Selecting Dietary-derived Bioactives which Protect Cartilage from Destruction in Osteoarthritis

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Abstract

Osteoarthritis (OA) is a disease that affects the whole joint, with progressive articular cartilage loss. Healthy cartilage must maintain a balance between extracellular matrix synthesis and degradation. Metalloproteinases (Matrix metalloproteinases, MMPs and a disintegrin and metalloproteinase domain with thrombospondin motifs, ADAMTSs) play an important role in cartilage degradation. The collagenases (MMP-1 and MMP-13) and aggrecanases (ADAMTS-4 and ADAMTS-5) are implicated in the development of OA because of their ability to degrade type 2 collagen and aggrecan. This project aims to identify compounds from the diet that could offer protection or slow the progression of OA via collagenase and/or aggrecanase inhibition.

Ninety-six dietary derived compounds selected from a Natural Product Library were screened at 10μM for IL1-induced and basal *MMP13* inhibition in SW1353 and C28/I2 cells. Several compounds inhibited *MMP13* at 10μM in both cell lines. Twenty compounds were selected for study in primary human articular chondrocytes. Of these compounds, apigenin and isoliquiritigenin showed the greatest inhibition of *MMP13* while also inhibiting *MMP1, ADAMTS4* and *ADAMTS5*. The effect of apigenin or isoliquiritigenin pretreatment on three pathways implicated in OA, the NFκB, TGFβ and Wnt pathways was analysed. Apigenin and isoliquiritigenin showed differences in the patterns of inhibition in the NFκB and TGFβ pathways. The Human Phospho-Kinase Array Kit was used to perform an unbiased dissection of the kinase pathways affected by apigenin and isoliquiritigenin. Apigenin and isoliquiritigenin had similar effects on many phosphorylation events, however there were kinase phosphorylation events that were specific to either apigenin or isoliquiritigenin treatment. Taken together these data suggest that the chondro-protective gene expression changes seen with apigenin and isoliquiritigenin treatment involves a complex network of signalling pathways that require further characterisation. If these compounds are able to get to the site of OA damage they may prevent progression of OA offering an alternative treatment.

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Abbreviations

AA	Adjuvant Arthritis
Adipo R1	Adiponectin receptor 1
AP	Apigenin
AP-1	Activator protein-1
APS	Ammonium Persulfate
ADAM	A Disintegrin And Metalloproteinase
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
BCA	Bicinchoninic acid
bFGF	Basic fibroblast factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CMC	Carboxymethylated Chitin
COX	Cyclooxygenase
CREB	The cAMP Response Element-binding Protein
Ct	Threshold Cycle
DMEM	Dulbecco's Modified Eagle's Medium
DMHP	Dimethoxy Hesperetin
DMM	Destabilisation of Medial Meniscus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGCG	Epigallocatechin-3-gallate

EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular signal-regulated Kinase
ESP	Epithiospecifier Protein
FAK	Focal Adhesion Kinase
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
GPI	Glycosylphosphatidylinositol
GSK-3	Glycogen Synthase Kinase 3
HDAC	Histone Deacetylases
HDL	High-density Lipoprotein
HSP27	Heat Shock Protein 27
HSP60	Heat Shock Protein 60
IGD	Interglobulin Domain
IGF	Insulin like Growth Factor
IKK	Inhibitor of Kappa B Kinase
IL1	Interleukin 1
IL6	Interleukin 6
IL1Ra	IL1 receptor antagonist
iNOS	Nitric Oxide Synthase
ISL	Isoliquiritigenin
JAK	Janus Kinase
JNK	c-JUN end terminal Kinases
JSN	Joint Space Narrowing
KOA	Knee Osteoarthritis
Lck	Lymphocyte-specific protein tyrosine kinase
LDH	Lactate Dehydrogenase

LDL	Low-density Lipoprotein
LPS	Lipopolysaccharide
Lyn	Lck/Yes novel tyrosine kinase
MAPK	Mitogen-activated protein kinases
MAPKKKs	MAPK kinase kinases
MDCK	Madin-Darby Canine Kidney Cells
MMP	Matrix Metalloproteinase
M-MLV	Moloney Murine Leukemia Virus
MT-MMP	Membrane-type MMP
NaBy	Sodium Butyrate
NFκB	Nuclease Factor kappa B
NGF	Nerve Growth factor
NHS	National Health Service
NO	Nitric Oxide
NSAID	Non-steroidal Anti-inflammatory Drug
NT	No Treatment control samples
OA	Osteoarthritis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHC	Primary Human adult articular Chondrocytes
PRAS40	Proline-rich AKT1 substrate 40
PUMA	p53-upregulated Modulator of Apoptosis
PVDF	Polyvinylidene Fluoride membrane
qRT-PCR	Quantitative Reverse Transcription PCR
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Polyacrylamide Gel Electrophoresis
SFN	Sulforaphane
siRNA	Short Interfering RNA

SNP	Single Nucleotide Polymorphism
SP1	Specificity Protein 1
Src	Proto-oncogene tyrosine-protein kinase Src
STAT	Signal Transducer and Activator of Transcription
SOX9	SRY(sex determining region Y) -type HMG (High mobility group box)
TBS	Tris-Buffered Saline
TBST	Tris-buffer saline with 0.1% (v/v) Tween-20
TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor beta
TIMP	Tissue Inhibitor of Metalloproteinase
TOR	Target Of Rapamycin
TUNEL	Immunohistochemistry and dUTP nick End Labeling
UTR	Untranslated region

Chapter 1: Introduction

1.1 Synovial Joint structure

The most common joint of the human skeleton is the synovial joint. A thin layer of hyaline cartilage (Figure 1.1) known as articular cartilage covers the end of bone providing the joint with a smooth surface for joint movement. A fibrous capsule encloses the joint, this capsule is lined with a synovial membrane. The cells that reside in the synovial membrane secrete synovial fluid into the synovial cavity helping to reduce friction and lubricate the joint. Ligaments hold the bones of the joint together.

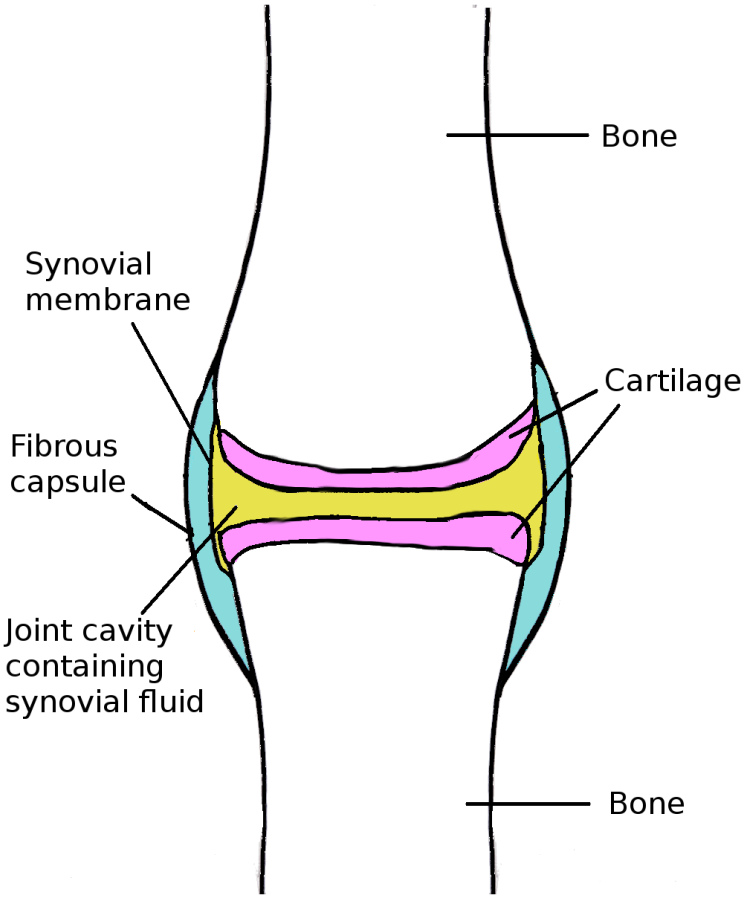


Figure 1.1. Diagram showing the structure of the synovial joint.

1.1.1 Articular cartilage and structure

Healthy articular cartilage provides the joint with a friction reducing weight-bearing surface. Articular cartilage is highly elastic able to deform and regain its shape. Articular cartilage distributes load evenly over the surface of the joint which avoids peak stresses occurring on the subchondral bone (James & Uhl, 2001). When compared with other soft tissues it has a low level of metabolic activity lacking nerves, lymphatic vessels and blood vessels.

Articular cartilage consists of four distinct regions of matrix (Figure 1.2). The first is the superficial zone; this area contains elongated and relatively inactive chondrocytes organized parallel to the articular surface. The matrix contains fine fibrils with very few polysaccharides. Chondrocytes produce lubricin in this surface layer which creates the smooth gliding surface the joint (Chang et al., 2009). The second zone is known as the transitional zone. The collagen fibrils of this layer are larger than those of the superficial zone and their orientation transitions from parallel to a columnar formation (James & Uhl, 2001), chondrocyte metabolic activity is also much more pronounced. This transitional and deeper zone contain high proteoglycan content and the prominent collagen fibres increase in diameter. The third zone is known as the deep zone. This zone also contains chondrocytes but they are larger, elongated and are organised in a columnar pattern perpendicular to the joint surface with the cells themselves containing lots of intermediate filaments. This zone also contains the largest collagen fibrils with the highest content of proteoglycans (Pearle et al., 2005). The final zone is the calcified zone that divides soft cartilage from subchondral bone. Here the chondrocytes contain almost no endoplasmic reticulum and little cytoplasm. Cartilage is anchored to subchondral bone via calcified collagen fibres (Clouet et al., 2009).

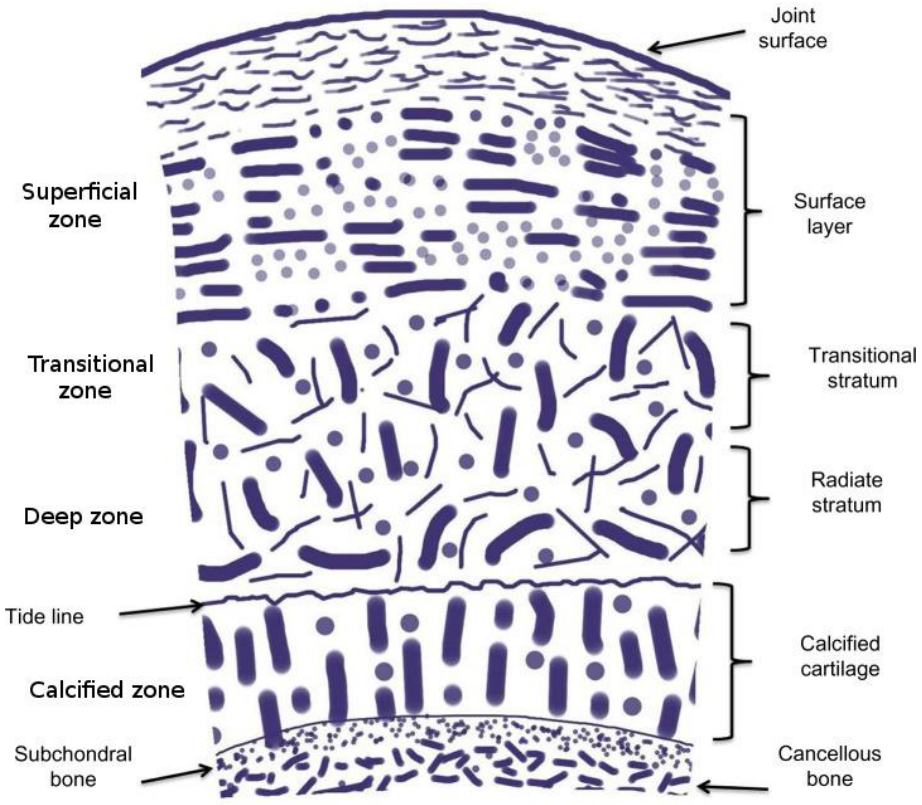


Figure 1.2. The four distinct regions of matrix. Schematic diagram of articular cartilage showing its different zones, compositions, and organisation, image adapted from (Levangie & Norkin, 2005).

1.1.2 The extracellular matrix structure and function

The extracellular matrix consists of three main matrices, the pericellular, territorial and interterritorial matrices (Figure 1.3). The territorial and interterritorial matrices are organised in a way to protect tissue from mechanical load. This matrix is rich in aggrecan, chondroitin sulphate proteoglycan and type 2 collagen (Vincent, 2013). The pericellular matrix surrounds the chondrocyte and is between 1-5µm thick (Poole, 1997) and rich in hyaluronan, proteoglycans and various aggregates of type 6 collagen (Knudson, 1993). The pericellular matrix has become an area of interest recently with research focusing on how the pericellular matrix stores growth factors and how the matrix responds to mechanotransduction (Vincent et al., 2007). Type 6 collagen is rich in the pericellular matrix and is an important molecule that forms a mechanical interface between type 2

collagen providing a link between the rigid interterritorial cartilage matrix and the chondrocyte (Söder et al., 2002). Together the chondrocyte and the surrounding pericellular matrix are collectively known as a chondron. Chondrons were classically described in 1925 by Benninghof as a 'fluid filled bladder' (Benninghoff, 1925). Type 6 collagen plays a major role in mechanical properties of the pericellular matrix. Type 6 collagen KO mice have an accelerated development of osteoarthritis, which may be due to the altered properties of the pericellular matrix which can no longer regulate the biomechanical environment of the chondrocyte (Alexopoulos et al., 2009).

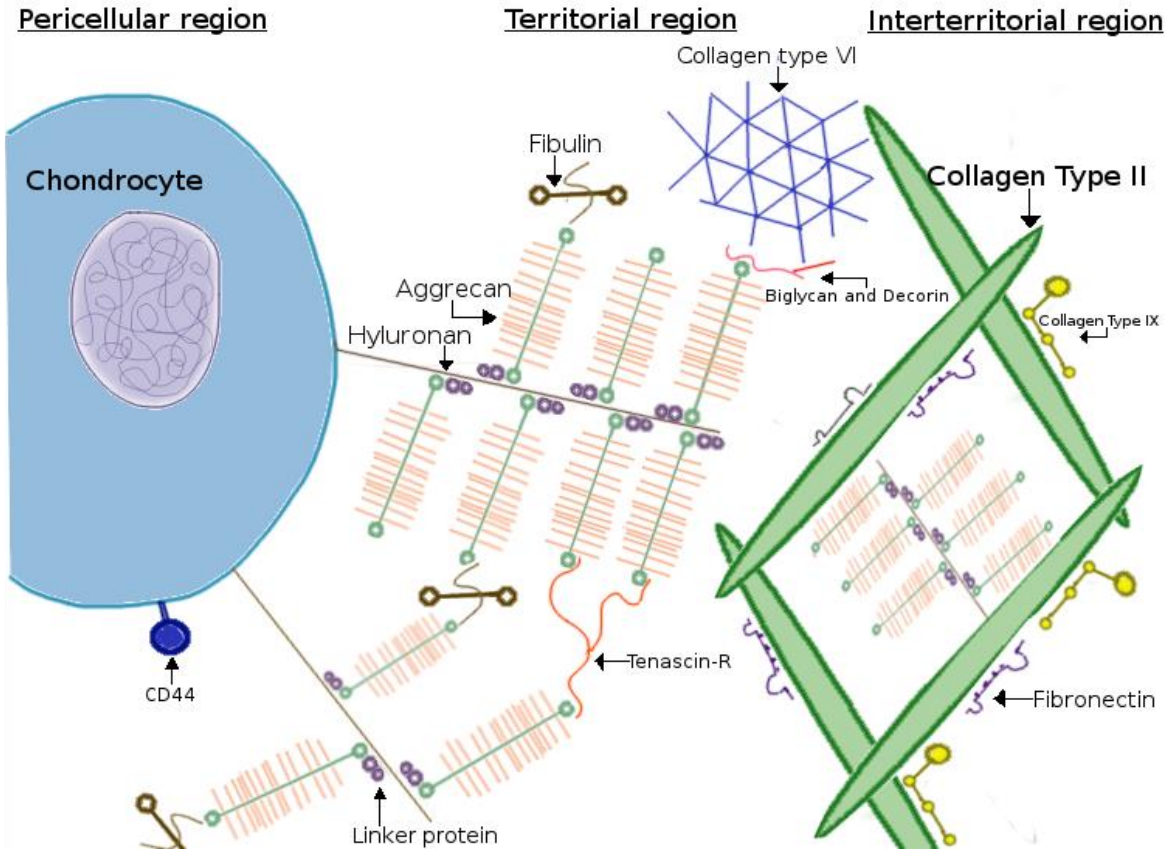


Figure 1.3. The assembly of aggregates and extracellular matrix components. Articular cartilage ECM consists of the pericellular, territorial and interterritorial regions. The territorial and interterritorial regions are rich in aggrecan and type 2 collagen. The pericellular and territorial regions consist of type 6 collagen which can interface with type 2 collagen. Fibulin and tenascin-R can associate with aggrecan. In the interterritorial region aggregates of hyaluronan and aggrecan can be found within type 2 collagen networks. The ECM is further strengthened by the non-collagenous protein COMP (cartilage oligomeric protein), type 9 collagen, fibronectin and decorin. Modified from (Dudhia, 2005), (Heinegård & Saxne, 2011).

Cartilage is composed of sparsely scattered chondrocytes and an extracellular matrix (ECM) comprised of, type 2 collagen along with various other minor collagens, proteoglycans (the most common being aggrecan), glycoproteins, glycosaminoglycans (GAGs), water and calcium salt. Healthy cartilage has a rapid proteoglycan turnover rate and a slow collagen turnover rate. Proteoglycans can be divided into two groups based on the nature of their core proteins, the larger hyaluronan-binding proteoglycans such as aggrecan and smaller proteoglycans including fibromodulin, biglycan, decorin and epiphycan (Iozzo, 1998). The larger proteoglycans are bound to hyaluronan non-covalently (Hardingham & Muir, 1974). The smaller proteoglycans core proteins consist of leucine-rich repeat structures, these structures can interact with cell membrane components, matrix glycoproteins and collagen (Iozzo, 1998).

Small proteoglycans may have a role in the correct spacing of collagen fibrils via the formation of glycosaminoglycan chains (Scott, 1991). Of these smaller glycoproteins biglycan and decorin contain either one or two chondroitin/dermatan sulphate chains, these chains potentially allow further interactions with other molecules (Bock et al., 2001). Decorin and biglycan mRNA has been detected in human bone, skin and periodontal ligament cells in culture (Fisher et al., 1989). Within hyaline cartilage from the knee, light and electron microscopical immunohistochemistry detected decorin in the interterritorial matrix and in vesicles in chondrocytes where it may modulate collagen metabolism (Miosge et al., 1994). In the same study the majority of biglycan was found in the pericellular matrix adjacent to chondrocytes, with both decorin and biglycan close or on collagen fibrils associating with globular structures of the matrix between collagen fibrils.

