells (Ji, 2006). The study also showed that CDDO-Im activated BMP-directed Smad signaling thus concluding that TGF-β and BMP pathways

contribute to the same mechanism of action of differentiation induced by CDDO-Im. These were among the first studies that established a relationship between triterpenoids and Smad signaling.

Since BMP and TGF-β are the primary factors involved in the process of chondrogenesis (Indrawattana, 2004), we speculated on the efficacy of triterpenoids as stimulators of BMP in mediating repair of both bone and cartilage. OA is a classic disorder with increased cartilage and bone destruction which led us to investigate the activity of synthetic triterpenoids in cartilage and bone formation as well as inhibition of cartilage destruction.

B.   Rexinoids

Rexinoids are defined as agents that bind selectively to the retinoid X receptors (RXRα, RXRβ and RXRγ), members of the nuclear receptor superfamily that regulate development, organ physiology and cell proliferation, differentiation and apoptosis (Liby, 2007). These receptors interact heterodimerically with many other members of the nuclear steroid receptor superfamily, and therefore have the ability to control the action of many steroid-like hormonal agents that modulates metabolism and cellular energetics (Mangelsdorf, 1995) (Shulman, 2005). In the past few years, rexinoids have shown great promise for both the prevention and treatment of cancer and chronic diseases. In contrast to synthetic triterpenoids, which are involved in regulating transcription factors that further modulate entire signaling pathways, rexinoids act as direct ligands for transcription factors. Great advances have been made in the synthesis of new rexinoids.

They do not have the toxic effects related to classical retinoids which make them appealing to use for the treatment of chronic diseases.

V. OA Model Systems

A. *In vivo* Animal Models

Experimental model systems that are used to simplify complex phenomena and to understand otherwise elusive processes (Richardson, 1984). An animal model for human disease can be defined as a homogenous set of animals which have an inherited, naturally acquired, or experimentally induced biological process, amenable to scientific investigation, that in one or more respects resembles the disease in humans (Pritzker, 1994).

The decision to use a particular animal model to study osteoarthritis is governed by criteria of relevance, appropriateness, and availability (Altman, 1990). Relevance refers to the comparability of the phenomenon studied in animals to the corresponding process in human disease. Appropriateness refers to the need to use a specific set of animals rather than simpler models to investigate the process in question while availability refers to practical factors such as adequacy of animal supply, the presence of controls, the ease of handling and environmental maintenance, as well as cost (Pritzker, 1994).

The complex pathobiologic changes of human joint disease, particularly OA, normally take several decades to develop and may be influenced by a multitude of genetic and environmental factors (Little, 2008). The need to clarify the molecular events that occur in joint tissues at the onset and during the progression of OA has necessitated

the use of models, which, although imperfect, can exhibit many of the pathologic features that characterize the human disease (Little, 2008). *In vitro* studies are most useful for defining specific molecular and cellular events in degradation of joint tissues such as cartilage. However, to fully understand the complex inter-relationship between the different disease mechanisms, joint tissues and body systems, studying OA in animal models is necessary (Little, 2008). A thorough understanding of the mechanism is required for development of anti-OA drugs, for which animal models have proven to be extremely useful. Before a promising new therapy can be initiated in the clinical environment, thorough testing in an alternate species is mandatory to ascertain optimal route of administration, dosage, safety and toxicity (Little, 2008).

Animal models of osteoarthritis (OA) commonly used in studying the pathogenesis of cartilage degeneration and potential therapeutic modulation of disease are generally either naturally occurring or surgically-induced (Bendele, 2001). Spontaneous OA occurs in the knee joints of various strains of mice (Walton, 1979) (Walton, 1977) (Sokoloff, 1962) and transgenic and mutant mouse models of OA have been developed and characterized (Helminen, 1993) (Fassler, 1994) (Takahashi, 1997) (Brewster, 1998). For example, STR/ORT and C57BL mice show spontaneous development of OA at an early age (Mason, 2001) (Wilhelmi, 1976). Mutations in genes encoding type II and type XI collagens, aggrecan and the multipass transmembrane protein (ANK) that controls pyrophosphate levels in cells have been detected in some mouse strains and they lead to development of early onset OA, if present in heterozygous state (Li, 1997). A study by Heliminen *et al* showed that an inbred line of transgenic mice expressing an internally deleted gene for type II collagen have degenerative changes of articular cartilage similar

to osteoarthritis (Heliminen, 1993). Biglycan and fibromodulin are small extracellular proteoglycans that are co-expressed in tendons, cartilage and bones (Helminen, 2002). Biglycan and fibromodulin single knockouts and mice deficient in both proteoglycans develop OA changes in the knee joints from 3 months of age onwards (Ameye, 2001). In OA, the involvement of MMP-13 is crucial for degradation of cartilage and development of human OA lesions (Shlopov, 1997). This finding has been utilized in the design of a novel animal model in which transgenic mice express the human MMP-13, which is specifically targeted to hyaline cartilage by the type II collagen promoter, and expression takes place under tetracycline regulated transcriptional control (Neuhold, 2001). Also, expression of truncated, kinase-deficient TGF-β type II receptor in transgenic mice, promotes terminal differentiation of chondrocytes and development of OA (Serra, 1997). A new transgenic animal model for OA that involves a deletion of BMP receptor type 1a displayed retention of webbing between some digits, lack of formation of some joints in the ankles, and premature osteoarthritis in other joints compared with normal wild-type mice (Mishina, 2002) (Young, 2005). These transgenic mouse strains are extremely important in understanding the involvement of various genes at different stages in the pathogenesis of OA.

Spontaneous OA also occurs in guinea pigs and macaques. The prevalence of OA in the guinea pig appears to be linked to laxity in the cruciate ligaments, suggesting a biomechanical driving force for OA development, and that this species is a model for secondary rather than primary disease (Quasnichka, 2006).

Induction of pathologic changes of OA can be induced by intraarticular injection of a variety of agents, including enzymes (papain, collagenase, trypsin, hyaluronidase),

cytokines (interleukin (IL)-1, TGF-β) and chemicals (monosodium iodoacetate (MIA)) in animals models like mouse, rat, rabbit and horse (Pritzker, 1994) (Van De Loo, 1994) (Beuningen, 2000) (Janusz, 2004). Many of these agents induce a significant acute local inflammation at the site of injection and thus may not replicate the naturally occurring sequence of the disease process of human OA (Little, 2008).

Surgically induced destabilization of joints is the most widely used induction method, where the underlying initiating mechanism is altered mechanical loading, one of the most common causes of secondary OA in humans (Bendele, 2001). Many induction methods actually copy known injuries in humans, such as meniscal injury as well as the molecular pathology (in cartilage e.g. ADAMTS cleavage of aggrecan, collagenase cleavage of collagen, chondrocyte early hypertrophic response etc) and histopathology that is observed in humans (Little, 2008). Surgically induced OA is carried out in various species of animal models such as mouse, rat, guinea pig, rabbit, dog, sheep and monkey (Moskowitz, 1973) (Smith, 2007).

B. Ex-vivo Models

Ex vivo organ culture models can provide information and insight concerning the mechanisms for functional events within cartilage, bone or synovium or selected cells within these tissues (Pritzker, 1994). These ex vivo models, inherently more highly controlled than animal models, are most useful for understanding short term biological events, and events isolated from the physiological influences of adjacent structures and general metabolism. However, the only limitation is that they can't simulate the structural

bone and cartilage changes which occur in joint tissues in animals over months to years (Pritzker, 1994).

The history of culturing bone explants goes back to the early 1930's, when Fell and Robison reported that the enzyme alkaline phosphatase played an important role in bone mineralization based on studies of chick bone fragments (Fell, 1934). Subsequently, Reynolds *et al.*, used bone explants to study collagen synthesis in bone (Reynolds, 1967) and to investigate the effects of a variety of agents on bone turnover such as vitamin A (Reynolds, 1968), Ascorbic acid (Reynolds, 1966), calcitonin (Reynolds, 1968), hydrocortisone (Reynolds, 1966) and biphosphonates (Reynolds, 1972) (Garrett, 2003). Over the past several years, many research groups have used rodent calvarial and long bone explants to study the effect of cytokines on bone resorption (Gowen, 1983) (Bertolini, 1986) and bone formation (Traianedes, 1998) (Mundy, 1999). An interesting study using mouse calvarial bones by Kusano *et al.*, demonstrated that Il-1 and IL-6 regulated MMP activity in bone resorption during the processes of bone modeling and remodeling (Kusano, 1998).

Ex vivo culture of mouse calvarial bones is an extremely effective method to observe changes in bone and cartilage under specific experimental conditions (Mohammad, 2008). This ex vivo culture system has advantages over osteoblast cell lines and tissue culture. The calvarial sections retain both the three-dimensional architecture of developing bone as well as a physiologically relevant array of cell types, including osteoblasts, osteoclasts, osteocytes and stromal cells, as well as mineralized matrix. In addition, the calvariae contain cells at all stages within the spatiotemporally complex

lineages leading to mature osteoblasts and osteoclasts (Mohammad, 2008). Ross Garrett, in his article on mouse calvarial organ cultures has perfectly described the use of cultured neonatal calvariae as an assay for agents with anabolic activity (Garrett, 2003). Experiments have been conducted to evaluate the effect of statins and BMP on new bone formation using mouse calvaria (Garrett, 2001). Also, calvarial organ cultures have been extremely useful in deciphering the role of osteoblast proteosome inhibitors in bone formation (Garrett, 2003). We have used this method extensively in our research to observe the anabolic activity of triterpenoids for new cartilage and bone formation.

C. In vitro Assays

In vitro assays are particularly advantageous in terms of costs, simplicity, a direct approach towards assessment of compound performance and ethical considerations (Polli, 2008). They offer a controlled environment to test specific cellular and molecular hypotheses (Polikov, 2008). Since there are several physiologic parameters to consider in the case of *in vivo* animal models, in vitro studies are very efficient in reducing experimental variation and assisting in the ease of experimental replication (Freshney, 2001).

In OA, the SW1353 chondrosarcoma cell line is an ideal system to evaluate the catabolic activity of chondrocytes (Mix, 2001). This cell line is used frequently as it behaves similarly to primary chondrocytes in terms of matrix metalloproteinase (MMP) expression in response to pro-inflammatory cytokines (Borden, 1996) (Mengshol, 2000) (Dinarello, 1996). The chondroprotective activities of various compounds have been

established using the SW1353 cell line. Lu *et al.*, demonstrated the chondroprotective role of sesamol that is exerted by inhibition of cytokine induced MMP expression through the signaling pathway of NF-κB in SW1353 cells (Lu, 2011). Similarly, Williams *et al.*, analyzed the chondroprotective effects of diallyl disulphide, a compound found in garlic using the SW1353 cell line (Williams, 2010). This compound also exerts its effects via repression of matrix degrading proteases. Thus, cytokine-stimulated SW-1353 cells serve as a practical culture system to study the pathologic expression of MMPs, which may be representative of MMP regulation in OA (Mix, 2001).