1.1.3 The structure and function of type 2 collagen

The collagens are a family of fibrous proteins. In mammals they constitute to 25% of the total protein mass (Erat et al., 2009). Collagen is one of the molecules involved in providing cartilage with its high tensile strength (Figure 1.4). Collagen forms the basic components of the ECM and is necessary for the structural integrity of vertebrate tissues (Liska et al., 1994). During muscular deformation collagen can store elastic energy (Silver et al., 2009), excess energy can then be transferred from the joint back to the attached muscles for dissipation (Silver et al., 2001). Collagen fibrils provide a biomechanical scaffold which

anchors macromolecules creating an environment for cell attachment, giving shape and form to a tissue (Kadler et al. 1996). Depending on the tissue, collagen fibrils can be arranged with different suprafibrillar architectures (Jokinen et al., 2004) with diameters in the range of 10-500nm (Holmes et al., 1992).

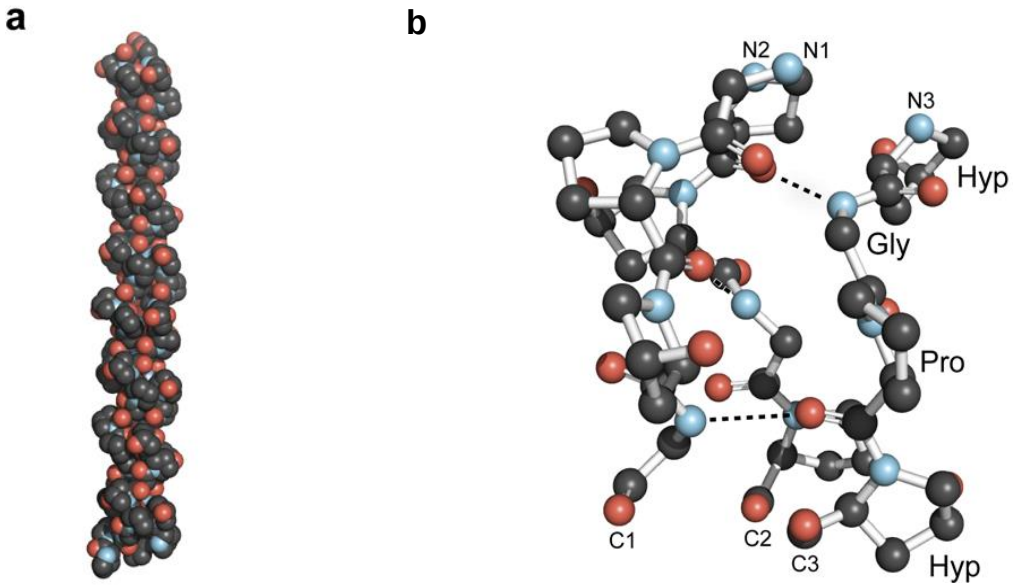


Figure 1.4. Structure of collagen (a) High resolution crystal structure of a collagen triple helix. **(b)** Ball and stick image of a segment of collagen triple helix. Showing the ladder of interstrand hydrogen bonds (Bella et al., 1994). Image adapted from (Shoulders & Raines, 2009).

Molecules of collagen consist of triple – stranded helical structures made from three α polypeptide chains, each α chain is made up of a left handed helix with an expanse of 18 amino acids per turn of the helix (Se-Kwon, 2013). The synthesis of collagens begins in the endoplasmic reticulum with the formation of three polypeptide chains forming procollagen (Jackson, 1978). The three α chains that form procollagen can be identical, such as with type 2 collagen which contains three α1 chains, or different such as type 1 collagen which contains two α1 chains and a single α2 chain. The composition of α chains consists of a series of triplet Gly-X-Y amino acid sequences. Glycine is the smallest amino acid allowing the three helical α chains to pack tightly together to form a right handed superhelix (Cao et

al., 2012). Proline is commonly the X amino acid, its ring structure stabilises the helical conformation in each α chain (John et al., 1999). Hydroxyproline is often in the Y position of the amino acid sequence. The hydroxyl groups of hydroxyproline form interchain hydrogen bonds that help stabilise the triple stranded helix (Hickman et al., 2000). The triple stranded helices can cross-link covalently at the N and C terminals via lysine residues forming collagen fibrils (Eyre et al., 2005). This cross-linking of helices is catalysed by lysyl oxidase. Pro collagen consists of distinct regions (Figure 1.5), the N-propeptide, C-propeptide, the α-chains and the flanking extrahelical telopeptides (Bulleid et al., 1997). Proteolytic processes help convert procollagen into its mature form by specific N terminus A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTSs) cleavage during and following secretion from the cell (Fukui et al., 2002). C-propeptides allow the procollagen to maintain its solubility prior to its assembly into insoluble polymers (Daviesz et al., 1997). N-propeptides are involved in the biogenesis, maturation and function (Bornstein, 2002) as well as influence fibril shape and diameter (Cluzel et al., 2000). The procollagen which is around 300nm inside the endoplasmic reticulum (Stephens, 2012) is then packed into Golgi-to-PM carriers (Canty & Kadler, 2005). Processing of procollagen has been thought of as an extra-cellular event as enzymes capable of converting procollagen to collagen are found in the medium of cultured cells (Kerwar et al., 1973). Fibrils were thought to form spontaneously as mature collagen self assembles, with various ECM components involved in the spatial regulation of fibrillogenesis (Kadler et al., 2008). However more recent research suggests that nonmuscle myosin 2 transports newly formed collagen fibrils at the plasma membrane and is fundamental to the development of collagen fibril-rich tissues (Kalson et al., 2013).

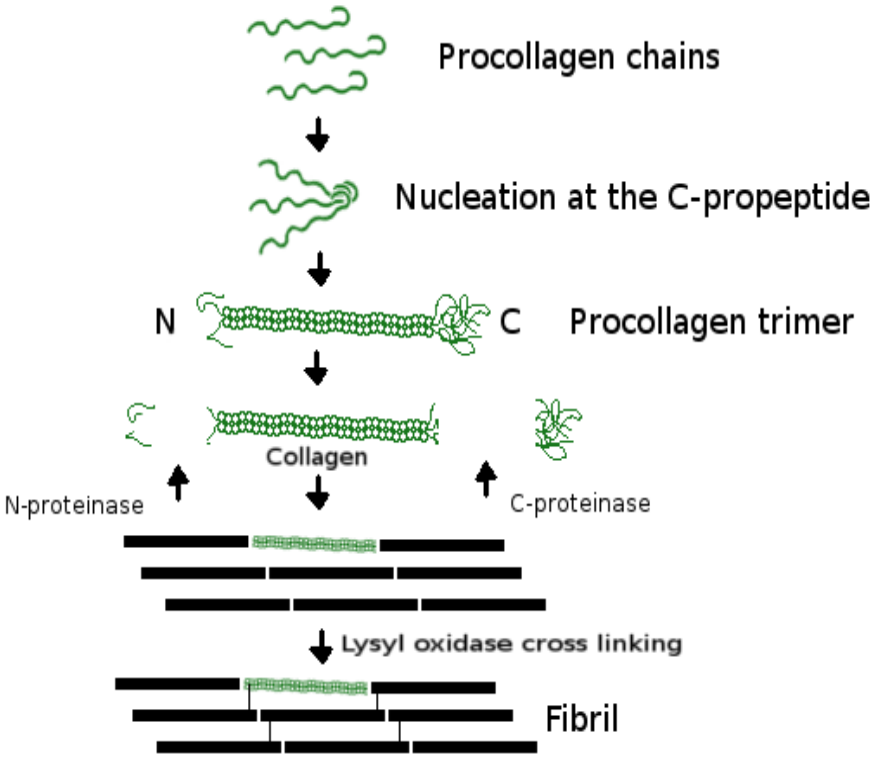


Figure 1.5. Collagen fibril formation. Procollagen consists of three 300nm long α chains that are synthesized in the endoplasmic reticulum. The procollagen α chains aggregate to form a rod like triple helix trimer flanked by a trimeric globular N-propeptide and a C-peptide domain. Metalloproteinases cleave the procollagen at the N and C terminal globular propeptides forming mature collagen. Mature collagen self assembles spontaneously into fibrils which are then cross-linked together by lysyl oxidase stabilizing the structure. Image adapted from (Kadler et al., 1996), (Canty & Kadler, 2005).

Type 2 collagen can interact with other cartilage specific collagens such as collagen types 9 and 11, aggrecan and other matrix proteins (Table 1) (Goldring, 2000).

Collagen Type	Structural features	% of total collagen
2	300nm-long fibrils $[\alpha1(II)]_3$	75%foetal, >90% adult
3	300nm-long fibrils $[\alpha1(III)]_3$	>10% adult
6	Periodic globular domains α1(VI), α2(VI), α3(VI)	Chondron basket microfilaments <1%
10	Hexagonal lattice $[\alpha1(X)]_3$	Hypertrophic cartilage only
11	Fibrillar α1(XI), α2(XI), α3(XI)	Fibril template 10% foetal, 3% adult
13	Transmembrane	(Transmembrane)
12/14	Fibril associated collagens with interrupted triple helices $[\alpha1(XII)]_3/[\alpha1(XIV)]_3$	Non-covalently fibril-associated collagens

Table 1. Collagens of cartilage tissue. Figure adapted from (Lodish, 2000) and (Eyre et al., 2006).

1.1.4 The structure and function of aggrecan

The most abundant proteoglycan present in cartilage is aggrecan (Mathews & Lozaityte, 1958). Aggrecan is a large aggregating proteoglycan. Proteoglycans are distinguished from other glycoproteins by the arrangement of their sugar side chains. At least one of the side chains of a proteoglycan is a glycosaminoglycan (GAG). Over 90% of the mass of aggrecan is comprised of substituted GAG chains (Kiani et al., 2002). Aggrecan interacts with a hyaluronic acid filament bound via globular domain 1 (Figure 1.6). Hyaluronic acid is a

ubiquitous repeating disaccharide unit of D-glucuronate and N-acetyl-D-glucosamine (H. Watanabe, 1997). Aggrecan GAG molecules contain chondroitin sulphate and keratin sulphate chains linked to a serine rich core protein. Chondroitin sulphate is a repeating disaccharide unit of D-glucuronic acid and N-acetyl-D-glucuronic acid. Keratin sulphate is a repeating disaccharide unit of D-galactose and N-acetyl-D-glucosamine. The sugar residues in GAGs can be sulphated giving them their negative charge. Aggrecan has a direct functional role in providing osmotic resistance to the cartilage, allowing cartilage to resist compression (Knudson & Knudson, 2001). The negative charge of aggrecan attracts cations causing water to be drawn into the molecule. In this hydrated form GAGs enable cartilage to absorb large pressure changes (Prydz & Dalen, 2000). This allows cartilage to sustain physiological levels of stress in the range of 2-12 mPA (Park et al., 2003). During compression most water leaves the GAG molecule but its strong negative charge prevents complete compression. Once pressure has been released GAGs become rehydrated. This mechanism allows cartilage to resist compression. This mechanism also causes the movement of interstitial fluid vital for the sustenance of chondrocytes in an avascular tissue (Y. Wang et al., 2012). A critical and early event in the pathogenesis of OA is the loss of aggrecan from cartilage, which is enzymatically mediated by aggrecanase activity (Tortorella & Malfait, 2008).

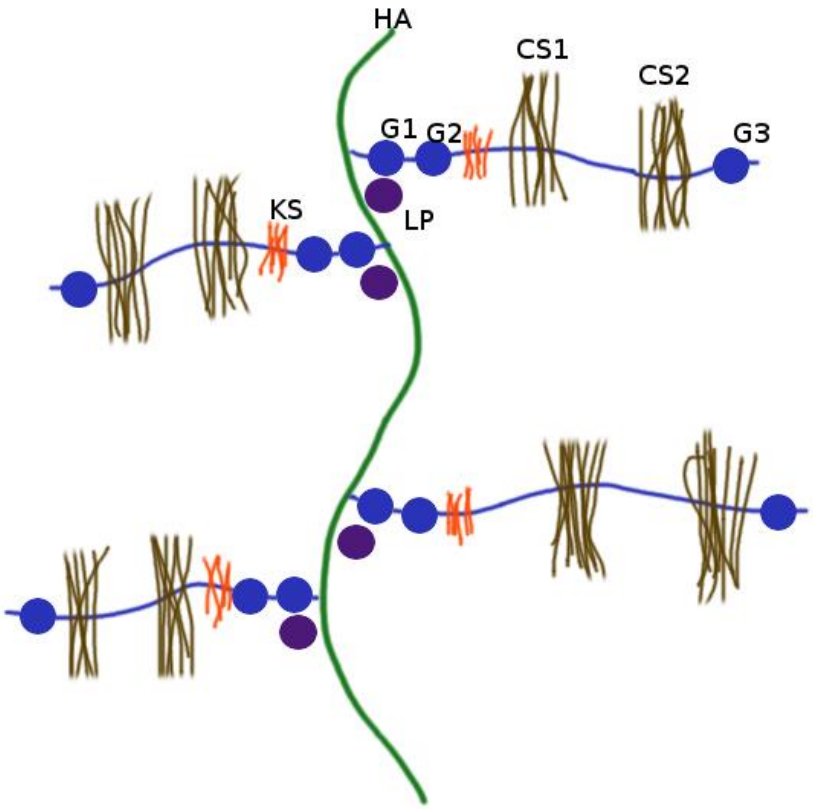


Figure 1.6 Schematic representation of aggrecan. HA: hyaluronic acid, CS1, CS2: chondroitin sulphate domains 1 and 2 KS: keratin sulphate; G1, G2, G3: globular domains; LP: link protein. Image adapted from (Jerosch, 2011).

1.1.5 Chondrocytes and their function in cartilage

The single cellular component of adult hyaline cartilage is the terminally differentiated chondrocyte. Chondrocytes account for 1-5% of the cartilage total volume (Jerosch, 2011). Both collagen and proteoglycan synthesis and degradation are mediated by chondrocytes (DeGroot et al., 2001). Chondrocytes arise from mesenchymal progenitors during skeletal development where they form the cartilage anlagen (Goldring et al., 2006). Mesenchymal cells condense expressing type 1, 2, 3 and 5 collagens with chondroprogenitor cells differentiating and expressing cartilage specific collagen types 2, 9, and 11 (Goldring, 2012). Chondrocytes then undergo proliferation, terminal differentiation, hypertrophy, eventually undergoing autophagy and apoptosis (Goldring et al., 2006).

After chondrogenesis occurs long bones develop from the cartilage analgen in a process known as endochondral ossification where cartilage matrix becomes calcified and chondrocytes switch to a hypertophic phenotype (Ferguson et al., 1998), (Poole, 1982). Adult chondrocytes survive in avascular tissue in a severely hypoxic environment (Xu et al., 2007). When cartilage is healthy chondrocytes have a low turnover of cartilage matrix proteins (Maroudas et al., 1998). Chondrocytes in healthy cartilage undergo anabolic processes (those that result in the synthesis of cartilage matrix components) and these are in equilibrium with catabolic processes (those that result in the normal turnover of matrix molecules) (Fukui et al., 2001). When a joint injury occurs chondrocytes attempt to repair cartilage by increasing anabolic processes however these repair attempts creates a cycle of anabolism and catabolism and eventually catabolic processes overpower the cells anabolic processes, this eventually results in cartilage erosion. Chondrocytes are responsible for the homeostasis of the ECM and can interact with the ECM via integrins and discoidin domain receptors (DDRs) (Aszodi et al., 2003), (van den Berg, 2011). Leucine-rich proteins, chondroadherin and PRELP (proline/arginine rich end leucine rich repeat protein) may be involved in interactions between chondrocytes and the surrounding matrix. These two leucine-rich proteins bind via membrane proteins such as syndecan and α2β1 integrin (Goldring, 2000). Chondrocyte senescence is likely to occur extrinsically through chronic stress as adult articular chondrocytes rarely if ever divide in normal tissue in vivo (Aigner et al., 2001). However in senescent chondrocytes β-gal activity has been reported to increase and telomere length decreases in oxidative induced senescence accelerated mice (Shimada et al., 2011). Aged chondrocytes are associated with the release of glycosaminoglycans and the production of reactive oxygen species, this results in the homeostasis of the ECM becoming more difficult to maintain for aged chondrocytes, leading to a breakdown and loss of cartilage as well as a decline in the proliferative and anabolic response to growth factors such as TGF-β (Loeser, 2009). Chondrocyte autophagic cell death has been reported to be higher in osteoarthritis patients when compared to healthy aged chondrocytes (Yin, 2013). These senescent and autophagic phenotypes of chondrocytes may contribute to age being a risk factor for osteoarthritis.

1.2 Osteoarthritis

Osteoarthritis (OA) is a disease that affects the whole joint with progressive and focal hyaline articular cartilage loss with accompanying morphological changes to the subchondral bone (Felson et al., 2000). Soft tissue structures in and around the joint are also affected, causing pain due to swelling and inflammation (Wise & Factors, 2007). Osteoarthritis causes a huge burden on many countries' healthcare systems. In the UK the costs of non-steroidal anti-inflammatory drug treatments (NSAIDS) are predicted to cost £44.85 million, with hip and knee replacement costs exceeding £850 million (A. Chen et al., 2012). The economic strain caused by OA is due to its high prevalence and effects of disability. The world's population is ageing and the prevalence of OA increases with age (Lawrence et al., 2008). The burden on healthcare systems will only increase with indirect costs from OA causing a loss of economic production over £3.2 billion (A. Chen et al., 2012).

1.2.1 Epidemiology

Osteoarthritis is the most common form of arthritis and is responsible for joint replacement surgery every 1.5 minutes in Europe (Wieland et al., 2005). In England there are 40 000 total hip replacements per year thought to be mainly due to the high prevalence of symptomatic hip osteoarthritis in the Western world (about 10% of people $\geqslant$60 years) (Dreinhöfer et al., 2006). In the UK approximately 8.5 million people suffer from OA with 6 million people in constant pain (Y. Wang et al., 2011). The disease limits movements in 80% of patients with 25% of these patients unable to perform daily activities affecting normal every day life (Kean et al., 2004). These disabilities often result in social isolation and depression (Rosemann et al., 2007).

The study of OA epidemiology is problematic due to the heterogeneity in definitions of the disease. The complex pathology of OA, the difficulty in detecting the diseases onset and grading its severity also make epidemiology challenging. Osteoarthritis is normally diagnosed via radiograph, MRI or arthroscopy and the severity of the disease can be graded using the Kellgren and Lawrence system (Kellgren & Lawrence, 1957). This grading system was developed in the 1950's by Kellgren and Lawrence when they undertook the first large scale epidemiological study of osteoarthritis (Felson et al., 2011). This grading system consists of five categories, starting with no radiographical evidence of OA with grade 0

through to grade 4 with increasing osteophyte development and joint space narrowing (JSN) severity (Kellgren & Lawrence, 1957). More recently a meta-anaylsis has revealed the prevalence of OA in the knee 23.9%, hip 10.9% and the hand 43.3% (Pereira et al., 2011).

1.2.2 Pathology

Cartilage loss is seen as the structural hallmark of OA and is visualized via joint space narrowing (JSN) using radiographs. Other hallmarks include bone changes in the form of subchondral sclerosis and osteophytes as well as changes to the ligaments, muscles, menisci, the afferent sensory system and the synovium (Roos et al., 2011). Muscle weakness is one of the earliest and most frequent findings in patients with OA (Palmieri-Smith et al., 2010). There are many characteristics of this debilitating disease such as focal erosive cartilage lesions.

Patients with radiographic OA with are reported to have a greater occurence and more cartilage lesions when compared to healthy subjects (Dunn et al., 2004), (Reichenbach et al., 2010), (Zarins et al., 2010). The cartilage lesions molecular characteristics change depending on joint location. Knee lesions generally show matrix degradation whereas ankle lesions display matrix synthesis (Moskowitz, 2007). This may potentially explain why knee OA is more common than ankle OA. Early up-regulation of matrix synthesis may help to control lesion progression whereas an increase in collagenase activity may result in a more common, severe pathology (Aurich et al., 2005).

During the development of OA micro-fractures in the subchondral bone occur. It is hypothesised that the failure to properly absorb impact leads to microdamage in the subchondral plate and calcified cartilage (Burr & Radin, 2003). This would result in the shock absorbing properties of the subchondral bone being negated (Lajeunesse et al., 2003). Stress-induced micro-fractures appears to be the first step in the development of subchondral cysts (Dürr et al., 2004). The cystic lesion itself is then induced by osteoclast reabsorbtion. The cysts can form on the bone during the early phases of OA however the prevalence of cysts in patients and whether they always lead to OA development is unclear. These sacs extrude from the joint and mainly consist of hyaluronic acid. Their formation can lead to pain and loss of mobility. Cysts can improve without medication however the symptoms often remain due to the damage cysts cause to the subchondral bone (Swagerty

& Hellinger, 2001). Unlike subchondral cyst formation osteophytes at the synovial joint margin are not linked to bone resorption. Antiresoprtive drugs such as zoledronate have failed to inhibit osteophyte formation and were unable to prevent or correct cartilage deterioration (Bagi et al., 2015). Osteophytes can be used to detect OA, whereas joint space narrowing, subchondral bone sclerosis and cysts are used to assess the severity (Kijowski et al., 2006). Osteophytes may be a repair mechanism helping to stabilise the joint however they also cause pain and loss of movement (Blom et al., 2004). Formation of osteophytes are thought to be caused by endochondral ossification (Brandt, 2003). This is the process by which avascular cartilage tissue is converted into vascularised bone tissue (Higgs, 2010).

1.2.3 Aetiology

There are many risk factors for OA some of which include, age, sex, genetics, obesity and joint injury but how these lead to increased risk remains uncertain (Blagojevic et al., 2010).

1.2.3.1 Aging

While old age is a major risk factor for OA, growing old does not inevitably result in OA, however in the USA 49.7% of adults 65 years or older suffer from reported doctor-diagnosed arthritis (Barbour, 2013). Aged chondrocytes have an increased susceptibility to cell death and respond poorly to growth factors (Anderson & Loeser, 2010). This results in chondrocytes being less successful in maintaining homeostasis in the articular cartilage. The imbalance between the catabolic and anabolic activities of the chondrocytes is in part caused by synovial inflammation. The homeostasis of the cartilage becomes imbalanced resulting in OA.

1.2.3.2 Gender

The Framingham Knee Osteoarthritis study, a population-based study of OA, reported that woman are 1.7 times more likely to develop knee OA than men (Felson et al., 1995). The reasons for this increase in incidence are not clear, however up until the age of 50 prevalence of OA between men and women are similar (Kellgren et al., 1963). It is hypothesised that hormonal changes brought on by the menopause may play a role in the increased incidence of OA in women past the age of 50 (Oliveria et al., 1995). Studies focusing on postmenopausal oestrogen intake have not been able to clearly define the

association of oestrogen and incident of knee and hip OA (Chaganti & Lane, 2011). Muscle weakness may unify the risk factors of sex, obesity, age and joint injury. Muscle strength is reduced after injury, is lower relative to body mass in people suffering from obesity, females have lower muscle strength than males and this strength simultaneously decreases with age (Roos, 2005).

1.2.3.3 Genetic factors

Genetic factors and heritability currently have a poorly defined role in the onset and propagation of OA. As early as 1955 Stetcher et al described Heberden's nodes as a hereditary disease (Stetcher, 1955). Heberden's nodes are firm swellings over the distal interphalangeal joints (Heberden, 1802) and are considered a marker for predisposition to OA (Stecher & Hersh, 1944). Various factors can contribute to the difficulty in analysing genetic association studies of OA. A lack of consensus on the definition of OA, how to compare OA from various joint sites together can make it difficult to group studies and to identify genes involved in the disease (Kerkhof et al., 2008). Despite these difficulties various single nucleotide polymorphisms (SNPs) have been associated with OA. Frizzled-related protein 3 is encoded by FRZB an antagonist of the Wnt signalling pathway (Valdes et al., 2007). Chondrocyte maturation, function and phenotype are regulated by the Wnt/β-catenin pathway and its vital for cartilage boundary definition, endochondral ossification and growth plate organisation (Tamamura et al., 2005). In a cohort study of 298 males and 305 females aged between 50-86 with a diagnosis of knee OA, with a control group of 300 men and 299 women an SNP in FRZB was associated with OA. The FRZB T/G haplotype was shown to be involved in the pathogenesis of knee and hip OA in women (Valdes et al., 2007). Data from the 1000 Genomes Project and a genome-wide association scan for OA identified SNP rs11842874 in MCF2L across 19,041 OA patients and 24,504 controls (Day-Williams et al., 2011). In human cells MCF2L regulates neurotrophin-3, a member of the nerve growth factor (NGF) family. Schwann cell migration is regulated by MCFL2 neurotrophin-3 induction (Z. Liu et al., 2009). Patients with moderate-to-severe OA of the knee were treated with Tanezumab, a humanised IgG2 monoclonal antibody directed against NGF, this treatment was associated with reduction in pain and an improvement in knee function (Lane et al., 2010). A genome-wide association study to identify new

susceptibility loci for OA (arcOGEN) found five genome-wide significant loci for association with OA (Zeggini et al., 2012).

1.2.3.4 Obesity

Research from the Framingham Knee Osteoarthritis Study associated overweight men and women with a higher risk for developing OA than participants that were not overweight (Felson et al., 2007). Obese individuals with a BMI greater than 30kg/m^2 were four times as likely to suffer from knee OA than intervals with a BMI below 25.0kg.m^2 (Magrans-Courtney et al., 2011). Wang et al., found that a continuing trend in obesity would present a loss of 2.2-6.3 million quality-adjusted life years (QALYs) in the UK. This loss in quality of life via an increase in obesity is due to non fatal diseases such as OA and hypertension becoming more prevalent (Wang et al., 2011). Obesity can contribute to OA in a variety of ways; biomechanical stress as well as hormonal and micro-nutritional changes could all potentially affect an individual's likelihood of developing OA. A report by the Arthritis Research Campaign titled "Osteoarthritis and obesity" explains that, "Excessive loading of the joint is the most important means by which obesity causes osteoarthritis". This direct cause and effect view of OA and mechanical stress may not accurately describe the role that obesity plays in the disease. Studies of OA in the hand have shown a clear association between obesity and incidence of OA, this association was also much stronger in women (Carman et al., 1994). There is an estimated 9-13% increased risk for the onset of the disease at the hand with every kilogram increase in body mass (Cicuttini et al., 1996). In these studies it appears that obesity is playing a role independent of mechanical stress. Systemic factors such as adipose tissue hormones (adipokines) could provide a metabolic link between osteoarthritis and obesity (Pottie et al., 2006). Traditionally adipose tissue was viewed primarily as a passive energy reserve, its identification as an endocrine organ began with the discovery and characterisation of the adipokine leptin (Zhang et al., 1994) and adiponectin in 1996.

Identified in 1996 (Maeda et al., 1996) adiponectin is an adipokine and its production correlates with several inflammatory markers (Francin et al., 2014). Females with erosive OA of the hands have an increased serum level of adiponectin (Filková et al., 2009).

Adiponectin has been found in the synovial fluid of females with knee OA and adiponectin was positively correlated with degraded fragments of aggrecan (Hao et al., 2011). The role that adiponectin plays in OA is a complicated one. The mRNA for a receptor of adiponectin (AdipoR1) is strongly associated with mRNA expression of type 2 collagen, aggrecan and Sox9 in OA articular cartilage (Francin et al., 2014). This suggests that adiponectin may be involved in cartilage repair attempts during OA. However the gene encoding adiponectin is closely correlated with *MMP13* gene expression. An immunoassay analysis of culture supernatants indicated that stimulation of primary human chondrocytes with physiologically relevant concentrations of the full length adiponectin hormone significantly increased MMP-13 activity (Francin et al., 2014). Further studies are needed to determine if adiponectin is protective in OA or if it actively participates in cartilage destruction.

Hormones such as leptin may have an important role in the pathogenesis of OA and obese individuals often suffer from leptin insensitivity. Osteoarthritis is more common in women than men and females aged between 32-60 have been shown to have serum leptin levels 2.3 times higher than males of the same age when corrected for fat mass (Bennett et al., 1997). High leptin expression levels have been shown to be associated with disease severity in OA patients (P. Zhang et al., 2015). Leptin strongly stimulates anabolic activity in chondrocytes inducing the synthesis of insulin like growth factor (IGF)-1 and TGF-β (Teichtahl et al., 2005). Activities of these proteins are thought to represent a repair mechanism for damaged cartilage.

1.2.3.5 Signalling pathways in OA

TGFβ is known to stimulate proteoglycan and collagen synthesis (Redini et al., 1988). The effects of TGFβ on the joint are more complicated than anabolic stimulation of cartilage alone. A strong decrease in the content of proteoglycan was found in the cartilage of rabbits knees when injected with TGFβ (Elford et al., 1992). Mice injected with TGFβ have increased proteoglycan degradation despite concomitant inhibition of IL1 stimulated degradation (van Beuningen et al., 1993). Active TGFβ has been shown to be present in the synovial fluid

of OA patients, at an average concentration of 4ng/ml (Fava et al., 1989). It was hypothesised that at these concentrations TGFβ could stimulate both aggrecan synthesis and degradation, resulting in increased aggrecan turnover (Moulharat et al., 2004). The resulting proteoglycan fragments produced by TGFβ-stimulated aggrecanases production could be the inflammatory trigger for cytokine production by chondrocytes (Pelletier et al., 2001) leading to an increase in metalloproteinase production and aggrecan and collagen degradation seen in OA. The balance of these cytokines is crucial for maintaining healthy articular cartilage.

Wnt signalling can also induce the expression of metalloproteinases in articular chondrocytes, promoting cartilage degradation and cartilage catabolism. Rabbit articular chondrocyte cultures subjected to experimental activation of β-catenin signalling by Wnt3A treatment showed a significant increase in metalloproteinase gene expression (Yuasa et al., 2008). Transgenic mice have been generated that express an inhibitor of β-catenin and T cell factor (ICAT) which was targeted to chondrocytes specifically using the *Col2a1* promotor. ICAT is a naturally occurring inhibitor that specifically disrupts TCF and beta-catenin interactions. Inhibition of the Wnt/β-catenin signalling pathway was shown to increase chondrocyte cell apoptosis and articular cartilage destruction in *Col2a1*-ICAT-transgenic mice (Zhu et al., 2008). These experiments show that both over and under activation of the Wnt/β-catenin can result in OA and a joint must have the correct homeostasis of the pathway to remain healthy. Without Wnt signalling β-catenin is targeted for proteasome-mediated destruction within the Glycogen synthase kinase 3 (GSK-3) destruction complex brought together by Axin/Axin2 and APC. In the presence of Wnt signalling Frizzled receptors inhibit the destruction of β-catenin, resulting in its accumulation and translocation to the nucleus (Yuasa et al., 2008). FRZB is a secreted Wnt antagonist, this gene has been knocked out in mice, this resulted in cartilage damage via increased activity and expression of matrix metalloproteinases which was associated with increased Wnt signalling (Lories et al., 2007).

IL1 is a pro-inflammatory cytokine that has been frequently studied and is found in the OA joint. During OA IL1 is primarily produced by chondrocytes (Goldring, 2000) and synovial fibroblasts (Sadouk et al., 1995). In OA joints IL1 localises to the superficial zones of OA at the site of cartilage degradation (Tetlow et al., 2001). IL1 promotes the synthesis of matrix

metalloproteinases in chondrocytes (Zayed et al., 2008) and can cause inflammation by inducing the expression of (COX-2) and nitric oxide synthase (iNOS), this can result in the synthesis of inflammatory mediators such as nitric oxide (NO) (Vuolteenaho et al., 2005), (Hung et al., 2008). IL1 can induce degradation of aggrecan (Ismail et al., 2015), proteoglycan (Nguyen et al., 1989) and type 2 collagen (Billinghurst et al., 2000), (Kozaci et al., 1997) in human chondrocytes. A local increase in IL1 receptor antagonist (IL1Ra) production in OA knee joints by intraarticular injection of transduced synovial cells has been shown to reduce the progression of experimentally induced OA lesions in a canine model (Pelletier et al., 1997). ILRa has also been shown to significantly improve clinical parameters of pain, preserve articular cartilage, and have beneficial effects on the histologic parameters of the synovial membrane and articular cartilage in an experimental equine OA model (Frisbie et al., 2002). Research into the inhibition of IL1R in humans is still rare. An intra-articular injection of 150mg anakinra (a recombinant form of human ILRa) has been shown to significantly reduce pain scores in patients suffering with knee OA (Chevalier et al., 2005). However this study lacked controls making it difficult to make a definitive conclusion on the efficacy of anakinra (Calich et al., 2010). In a randomized, double blind, placebo controlled study a 10mg intraarticular injection of anakinra in patients with OA of the knee was not associated with improvements in OA symptoms compared with placebo (Chevalier et al., 2009). For cartilage to remain healthy there must be a balance between matrix synthesis and degradation. How this balance is maintained is poorly understood, but anabolic and catabolic cytokines appear to have important roles (Buckwalter et al., 2005). When this balance is lost several key metalloproteinases are upregulated and cartilage destruction occurs.

1.3 The Metalloproteinases

There are six classes of proteinases (aspartic, cysteine, glutamic, metallo-, serine and threonine), these are classified according to their catalytic mechanisms (Rawlings et al., 2004), (Shen & Chou, 2009). Metalloproteinases contain a metal ion at their active site. The metzinicin superfamily of metalloproteinases are defined by the HEXXH motif, a histidine as the third zinc ligand and a right hand met-turn. This superfamily includes the astacin, adamalysins, serralysin and the matrixin subfamilies (Hooper, 1994). These proteases can degrade all ECM components (P. Lu et al., 2011). While many classes of

proteases are upregulated in OA the current dogma is that a disintegrin and metalloproteinase domain with thrombospondin motis (ADAMTSs) degrade aggrecan (Verma & Dalal, 2011) and following this critical step a diverse range of matrix metalloproteinases continue to degrade major components of the ECM (G. Smith, 2006).

1.3.1 ADAMs and ADAMTSs

The ADAMs (Disintegrin And Metalloproteinase domain) and ADAMTSs (disintegrin, a metalloproteinase and thrombospondin motifs) are adamalysins. The ADAMs family include both transmembrane and secreted proteins, ADAM-9, ADAM-10 and ADAM-28 can be alternatively spliced to be either secreted or membrane-bound (Hotoda et al., 2002), (Yavari et al., 1998) (Roberts et al., 1999). Both transmembrane and secreted ADAMs are involved in the cleavage of the ectodomain of many receptors, signalling molecules, cytokines and growth factors (Edwards et al., 2008). Membrane-bound ADAMs proteolytically release the extracellular portions of other transmembrane proteins in a process known as shedding (Moss & Lambert, 2002). The ADAMs domain structure consists of a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain and a cytoplasmic tail (Seals & Courtneidge, 2003). It is thought that it is the cysteine-rich domain that is involved in the regulation of ADAMs specific functions (K. Smith et al., 2002).

While the majority of ADAM proteinases are transmembrane proteins the ADAMTSs have no transmembrane domains and are therefore secreted molecules that can bind to the ECM (Kaushal & Shah, 2000). ADAMTSs proteins share sequence similarity to the ADAMs (A disintegrin and metalloproteinase) (Nicholson et al., 2005). ADAMTS aggrecanases were discovered by the end sequences of cleaved aggrecan (termed neo epitopes) found in culture medium and synovial fluid in the absence of matrix metalloproteinase activity. There are 19 known ADAMTSs numbered 1-20 with no ADAMTS-11 and the function and substrates of many of the ADAMTSs are yet to have been characterised (Table 2). ADAMTS-2, -3 and -14 are pro collagen N-propeptidases these are essential for the maturation of triple helical collagen fibrils and are required for fibril assembly (Colige et al., 2002). ADAMTS-1, -4, -5, -8, -9 and -15 have been shown to degrade aggrecan matrix proteoglycan with varying efficeniency (Kuno et al., 2000), (Tortorella, 1999), (Tortorella et al., 2002),

(Collins-Racie et al., 2004), (Somerville et al., 2003), (Yamaji et al., 2001). Of these ADAMTS-4 and ADAMTS-5 are the most efficient aggrecanases (Abbaszade et al., 1999), (Tortorella et al., 1999) with ADAMTS-5 reported as being 1,000 times more active than ADAMTS-4 under physiological conditions (Gendron et al., 2007). These aggrecanases cleave aggrecan at a number of sites in the proteins core, cleavage at the interglobulin domain (IGD) between the N-terminal G1 and G2 globular domains predominantly occurring within diseased tissue resulting in loss of the entire GAG containing region (Little et al., 2007) (Sandy et al., 1992) (Sandy, 2006).

Gene	**Location**	**Factors inducing or (repressing) expression**	**Substrates**
ADAMTS1	21q21	Progesterone, Brg1, IL1, S100A8, S100A9, TNFα	Aggrecan, versican, syndecan 4, TFPI-2, semaphorin 3C, nidogen-1, −2, desmocollin-3, dystroglycan, mac-2, gelatin (denatured collagen type I), amphiregulin, TGF-α, heparin-binding EGF
ADAMTS2	5q35	Glucocorticoids (in monocytes), IL-6	Fibrillar procollagens types 1-3 and V
ADAMTS3	4q21		Fibrillar procollagen type 2, biglycan
ADAMTS4	1q23	IL1 + oncostatin M, TNFα, S100A8, S100A9, leptin, IL-6	Aggrecan, versican, reelin, biglycan, brevican, matrilin-3, α2-macroglobulin, COMP
		(HDAC inhibitors, pentosan polysulfate)	
ADAMTS5	21q21	IL1, TNFα, S100A8, S100A9, leptin, IL-6	Aggrecan, versican, reelin, biglycan, matrilin-4, brevican, α2-macroglobulin
		(HDAC inhibitors)	
ADAMTS6	5q12	TNFα,	-
ADAMTS7	5q24	PTHrP	COMP
		(miR-29a/b)	
ADAMTS8	11q24		Aggrecan
ADAMTS9	3p14	TNFα, IL1 + oncostatin M, leptin	Aggrecan, versican
		(HDAC inhibitors)	
ADAMTS10	19p13		Fibrillin-1
ADAMTS12	5p13		COMP
ADAMTS13	9q34	(IL1)	vWF
ADAMTS14	10q22		Fibrillar procollagen type I (pNα1 and pNα2 chains)
ADAMTS15	11q24		Aggrecan, versican
ADAMTS16	5p15	Follicle stimulating hormone; forskolin (cAMP);	-
		Transcription factors: Wilm's tumor-1; Egr-1, Sp1	
ADAMTS17	15q26		-
ADAMTS18	16q23		-
ADAMTS19	5q23		-
ADAMTS20	2q12		Versican

Table 2. ADAMTS genes. Table showing the chromosomal positions, expression-inducing factors and substrates of the ADAMTSs Table adapted from (Kelwick et al., 2015).