ATDC5, a murine chondrocytic cell line widely used as a monolayer culture system to study chondrogenic differentiation is comparable to the clinically suitable options of human articular chondrocytes and adult mesenchymal stem cells from human bone marrow (Tare, 2005). Tare *et al*, used the ATDC5 cell line to observe the chondrogenic differentiation potential of these cells in the presence of growth factors like insulin and TGF-β (Tare, 2005). This cell line serves as an ideal system to study the differentiation potential of chondrocytes induced by various factors as well as compounds. Ben-Eliezer *et al.*, demonstrated that leptin regulates chondrogenic differentiation in ATDC5 cell line through JAK/STAT and MAPK pathways (Ben-Eliezer, 2007). Similarly, the effect of glucocorticoid on chondrogenesis, differentiation and apoptosis was observed using the ATDC5 cell line (Mushtaq, 2002). The sequential progression of differentiation mediated by BMP-2 has also been depicted using this cell line (Shukunami, 1998). Thus, ATDC5 cell line is a practical option to analyze the anabolic activity of different compounds, which in our case are triterpenoids.

MATERIALS AND METHODS

A. Reagents

The synthesis of the triterpenoids, CDDO-Im and CDDO-EA, has been described (Liby, 2007) (Sporn, 2011). All other chemicals were from Sigma-Aldrich (Carlsbad, CA).

B. Cell Culture

Human chondrosarcoma cell line SW 1353 was obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at $37\,^{\circ}C$ and 5% CO_2. Cells were grown to confluence, washed with sterile PBS and placed in 60mm plates for experiments. As described previously (Mix, 2004), cells were treated with 1 ng/ml TNF and IL-1 for 24 h for induction of MMPs. Where indicated, the cells were treated with 1nM, 10 nM or 100 nM CDDO-Im or CDDO-EA either alone or with cytokines and experiments were conducted in a dose dependent manner. Cells were harvested for RNA.

C. Analysis of bone forming activity in organ cultures of murine calvarial bones.

The technique for studying organ cultures of neonatal murine calvarial bones has been described in detail previously (Garrett, 2003). Pregnant (timed) ICR Swiss White

mice were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN). On day 0, calvarial bones were removed from the calvariae of 4-day-old ICR Swiss mice and then cultured in Biggers, Gwatkins, Judah medium (BGJ medium) supplemented with 1mg/ml of bovine serum albumin (Cohn Fraction V), 100 U/ml each of penicillin/streptomycin and 0.292 mg/ml of glutamine. Bones were treated on day 1 with or without test compounds. On day 4, the medium was replaced with fresh medium containing test compounds (50 nM up to 500 nM). Calvaria were collected on day 7 and stored in 1ml Trizol at -80°C for further RNA analysis or fixed in 10% buffered formalin for 24h and transferred to 80% ethanol for histologic analysis.

D. Histologic Analysis of murine calvarial bones.

After fixation in 10% buffered formalin for 24 h, calvaria were decalcified in 14% EDTA overnight, embedded in paraffin and sectioned at 4 μm. Sections were stained either with modified hematoxylin and eosin or with toluidine blue (1% in 70% ethanol for 20 minutes, followed by destaining in 70%, 90% and 100% ethanol for 15 seconds), placed in xylene three times and then mounted. The effects of bone formation were evaluated by histomorphometric assessment using Zeiss AxioCam HRc camera fitted to a Zeiss Axioscope 2 plus microscope. Procedures for immunofluorescence staining have been described earlier (Medici, 2010). Primary antibodies against collagen type II (AB746P, Millipore, Billerica, MA) were used at 1: 100 dilution; AlexFluor secondary antibodies (Invitrogen, Carlsbad, CA) at 1:200 dilution. For nuclear staining To-PRO-3 Iodide (T3605, Invitrogen, Carlsbad, CA) was used.

E. Quantitative reverse transcription – polymerase chain reaction (RT-PCR) analysis.

To determine the changes of mRNA levels by triterpenoids and cytokines, we utilized quantitative RT-PCR analysis. The cells were incubated with compounds for indicated period and the cells were then lysed with Trizol to extract RNA. In the case of murine calvarial bones, the calvaria was homogenized and centrifuged at 4000 rpm for 10 minutes. Supernatant was transferred to new eppendorf tubes for the RNA extraction procedure. RNA was reverse transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The cDNA was used for quantitative PCR which was run on ABI Prism 700 sequence detection system. The primers for the experiments were obtained from Applied Biosystems (Foster City, CA).

PART I

Triterpenoids CDDO-IM and CDDO-EA induce chondrogenesis in new born mouse calvaria

1.1. Introduction

Chondrogenesis is the earliest phase of skeletal development, involving mesenchymal stem cell (MSC) recruitment and migration, condensation of progenitors, and chondrocyte differentiation, and maturation resulting in the formation of cartilage and bone during endochondral ossification (Goldring, 2006). It needs the stimulation with cell growth factors to make the multipluripotent MSCs differentiate into chondrogenic lineage (Indrawattana, 2004). The transforming growth factor- β (TGF-β) family, bone morphogenetic proteins (BMPs) and insulin growth factors (IGFs) are extremely important growth factors required in the process of chondrogenesis (Richter, 2009). Indrawattana *et al* showed that the combined growth factors TGF-β3 and BMP-6 or TGF-β3 and IGF-1 were more effective for chondrogenesis induction compared to them alone (Indrawattana, 2004).

The process of chondrogenesis that involves sequential differentiation and maturation from chondroprogenitors to hypertrophic chondrocytes is regulated by transcription factors such as the Sry-type high-mobility group box (Sox) genes, the basic helixloop-helix (bHLH) transcription factor Scleraxis (Scx) and the runt-related Runx genes (Furumatsu, 2010). The Sox E protein, Sox9, which encodes a high mobility group (HMG) DNA-binding domain has been identified as the master transcription factor in

chondrogenesis (Kamachi, 2000) (Bernard, 2010). Sox9 interacts with Sox5 and Sox6 to activate the collagen II and aggrecan genes during cartilage formation (Sekiya, 2000) (Ikeda, 2005). Tensile strength of the cartilage is due to collagen type II fibrils (Goldring, 2010). Aggrecan, a large chondroitin sulfate proteoglycan is extremely important to maintain the structure of cartilage (Sekiya, 2000).

Ex vivo culture of mouse calvarial bones is an extremely effective method to observe changes in bone and cartilage under specific experimental conditions (Mohammad, 2008). New synthetic triterpenoids have been synthesized and developed as anti-inflammatory agents (Liby, 2007) (Sporn, 2011). Synthetic tritepenoids are also known to induce differentiation in many different cell types in monolayer culture (Suh, 1999), but they have not previously been evaluated for chondrogenic activity (Suh, 2012) (Refer to figure 1.1 for the structure of CDDO-Im and CDDO-EA). In the past few years, rexinoids have shown tremendous potential for the prevention and treatment of cancer because of their anti inflammatory activity (Liby, 2007). However, they also have not been studied for cartilage formation activity as of yet either.

Numerous preclinical, epidemiologic, and clinical studies have suggested the benefits of vitamin D and its analogues for the prevention and treatment of cancer (Lee, 2008). The ligand for vitamin D receptor, 1α,25-dihydroxyvitamin D3 (1α 25(OH)$_2$D3); the key hormone in calcium/phosphate homeostasis is a hormonally active metabolite synthesized from vitamin D3 predominantly through hydroxylation by a 25-hydroxylase in the liver and a 1α-hydroxylase in the kidney (Deeb, 2007) (Lee, 2008). Previously, there has been no chondrogenic activity reported with this vitamin D analog.

There is a strong rationale for the potential use of synthetic triterpenoids as chondrogenic agents, since they are already known to enhance signalling by both TGF-β and BMPs (Suh, 2003), and both of these cytokines and their signalling proteins (Smads) are known to play important roles in chondrogenesis (Song, 2009) (Suh, 2012). Here, we determined whether the two new synthetic triterpenoids, namely CDDO-EA and CDDO-Im have the ability to induce chondrogenesis in mouse calvarial organ cultures both histologically and with classic markers of cartilage in a dose and time dependent manner. We have also evaluated the effect of LG100268, a rexinoid as well as vitamin D (1α $25(OH)_2$ D3) for the induction of chondrogenesis. SOX9 and collagen type II are two of the main markers that are suggestive of new cartilage formation.

1.2. Results

A. CDDO-Im and CDDO-EA induced chondrogenesis in mouse calvarial organ cultures (immunohistological results)

For the first two sets of immunohistological data, more than 300 individual calvarial organ cultures were performed (Fig 1.2 and 1.3). Calvarial bones were isolated from 4 day old mice and treated with 200 nM of CDDO-Im and CDDO-EA for 7 days. Induction of cartilage is not seen on control sections stained with either H&E or toluidine blue (Fig 1.2). In contrast, metachromatic toluidine blue staining is observed when the calvaria are treated with CDDO-Im and CDDO-EA. With toluidine blue, bone stains orthochromatically (blue). In the experimental H&E stains, the light purple area is greatly enhanced compared to the control suggestive of new cartilage formation (Fig 1.2). These

results were obtained from three separate experiments. Figure 1.3 shows immunohistochemically that CDDO-Im and CDDO-EA (200 nM) both induce the formation of type II collagen (collagen IIα1), which is not seen in the control sections (Fig 1.3).

B. CDDO-Im and CDDO-EA induced chondrogenesis in mouse calvarial organ cultures (RNA results)

In addition to the histologic analysis of the calvarial cultures, we have investigated some mechanistic aspects of the action of both CDDO-EA and CDDO-Im in the calvaria. After 7 days of culture, RNA was isolated from the calvaria using homogenization and quantitative RT-PCR analysis was performed for more than 15 different markers, including: SOX9, collagen IIα1, all three isoforms of TGF-β, BMPs 2 and 4, BMP receptor II, Smads 3, 4, 6, and 7, tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), and matrix metalloproteinase-9 (MMP-9). Table 1.1 shows that all of these markers (except MMP-9) are significantly up-regulated by both triterpenoids, when the calvaria were treated at either the 200 or the 500 nM dose. The 50 nM dose and was generally ineffective. In contrast, both triterpenoids are strong inhibitors of the expression of MMP-9; CDDO-EA (200 nM), caused almost 80% inhibition of the expression of this metalloproteinase (Table 1.1) (Suh, 2012).

C. CDDO-EA induced chondrogenesis in mouse calvarial cultures in a time dependent manner

This set of experiments are only conducted with CDDO-EA after evaluating its high potency from Fig 1.2 and 1.3. Calvarial bones were isolated from 4 day old mice and treated with 200 nM of CDDO-EA for 7 days. No new cartilage is seen on control sections stained with either H&E or toluidine blue (Fig 1.4 and 1.5). In contrast, day 4 samples treated with CDDO-EA showed the beginning of some cartilage formation. Day 7 samples demonstrated a substantial increase in cartilage as compared to day 4 samples (Fig 1.4 and 1.5). Day 10 samples were collected; however, due to technical difficulties; the samples were lost.

Similar to previous sections, we wished to investigate the mechanistic aspects of the action of CDDO-EA. After 4 and 7 days of culture, RNA was isolated from the calvaria and quantitative RT-PCR analysis was performed for 7 different markers including SOX9, collagen IIα1, Smad 6 and 7, TIMP 1 and 2 as well as MMP-9. Besides MMP-9, all of the markers were significantly upregulated on day 4 and day 7. A decrease in induction is observed on day 10 for SOX9 and Collagen II. No significant differences were observed in the expression levels of MMP-9 (Table 1.2).

D. Effect of rexinoid LG100268 and vitamin D (1α 25(OH)$_2$ D3) in mouse calvaria.

To investigate the effects of rexinoid LG100268 and 1α 25(OH)$_2$ D3, calvaria were treated with 100 nM of LG100268 and 10 nM of 1α 25(OH)$_2$ D3 along with 200 nM of CDDO-EA. Combination treatment with CDDO-EA and LG100268 or 1α 25(OH)$_2$ D3 was also performed. RNA was isolated from the calvaria and quantitative RT-PCR analysis was performed for 5 different markers including SOX9, collagen IIα1, TIMP 1 and 2 and MMP-9 (Fig 1.6 and 1.7). LG100268 significantly downregulated SOX9 and

collagen IIα1 expression (Fig 1.6). The combination of CDDO-EA and LG100268 however, moderately upregulated SOX9 and collagen IIα1 levels. 1α 25(OH)$_2$ D3 showed no significant effects when treated alone as well as with CDDO-EA (Figure 1.6). As shown in figure 1.7, LG00268 downregulated the expression of MMP inhibitor TIMP-2 while MMP-9 expression showed no significant differences. Vitamin D (1α 25(OH)$_2$ D3) had no significant effects on the expression levels of TIMP-1, TIMP-2 and MMP-9 as the dose of 10 nM.

1.3 Discussion

Our results clearly show that synthetic triterpenoids CDDO-Im and CDDO-EA induce chondrogenesis in calvarial organ cultures, as evaluated both morphologically and with biochemical markers of the expression of the cartilage phenotype (Suh, 2012). These results are very evident in the dose and time dependent experiments. The metachromatic toluidine blue purple staining, seen in Figure 1.2, is indicative of the ability of CDDO-Im and CDDO-EA to induce the formation of proteoglycans, such as aggrecan, which are vital for composition of articular cartilage (Roughley, 2001).

With respect to the dose dependent assays, 200 nM of CDDO-Im or CDDO-EA seem to be optimal. Treatment with 50 nM triterpenoid yielded only marginal induction of chondrogenesis, while treatment with 500 nM synthetic triterpenoids gave somewhat variable results (Table 1.1). Treatment with 1 μM triterpenoid was invariably toxic to the organ cultures (Suh, 2012). These experiments helped us identify an optimal dose for future mechanistic experiments.

In the case of the time dependent assays, day 4 and day 7 after 200 nM of CDDO-EA treatment showed positive results. However, the slight reduction in cartilage markers on day 10 (Table 1.2) might be indicative of hypertrophy of chondrocytes. Chondrogenesis is the process that results in the formation of the cartilage intermediate, or anlagen, and leads to endochondral ossification during skeletal development (Goldring, 2006). It is possible that by day 10 chondrocytes begin to undergo hypertrophy to form new bone. Futher mechanistic studies are required to confirm this speculation.

Figure 1.6 and 1.7 clearly show that rexinoid LG100268 downregulates SOX9 and collagen type II expression when compared to control and CDDO-EA. It is a possibility that rexinoids inhibit cartilage formation thus causing an opposite effect when compared with triterpenoids. Further mechanistic studies are required to confirm this finding. The increase in SOX9 and collagen type II expression by combination treatment of LG100268 and CDDO-EA can be attributed to CDDO-EA. Vitamin D does not seem to have any significant effects on induction of chondrogenesis at the dose tested (10 nM).

In this section, we have not extensively addressed the underlying mechanisms whereby the triterpenoids induce the chondrogenic phenotype. Although it is well known that synthetic triterpenoids can induce differentiation (myeloid, adipogenic, and neuronal) in a variety of cells in culture (Suh, 1999), the molecular mechanisms involved are still obscure (Suh, 2012). This is undoubtedly a reflection of the fact that synthetic triterpenoids are multifunctional agents that interact with many molecular targets in the cell (Sporn, 2011). In almost all cases, it has been shown that the binding of any synthetic triterpenoid to its protein target involves an active cysteine residue in the target (Sporn, 2011) (Liby, 2007). Targets of synthetic triterpenoids that are already known include

Keap1/Nrf2, IκB kinase/NFκB, proteins that are involved with estrogen receptor signalling, insulin receptor signalling, glucocorticoid receptor signalling, JAK/Stat signalling, PTEN signaling, retinoic acid receptor activation, IGF-1 signalling, and the actions of several interleukins, as well as proteins associated with the actin-cytoskeleton of the cell (Tore, 2011) (Suh, 2012). Further mechanistic studies are required to decipher the exact pathway used by triterpenoids to exert their effects.

Osteoarthritis is a disease that primarily involves degradation of cartilage along with changes in the subchondral bone as well as synovial inflammation (Arden, 2006). There could be numerous benefits if all the results discussed here could potentially be translated into studies *in vivo*. However, there are many complications that could possibly arise such as an appropriate dosage for *ex vivo* and *in vitro* studies might be too toxic in vivo or not beneficial at all. Extensive studies in *in vivo* models of osteoarthritis need to be carried out to establish a positive correlation between triterpenoids and chondrogenesis *in vivo*. This study provides optimism for more research in the field and emphasizes on the potential use of triterpenoids for treatment of osteoarthritis.

1.3. Summary

In this part, we have successfully demonstrated that CDDO-Im and CDDO-EA induce chondrogenesis in mouse calvarial organ culture by upregulating important cartilage markers. 200 nM of CDDO-Im and CDDO-EA was more effective compared to 50 nM and 500 nM. Time dependant studies showed positive effects of CDDO-EA on day 4 and day 7 after treatment however, day 10 showed a slight decrease in the

expression levels of cartilage markers. Rexinoids downregulate the expression of SOX9 and collagen type II while vitamin D (1α $25(OH)_2$ D3) did not have any significant effects at the dose tested.

Oleanolic acid

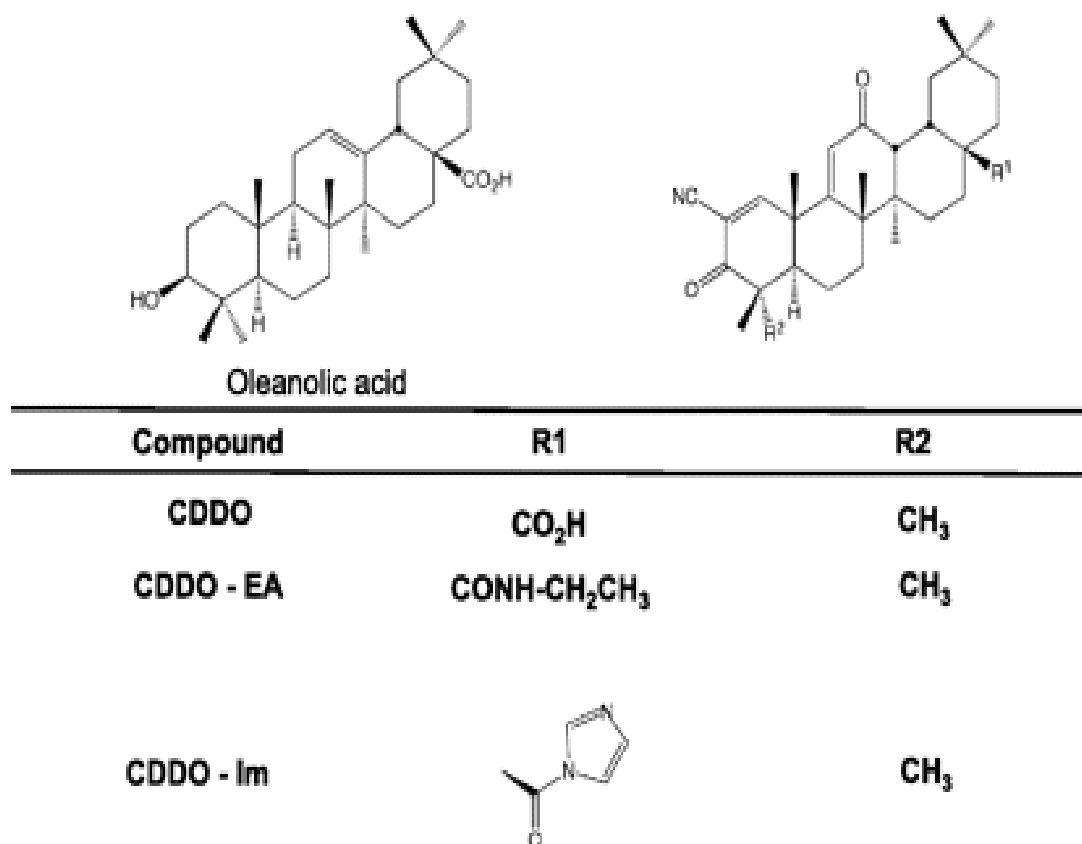| Compound	R1	R2
CDDO	CO_2H	CH_3
CDDO - EA	$CONH\text{-}CH_2CH_3$	CH_3
CDDO - Im		CH_3