The molecular structure of the ADAMTS proteins can be subcategorized into various domains and motifs (Figure 1.7). Initially synthesised as inactive pre-proenzymes the domains from the N-terminus to the C-terminus consists of a signal peptide, pro-domain, a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich domain, a spacer

region and a C-terminal containing variable numbers of thrombospondin repeats with additional modules (Porter et al., 2005). The pro domain retains the enzymes latency and is thought to also be potentially important for correct protein folding and secretion. While the majority of ADAMTSs are synthesised as zymogens that are activated through the removal of the pro domain both ADAMTS-7 and -13 retain activity with the pro domain attached (Somerville et al., 2004) (Majerus et al., 2003).

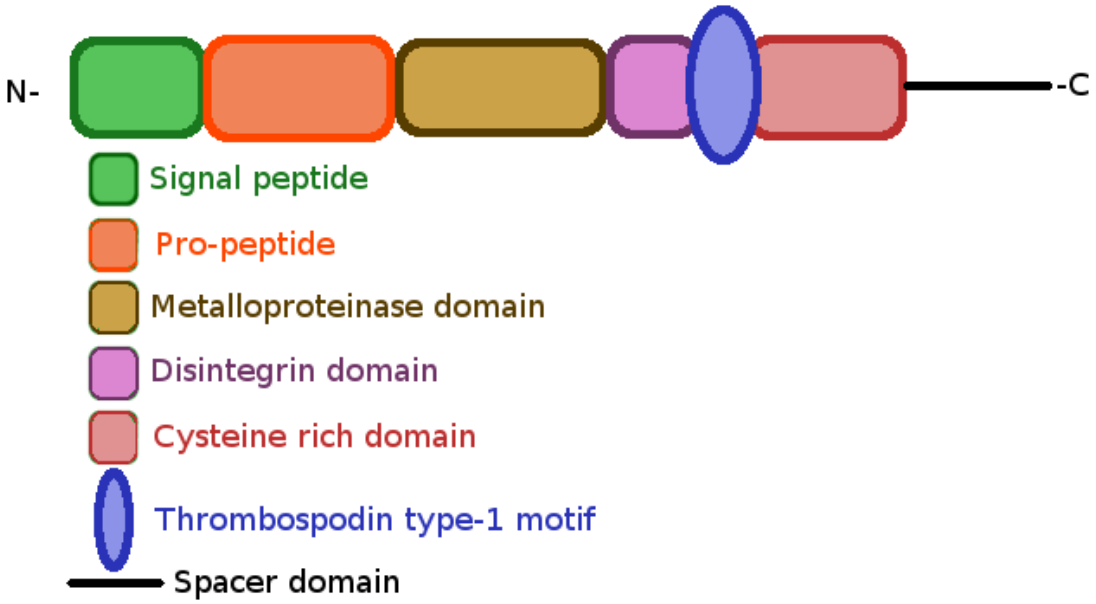


Figure 1.7. Common structure of ADAMTSs.

ADAMTSs appear to play a role in the development of OA. ADAMTS-5 knock out mice had a significant reduction in the severity of cartilage destruction after surgically induced instability when compared with wild type mice (Glasson et al., 2005a). Interestingly deletion of ADAMTS-4 does not confer a similar protection as seen in ADAMTS-4 mice (Gendron et al., 2007). ADAMTS-5 has also been shown to be the major aggrecanase in mouse cartilage. In a mouse model of inflammatory arthritis ADAMTS-5 was deleted, this protected mice against aggrecan loss and cartilage erosion (Stanton et al., 2005).

Whether ADAMTS-5 is the principal aggrecanase in human cartilage is still unclear (Fosang et al., 2008). Two likely deleterious nonsynonymous single-nucleotide polymorphisms

(SNPs) were identified in ADAMTS-5 using bioinformatic analysis. These genetic variations were examined in 2715 patients with knee, hip, or hand OA and in 1185 OA-free controls. Genetic variation in ADAMTS-5 appeared to have no participation in OA susceptibility (Rodriguez-Lopez et al., 2008). These results indicated that further exploration is needed to determine whether aggrecanase ADAMTS-5 in human OA is as critical as in mouse models.

1.3.2 Matrix Metalloproteinases

Matrix Metalloproteinases (MMPs) also called matrixins are enzymes responsible for the cleavage of matrix proteins such as collagen, laminin and fibronectin as well as many other substrates (Table 3). The MMP family is composed of 24 matrixin genes including a duplicate of MMP-23 resulting in 23 MMPs in humans (Nagase et al., 2006). MMP1 was discovered in a tadpole tail during metamorphosis (Gross & Lapiere, 1962) and was the first secreted member of a family of enzymes to be discovered (Maskos, 2005). The sequence homology with MMP-1, the propeptides cysteine switch motif, and the zinc binding motif (HEXXHXXGXXH) in the active site are the signatures used to assign proteinases to this family (Visse & Nagase, 2003). The prodomain is an amino-terminal signal sequence that directs the molecule to the endoplasmic reticulum (Figure 1.8). The propeptide domain contains the zinc-interacting thiol group that maintains the enzyme in its inactive zymogen form. This pro-domain consists of about 80 amino acids containing a conserved PRCG(V/N)PD amino acid sequence that interacts with the catalytic zinc ion to suppress its own proteolytic activity (cysteine switch) (Kim & Joh, 2012). Conformational changes occur when the pro-domain is removed and the zinc ion binds to water allowing for hydrolysis of peptide bonds when the MMP binds with its substrate (Van Wart & Birkedal-Hansen, 1990). Cleavage of the prodomain can be mediated by MMPs themselves in a process called autolysis or by another proteinase, allosteric perturbation of the zymogen or modification of the free thiol by experimental or physiological means (Tocchi & Parks, 2013) (Springman et al., 1990).

The catalytic domain contains the zinc binding site, 2-3 calcium ions that maintain the structure of the active site and a conserved methionine forming part of the ‘met-turn’ structure (Bode et al., 1993). The catalytic domain consists of 160-170 amino acids making

three α-helices a five-stranded β-sheet and bridging loops (Tallant et al., 2010). The haemopexin domain-containing MMPs have a haemopexin like domain at the C-terminus that is connected to the catalytic domain by a proline-rich hinge domain, these domains mediate interactions with the tissue inhibitors of metalloproteinases, proteolytic substrates and cell surface molecules (Egeblad & Werb, 2002). MMP-7, 26 and 23 are exceptions to this structure as they contain no hinge or haemopexin domains (Lohi et al., 2001).

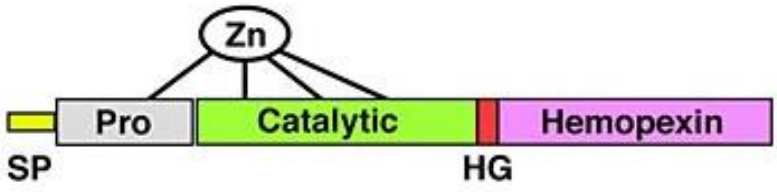


Figure 1.8. Common structure domain of an MMP. SP – signal peptide and Pro – domain at the N terminus. Catalytic site is zinc bound, HG – hinge domain, with a Haemopexin domain at the C terminus. Figure adapted from (Brauer, 2006).

MMPs are grouped into the membrane-type MMPs (MT-MMPs) (MMP-14 to MMP-17 and 24, 25 or MT1-6-MMP), the stromelysins (MMP-3, 10, 11), a heterogeneous subgroup including matrilysin (MMP-7), gelatinases (MMP-2, 9) and the collagenases (MMP-1, 8, 13) (Y.E. Chen, 2004) (Table 3).

MMP	COMMON NAMES	MATRIX SUBSTRATE
MMP-1	Collagenase-1, fibroblast collagenase	Type 3>1>2>7>10 collagen gelatin, aggrecan, entactin, tenascin, Pro-MMP1, Pro-MMP2
MMP-8	Neutrophil collagenase, collagenase-2	Type 1>2>3,7,10 collagen gelatin, aggrecan, Pro-MMP8
MMP-13	Collagenase-3	Type 2>3>1, 7,10 collagen, gelatin, entactin, tenascin, aggrecan
MMP-3	Stromelysin-1, SL-1, Transin-1	Aggrecan, laminin, fibronectin, non triple helical regions of type 2, 3, 4, 5, 9, 10 and 11 collagen, gelatin, Pro-MMP1, 3, 7, 8, 9 and 13
MMP-10	Stromelysin-2, SL-2, Transin-2	Fibronectin, Pro-MMP1, 8 and 10
MMP-11	Stromelysin-3, SL-3, ST3, STMY3	Fibronectin, laminin, aggrecan, type 6 collagen
MMP-19	Stromelysin-4, SL-4	Native type 4 collagen, gelatin, laminin, fibronectin, tenascin, fibrin/fibrinongen
MMP-7	Matrilysin, mat, PUMP-1, uterine metalloproteinase	Fibronectin, laminin, non helical segments of type 4, 5, 9, 10 and 11 collagen gelatin, aggrecan, fibrin/fibronectin
MMP-26	Endometase, matrilysin-2	Native type 4 collagen, gelatin, fibronectin, fibrin/fibrinogen, Pro-MMP9
MMP-2	Gelatinase A, 72-kDA gelatinase	Gelatin, type 1, 4, 5, 7, 10 and 11 collagen, elastin, fibronectin, laminin-5, aggrecan, vitronectin
MMP-9	Gelatinase thermometer, 92-KDa gelatinase	Gelatin, type 1, 4, 5, 7, 10, 11 collagen, elastin, fibronectin, laminin, aggrecan, vitronectin, Pro-MMP2, 9 and 13
MMP-14	MT1-MMP	Type 1,2,3 collagen, gelatin, fibronectin, vitronectin, aggrecan, Pro-MMP2 and 13
MMP-15	MT2-MMP	Proteoglycan, Pro-MMP2
MMP-16	MT3-MMP	Native collagen type 3, fibronectin, Pro-MMP2

MMP-17	MT4-MMP	Gelatin, fibrilin/fibronectin, Pro-MMP2
MMP-24	MT5-MMP	Fibronectin, proteoglycans, gelatin
MMP-25	MT6-MMP	Type 4 collagen, gelatin, fibronectin, pro-MMP2, pro-MMP9
MMP-20	Enamelysin	Amelogenin
MMP-23	Cysteine array MMP femalysin	Gelatin
MMP-27		No matrix defined substrates
MMP-28	Epilysin	Casein

Table 3. MMP genes. Table to summarise MMPs and their substrates. Table adapted from (S. Shapiro, 1998), (Egeblad & Werb, 2002).

The collagenases consist of MMP-1, 8, 13. These enzymes are able to cleave interstitial collagens 1, 2 and 3 into specific ¾- and ¼- collagen fragments (Sorsa et al., 1989). MMP-2 and MMP-14 have more recently been shown to make this specific cleavage but with less catalytic efficiency (Knauper et al., 1996), (Ohuchi et al., 1997), (d'Ortho et al., 1997). Collagenases are important for ECM remodelling during tissue development and regeneration. The mechanism of collagen breakdown that MMPs facilitate is fundamental in normal healthy cells and is involved in tissue reabsorption and remodelling, cell proliferation and migration, chemotaxis and apoptosis (Nagase & Woessner, 1999). Disruption of this homeostasis can result in diseases such as OA (Israel et al., 1998) and tumour metastasis (Ala-aho & Kähäri, 2005).

The stromelysins consist of MMP-3, 10 and 11. Both MMP-3 and MMP-10 digest proteoglycan core proteins, laminin, fibronectin, elastin, gelatin and type 3,4,5,7 and 9 collagen (D. Li, 2003). Both MMP-3 and MMP-10 participate in proMMP activation. For the maturation of proMMP-1 into the fully active MMP-1 it is crucial for MMP-3 to cleave the Gin80-Phe81 bond (Suzuki et al., 1990). The third stromelysin is MMP-11, however this MMP can only weakly degrade ECM components apart from type 6 collagen (Murphy et al.,

1993). The furin recognition sequence is present in MMP-11 suggesting its activated intracellularly (Pei & Weiss, 1995).

The matrilysin group consists of MMP-7 and MMP-26. These MMPs lack a haemopexin domain. As well as degrading ECM components matrilysins have been shown to process cell surface molecules such as E-cadherin in Madin-Darby canine kidney cells (MDCK). The MDCK cells were treated with 100ng/ml of matrilysins for 2 hours, which stimulated E-cadherin fragments to be released (Noe et al., 2001). Unusually for an MMP, MMP-26 was found to be stored intracellularly in MCF7 breast carcinoma cells (Marchenko et al., 2004).

Gelatinases include MMP-2 and MMP-9. Gelatinases are the main enzymes for the digestion of gelatin which is the product of collagen after its degradation by the collagenases. These MMPs have three repeats of a fibronectin type 2 motif in the metalloproteinase domain. These repeats help the gelatinases bind to gelatin, collagens and laminin (Allan et al., 1995).

There are two groups of Membrane type MMPs (MT-MMPs). There are the type 1 transmembrane proteins such as MMP-14, 15, 16 and 24 which have a carboxy terminal, single span transmembrane domain and a short cytoplasmic tail (Fillmore et al., 2001). Finally there are the glycosylphosphatidylinositol (GPI)-anchored MMPs such as, MMP-17 and MMP-25. These anchors are located downstream of the haemopexin-like domain (Sohail et al., 2008). These MT-MMPs all have a RX(R/K)R furin recognition sequence at the propeptide C-terminus (Nagase et al., 2006). This allows furin to activate the MT-MMPs intracellularly, where they can then be expressed on the cell surface (Thomas, 2002).

1.3.3 The regulation of Matrix Metalloproteinases

MMP expression is largely regulated transcriptionally (Figure 1.9). Trans-activators such as activator protein-1 (AP-1), PEA3 (polyoma enhancer A binding protein-3), SP1 (specificity protein 1), β-catenin/TCF4 and nuclear factor kappa B (NFκB) can regulate MMPs through their cis elements (Figure 1.10). NFκB is a trans-activator that can regulate metalloproteases and mediates articular chondrocytes responses to proinflammatory cytokines such as IL1. The inhibitory κB (IκB) protein associates with NFκB in the cytoplasm keeping the protein in its inactive form. IL1 stimulation results in a signaling cascade leading

to the release of NFκB from IκB. Activated NFκB then translocates to the nucleus binding to its consensus DNA sequence within target genes resulting in their transcription. NFκB plays a central role in the development of OA. Cartilage damage has been shown to decrease in a surgically induced mouse OA model when treated with siRNA that inhibits NFκB/p65 (Chen et al., 2008). IκB overexpression decreases MMP-1, 3, 9, 13 mRNA and protein levels (Amos et al., 2006) and IL1 stimulated *ADAMTS4* gene expression in human OA synovial fibroblasts (Bondeson et al., 2007).

MMP promoters can be placed into three groups based on the composition of the cis-elements. The first group contains MMP-1, 3, 7, 9, 10, 12, 13 and 19, these MMPs contain TATA boxes at around -30bp with AP-1 sites around -70bp. MMP8, 11 and 21 contain the TATA box but no AP-1 sites in the proximal promoter. Finally MMP-2, 14 and 28 contain neither TATA boxes or AP-1 sites in the proximal promoter (Gosset et al., 2010). This last group's expression is determined by SP1 transcription factors that bind to a proximal GC group (Yan & Boyd, 2007).

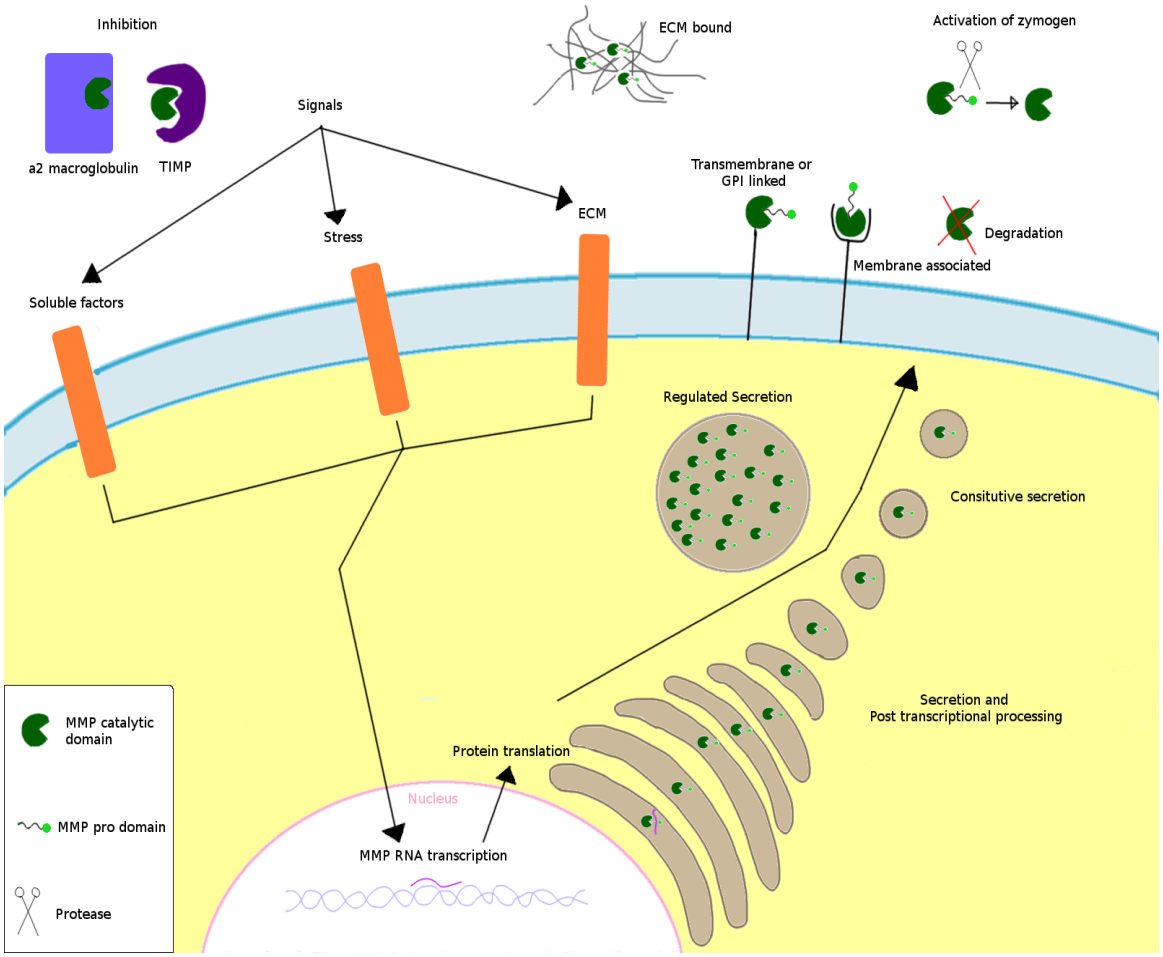


Figure 1.9. MMP regulation. MMP function is regulated at many levels. This regulation occurs at the RNA transcription and protein levels but also by the secretion, intracellular trafficking, subcellular or extracellular localization, activation of the zymogen form, expression of their endogenous protein inhibitors of metalloproteinases (TIMPs) and α2-macroglobulin and protease degradation. Figure adapted from (Page-McCaw et al., 2007).

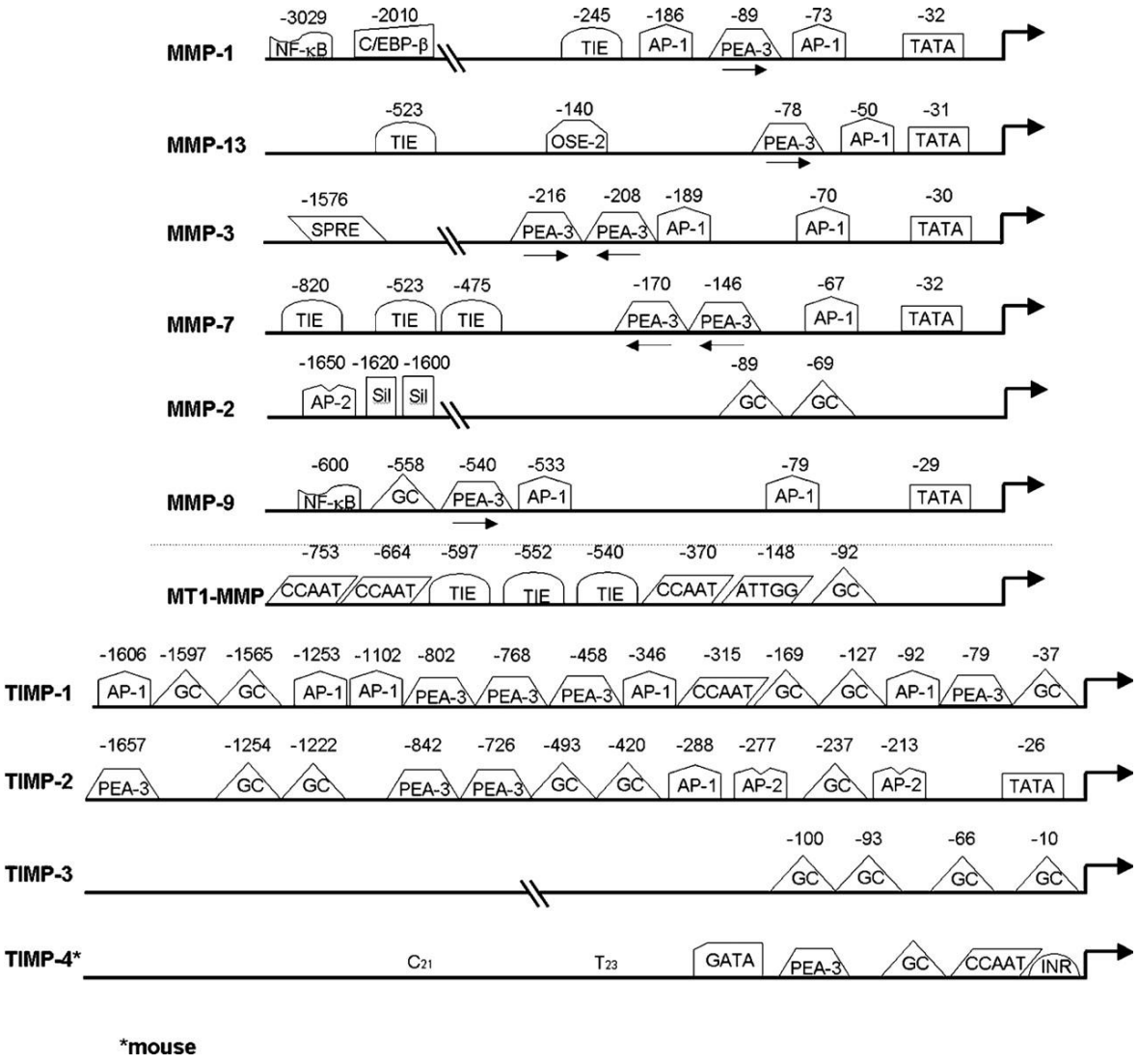


Figure 1.10. MMP Promoters. Diagram showing cis elements in human MMP and TIMP promoters. C/EBP-β, CCAAT enhancer-binding protein. TIE, TGF-β inhibitory element. AP-1, activator protein-1. PEA-3, polyoma enhancer A binding protein-3. OSE-2, osteoblastic cis-acting element. TATA, TATA box. AP-2, activator protein-2. Sil, silencer. GC, Sp-1 binding site. NF-κB, NF-κB binding site. CCAAT, CCAAT box. GATA, GATA binding site. INR, initiator-like sequence. Taken from (Deschamps & Spinale, 2006).

Cytokines and growth factors such as IL1, tumour necrosis factor α (TNFα) and basic fibroblast factor (bFGF) convergently transactivate MMP promoters via AP-1 and PEA3 through p38, c-Jun N-terminal kinases (JNK), mitogen-activated protein kinases (MAPK) and protein kinase C (PKC) signalling (Goldring et al., 2011), (S. Ahmed et al., 2003), (S. Ahmed et al., 2005), (Tower et al., 2003), (Muddasani et al., 2007), (Im et al., 2007). Cell surface

receptors such as integrins can activate focal adhesion kinase (FAK), which can potentially interact with the MAPK pathway leading to trans activation of AP-1 and/or PEA3 resulting in MMP expression. (Yan & Boyd, 2007).

There has been some evidence of epigenetic control of MMPs in the form of methylation (Barter et al., 2012). In one study the promoter of MMP-9 was methylated and silenced in lymphoma cell line (Chicoine et al., 2002). Another study used wild type colon cancer cells to demonstrate that demethylating agents could yield an induction of MMP-3 expression (Couillard et al., 2006). Histone deacetylase inhibitors trichostatin A (TSA) and sodium butyrate (NaBy) have been shown to repress MMP-1 and MMP-13 at the mRNA and protein levels in SW1353 cells (Young et al., 2005).

MMPs are synthesised as pre-proenzymes. During translation the signal peptide is removed generating the zymogen. An important regulatory step of MMP activity is the activation of the zymogen (Nagase et al., 2006). There are also MMPs that are processed into the active form at the cell membrane. An example of this form of activation is the MT-MMP mediated cascade that activates proMMP-2 (Sato et al., 1994).

1.3.4 Tissue inhibitors of metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) have a molecular weight of 21-30kDA, consist of 184-194 amino acids and are inhibitors of MMPs (Yadav et al., 2011). While TIMPs inhibit the activity of all MMPs tested so far (Nagase et al., 2006) with the exceptions of MT1-MMP and MT3-MMP for TIMP1 (Will et al., 1996), (Shimada et al., 1999) TIMP3 can also inhibit ADAMTSs. There are four human inhibitors in the TIMP family, TIMP1-TIMP4, these inhibitors are expressed in a range of different tissues. Many of the functional interactions between MMPs and TIMPs have been established by crystallographic structure analysis, crystal structures for MT1-MMP/TIMP2 and MMP-3/TIMP1 have both been solved and described (Fernandez-Catalan et al., 1998), (Gomis-Rüth et al., 1997). TIMPs have 12 cysteine residues that have been shown to form disulphide bonds that form a 6 loop structure (Figure 1.11). The N-terminal and VIRAK amino acid sequence is conserved in all TIMPs (Caterina et al., 1998).

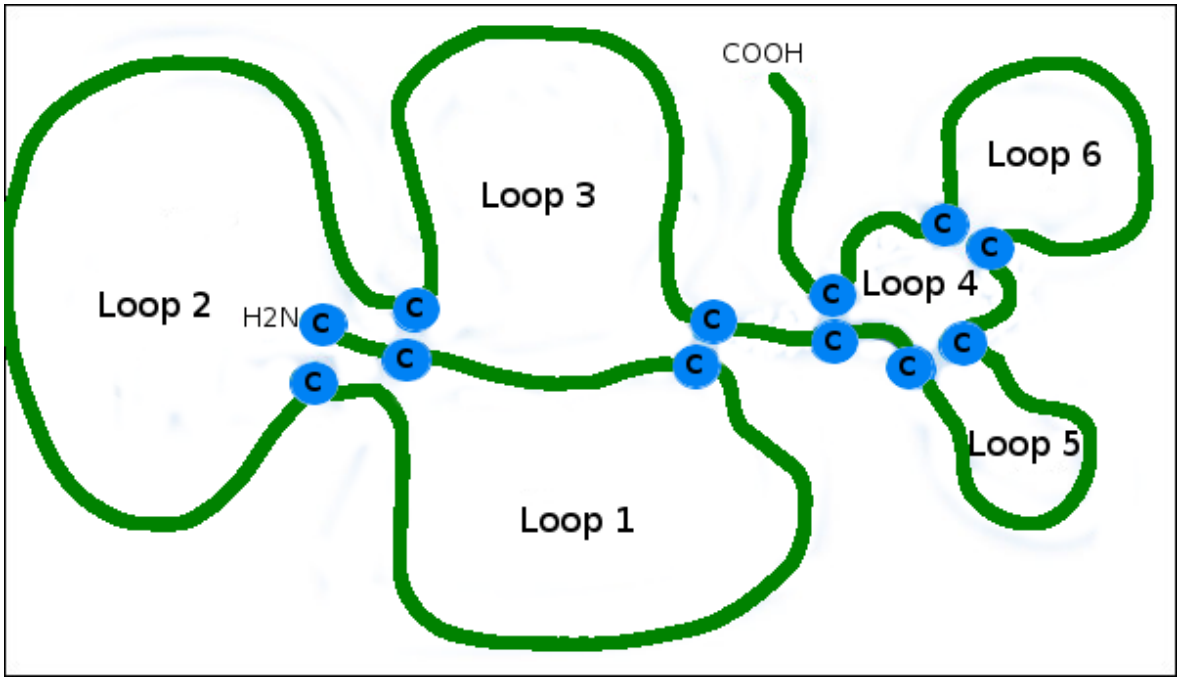


Figure 1.11. Structure of TIMP. Diagram shows the six loop structure of TIMP1. Image adapted from (Murphy & Willenbrock, 1995).

The catalytic zinc ion of a MMP is bidentate-chelated by the N-terminal amino group and the carbonyl group of the TIMP, this expels the water molecule bound to the zinc atom inactivating the MMP (Visse & Nagase, 2003). TIMPs bind tightly to MMPs in a 1:1 ratio, meaning that if MMP production is below TIMP protein levels ECM degradation is prevented (Cawston & Wilson, 2006).

While all TIMPs share basic similarities they also exhibit different structural features, expression patterns and biochemical properties, this may suggest that various TIMPs may have distinct and particular roles in vivo (Baker et al., 2002). TIMPs have also been reported to have activities other than MMP inhibition, these biological activities include promoting cell, promoting cell proliferation, anti-angiogenic, pro and anti-antiapoptotic and synaptic plasticity activities (Brew & Nagase, 2010) as well as activation of some proMMPs (Bernardo & Fridman, 2003). In isolated rabbit mature osteoclasts TIMP1 and TIMP2 stimulated bone-resorbing activity. Bone-resorbing activity was stimulated at lower concentrations of TIMPs (~ng/ml) than concentrations needed to inhibit bone-resorbtion (~µg/ml). This activity appeared to be MMP independent as MMP inhibitors BE16627B and R94138 could not mimic the osteoclast activity caused by TIMPs (Sobue et al., 2001).

TIMPs -1, -2 and -4 are all soluble proteins whereas TIMP-3 is associated with the ECM through its C-terminal domain which recognises and binds to heparan sulphate in its glycosylated (27KDa) and unglycosylated (24KDa) forms (M. Lee et al., 2007) (W. Yu et al., 2000). Of the four TIMPs TIMP-3 has the broadest inhibition spectrum and is the most potent inhibitor of the ADAMTS family (Brew & Nagase, 2010). There are also more subtle differences between the affinities of different TIMPs for other MMPs. TIMP-2 and -3 are weaker inhibitors than TIMP-1 for MMP-3 and MMP-7 which contrasts with their affinities for other MMPs (Hamze et al., 2007). TIMP-4 expression is selectively elevated in the human heart but has also been shown to be increased in human osteoarthritic femoral head cartilage, but while TIMP-4 may be increased as a defence mechanism against MMPs, these levels may not be enough to prevent cartilage damage in OA (Greene et al., 1996) (Huang et al., 2002).

The expression of TIMPs are carefully regulated during normal physiological conditions and are upregulated in response to cartilage growth factors after joint damage, however this upregulation is not sustained whereas the MMP upregulation is sustained which can result in collagen destruction (Cawston et al., 1999).

Inactivation of TIMPs can occur by neutrophil elastase, trypsin, and chymotrypsin, resulting in the degradation of TIMP into small fragments (Itoh & Nagase, 1995).

1.3.5 Matrix metalloproteinases in osteoarthritis

MMPs play an important role in articular cartilage degradation (Haslauer et al., 2013), (Poole et al., 2003). The collagenases are important in the development of OA because of their ability to degrade type 2 collagen, the predominant collagen in cartilage. While MMP-8 can cleave type 2 collagen it has a higher specificity for type I collagen. MMP-8 is a major collagenase in tissues such as human dentin, highly expressed in human neutraphils and potentially important in cardiovascular disease, periodontis and breast cancer (Sulkala et al., 2007), (Gioia et al., 2002), (Sorsa et al., 2011), (Decock et al., 2008). However MMP-8 appears to play a minor role in articular cartilage chondrocytes with no significant upregulation of *MMP8* in OA cartilage in vivo nor by IL1 in vitro (Stremme et al., 2003).

MMP's ADAMTSs and TIMP expression have been profiled in three comprehensive screens of end stage OA (Kevorkian et al., 2004) (Davidson et al., 2006) (Swingler et al., 2009). Kevorkian et al and Davidson et al screened for metalloproteinase gene expression in OA femoral head cartilage versus normal femoral head cartilage. The screen by Davidson et al also included gene expression from the synovium of the joint. Swingler et al screened the entire degradome in OA versus normal femoral head cartilage. All three screens showed anomalistic gene expression for ADAMTSs and MMPs in OA cartilage. Aggrecanases *ADAMTS4* and *5* were shown to be decreased in cartilage and synovium however aggrecan loss via proteolysis is an early event in OA so mRNA expression may only be detected in earlier OA samples than the tissues used in these studies. *MMP1* was also decreased in the screens while *MMP13* was one of the most significantly increased in all three screens, helping to support the theory that *MMP13* is the key collagenase involved in collagen type 2 breakdown and the progression of OA.

MMP-1 and MMP-13 are able to cleave native fibrillar collagen and both contribute to the pathological cleavage of collagen fibrils in OA (Burrage et al., 2006). Animal models have shown the potential protective effects that *MMP1* inhibition may have in OA however the lack of a well characterised murine homologue has significantly limited the study of *MMP1* and the development of MMP-targeted therapeutics (Foley & Kuliopulos, 2014). Elevated MMP-1 activity has been reported in equine synovial OA joints (Brama et al., 2010). Twenty rabbits that underwent anterior cruciate ligament transection to induce OA had significantly reduced MMP-1 at the mRNA and protein level when treated with carboxymethylated chitin (CMC). Cartilage degradation was less severe in CMC treated rabbits and it was suggested that this was due to MMP-1 inhibition (Hongbin et al., 2004). Selective inhibitors have shown that MMP-13 may be the collagenase predominantly responsible for collagen release from human OA cartilage (Billinghurst et al., 1997). Selective inhibitors that could inhibit MMP-8 and MMP-13 but not MMP-1 were used on articular cartilage samples from 11 patients with OA and 5 adults without OA. A collagenase-generated neoepitope was used to reflect cleavage of type 2 collagen. The neoepitope was used to show that the inhibitor for MMP-8 and MMP-13 but not MMP-1 was able to inhibit digestion of type 2 collagen in OA cartilage (Dahlberg et al., 2000). MMP-13 appears to be the collagenase with the highest specificity for type 2 collagen with the

most efficient cleavage of the protein (Minond et al., 2006). The inhibition of MMP-13 has been studied in animal models of OA. In one model *MMP13* knock out mice were surgically induced for knee OA. The *MMP13* knock out mice had reduced cartilage erosion in the presence of aggrecan depletion 8 weeks after surgery (Little et al., 2009). Immunohistochemistry and dUTP nick end labeling (TUNEL) staining was used to detect aggrecan degradation. Although inhibition of *MMP13* may have prevented cartilage degradation via reduced type 2 collagenolysis other mechanisms were also suggested. Reduced degradation of other MMP-13 substrates such as fibromodulin or type IX collagen could participate in the reduction of cartilage erosion. MMP-13 is also able to activate other MMPs such as proMMP-9, activation of these MMPs may contribute to cartilage erosion (Knäuper et al., 1997). It is possible that MMP-13 could regulate inflammatory cytokine and chemokine activity with roles in cartilage degradation (Manicone & McGuire, 2008). Taken together this research suggests that *MMP13* inhibition could have a potential therapeutic effect in OA.

1.4 Current Treatments

Current treatments for OA have focused on anti-inflammatory drugs and pain relief rather than addressing the degradation of cartilage.

As OA is associated with muscle weakness and obesity, current treatments have included lifestyle changes to the patient's traditional diet and exercise regime. This form of treatment is useful as physical inactivity can also act as a severe risk factor for developing OA (Vijay et al., 2002). When body weight is reduced pain is reduced and functional capacity of the joint is improved. Alleviating obesity through these methods has been suggested to be among the most beneficial methods of relieving pressure on OA joints (Magrans-Courtney et al., 2011).

Clinicians routinely recommend that adults with OA strengthen muscles with exercise as strengthening has been linked with a reduction in pain and morbidity in patients (O'Grady, 2001). However individuals with local joint abnormalities such as maligned or lax knees have an increased likelihood of tibiofemoral osteoarthritis progression with greater quadriceps strength (Sharma et al., 2003). It is possible that by building up quadriceps

strength the greater joint reaction force exerted by the muscle exceeds the stabilizing benefit gained, resulting in osteoarthritis progression.