Figure 1.1 Structures of CDDO, CDDO-EA and CDDO-Im (Pitha-Rowe, 2009).
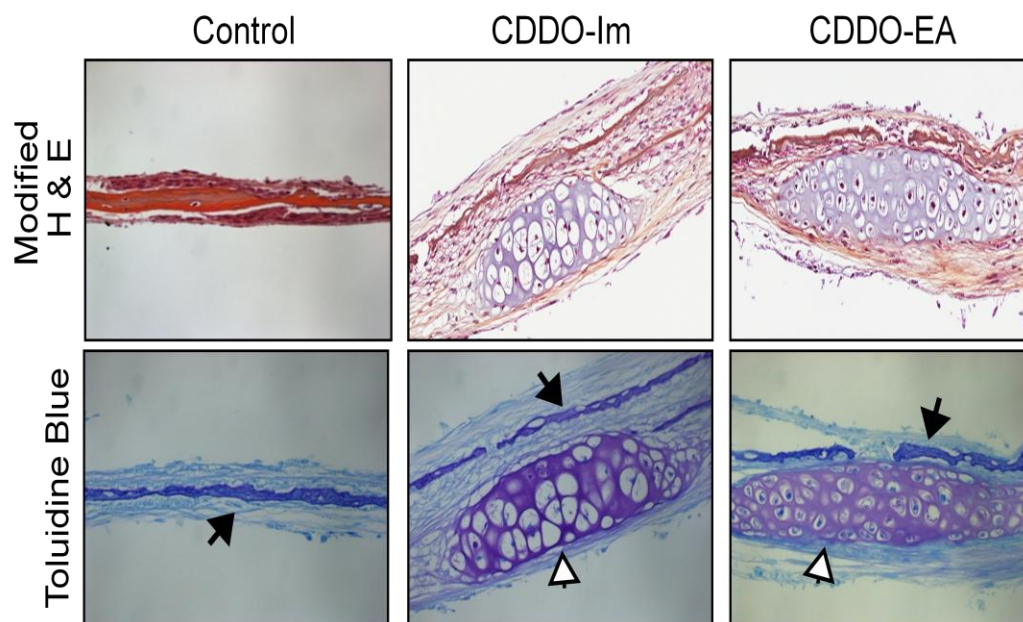
Fig 1.2. Histology of calvaria after 7 days of culture.
CDDO-Im and CDDO-EA, 200 nM. Toluidine blue stains bone orthochromatically (blue staining, noted by black arrowheads); cartilage is metachromatic (purple staining, noted by open arrowheads). Essentially similar results were found in 3 separate experiments (Suh, N; Paul S; Lee, H.J; Yoon, T; Shah, N; Son, A; Reddi, H; Medici, D and Sporn, M. "Synthetic Triterpenoids, Cddo-Imidazolide and Cddo-Ethyl Amide, Induce Chondrogenesis." *Osteoarthritis Cartilage* (2012)).

Fig 1.3. Calvarial cultures stained for type II collagen (COL2A1).

Details for fluorescence immunohistochemistry are in the Materials and Methods section. Collagen is stained red (Suh, N; Paul S; Lee, H.J; Yoon, T; Shah, N; Son, A; Reddi, H; Medici, D and Sporn, M. "Synthetic Triterpenoids, Cddo-Imidazolide and Cddo-Ethyl Amide, Induce Chondrogenesis." *Osteoarthritis Cartilage* (2012)).

Control   **CDDO-EA**

Day 4

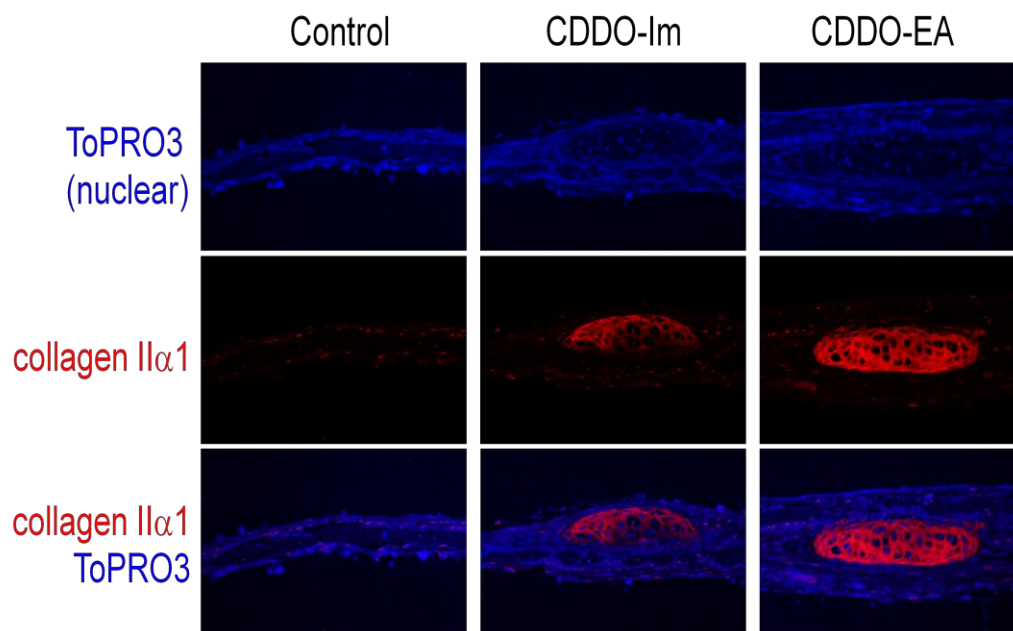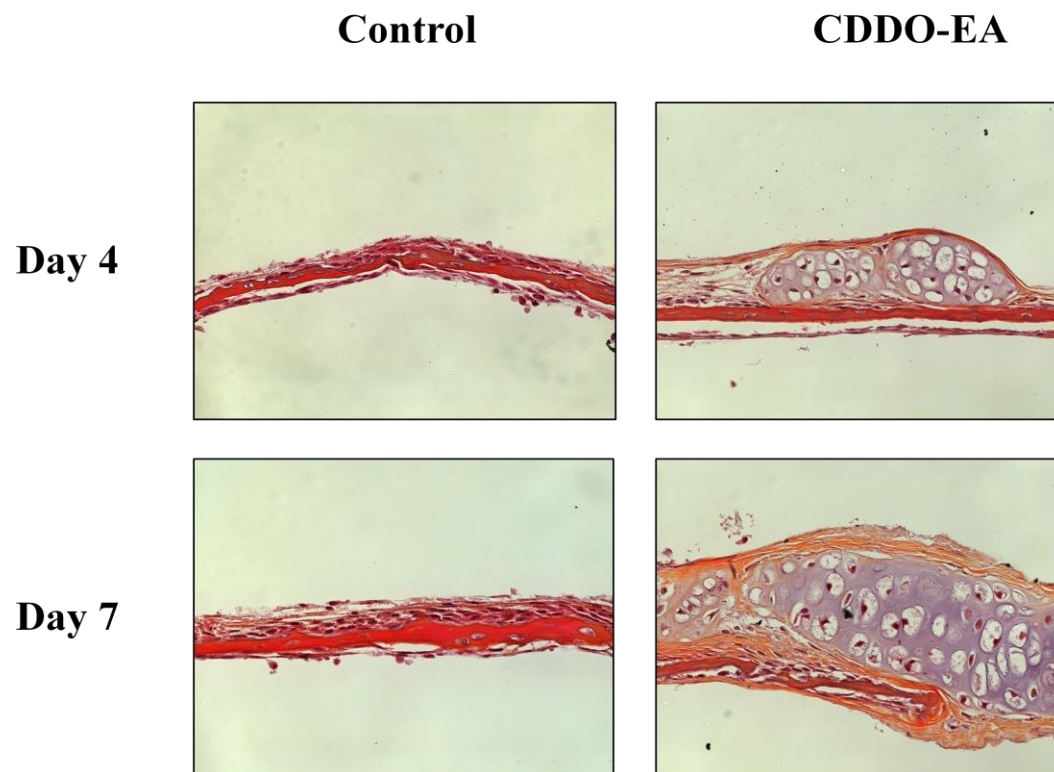

Day 7

Figure 1.4. H&E staining after 4 and 7 days in culture. Calvarial tissue was treated with 200 nM of CDDO-EA
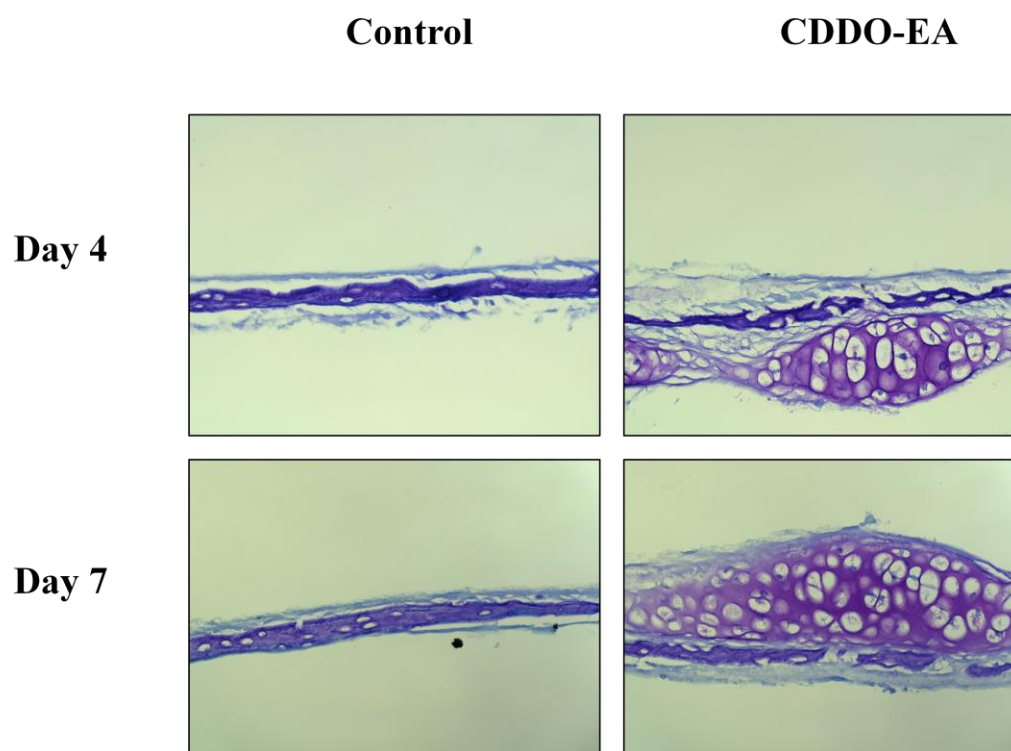
Figure 1.5. Toluidine blue staining after 4 and 7 days in culture. Calvarial tissue was treated with 200 nM of CDDO-EA

A

SOX-9

B

Collagen II alpha I

Figure 1.6. Effect of LG100268 and 1α 25(OH)$_2$ D3 and combination treatment with CDDO-EA in mouse calvaria.

Calvaria was treated with 200 nM of CDDO-EA, 100nM of LG100268 and 10nM of 1α 25(OH)$_2$ D3 for 7 days. Total RNA was isolated and mRNA levels for SOX9(A) and collagen type II (B) were measured using quantitative RT-PCR analysis described in the Materials and Methods section. Signals were normalized to GAPDH (**1**-Control; **2**-CDDO-EA; **3** -LG100268; **4** -1α 25(OH)$_2$ D3; **5** – LG+CDDO-EA and **6** -1α 25(OH)$_2$ D3 + CDDO-EA). N = 4 calvaria per treatment group. Error bars represent standard errors and p values were calculated using ANOVA followed by Dunnetts multiple comparison test (* p<0.05, ** p<0.01).

A

TIMP-1

B

TIMP-2

C

MMP-9
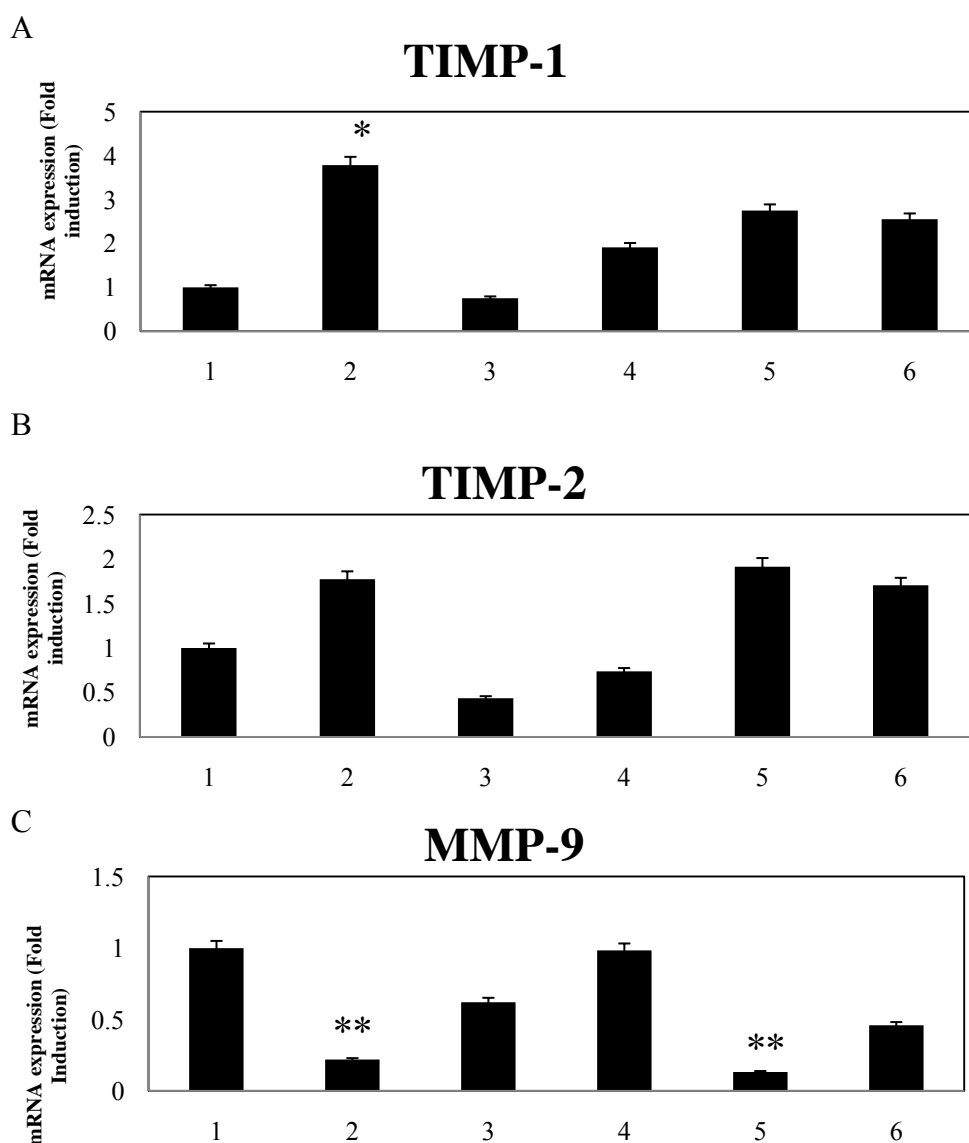
Figure 1.7. Effect of LG100268 and 1α 25(OH)$_2$ D3 and combination treatment with CDDO-EA in mouse calvaria.

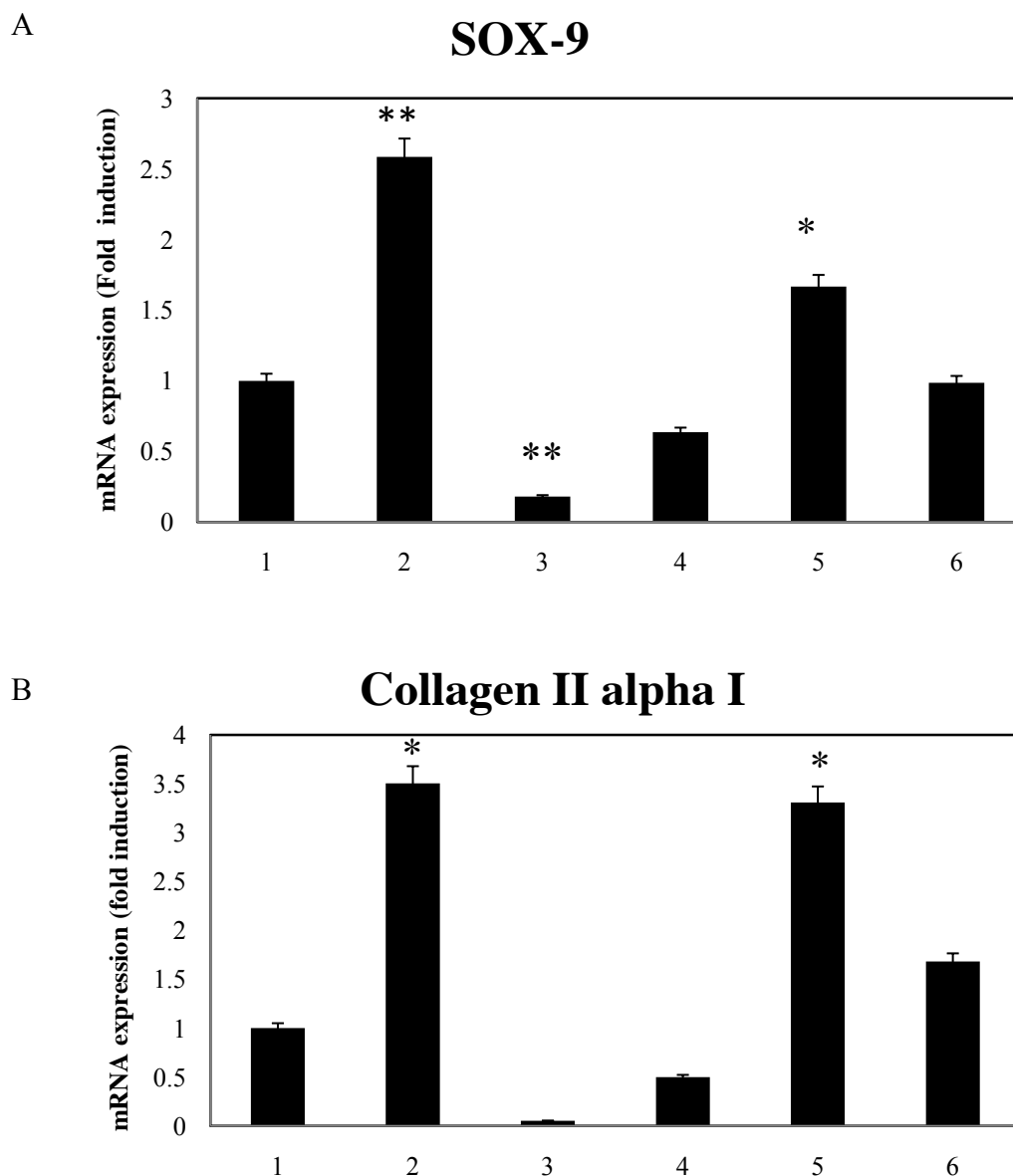Calvaria was treated with 200 nM of CDDO-EA, 100nM of LG100268 and 10nM of 1α 25(OH)$_2$ D3 for 7 days. Total RNA was isolated and mRNA levels for TIMP-1(A) TIMP-2 (B) and MMP-9 (C) were measured using quantitative RT-PCR analysis described in the Materials and Methods section. Signals were normalized to GAPDH. (**1**-Control; **2**-CDDO-EA; **3** -LG100268; **4** -1α 25(OH)$_2$ D3; **5** – LG+CDDO-EA and **6** -1α 25(OH)$_2$ D3 + CDDO-EA). N = 4 calvaria per treatment group. Error bars represent standard errors and p values were calculated using ANOVA followed by Dunnetts multiple comparison test (* $p<0.05$, ** $p<0.01$).

Table 1.1. Quantitative RT-PCR analysis on calvaria

Gene	CDDO-Im			CDDO-EA		
	50 nM	200 nM	500 nM	50 nM	200 nM	500 nM
SOX-9	1.26 ± 0.08	1.33 ± 0.05	$1.83 \pm 0.13**$	1.64 ± 0.17	$2.62 \pm 0.33**$	$3.12 \pm 0.38**$
COL2A1	1.45 ± 0.09	1.32 ± 0.23	1.72 ± 0.39	$4.12 \pm 1.78*$	$5.45 \pm 1.13**$	3.18 ± 0.71
TGF-β1	0.96 ± 0.07	1.08 ± 0.12	$1.46 \pm 0.12*$	1.17 ± 0.09	$1.47 \pm 0.24*$	$1.74 \pm 0.17**$
TGF-β2	1.17 ± 0.05	1.15 ± 0.09	1.17 ± 0.10	$1.36 \pm 0.13*$	$1.61 \pm 0.11**$	$1.88 \pm 0.09**$
TGF-β3	1.30 ± 0.07	$1.52 \pm 0.16**$	$1.66 \pm 0.13**$	$1.39 \pm 0.07*$	$1.56 \pm 0.13**$	$1.44 \pm 0.11**$
BMP-2	1.17 ± 0.07	1.60 ± 0.19	$3.14 \pm 0.53**$	1.53 ± 0.20	$2.52 \pm 0.29**$	$5.66 \pm 0.67**$
BMP-4	0.97 ± 0.05	0.98 ± 0.06	1.35 ± 0.22	1.28 ± 0.12	1.47 ± 0.13	$2.49 \pm 0.35**$
BMPRII	1.09 ± 0.06	1.22 ± 0.11	$1.46 \pm 0.14*$	$1.50 \pm 0.14*$	$1.71 \pm 0.19**$	$2.24 \pm 0.22**$
Smad3	0.97 ± 0.07	0.90 ± 0.05	1.35 ± 0.19	1.14 ± 0.12	1.35 ± 0.07	$1.93 \pm 0.16**$
Smad4	0.96 ± 0.06	1.03 ± 0.09	1.26 ± 0.09	1.06 ± 0.03	$1.37 \pm 0.12**$	$1.89 \pm 0.12**$
Smad6	0.95 ± 0.05	1.23 ± 0.14	$1.77 \pm 0.19**$	1.09 ± 0.04	$1.77 \pm 0.24**$	$2.85 \pm 0.31**$
Smad7	1.05 ± 0.07	1.32 ± 0.14	$1.78 \pm 0.18**$	1.28 ± 0.10	$1.86 \pm 0.19**$	$3.06 \pm 0.28**$
TIMP-1	1.43 ± 0.19	1.83 ± 0.18	$3.00 \pm 0.65**$	1.88 ± 0.34	$2.85 \pm 0.38**$	$4.65 \pm 0.38**$
TIMP-2	1.24 ± 0.10	1.67 ± 0.19	$2.62 \pm 0.29**$	$1.78 \pm 0.18*$	$2.44 \pm 0.31**$	$4.09 \pm 0.36**$
MMP-9	0.73 ± 0.16	$0.44 \pm 0.12**$	$0.26 \pm 0.03**$	$0.49 \pm 0.09**$	$0.22 \pm 0.03**$	$0.19 \pm 0.06**$

N = 20 calvaria for control group and N = 12 calvaria per treatment group. Statistical significance was performed with ANOVA followed by Dunnett's multiple comparison test using GraphPad Prizm 4.0 software. Data are represented as average +/- S.E. (* $p<0.05$, ** $p<0.01$). All values have been normalized to the 20 control cultures, assigned a value of 1.00

Table 1.2. Quantitative RT-PCR analysis for day 4, day 7 and day 10 after treatment with CDDO-EA (200 nM) in mouse calvaria.