Acetaminophen (paracetamol) is a weak inhibitor of the synthesis of prostaglandins recommended as a first-line oral analgesic for knee OA (Zhang et al., 2008). (Jordan, 2003). A six week randomised control test in 25 patients showed that pain at rest for knee OA significantly improved with paracetamol over placebo (Jordan, 2003). Paracetamol's action at the molecular level is still unclear (Graham & Scott, 2005), but it appears to raise the patients pain threshold through a central rather than a peripheral mechanism (Zhang et al., 2004). However meta-analysis of clinical data has shown that both non steroidal anti-inflammatory drugs and COX-2 inhibitors are more effective at pain relief and restoring physical function than acetaminophen (Neame et al., 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) are small molecule drugs used to treat OA. They are effective for treating pain and are commonly used if the patient does not respond well to acetaminophen. NSAIDs target the isoforms of cyclooxygenase COX-1 and COX-2. COX-1 is expressed in many different cell types and COX-2 is induced more specifically at sites of inflammation. There are 2 subsets of NSAIDs, non-selective NSAIDs that inhibit both COX-1 and COX-2 and selective NSAIDs (coxibs) that target only COX-2. NSAIDs provide pain relief and have anti-inflammatory effects due to their ability to inhibit prostaglandin synthesis. COX enzymes are able to convert arachidonic acid into PGH2. PGH2 is then further catalysed by synthases into 5 bioactive prostaglandins, (PGE2, PGI2, PGF2a, PGD2, and thromboxane A2) (Chen et al., 2008). These prostaglandins then contribute to peripheral nerve sensitisation through protein kinase A mediated phosphorylation of sodium channels in nociceptor terminals, causing increased excitability and reducing the pain threshold (Wieland et al., 2005). When compared to paracetamol NSAIDs have generally had better efficacy but increased gastrointestinal side effects. For a 2 year randomised control trial Naproxen (a non-specific NSAID) was compared to acetaminophen in 178 patients. Although Naproxen led to greater reductions in pain than acetaminophen patient drop out was high due to acetaminophens lack of efficacy and adverse gastrointestinal side effects caused by Naproxen (Williams et al., 1993). As COX-1 is found in many different cell types it is thought that by only inhibiting the more specific COX-2 targets there would be less gastrointestinal side effects. However COX-2 is also present in

high levels in the kidney potentially causing hypertension and fluid retention in patients (Petit-Zeman, 2004). COX-2 inhibitors have also been linked with myocardial complications. In a comparison between 8076 patients taking either Naproxen or Rofecoxib (a selective inhibitor of COX-2) fewer clinically important upper gastrointestinal events were found when using Rofecoxib. However Rofecoxib also increased myocardial infarction rates (Krotz et al., 2005).

Intra-articular corticosteroid injections are used to deliver steroids directly to a joint with OA while avoiding most of the systemic effects caused by oral steroids. Oral steroids can cause peptic ulceration, aggravate diabetes, osteoporosis and muscle weakness. Injections of corticosteroids have been shown to significantly improve symptoms in a randomised trial of 101 patients. Patients were given either a placebo injection, an injection of corticosteroids or an injection of hyaluronic acid. While the corticosteroids had a clinical effect when compared to placebo it was short-lived with no protective effect seen at a 3 month follow up. The injections of hyaluronic acid caused no statistically significant change in patient outcome (Qvistgaard et al., 2006). Corticosteroid and hyaluronic acid intra-articular injections can cause pain with injection, cause post-injection flare, fat atrophy and can introduce infection into the joint (Kruse, 2008). A retrospective cohort study of 80 patients has shown the potential adverse immunosuppressive effect intra articular injections can have, seen by their effect on the eventual outcome of total joint replacement due to increased incidence of post operative joint infection (Kaspar & de V de Beer, 2005).

Osteotomy is surgery involving reshaping of bones. Osteotomy of the knee changes the position of the joint so that the weight bearing on the joint is on a healthy area of cartilage. Valgus high tibial osteotomy has been shown to improve joint function and reduce pain in the knee in eleven trials (Brouwer et al., 2005). Long term osteotomy cannot stop the degenerative process with the majority of patients eventually requiring total knee arthroplasty (van Raaij et al., 2007). Arthroplasty is a surgical procedure that replaces the arthritic joint surface with prosthesis. Joint replacement is used primarily in patients with late stage arthritis (Shen et al., 2011). With any surgery there are inherent risks such as bleeding, blood clots, breathing complications and infection as well as heart attack and stroke.

Synthetic MMP inhibitors such as S-34291 a wide spectrum inhibitor with preferential selection on MMP-13 and MMP-1 have successfully prevented the loss of cartilage ex vivo and in a guinea pig model (Cawston & Wilson, 2006). While several MMP inhibitors have shown impressive results in animal models Ro32-3555 (Trocade) a collagenase selective inhibitor did not prevent progression of joint damage in patients with rheumatoid arthritis (Close, 2001). Broad-spectrum synthetic inhibitors have also been linked to side effects such as musculoskeletal pain and tendonitis (Drummond et al., 1999). Selective MMP-13 inhibitors have shown chondro-protective effects in animals (Li et al., 2011) and could potentially offer a treatment for OA.

Although there are many different treatments available for OA there is a need for effective disease-modifying drugs. A new approach to treatment is needed. Prevention or slowing of OA via dietary intervention or supplementation is an attractive alternative.

1.5 Diet derived bioactives as a potential therapy

Pharmacological intervention for OA has had a lack of clinical efficacy. This may be due to pharmacological treatments typically only having a single mode of action and OA being a multifactoral disease. For this reason nutrition can be seen as an attractive alternative treatment as foods typically contain multiple bioactives that can interact with multiple cellular pathways (Ameye & Chee, 2006). Bioactive compounds are substances found in the diet that have an effect on living tissue. They are an attractive alternative strategy for treating OA due to their low side effect profile. Nutrition is potentially a modifiable factor that could have a significant impact on OA. Pharmaceuticals are normally prescribed after the onset of disease. Bioactive compounds and changes to the diet can be used to help prevent disease. Williams et al showed that a diet high in fruit and vegetables had a protective association with radiographic hip OA. Allium vegetable consumption was shown to have a protective association with hip OA. Diallyl disulphide is a compound found in alliums and it was shown that this compound could repress expression of MMPs in SW1353 chondrosarcoma cells (Williams et al. 2010). This form of treatment could potentially delay the onset of OA as well as reduce the severity and progression of the disease. Detailed molecular studies ahead of a full clinical trial are required in order optimally to design trials

to examine potential efficacy. There is currently limited data on the inter-relationship between diet and OA. Currently there is a large amount of variability in studies e.g. in animal models.

While a dietary intake approach would be optimal in order to relate to human exposure many studies use intra-articular injection and many use concentrations of compounds that would not be achievable through diet or supplementation. Intervention trials in man have variable numbers of patients, the length of time of each study can be vastly different and many of the studies are only of a short duration. Before dietary advice can be given for the prevention of OA, better quality studies need to be completed. Diet derived bioactive compounds with potential health benefits can be classed into several different groups. These groups consist of flavonoids (and related compounds), carotenoids, plant sterols, glucosinolates and others.

1.5.1 Flavonoids

Flavonoids are diphenylpropanes possessing 15 carbon atoms containing two benzene rings joined by a 3 linear carbon chains (Crozier et al., 2006). Flavonoids occur in plants and are common components of the human diet (Cao et al., 1997). There are several subcategories of flavonoids including flavan-3-ols, anthocyanins, flavonols, flavones, isoflavones and flavanones (Figure 1.12).

Flavonoids have been shown to have anti-platelet, anti-lipoperoxidant, anti-tumoral, anti-ischemic anti-inflammatory effects (Tzeng et al., 1991), (Joyeux et al., 1995), (Parajuli et al., 2009), (Leonardo & Doré, 2011). Flavonoids have also been shown to have the potential to protect against collagen matrix breakdown via inhibition of MMPs (M. Ahmed et al., 2005). Human chondrocytes from OA cartilage were incubated with epigallocatechin-3-gallate (EGCG) 20-100µM before with or without 5ng/ml of IL1 for 24 hours. EGCG inhibited the expression and activities of MMP-1 and MMP-13 in a dose dependent manner. It was hypothesized that EGCG had this effect due to inhibition of transcription factors such as NFκB at the mRNA level (S. Ahmed et al., 2004). In another study SW1353 cells were stimulated with IL1 at 10ng/ml for 24 hours before being treated with apigenin, a plant derived flavonoid at 25-5µM for 6 hours. Apigenin strongly inhibited *MMP13* induction. SW1353 cells were then treated with apigenin at 5-25µM for 2 hours before IL1 treatment

for 30 minutes. Cells were then lysed and nuclear extract was prepared for the electrophoretic mobility shift assay (EMSA). Apigenin did not reduce NFκB activation, however AP-1 was inhibited from binding to its corresponding DNA site and the translocation of c-fos was also inhibited.

1.5.2 Flavan-3-ols

Of the flavonoids the flavan-3-ol subclass is the most complex. The structures of flavan-3-ols can range from complex polymeric compounds such as procyanidins to simple monomeric catechins (Neilson & Ferruzzi, 2011). Flavon-3-ols such as catechins and procyanidins have been found in green and black tea as well as red and white wine (Auger et al., 2004). While not dietary derived Pycnogenol is a pine bark extract rich in procyanidins (D'Andrea, 2010). Pycnogenol has been reported to inhbit the activation of NFκB and the activity of MMPs (Grimm et al., 2006) (Grimm et al., 2004). While Pycnogenol has had positive outcomes in small trials (Belcaro et al., 2008), (Cisár et al., 2008) a Cochrane review of Pycnogenol stated that it was not possible to reach definite conclusions on either efficacy or safety of Pycnogenol (Schoonees et al., 2012).

1.5.3 Anthocyanins

Anthocyanins are sugar conjugates of anthocyanidins (Crozier et al., 2006). Anthocyanins often give pigmentation to fruit flesh and foliage (Chagne et al., 2012). Anthocyanins are thought to pigment plants to protect them from excess light and insect herbivory (Karageorgou & Manetas, 2006). Anthocyanins are thought to pigment plants to protect them from excess light and insect herbivory (Karageorgou & Manetas, 2006). Delphinidin an anthocyanidin found in pigmented fruits and vegetables has been shown to be an inhibitor of IL1 induced production of cartilage-degrading molecule PGE2 via inhibition of *COX-2* expression. Delphinidin was shown to block the activation of NFκB by suppressing the activation of upstream kinases NFκB-inducing kinase and IL1 receptor-associated kinase-1 in human OA chondrocytes (Haseeb et al., 2013).

1.5.4 Flavonols

Flavonols are commonly consumed in various teas, fruits and vegetables (Hertog et al., 1993). Myricetin, quercetin and kaempferol are commonly found as glycosylated flavonols.

During the fermentation of wine the glycosylated compounds are often released as free flavonols (Tsanova-Savova & Ribarova, 2002). The flavonol quercetin has been shown to prevent cartilage degradation in tissue explants. The loss of total aggrecan in cartilage explants maintained in catabolic medium for 6 days containing either 10-50µM of quercetin was examined. Quercetin suppressed total aggrecan loss in a dose dependent manner. Cartilage explant RNA was extracted and analysed using quantitative real time PCR (qRT-PCR). Quercetin suppressed IL1 stimulated expression of *ADAMTS4* and basal expression of *ADAMTS5*. Quercetin may protect cartilage from stimulated aggrecan loss by inhibiting the expression of *ADAMTS4* and *ADAMTS5* (Lay et al., 2012).

1.5.4 Flavones

Flavones are less common than flavonols. Celery and parsley are both edible sources of flavones containing luteolin and apigenin (Manach et al., 2004). Luteolin has been shown to inhibit IL1 induced *MMP13* via inhibition of IL1-induced extracellular signal-regulated Kinase (ERK) activation in a dose-dependent manner in osteoblasts (Yang et al., 2012). SW982 hyman synovial cells treated with 1-10µM luteolin over 24 hours had reduced IL1 induced MMP-1 production via inhibition of phosphorylated JNK and p38 levels (Choi & Lee, 2010). Flavones in a polymethoxylated form include nobiletin and tangeritin, found in citrus fruits (Swift, 1960), (Takanaga et al., 2000). Nobiletin is a polymethoxyflavone found in citrus fruit peel (S. Guo et al., 2012). Nobiletin has been shown to inhibit gene expression of IL1 and IL6 in mouse J774A.1 macrophages (Lin et al., 2003). Tangeritin has been shown to have both inhibit *MMP2* and *MMP9* in human brain tumour cell lines *in vitro* (Rooprai et al., 2001). MMP-2 and MMP-9 are gelatinases, however they also activate proMMP-13 a collagenase. Inhibition of *MMP2* and *MMP9* could reduce the activiation of MMP-13 preventing degradation of type 2 collagen.

1.5.5 Isoflavones

Isoflavones have the B-ring attached at C3 rather than the C2 position (Figure 1.12). Isoflavones are found in leguminous plants with high concentrations in soyabean (Sung et al., 2004). Genistein from soy protein has been shown to reduce serum total and low density lipoprotein (LDL)-cholesterol in humans (van der Schouw et al., 2000). Currently the data for genistein and OA are limited, *in vitro* and preclinical trials have not been able

to clearly define whether genistein has a protective effect on articular cartilage (Henrotin et al., 2011). While genistein has been reported to have no effect on cartilage metabolism (Claassen et al., 2008), it has been reported to inhibit COX-2 production giving genestin an anti-inflammatory effect in human chondrocytes (Hooshmand et al., 2007). It has been hypothesised that there is a relationship between OA and a changes to estrogen metabolism in menopausal women. As genistein shares a similar ring system with oestrogen it could have the capacity to alter the function of the reproductive system (Jeschke et al., 2005).

1.5.6 Flavanones

Flavanones have a chiral centre at C2 with no $\Delta^{2,3}$ double bond (Figure 1.12). In most flavanones the C-ring is attached to the B-ring at C2. Flavanones such as hesperidin and narirutin are commonly found in sweet oranges, tangerines and tangors (Peterson et al., 2006). 7, 3'-dimethoxy hesperetin (DMHP) a derivative of the parent compound hesperidin which has been reported to have have anti-inflammatory properties. Adjuvant arthritis (AA) was induced in rats and DMHP was administered intragastrically once a day from 12-21 days after AA induction. DMHP significantly inhibited hind paw swelling and arthritis index. The expression of *IL6* in AA rat synovium was suppressed in a dose dependent matter with DMHP treatment (Li et al., 2012).

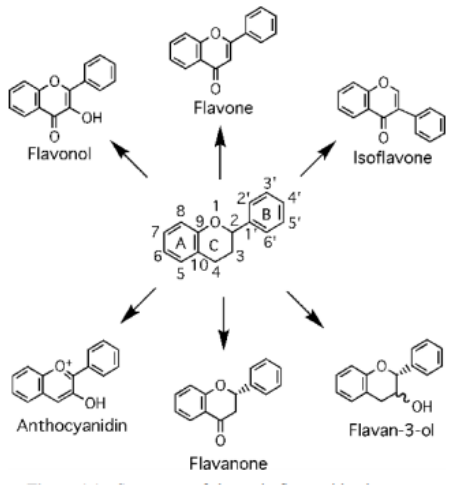


Figure 1.12. Structures of Flavonoid subgroups. General features of flavonoid structures. Image adapted from (Crozier et al., 2006).

1.5.7 Carotenoids

Carotenoids are a diverse group of tetraterpenes. β-carotene (Figure 1.13) is the most widely known tetraterpene in plants and is a direct precursor to vitamin A (Weeks, 1986). Vitamin A and its derivatives, retinoids, are known to have profound effects on cartilage and the skeleton and may contribute to osteoarthritis (Davies et al., 2009). The diverse set of structures that carotenoids have results in the compounds having a wide range of colours. Carotenoids give red and yellow colours to many fruits, vegetables, flowers, roots, algae, yeasts mushrooms and egg yolks (Isler et al., 1967). Carotenoids are apolar lipophylic molecules that are normally insoluble in water (Ziouzenkova et al., 1996), (Polyakov et al., 2009). Dietary sources of carotenoids such as lutein, lycopene, β-crytoxantin were found in foods such as carrots, citrus fruits, spinach, peas and tomatoes (O'Neill et al., 2001). Dutta, Chaudhuri, and Chakraborty described the health promoting functions of carotenoids as being involved in prevention of cancer, cardiovascular disease, cataract formation and macular degeneration. Epidemiological studies have shown that consuming carotenoids such as β-crytoxanthin in the form of a glass of freshly squeezed orange juice a day is associated with a reduced risk of developing inflammatory disorders such as rheumatoid arthritis (Pattison et al., 2005). Phytotene and phytofluene are colourless carotenoids that have been able to inhibit *MMP1* expression in IL1 induced fibroblasts at 15µg/ml (Fuller et al., 2006). Although this inhibition of collagenase was seen in fibroblasts carotenoids may have the potential to downregulate MMPs in chondrocytes.

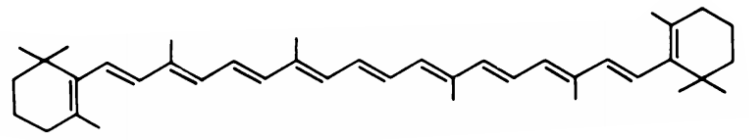


Figure 1.13. Structure of β-carotene. β-carotene is a widely known tetraterpene in plants. Image adapted from (Sies & Stahl, 1995).

1.5.8 Plant sterols

Plant sterols are found in all plants and like carotenoids are terpenoids (Figure 1.14). Plant sterol esters have been added to foods such as spreads. These reduced fat spreads containing plant sterols have been showed to reduce low density lipids (LDL) and high density lipids (HDL) cholesterol as well as triacylglycerol levels in subjects on a 5 week diet when compared to control diet groups (Maki et al., 2001). The plant sterol stigmasterol has shown potential anti-osteoarthritic properties. Human primary OA chondrocytes stimulated IL1 were pre-incubated with 20µg/ml of stigmasterol for 48 hours. *MMP3*, *MMP13* and *ADAMTS4* was significantly reduced. IKβ protein levels was reduced by stigmasterol at 20µg/ml (Gabay et al., 2010). These results suggest that plant sterols such as stigmasterol can inhibit MMPs and ADAMTSs involved in OA induced cartilage degradation in part through the inhibition of the NFκB pathway.

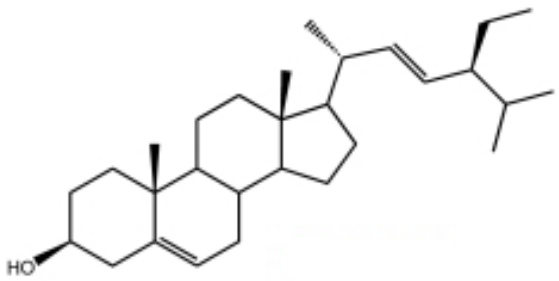


Figure 1.14. Structure of Stigmasterol. Stigmasterol is a major constituent of the sterol profiles of plant species. Image adapted from (Dufourc, 2008).

1.5.9 Glucosinolates

Glucosinolates are β-thioglucoside N-hydroxysulphates containing a variable side chain (Crozier et al., 2006), (Figure 1.15). Glucosinolates are found in hundreds of cruciferous vegetables (Vaughan et al., 1976) and many are precursors to isothiocyanates such as sulforaphane (SFN) (Figure 1.15). Vegetables such as broccoli are high in glucosinolates and often present in the human diet. When the vegetable is chopped or chewed the

glucosinolates are exposed to the enzymatic action of myrosinase. Myrosinase coexists in crucifers and once in contact with glucosinolate it hydrolysis its thioglucosidic bond releasing isothiocyanates, nitrile or thiocyanates causing the bitter taste of the vegetables (T. Shapiro et al., 2001), (Crozier et al., 2006). Bacterial myrosinase present in the human colon can also hydrolyse glucosinolates (Johnson, 2002). Mild cooking of cruciferous vegetables can denature the epithiospecifier protein (ESP) that normally prevents isothiocyanate formation, instead ESP promotes formation of nitriles (Traka & Mithen, 2008). This cooking method results in more of the beneficial isothiocyanates but prolonged heating may also denature myrosinase (Matusheski et al., 2004). While prolonged cooking does not seem to effect glucosinolates themselves they can leech into water if boiled (López-Berenguer et al., 2007). Studies have shown that isothiocyanates could potentially protect against cancer, diabetes and renal damage as well as neurodegenerative, ocular, respiratory and cardiovascular disorders (Seow, 2002), (Ragheb et al., 2009), (Nyengaard et al., 2004), (Trinh et al., 2008), (Gao et al., 2001), (Riedl et al., 2009), (Angeloni et al., 2009).