Gene	Day 4	Day 7	Day 10
SOX9	1.73 ± 0.05	$2.81 \pm 0.08**$	$1.92 \pm 0.08*$
Collagen 2α1	$4.58 \pm 0.26**$	$6.5 \pm 0.51**$	$5.49 \pm 0.74**$
Smad6	1.25 ± 0.21	$1.91 \pm 0.09**$	$1.51 \pm 0.142*$
Smad7	$1.64 \pm 0.201*$	$2.15 \pm 0.16**$	$1.64 \pm 0.03*$
TIMP-1	1.65 ± 0.14	$2.54 \pm 0.17**$	$2.03 \pm 0.04*$
TIMP-2	$1.34 \pm 0.06*$	$2.44 \pm 0.06**$	$2.35 \pm 0.12**$
MMP-9	0.19 ± 0.18	0.22 ± 0.16	0.20 ± 0.05

N = 4 calvaria for control and treatment group. Statistical significance was performed with ANOVA followed by Dunnett's multiple comparison test using GraphPad Prizm 4.0 software. Data are represented as average +/- S.E. (* $p<0.05$, ** $p<0.01$). All values have been normalized to the 4 control cultures, assigned a value of 1.00

PART II

Triterpenoids CDDO-EA and CDDO-Im suppress cytokine induced MMP expression in SW 1353 human chondrosarcoma cell line.

2.1. Introduction

Interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and matrix metalloproteinsases (MMPs) play important roles in the pathogenesis of OA (Shi, 2004). MMPs belong to a family of zinc-dependent enzymes that mediate the turnover of extracellular matrix proteins by its proteolytic activity (Mix, 2001). Low physiologic levels of MMPs are involved in normal development, wound healing, and reproduction. However, aberrant regulation of MMPs and high levels of expression have been implicated as a significant factor in OA as well as RA (Mix, 2001). 3 members out of the 20 member MMP family are particularly important as they are highly effective at cleaving fibrillar collagen, the most abundant component of the extracellular matrix (Jeffery, 1998). Collagenase 1(MMP-1) is expressed most ubiquitously and is found in a variety of cells, including fibroblasts, endothelial cells, smooth muscle cells, chondrocytes, and multiple tumor types (Jeffery, 1998) (Vincenti, 1996). MMP-9 is extremely important for normal tissue remodeling (Okada, 1995). Collagenase 3 (MMP-13) exhibits the broadest substrate specificity of the collagenases, with the highest activity against type II collagen, the main collagen in cartilage (Freije, 1994). After secretion and activation, MMP activities can be down-regulated by inhibition or degradation. Specific endogenous inhibitors, the family of tissue inhibitors of

metalloproteinases (TIMPs 1–4), bind to individual MMPs with high affinity (Dreier, 2004).

The pathologic expression of MMP-1 and MMP-13 can be increased by inflammatory cytokines IL-1β and TNF-α (Mauviel, 1993) (Vincenti, 1996). These cytokines are involved in the progression of OA by inducing the expression of MMPs in synovial fibroblasts and chondrocytes (Dinarello, 1996) (Arend, 1995). Shi *et al.*, examined the differential effects of IL-1β and TNF-α on proinflammatory cytokine and matrix metalloproteinase expression using SW1353 cells (Shi, 2004). They also established an important role for IL-1β in terms of joint inflammation and cartilage destruction in OA.

Previous studies have formed a relationship between triterpenoids and pro inflammatory cytokine induced MMP expression. Mix et al., demonstrated that CDDO is a novel inhibitor of MMP-1 and MMP-13 gene expression mediated by inflammatory cytokines (Mix, 2001). Thus, CDDO may have therapeutic potential for the inhibition of joint degradation in osteoarthritis. Another study by the same group focused on the mechanism used by CDDO to exert its effects. Elliott *et al.*, reiterated the finding that CDDO inhibits IL-1-induced MMP-1 and MMP-13 expression in human chondrocytes. It also inhibits the expression of Bcl-3, an IL-1-responsive gene that preferentially contributes to MMP-1 gene expression (Eliott, 2003).

CDDO shows promise as a therapeutic option against joint destruction in OA; however there have been no studies investigating the role of CDDO derived triterpenoids namely CDDO-EA and CDDO-Im in the catabolic pathway of OA. Since CDDO-EA and CDDO-Im have been found to be more potent compared to their parent compound (Place,

2003), we investigated their effects on IL-1β and TNF-α induced MMP-1, MMP-9 and MMP-13 expression using SW1353 chondrosarcoma cell line.

2.2. Results

A. Effect of TNF-α and IL-1β on MMP gene expression in SW1353 cells.

We investigated the ability of TNF-α and IL-1β to induce MMP expression in SW1353 chondrosarcoma cells. Also, we wanted to establish an appropriate dose for the cytokines to use in further experiments. In our study, cells were treated with 1 ng/ml or 10 ng/ml of TNF-α and IL-1β for 24 hours prior to harvest. Total RNA was isolated and mRNA levels for MMP-1, MMP-9 and MMP-13 was measured using quantitative PCR analysis. MMP-1 and MMP-13 expression was highly upregulated after cytokine treatment (Fig 2.1). Treatment with 10 ng/ml of TNF-α showed a 30 fold and 60 fold increase in MMP-1 and MMP-13 expression respectively. MMP-9 expression was not as drastic with a 10 fold increase by 1 and 10 ng/ml of TNF-α. In the case of IL-1β, MMP-1 showed a similar 25 fold induction with 1 and 10 ng/ml of the cytokine. However, MMP-9 and MMP-13 expression levels, even though induced, were lower compared to MMP-1 (Fig 2.1).

B. Effect of CDDO-Im and CDDO-EA on TNF-α induced MMP expression in SW1353 cells.

In order to understand the effect of synthetic triterpenoids on cytokine-induced expression of MMPs, RNA samples after treatment of SW1353 cells with 1ng/ml TNF-α

and/or CDDO-Im and CDDO-EA were analyzed by quantitative RT-PCR for the induction of mRNA levels of MMP-1, MMP-9 and MMP-13. CDDO-Im strongly inhibited MMP-1, MMP-9 and MMP-13 induction by TNF-α in a dose dependant manner (Fig 2.2). Similar results were observed for CDDO-EA in the case of all three markers. One hundred nM of CDDO-Im or CDDO-EA showed potent inhibitory effects (Fig 2.2). We also measured the mRNA levels of anabolic markers like SOX9, Collagen II, TIMP 1-3 and Smad 6 and 7, but the results were not significant (Table 2.1)

C. Effect of CDDO-Im and CDDO-EA on Il-1β induced MMP expression in SW1353 cells.

In order to understand the effect of synthetic triterpenoids on cytokine-induced expression of MMPs, RNA samples after treatment of SW1353 cells with 1ng/ml IL-1β and/or CDDO-Im and CDDO-EA were analyzed by quantitative RT-PCR for the induction of mRNA levels of MMP-1, MMP-9 and MMP-13. CDDO-Im strongly inhibited MMP-1, MMP-9 and MMP-13 induction by IL-1β in a dose dependant manner (Fig 2.3). However, the effects of CDDO-EA were not as potent especially in MMP- 1 and MMP-9. In the case of MMP-13, 100 nM of CDDO-EA was potent enough to downregulate its expression (Fig 2.3). We also measured the mRNA levels of anabolic markers like SOX9, Collagen II, TIMP 1-3 and Smad 6 and 7, but the results were not significant (Table 2.2)

2.3. Discussion

This part addresses induction of MMP-1 and MMP-13 by inflammatory cytokines and demonstrates that synthesis of these enzymes can be suppressed by the synthetic triterpenoids CDDO-Im and CDDO-EA. Using SW1353 human chondrosarcoma cells to investigate cytokine-mediated expression of MMPs, we have shown that cytokines IL-1β and TNF-α induce MMP expression (Fig 2.1). Other studies have observed the effect of cytokines on different MMP levels and have reached similar conclusions (Mix, 2001) (Mauviel, 1993) (Borden, 1996). This finding emphasizes the importance of pro-inflammatory cytokine inhibitors for the inhibition of MMP expression thus preventing joint destruction in OA. IL-1β and TNF-α inhibitors are currently in clinical trials under the DMOAD program for the treatment of OA (Hunter, 2011).

There were significant differences between the induction levels of TNF-α and IL-1β. TNF-α consistently upregulated MMP-1, MMP-9 and MMP-13 gene expression. IL-1β strongly upregulated MMP-1 expression however, MMP-9 and MMP-13 induction was rather weak (Fig 2.1). This result further emphasizes differential activity of cytokines as described by Shi *et al*., 2004. TNF-α and IL-1β are extremely specific and they each activate a distinct set of genes in chondrosarcoma cells (Shi, 2004). They observed differential regulation by TNF-α and IL-1β in terms of MMP-1 and MMP-13 expression which further confirms our findings.

Previous findings demonstrate the selectivity of triterpenoids among the MMP family (Mix, 2001). We observed that CDDO-Im and CDDO-EA are also highly selective among MMP family members, with MMP-13 and MMP-1 being the major targets of inhibition. The cytokine induction was 16 and 30 fold for MMP-1 and MMP-13

respectively (Fig 2.2). CDDO-Im and CDDO-EA effectively suppressed MMP expression in both the cases. The inhibition of MMP-13 by CDDO-Im and CDDO-EA may be particularly significant because this enzyme is not abundant in normal physiology and seems to be more restricted to pathologic conditions such as OA and RA (Huebner, 1998) (Billinghurst, 1997) (Mitchell, 1996) (Reboul, 1996) (Mix, 2001). Targeting cytokine responsive MMPs may selectively block the pathologic expression of these enzymes, thus avoiding the inhibition of constitutive MMPs required for normal physiology (Mix, 2001).

Dose establishment is crucial for our experiments. Since, 1 ng/ml of TNF-α and IL-1β was efficient for induction of MMP levels, we chose to treat with a lower dose of the cytokines. Also, we show that 100 nM of CDDO-Im and CDDO-EA strongly downregulated MMP levels compared to the lower doses (Fig 2.2 and 2.3). This experiment helped us establish an effective dose for future experiments.

Previous reports have mentioned the ability of CDDO to repress the expression of cytokine inducted MMPs (Eliott, 2003) (Mix, 2001). Here, we successfully demonstrate that CDDO-EA and CDDO-Im are as potent and specific as their parent compound. Future experiments will involve deciphering the mechanism used by these compounds to exert their effects.

2.4 Summary

Pro-inflammatory cytokines TNF-α and IL-1β significantly increase MMP expression in SW1353 human chondrosarcoma cells. The cytokines are extremely specific as they differentially regulated the expression of MMPs. Synthetic triterpenoids

CDDO-Im and CDDO-EA inhibit the cytokine induction of MMP-1, MMP-9 and MMP-13 in a dose dependant manner. However, the mechanism of action remains elusive. This part of the study assigns a novel function to CDDO-Im and CDDO-EA and provides support for its potential therapeutic application in the catabolic pathway of OA.
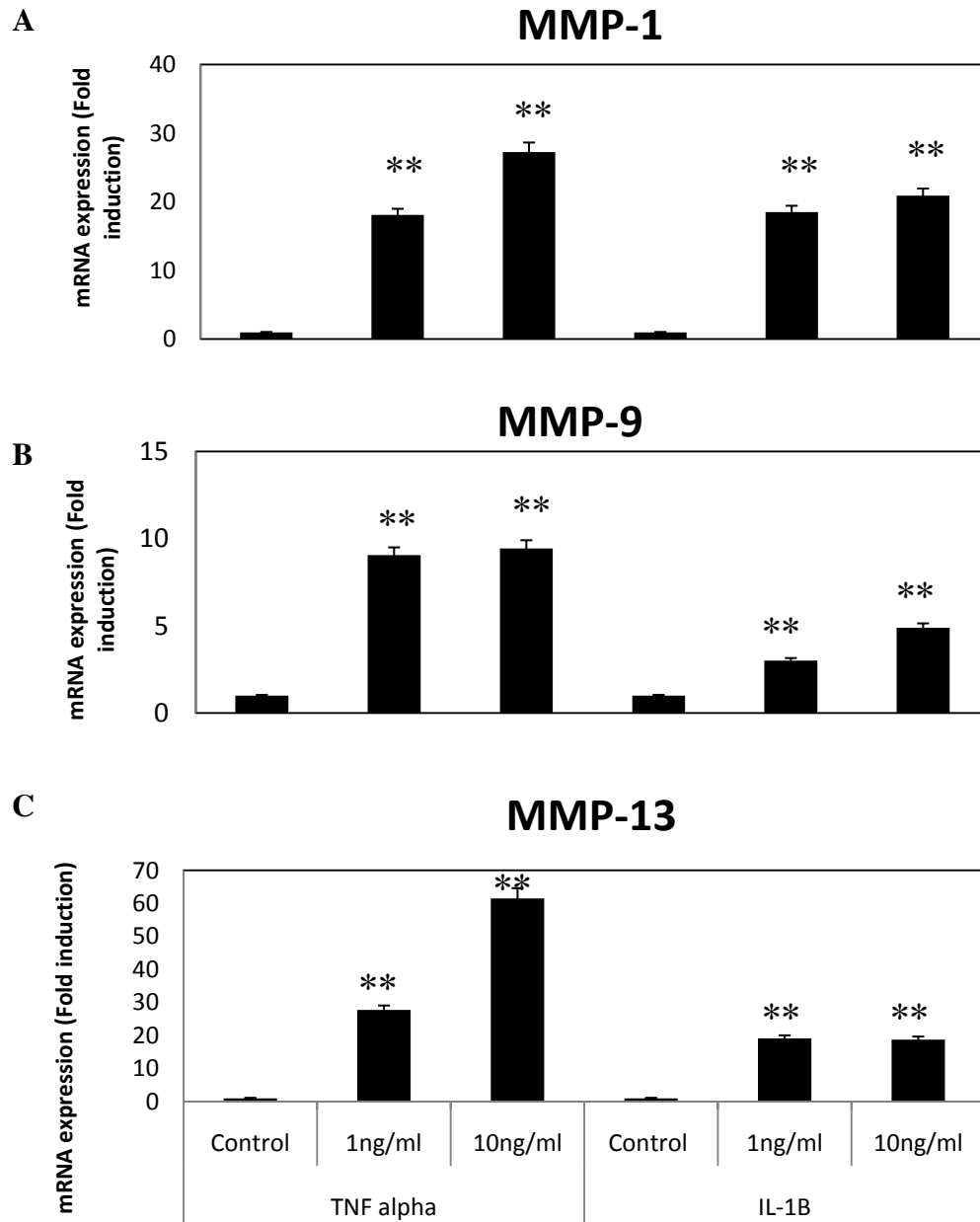
A

MMP-1

B

MMP-9

C

MMP-13

Figure 2.1. TNFα and IL-1β induces MMP activity in SW1353 chondrosarcoma cells in a dose dependant manner. SW1353 cells (4 X 10^5 cells/60mm dish) were treated with 1 ng/ml or 10 ng/ml of TNFα and IL-1β for 24 h prior to harvest. Total RNA was isolated and mRNA levels for MMP-1(A), MMP-9 (B) and MMP-13 (C) were measured using quantitative RT-PCR analysis described in the Materials and Methods section. Signals for all 3 MMPs were normalized to GAPDH. N = 4 per treatment group. Error bars represent standard errors and p values were calculated using ANOVA followed by Dunnetts multiple comparison test (* $p<0.05$, ** $p<0.01$).
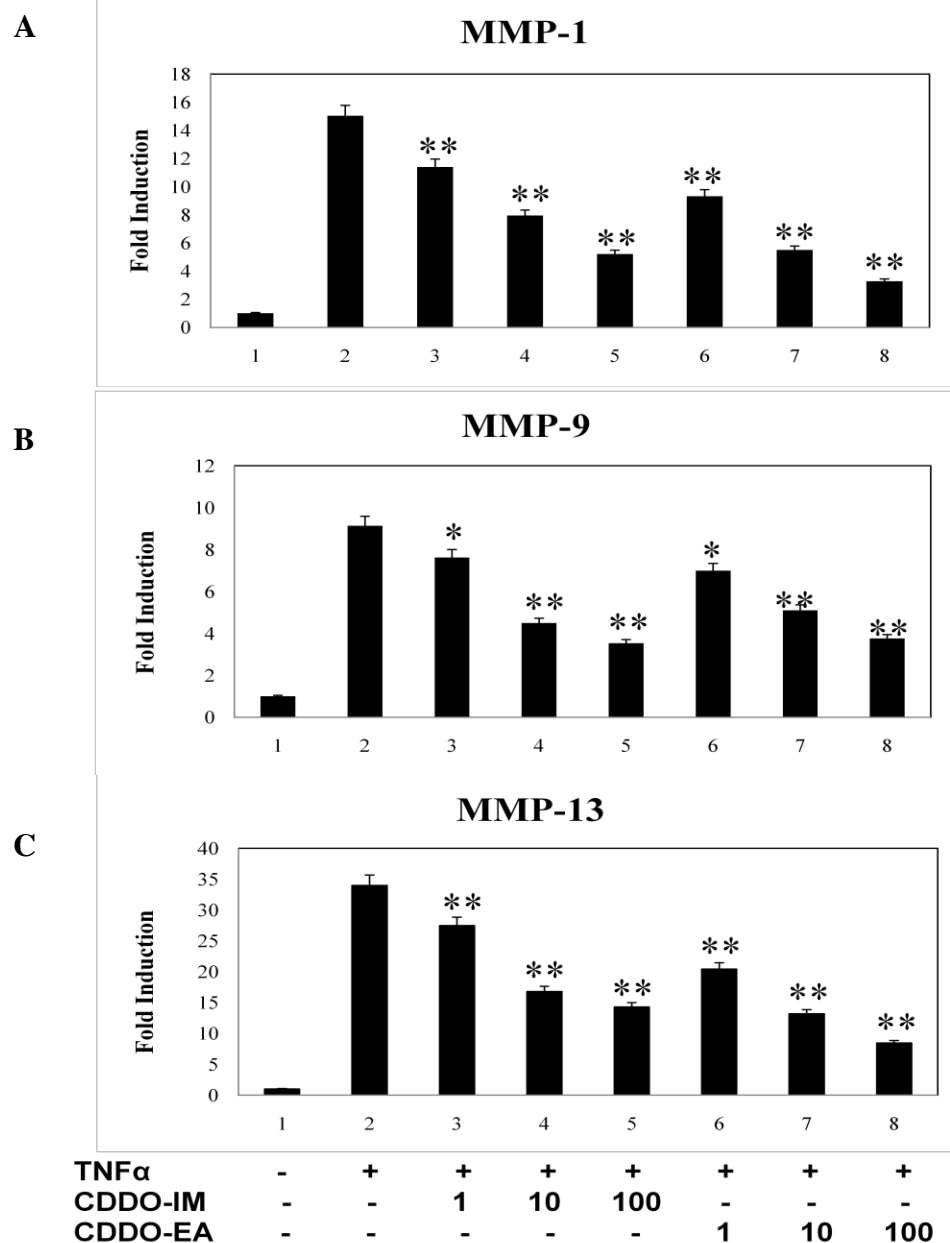
A

MMP-1

B

MMP-9

C

MMP-13

TNFα	-	+	+	+	+	+	+	+
CDDO-IM	-	-	1	10	100	-	-	-
CDDO-EA	-	-	-	-	-	1	10	100

Figure 2.2. CDDO-Im and CDDO-EA downregulate the mRNA expression of TNF-α induced MMPs. SW1353 cells (4 X 10^5 cells/60mm dish) were treated with 1ng/ml TNF-α alone or together with different concentrations of CDDO-Im and CDDO-EA for 24 h. Total RNA was isolated and mRNA levels for MMP-1(A), MMP-9 (B) and MMP-13 (C) were measured using quantitative RT-PCR analysis. Signals for all 3 MMPs were normalized to GAPDH. N = 4 per treatment group. Error bars represent standard errors and p values were calculated in comparison to TNF-α control (2) using ANOVA followed by Dunnetts multiple comparison test (* p<0.05, ** p<0.01).