Isothiocyanates such as SFN have been shown to have potential anti-osteoarthritic effects. Human primary chondrocytes treated with 1-20µM of SFN and stimulated with IL1 an hour later for 24 hours had a reduced production of PGE2 and NO protein levels as well as *MMP1* and *MMP13* mRNA. At 5µM SFN inhibited COX-2 and iNOS at the mRNA and protein levels. At 10µM SFN significantly reduced IL1 stimulated NFκB promoter luciferase activity (H. Kim et al., 2012). This research suggests that SFN is capable of inhibiting a range of catabolic mechanisms in articular chondrocytes.

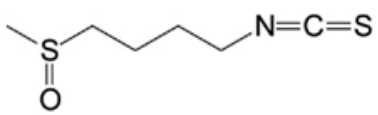


Figure 1.15. Structure of Sulforaphane. Suforaphane is a plant derived isothiocyanate obtained in the diet through consumption of cruciferous vegetables. Image adapted from (Boddupalli et al., 2012)

1.6 Conclusion and Aims

There are many compounds present in the habitual diet which have been shown to have activity in both laboratory models of osteoarthritis though this has not been convincingly proven in human disease to date. Current treatments for OA such as painkillers and anti-inflammatories are unable to prevent the progression of the disease. Joint replacement is offered to patients at end-stage disease, at large financial burden to the National Health Service (NHS). MMP-13, MMP-1, ADAMTS-4 and ADAMTS-5 have been shown to be a key collagenases and aggrecanases in cartilage degradation. We hypothesise that bioactives from the habitual diet will be chondro-protective. This project aims are to:

1. Build a custom dietary derived library of compounds from a Bioactive Compound library

2. Screen compounds from the custom built dietary derived library for inhibition of IL1-induced and basal *MMP13* expression and cytotoxicity in SW1353 and C28/I2 cells.

3. Select 20 compounds from the custom built dietary derived library for inhibition of IL1-induced and basal *MMP13* and *MMP1* expression in primary chondrocytes.

4. Complete dose-response curves for two candidate compounds from primary cell experiments looking at effects on *MMP13, MMP1, ADAMTS4* and *ADAMTS5* and screen for cell cytotoxicity.

5. Use cell based luciferase assays to analyse the two candidate compounds mechanism of action, exploring pathways implicated in cartilage homeostasis and OA the NFκB, TGFβ and Wnt pathways.

6. Perform an unbiased dissection of the kinase pathways affected by the two candidate compounds in primary chondrocytes by using the Human Phospho-Kinase Array.

Chapter 2: Materials and Method

2.1 Materials

2.1.1 Cell lines

2.1.1.1 SW1353

The SW1353 human chondrosarcoma cell line was obtained from American Type Culture [ATCC (Manassas, VA, USA)].

2.1.1.2 C28/I2

The immortalized human juvenile costal chondrocytes cell line C28/I2 were provided by Prof. Mary Goldring, Hospital for Special Surgery, New York. The C28/I2 cells were established by transduction of primary cultures of juvenile costal chondrocytes with vectors encoding simian virus 40 large T antigen (Goldring et al., 1994).

2.1.1.3 Primary Articular Chondrocytes

Primary human articular chondrocytes were isolated from the cartilage of patients with knee OA (KOA) who underwent knee replacement surgery at the Norfolk and Norwich University Hospital in collaboration with Prof. Simon Donell (Consultant Orthopaedic Surgeon and Honorary Professor, University of East Anglia, UK). Cartilage from KOA was removed from the entire surface of both tibial plateaus and femoral condyles including medial and lateral compartments. Cartilage samples were removed from the joint using a sterile scalpel and washed in sterile phosphate buffered saline (PBS) solution [Life Technologies (Glasgow)] containing 100U/ml penicillin, 100µg/ml streptomycin [Sigma-Aldrich (Dorset, UK)] and cut into 2-5mm pieces. These pieces were then incubated at 37°C in a shaker overnight in digestion media 0.1% (w/v) collagenase type 1A (Sigma-Aldrich) and 0.4% (w/v) Hepes (Sigma-Aldrich) in 50ml Dulbecco's modified Eagle medium (DMEM + GlutaMAX), (Life Technologies) containing 100U/ml penicillin, 100µg/ml streptomycin (Sigma-Aldrich). The digestion mixture was filtered through a cell strainer (BD Falcon) and centrifuged at 180g for 5 minutes. The cell pellet was washed in DMEM + GlutaMAX (Life technologies) media containing 100U/ml penicillin and 100µg/ml streptomycin (Sigma-

Aldrich). Cells were cultured in DMEM + GLUTAMAX (Life Technologies) containing 10% (v/v) foetal calf serum (FCS) [American Type Culture Collection (ATCC) (Washington, USA)], 2mM glutamine, and 100 IU/ml penicillin and 100ug/ml streptomycin (Sigma-Aldrich). Cells were seeded at $4x10^4$ cells/cm^2 and incubated at 37°C, 5% CO_2 (v/v) and allowed to adhere for ~8 days. The media was changed following adherence, and at 80-90% confluency, cells were either frozen and stored in liquid nitrogen, or utilised in in vitro assays up to passage 1.

2.1.2 Diet derived bioactives

2.1.2.1 Compound screens

Dietary-derived compounds (Sigma-Aldrich) were selected from the literature for suspected anti-inflammatory effects. Ninety six compounds were selected from Bioactive Compound Library [Stratech (Suffolk, UK)]. For compound list see Appendix. Compounds were stored in dimethyl sulfoxide (DMSO) (Sigma-Aldritch) at 10mM at -80°C.

2.1.2.2 Selected compounds

Aloe emodin, apigenin and isoliquiritigenin were purchased from (Sigma-Aldrich) and made into 50mM stocks with DMSO for use in all dose response and time course experiments.

2.1.3 Cytokines

Recombinant human interleukin-1 (IL1), Wnt3a and transforming growth factor beta (TGFβ) were purchased from R&D Systems (Abingdon, UK), reconstituted in phosphate-buffered saline solution (PBS) containing 0.1% (w/v) bovine serum albumin and stored at -80°C (as recommended by supplier).

2.1.4 Immunoblotting

All antibodies used were rabbit polyclonal unless otherwise stated. (Total p38 Antibody #9212), [p-p38 (Thr180/Tyr182) Antibody #4511S], (total JNK Antibody #9258S), [pJNK (Thr183/Tyr185) Antibody #4668S], [total Erk (Erk1/2) Antibody #9102] and [p-Erk (Erk1/2) (Thr202/Tyr204) Antibody #9101S] were purchased from Cell Signaling Technology (Beverly MA, USA). Secondary antibody used was a goat polyclonal anti-rabbit IgG, alkaline

phosphatase for fluorescent detection [Abcam, (Cambridge)]. Horseradish peroxidase-conjugated anti-mouse (PO260) secondary antibodies were purchased from Dako UK Ltd (Cambridgeshire, UK).

Polyvinylidene fluoride membrane (PVDF) membrane was purchased from Millipore (Watford, UK). Sodium dodecyl sulphate (SDS), N,N,N,N'-Tetramethylethylenediamine (TEMED), extra thick filter paper, protein standard Precision Plus dual colour, gel rigs, electrophoresis tanks and a Trans-Blot SD Semi-dry electrophoretic transfer cell were all purchased from Bio-Rad Laboratories (Hemel Hempstead, UK). Ammonium persulphate (APS), polyoxyethylenesorbitan monolaurate (Tween-20), bovine serum albumin (BSA) were purchased from Sigma Aldrich (Dorset, UK). Non-fat dry milk powder was purchased from Fisher (Leicestershire, UK).

2.2 Methods

2.2.1 Cell culture

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) + GlutaMAX (Life Technologies) containing 10% v/v FCS (ATCC) , 2mM glutamine, and 100 IU/ml penicillin and 100ug/ml streptomycin (Gibco).Cells were incubated at 37°C in a humidified atmosphere of 5%v/v CO2. Every 3 to 4 days media was replaced until cells reached confluence. Confluent cells were passaged using trypsin-1mM ethylenediaminetetraacetic acid (EDTA) (Life Technologies). For long term storage, cells were cryogenically frozen via trypsinisation, centrifuged at 130g for 5 minutes and resuspended in cryo-preservation medium (90% v/v FCS, 10% v/v DMSO) before slow freezing (at approximately 1°C per minute) in a cell freezing chamber at -80°C. For longer term storage cells were transferred to liquid nitrogen.

2.2.1.1 Cell treatments

For qrRT-PCR experiments cells were plated at $10x10^3$ cells per well in a 96 well plate (Fisher Scientific) in DMEM + GLUTAMAX (life technologies) [+10% (v/v) heat inactivated FCS (ATCC) and left to adhere overnight followed by a 12-hour serum starvation (DMEM alone). Compounds of interest were added an hour before stimulation with 5ng/ml IL1 (R&D Systems Europe Ltd, Abingdon, UK).

For cloning SW1353 cells were plated onto a 96-well plate (Fisher Scientific) at $6x10^3$ per well in DMEM + GLUTAMAX (life technologies) [+10% (v/v) heat inactivated FCS (ATCC) and left to adhere overnight at 37°C, 5% (v/v) CO_2 followed by a 12-hour serum starvation (DMEM alone). Compounds or interest were added an hour before stimulation with IL1, wnt3a or TGF-β (R&D Systems).

For Western blot analysis Primary KOA chondrocytes or SW1353 cells were plated at $2x10^5$ cells per well in a 6 well plate (Fisher Scientific) in DMEM + GLUTAMAX (Life Technologies) [+10% (v/v) heat inactivated FCS (ATCC) and left to adhere overnight at 37°C, 5% (v/v) CO_2 followed by a 12-hour serum starvation in phenol free DMEM (life technologies). Compounds of interest were added an hour before stimulation with IL1 (R&D Systems).

2.2.1.2 Cytotoxicity assay cell necrosis

Cytotoxicity of compounds were assessed using the CytoTox 96® Non-Radioactive Cytotoxicity as described by the manufacturer's instructions [Promega (Hampshire, UK)]. The CytoTox 96® Assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay, which results in conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. Visible wavelength absorbance data are collected using a 96-well plate reader. Data was presented as percentage of cell death (necrosis) relative to total cell death control cell lysis ± mean standard deviation. Total cell death control (100% cell death) calculated by freeze thawing no treatment chondrocytes twice and measuring LDH released.

2.2.1.3 Cytotoxicity assay cell apoptosis

Cytotoxicity of compounds were assessed using the Caspase-Glo® 3/7 Assay as described by the manufacturer's instructions (Promega). The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in cultures of adherent cells. Total cell death control (100% cell death) calculated by treating cells with 1µM stuarosporine for 6 hours. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a

substrate of luciferase used in the production of light. The Caspase-Glo® 3/7 Reagent is optimized for caspase activity, luciferase activity and cell lysis. Addition of the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal.

2.2.2 cDNA synthesis – Cells-to-cDNA II with MMLV RNA

Cells were washed in ice-cold PBS twice. Cells were harvested into 30 µl of Cells-to-cDNA II cell lysis buffer [Ambion, Applied Biosystems, (Warrington, UK)]. The lysate was transferred to a 96-well PCR plate (Applied Biosystems) and incubated at 75°C for 15 minutes. 2U DNase I (Ambion) and 10x DNase I buffer (Ambion) were added to each well and incubated at 37°C for 15 minutes followed by 75°C for 5 minutes. DNase I-treated lysates (8 µl) were transferred to a fresh 96-well PCR plate. Lysates were primed for reverse transcription using 10 mM dNTP mix (2.5 mM of each) [Bioline (London, UK)] and 200ng random primers (Invitrogen) at 70°C for 5 minutes. Reverse transcription was carried out in a volume of 20µl using 100U M-MLV reverse transcriptase (Invitrogen) and 20U RNasin ribonuclease inhibitor (Promega) according to the manufacturer's instructions (Promega). Samples were incubated at 37°C for 50 minutes then 75°C for 15 minutes. To each well, 30µl analytical grade H_20 (Sigma-Aldrich) was added and cDNA stored at -20°C.

2.2.3 Quantitiative Real Time PCR (qRT-PCR)

2.2.3.1 Standard Probe-based Real-Time qRT-PCR

Relative quantification of genes was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems) with MicroAmp optical 96-well plates (Applied Biosystems) using selected primer probe sets (see Table 4) in accordance with the manufacturer's protocol. The constitutively expressed 18S rRNA housekeeping gene was used as the control for relative mRNA gene expression. The threshold cycle (Ct) is the cycle number during an RT-PCR run where the signal is detectable above baseline. Samples were excluded from further analysis if their 18S rRNA Ct level exceded 1.5Ct ± the median 18S rRNA Ct to ensure quality of data. Our lab has previously established 18S rRNA to be the optimal housekeeping gene for use with chondrocyte cell lines and cartilage samples. For genes of interest 5ng of cDNA was used per reaction, for 18S rRNA 1ng of cDNA was used

per reaction. 2x Taqman Universal PCR Master Mix (Applied Biosystems), 100nM forward and reverse primers (Sigma-Aldrich) and 200nM specific probe (Sigma-Aldrich) was added to each reaction. PCR reactions used 5µl of reverse-transcribed RNA (for 18S rRNA analysis a 10-fold dilution of cDNA was used), 2x TaqMan Universal Master Mix (Applied Biosystems), 100nM of each primer and 200nM of probe in a total volume of 20µl. Conditions for the PCR reaction were as follows: 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Relative quantification is expressed as $2^{-\Delta Ct}$, where ΔCtT is Ct (target gene) – Ct (18S). Microsoft Excel was used to analyse the data.

Gene	Sequences	Reference
MMP1	Forward Primer: 5'-AAGATGAAAGGTGGACCAACAATT -3' Reverse Primer: 5' -CCAAGAGAATGGCCGAGTTC -3' Probe: 5'- FAM-CAGAGAGTACAACTTACATCGTGTTGCGGCTC-TAMRA -3'	(Nuttall et al., 2003)
MMP13	Forward Primer: 5'- TTCCGCCTGTCTCAAGATGATAT -3' Reverse Primer: 5'- AAAGGACAAAGCAGGATCACAGTT -3' Probe: 5'- FAM-TCAGTCCCTCTATGGACCTCCCCCTGAC-TAMRA -3'	(Nuttall et al., 2003)
ADAMTS4	Forward Primer: 5'- CAAGGTCCCATGTGCAACGT -3' Reverse Primer: 5'- CATCTGCCACCACCAGTGTCT -3' Probe: 5'- FAM-CCGAAGAGCCAAGCGCTTTGCTTC-TAMRA -3'	(Porter et al., 2005)
ADAMTS5	Forward primer: 5'-TGTCCTGCCAGCGGATGT-3' Reverse primer: 5'-ACGGAATTACTGTACGGCCTACA-3' Probe: 5'- FAM-TTCTCCAAAGGTGACCGATGGCACTG-TAMRA -3'	(Porter et al., 2005)
18S rRNA	Forward primer: 5'-GCCGCTAGAGGTGAAATTCTTG-3' Reverse primer: 5'-CATTCTTGGCAAATGCTTTCG-3' Probe: 5'-ACCGGCGCAAGACGGACCAG-3'	(Corps et al., 2006)

Table 4. Quantitative Real Time PCR primer probe sets. Primer and probe sets listed for the genes analysed using qRT-PCR. FAM was the fluorophore used and TAMRA was the quencher. All other primers were designed using the Universal Probe Library Assay Design Center by Roche Applied Science and used with probes from the Universal Probe Library.

2.2.3.2 Universal Probe-based Real-Time qRT-PCR

The short 8-9 nucleotide-long locked nucleic acid probes give the Universal Probe library [Roche Applied Science (West Sussex)] wide transcript coverage. *HO-1* and *AXIN2* were designed using the Universal Probe Library (Roche). The PCR reaction mix contained Taqman 2x mastermix, 100nM of forward and reverse primer and 200nM of fluorescent probe (in a final volume of 25µl). PCR cycles the same as Standard Probe-based Real-Time qRT-PCR.

2.2.4 Sub-cloning

The smad2/3/4 signalling reporter (CAGA12-luc) contains 12 binding sites of the Smad2/3/4 binding consensus upstream of the pGL3 gene, this reporter was a gift from Dr Andrew Chantry, University of East Anglia, UK and is described in (Pais et al., 2010).

The NFκB signalling reporter (κB) vector contains 5 binding sites of NFκB upstream of the firefly luciferase-encoding gene pGL3, this reporter was a gift from Prof. Derek Mann, (Newcastle University, UK), (originally from Prof. Ronald Hay, University of Dundee, UK).

The canonical Wnt signalling reporter (TOPFLASH) vector contains 7 binding sites of TCF/LEF upstream of the firefly luciferase encoding gene in the pTAL-Luc vector, this reporter was a gift from Prof. Andrea Munsterberg (University of East Anglia, UK), the reporter was originally from Prof. Randall Moon (University of Washington, USA).

2.2.4.1 Preparation of competent DH5α cells Plasmid extraction (Qiagen mini preps)

E.coli DH5α bacterial colonies were spread onto LB/Agarplates supplemented with 100µg/µl ampicillin (Sigma-Aldrich) and incubated overnight at 37°C. One colony was inoculated into 5ml LB broth [1% w/v Tryptone (Fisher Scientific), 2% w/v Yeast Extract (Fisher Scientific) and 0.5% w/v Sodium Chloride (Fisher Scientific] and incubated overnight at 37°C. Once cells reached optimal density (OD600 = 0.6) they were chilled on ice for 10 minutes in 50ml Falcon tubes (Fisher Scientific). Cells were centrifuged at 1000 x g for 10 minutes at 4°C and supernatant was removed. The cell pellet was re-suspended in 5ml 0.1M CaCl (Fisher Scientific) and incubated on ice for 10 minutes before being centrifuged at 1500g, 4°C for 10 minutes where the supernatant was then removed. The pellet was re-suspended gently into 1ml ice cold 0.1M CaCl (Fisher Scientific) and glycerol (400µl / ml) (Fisher Scientific) and stored frozen at -80°C.

2.2.4.2 Plasmid extraction (Qiagen mini preps)

The protocol was followed as per manufacturer's instructions and all buffers described are from [Qiagen (Manchester)]. 1.5ml bacterial cells were spun at 10,000 x g for 5 minutes and the supernatant removed. The bacterial pellet was re-suspended in 250µl mini-prep buffer P1 to this 250µl mini-prep buffer P2 was added and mixed by inversion. 350µl N3

buffer was added, mixed by inversion and centrifuged at 10,000 x g for 10 minutes. All supernatant was then transferred into QIAspin columns and centrifuged for 1 minute at 10,000 x g with flow through removed. 750µl of PE buffer was added to the column and spun at 10,000 x g for 1 minute and flow through was discarded. Plasmid was then eluted from QIAspin column with 30µl of analytical grade water by centrifugation at 10,000 x g for 1 minute.