A

MMP-1

B

MMP-9

C

MMP-13

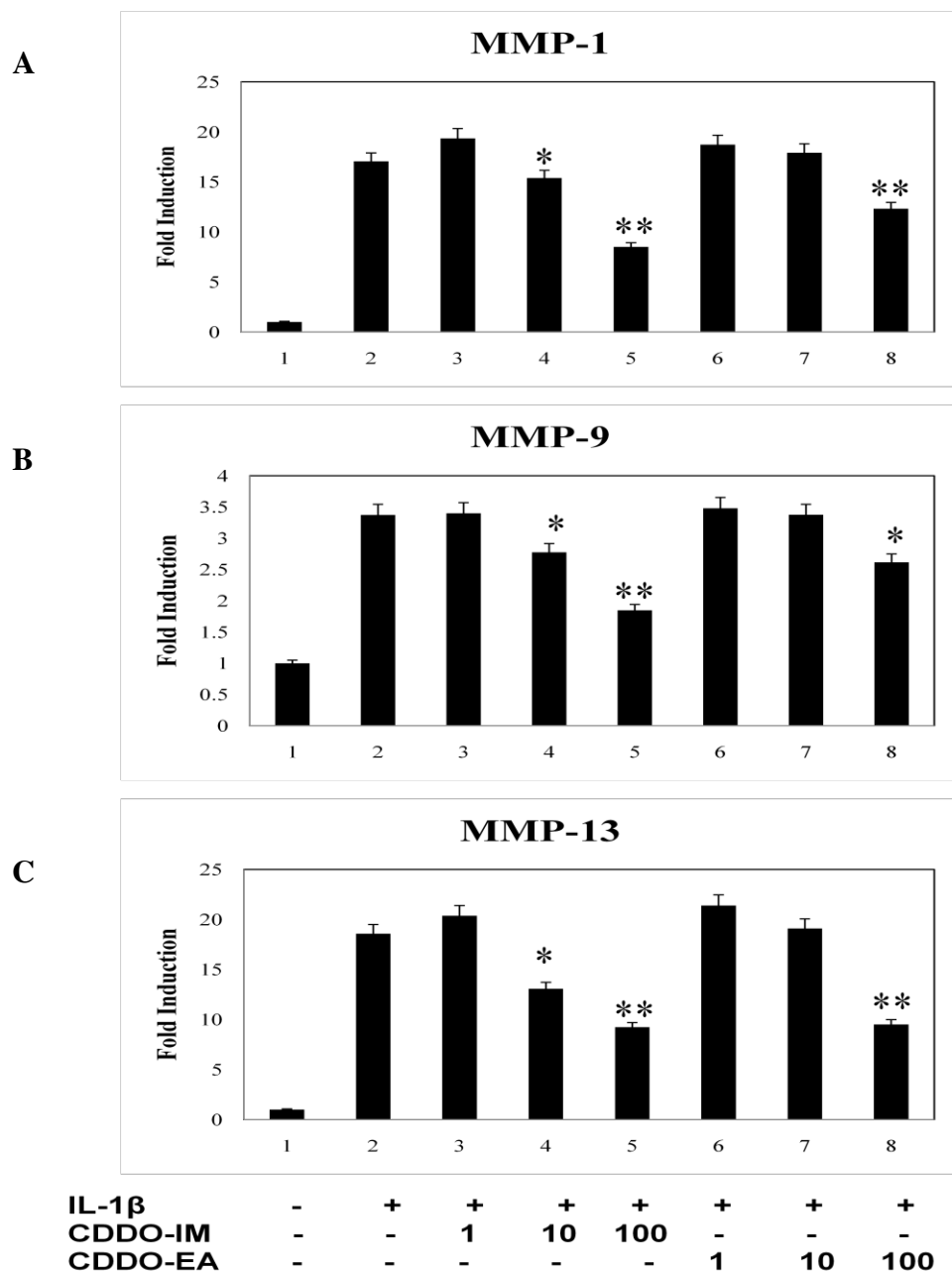| IL-1β | - | + | + | + | + | + | + | + |
| CDDO-IM | - | - | 1 | 10 | 100 | - | - | - |
| CDDO-EA | - | - | - | - | - | 1 | 10 | 100 |

Figure 2.3. CDDO-Im and CDDO-EA downregulate the mRNA expression of IL-1β induced MMPs. SW1353 cells (4 X 10^5 cells/60mm dish) were treated with 1ng/ml IL-1β alone or together with different concentrations of CDDO-Im and CDDO-EA for 24 h. Total RNA was isolated and mRNA levels for MMP-1(A), MMP-9 (B) and MMP-13 (C) were measured using quantitative RT-PCR analysis. Signals for all 3 MMPs were normalized to GAPDH. N = 4 per treatment group. Error bars represent standard errors and p values were calculated in comparison to IL-1β control (2) using ANOVA followed by Dunnetts multiple comparison test (* $p<0.05$, ** $p<0.01$).

Table 2.1. Quantitative RT-PCR analysis for TNF-α in SW1353 cells

Gene	TNF-α	TNF-α + CDDO-Im (1nM)	TNF-α + CDDO-Im (10 nM)	TNF-α + CDDO-Im (100 nM)	TNF-α + CDDO-EA (1 nM)	TNF-α + CDDO-EA (10 nM)	TNF-α + CDDO-EA (100nM)
SOX9	0.84 ± 0.02	1.08 ± 0.4	0.52 ± 0.002	0.49 ± 0.001	0.66 ± 0.012	0.57 ± 0.004	0.46 ± 0.003
NRF2	1.02 ± 0.01	0.37 ± 0.02	0.67 ± 0.0002	0.86 ± 0.007	0.75 ± 0.0002	0.86 ± 0.005	0.86 ± 0.006
Collagen II	0.63 ± 0.0003	0.74 ± 0.015	0.67 ± 0.01	0.75 ± 0.12	0.85 ± 0.017	0.76 ± 0.0008	0.67 ± 0.002
SMAD 6	0.83 ± 0.009	0.95 ± 0.02	0.71 ± 0.003	0.72 ± 0.013	1.014 ± 0.01	0.71 ± 0.008	0.57 ± 0.005
SMAD 7	0.94 ± 0.0001	0.94 ± 0.01	0.78 ± 0.004	0.85 ± 0.008	0.911 ± 0.005	0.82 ± 0.003	0.86 ± 0.005

$N = 2$ for control and treatment group. All values have been normalized to the 2 control plates, assigned a value of 1.00. This data is preliminary and the statistical analysis was not performed because of low sample number.

Table 2.2. Quantitative RT-PCR analysis for IL-1β in SW1353 cells

Gene	IL-1β	IL-1β + CDDO-Im (1nM)	IL-1β + CDDO-Im (10 nM)	IL-1β + CDDO-Im (100 nM)	IL-1β + CDDO-EA (1 nM)	IL-1β + CDDO-EA (10 nM)	IL-1β + CDDO-EA (100)
SOX9	0.78 ± 0.003	0.64 ± 0.5	0.53 ± 0.023	0.49 ± 0.01	0.74 ± 0.03	0.78 ± 0.04	0.41 ± 0.05
NRF2	0.82± 0.02	1.09 ± 0.03	1.21 ± 0.01	0.84 ± 0.006	1.05 ± 0.1	1.12 ± 0.03	0.91 ± 0.13
Collagen II	0.84 ± 0.03	0.82 ± 0.16	0.90 ± 0.04	0.83 ± 0.14	0.84 ± 0.12	1.1 ± 0.01	0.89 ± 0.003
SMAD 6	0.91 ± 0.1	1.21 ± 0.01	0.93 ± 0.03	0.95± 0.016	1.06 ± 0.01	1.09 ± 0.0003	0.85 ± 0.002
SMAD 7	0.82 ± 0.2	0.91 ± 0.01	1.3 ± 0.06	0.92 ± 0.01	0.87 ± 0.003	0.85 ± 0.1	0.78 ± 0.001

N = 2 for control and treatment group. All values have been normalized to the 2 control plates, assigned a value of 1.00. This data is preliminary and the statistical analysis was not performed because of low sample number.

CONCLUSION

We have investigated the effect of CDDO-Im and CDDO-EA in the anabolic as well as catabolic pathways of osteoarthritis using mouse calvarial organ cultures and SW1353 chondrosarcoma cell line. CDDO-Im and CDDO-EA potently induce chondrogenesis in mouse calvaria by upregulating the expression of primary cartilage markers in a dose and time dependant manner. We also determined the ability of rexinoid LG100268 and the vitamin D (1α $25(OH)_2$ D3) to induce chondrogenesis in mouse calvaria. LG100268 downregulated the expression of cartilage markers which might possibly suggest an opposite effect compared to triterpenoids in mouse calvaria. 1α $25(OH)_2$ D3 showed no significant effects at the dose tested. CDDO-Im and CDDO-EA were capable of suppressing pro-inflammatory cytokine-induced MMP expression in SW1353 cells. Since MMPs are involved in degradation of cartilage, it provides a novel function to triterpenoids for the treatment of diseases involving MMP deregulation. These results suggest that CDDO-Im and CDDO-EA can be considered to be useful agents potentially for treatment of osteoarthritis.

FUTURE DIRECTIONS

We have some ongoing experiments investigating the effect of triterpenoids and rexinoids in mesenchymal stem cell differentiation. Mesenchymal stem cells (MSCs) or bone marrow stromal stem cells are a prototypical adult stem cell with capacity for self-renewal and differentiation with a broad tissue distribution (Williams, 2011). Evidence for a population of cells with multilineage mesodermal differentiation capacity was first demonstrated by Friedenstein and colleagues in seminal studies that demonstrated the ability of mesenchymal populations to generate cartilage, bone, myelosupportive stroma, adipocytes, and fibrous connective tissue (Friedenstein, 1968). They participate in different functions like organ homeostasis, wound healing, and successful aging (Williams, 2011).

With an increasing aging population, clinical imperatives to augment and facilitate skeletal tissue lost as a consequence of trauma or degeneration have led to increased interest in these progenitor cells (Oreffo, 2005). From a therapeutic perspective, and facilitated by the ease of preparation and immunologic studies, MSCs are emerging as an extremely promising therapeutic agent for tissue regeneration (Williams, 2011). The lack of efficient modalities of treatment for large chondral defects has prompted research on tissue engineering and promising therapies rely on the use of MSCs which can differentiate towards chondrocytes (Richter, 2009). MSC from bone marrow were purchased from ScienCell (Carlsbad, CA) and RNA as well as protein assays are currently being conducted to test the hypothesis that triterpenoids induce differentiation in mesenchymal stem cells. Expression of various cartilage markers will be analyzed.

We plan to execute several studies to decipher the mechanism triterpenoids use to exert their anabolic as well as catabolic effects. There are several cellular pathways that are regulated by triterpenoids (Liby, 2007). We wish to use primary bone marrow mesenchymal stem cells to examine these pathways. Currently, we are working on optimizing the protocol for isolation of bone marrow stromal cells from mice. We plan to execute several studies to observe the effect of triterpenoids on the Nrf2 pathway.

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