2.2.4.3 SW1353 transfection

SW1353 cells were plated onto a 96-well plate (Fisher Scientific) at $6x10^3/cm^2$ well in DMEM + GLUTAMAX (life technologies) [+10% (v/v) heat inactivated FCS (ATCC) and left to adhere overnight at 37°C, 5% (v/v) CO_2. Transfections were carried out using 100ng plasmid DNA, 500ng Lipofectamine 2000 (Fisher Scientific) and 50ng of Renilla plasmid for 24 hours at 37°C, 5% (v/v) CO_2 in serum-free culture medium (DMEM alone). Cells were then treated for 6 hours. The Promega Dual Luciferase Reporter Assay was used to measure luciferase activity according to manufacturer's instructions. Media was removed from wells of 96 well tissue culture plate (Fisher Scientific), and cells washed with cold sterile PBS solution (Life Technologies). Cells were treated with 50µl of lysis buffer (Promega), scraped and incubated for 30 minutes at room temperature. 10µl was transferred to an opaque 96 well plate and 50µl of luciferase assay reagent II was added and firefly luciferase activity measured immediately on a spectrophotometer [EnVision 2103 Multilabel plate reader (Perkin Elmer)] (Luminescence 700 setting, 560nm) using Wallac EnVision Manager software. 50µl of Stop-andGgo solution (Promega) was added and the plate was measured for a second time to measure renilla activity (Luminescence 700 setting on the Envision plate reader, 560nm). Firefly luciferase relative light units were normalised to Renilla relative light units to account for the transfection efficiency.

2.2.5 Western blotting

Primary KOA chondrocytes or SW1353 cells were plated at $2x10^5$ cells per well in a 6 well plate (Fisher Scientific) in DMEM + GLUTAMAX (life technologies) [+10% (v/v) heat inactivated FCS (ATCC) and left to adhere overnight at 37°C, 5% (v/v) CO_2 followed by a 12-hour serum starvation in phenol free DMEM (Life Technologies). Compounds of interest were added an hour before stimulation with IL1 (R&D Systems). For MMP analysis media

was harvested and frozen at -20°C. For all other experiments whole cells were washed twice in sterile ice cold PBS (Life Technologies) cell lysates were harvested and scraped into 100µl ice cold radioimmunoprecipitation assay buffer (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate, 10 mM NaF, 2 mM Na_3VO_4, 1 protease inhibitor cocktail (Fisher Scientific). Samples were centrifuged for 10 minutes at 300g. Supernatant was collected and stored at -20°C.

2.2.5.1 Bicinchoninic acid (BCA) protein quantification assay

Protein concentrations of lysate samples as well as conditioned media were assessed using the bicinchoninic acid (BCA) protein quantification assay as described by the manufacturer's instructions (Life technologies) in order to normalise each sample to cellular protein levels before SDS-PAGE analysis. A standard curve of known protein bovine serum albumin (BSA) was compared to individual samples to determine their total cellular protein concentrations. Increasing amounts of BSA (0.3125-10mg/ml) and equivalent volume of samples were plated into 96-well plates. Each well had 200µl of BCA reagent (50 parts of reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartate in 0.1M sodium hydroxide) to 1 part of reagent B (solution of 4% copper sulfate pentahydrate) added to each well. Samples were then incubated for 30 minutes at 37°C. Absorbance was determined at 550nm using [EnVision 2103 Multilabel plate reader (Perkin Elmer)]. The concentrations of each samples were determined from a standard curve that was constructed with the OD values obtained from the known standard (BSA).

2.2.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A running gel was created by combining 10% v/v acrylamide [BIO-RAD (Hertfordshire)], 365mM Tris-HCl (pH 8.8) (Sigma Aldrich), 0.1% w/v SDS, 0.05% w/v ammonium persulphate (APS) and 0.04% w/v tetramethylethylenediamine (TEMED) (BIO-RAD). This was left at room temperature to polymerise. A stacking gel was made by combining 4% v/v acrylamide, 365mM Tris-HCl (pH 8.8), 0.1% w/v SDS, 0.1% w/v APS and 0.04% v/v TEMED, stacking gel was poured onto running gel. A comb was added to create loading wells and the gel was left to polymerise. The SDS-PAGE equipment was assembled and running buffer (25mM Tris-HCl, 200mM glycine and 0.1% w/v SDS) was poured into the tank. Samples were mixed with 5x sample buffer (10% w/v SDS, 10nM β-mercaptoethanol, 20% v/v

glycerol, 0.2M Tris-HCl (pH 6.8), 0.05% w/v Bromophenol Blue) at a ratio of 4:1 and incubated at 100°C for 5 minutes. Samples were then loaded to SDS-PAGE gel and ran at 200v for 45 minutes separating protein samples.

2.2.5.3 Semi-dry blot transfer

Protein samples were then transferred to Polyvinylidene Fluoride (PVDF) membrane at 10v for 30 minutes using the semi-dry blot technique in transfer buffer (buffer (0.29% w/v Glycine, 0.58% w/v Tris, 0.037% w/v SDS and 20% w/v Methanol). Samples on PVDF membranes were then covered with blocking solution (5% w/v milk powder, Tris-buffered saline solution (TBS- 150mM NaCl, 100mM Tris, pH 7.5)) for 1 hour at room temperature. Samples were then washed in wash buffer (TBS, 0.1% w/v Tween 20) and incubated overnight with primary antibody in antibody dilution buffer (TBS, 0.1% v/v Tween 20 and 2.5% w/v milk) (see immunoblotting for primary antibody details). Membranes were then washed three times using wash buffer and incubated with secondary antibody in antibody dilution buffer. Secondary antibodies used were a horseradish peroxidase-conjugated secondary antibody (Dako) for Chemiluminescent detection and a goat polyclonal anti-rabbit IgG (Abcam) for fluorescent detection in the Odyssey Infrared Imaging System [Li-COR (Cambridge UK].

2.2.6 Human Phospho-Kinase Antibody Array

The analysis of phosphorylation profiles of 43 kinases and their protein substrates was completed with the Human Phospho-Kinase Array as described by the manufacturer's instructions (R&D Systems). Capture and control antibodies were spotted in duplicate on nitrocellulose membranes. Cell lysates were diluted and incubated overnight with the Human Phospho-Kinase Array. The array was washed to remove unbound proteins followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied and a signal was produced at each capture spot corresponding to the amount of phosphorylated protein bound allowing semi-quantitfication of phosphorylated representatives of the MAP Kinases, Src family and Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) JAK/STAT pathways amongst others.

2.2.7 Statistical analysis

Microsoft Excel (2008) for Mac was used to analyse data. Students t-test and one-way ANOVA with Dunnett's post test was performed using GraphPad Prism version 6 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. $p<0.05$ was chosen as the cut off for statistical significance. Student's t-test was used to test the difference between two groups. One-way ANOVA was used when testing for differences between ≥3 groups.

Chapter 3: Dietary Derived Bioactive Screen

3.1 Introduction

As discussed in the chapter 1, metalloproteinases play a pivotal role in the destruction of cartilage seen during OA. In particular, *MMP13* has been considered critical for OA progression. Pharmacologic inhibition of *MMP13* has been shown to be an effective strategy to decelerate articular cartilage loss in a murine model of injury-induced knee OA (Wang et al., 2013). Few pharmaceutical companies now consider OA as a disease area. This is in part due to issues with drug toxicity but also because OA generally progresses slowly and there are no validated biomarkers for cartilage destruction (the only FDA approved end point in a clinical trial is joint space narrowing which is assessed by x-ray) (Kraus, 2012). A potential alternative strategy is to focus on compounds from the habitual diet that may prevent the onset or slow the progression of OA. Williams et al., showed that a diet high in fruit and vegetables had a protective association with radiographic hip OA. Osteoarthritic chondrocytes have been shown to produce MMPs that can result in degenerative changes of the cartilage matrix in OA (Tetlow et al., 2001).

As MMP gene expression is primarily regulated at the transcriptional level (Fanjul-Fernández et al., 2010) a useful initial readout of *MMP13* mRNA levels in SW1353 cells can be used to select bioactive compounds for further study. Previous data published by our laboratory has shown that SFN significantly inhibited cytokine-induced *MMP13* expression at 5μM in a dose-dependent manner in SW1353 cells (Davidson et al., 2013).

We hypothesise that bioactives in the habitual diet will be chondro-protective. The aims of this chapter are:

- Design a custom dietary derived library of 96 dietary derived bioactive compounds (see Appendix for compound list) from a list of natural products.

- Screen compounds for potential toxic effects using a cell necrosis cytoxicity assay.

- Use qRT-PCR to screen 96 dietary derived bioactives for inhibition of IL1-induced and basal *MMP13* expression in SW1353 cells.

- Rescreen these compounds in the C28/I2 cell line to eliminate compounds with cell line specific effects.

- Select compounds that show MMP13 inhibitory effects in both cell lines for further analysis in primary chondrocytes.

3.2 Results

3.2.1 Designing the custom dietary derived library

The original Bioactive Compound Library contained a collection of bioactive chemical compounds from synthetic and natural sources. Only compounds that were produced by living organisms were selected from this list creating the Natural Product Library. Ninety six dietary derived compounds were selected from the Natural Product Library and compiled into a custom-built library in a 96-well plate format. Criteria for compound selection shown in Figure 3.1.

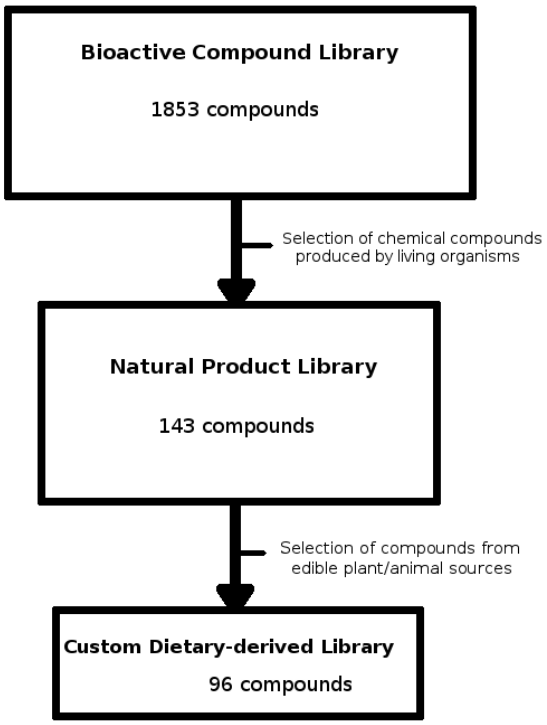


Figure 3.1. Custom dietary derived compound library design. In order to be selected for the 96-well plate custom library the natural products had to be dietary derived. The library was designed to contain bioactive compounds with a range of chemical structures including flavonoids, anthocyanins, terpenoids, carotenoids and other polyphenols as well as precursors of dietary compounds such as polydatin (a natural precursor of resveratrol).

3.2.2 Cell cytotoxicity screen

3.2.2.1 Dietary derived compounds effects on cell necrosis - Lactate dehydrogenase assay

SW1353 chondrosarcoma cells were cultured and plated as described in section 2.2.1.1. SW1353 cells were treated with one of 96 dietary-derived compounds described in section 2.1.2.1 at 10µM for 6 hours. The CytoTox 96® Non-Radioactive Cytotoxicity assay was used as described by the manufacturer's instructions (see Chapter 2 section 2.7.2 for more details). LDH was detected in harvested supernates as a measure of compound toxicity. Compounds from the custom built dietary derived library had no significant effect on cell necrosis compared to no treatment control (Figure 3.2).

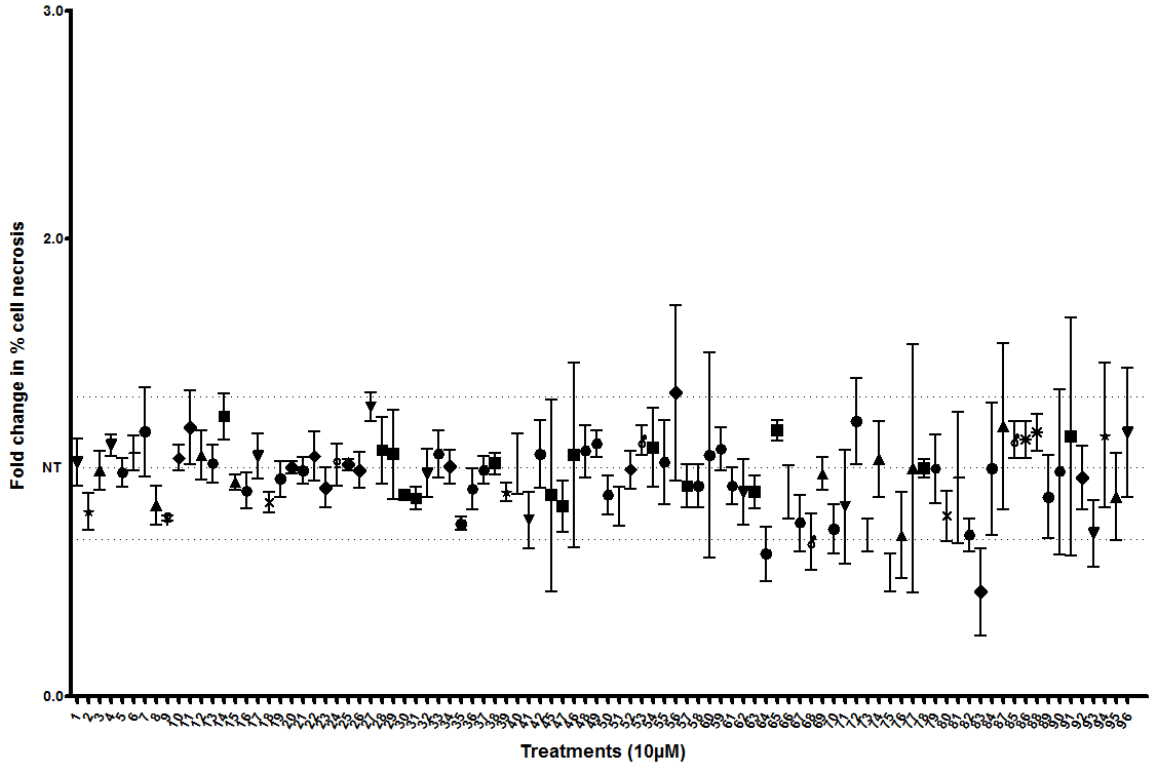


Figure 3.2. Fold change in percentage cell necrosis relative to untreated control samples in SW1353 cells. SW1353 cell line pre-treated with 10µM of one of 96 diet derived compounds (see Appendix for compound list). Compounds effect on fold change in % cell necrosis relative to untreated control was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with compounds for 6 hours. Data plotted as mean ± SEM, n=3. Dotted line represents cell necrosis in untreated control sample cells with ± standard deviation.

3.2.3 Bioactive compound screen in SW1353 cells

3.2.3.1 SW1353 IL1 induced *MMP13* expression screen

SW1353 chondrosarcoma cells were cultured and plated as described in section 2.2.1.1. SW1353 cells were treated with one of 96 dietary-derived compounds described in section 2.1.2.1 at 10µM for 6 hours. Compounds of interest were added an hour before stimulation with 5ng/ml IL1. Emodin, ursolic acid, oleanolic acid, luteolin, polydatin, apigenin, neohesperidin dihydrochalcone, l-carnitine and isoliquiritigenin significantly inhibited IL1 induced *MMP13* expression in SW1353 cells (p<0.05-0.0001) (Figure 3.3). DL-Carnitine hydrochloride significantly increased IL1 induction of *MMP13* expression in SW1353 cells (Figure 3.3).

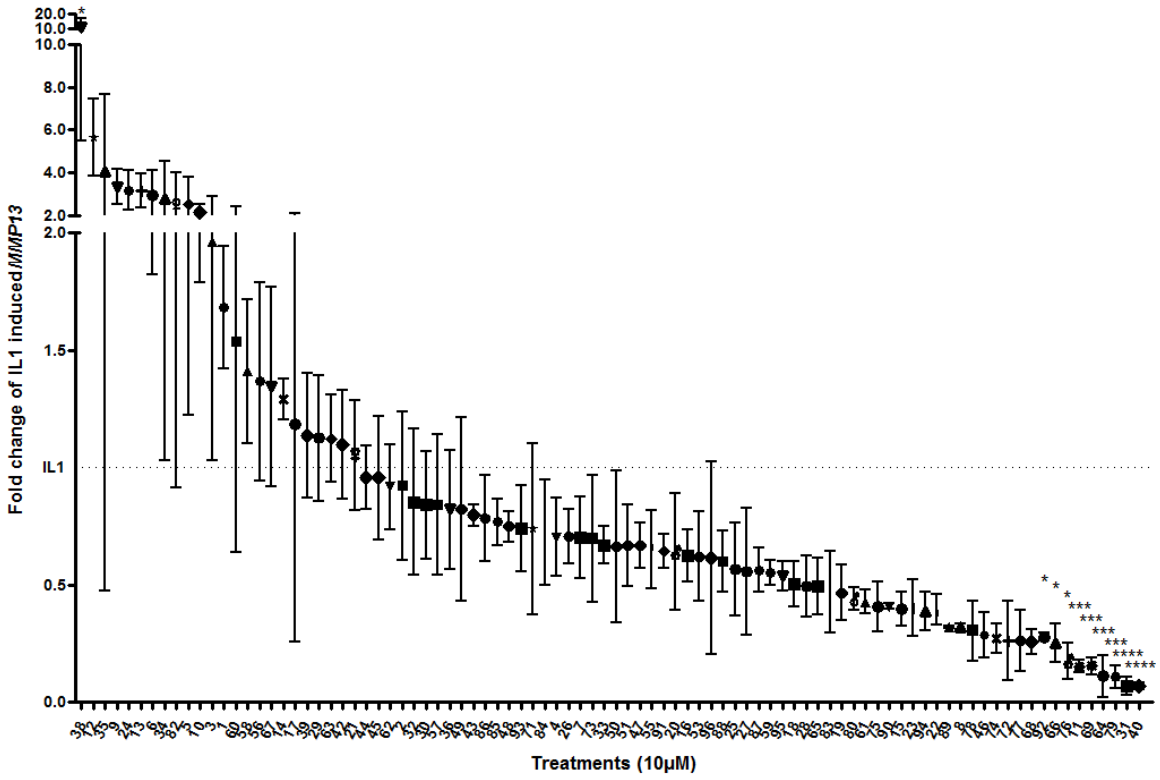


Figure 3.3. Fold change in *MMP13* gene expression normalised to 18s relative to IL1 treated control samples in SW1353 cells. SW1353 cell line pre-treated with 10µM of one of 96 diet derived compounds (see Appendix for compound list). Compounds effect on IL1 induced *MMP13* levels was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. and plotted as mean ± SEM, n=3. Dotted line represents IL1 treatment alone. * p<0.05, *** p<0.001, **** P<0.0001

3.2.3.2 SW1353 Basal *MMP13* expression screen

SW1353 chondrosarcoma cells were cultured and plated as described in section 2.2.1.1. SW1353 cells were treated with one of 96 dietary-derived compounds described in section 2.1.2.1 at 10µM for 6 hours without IL1 stimulation. Myricetin, theobromine and sesamin significantly inhibited basal *MMP13* expression in SW1353 cells ($p<0.01$) (Figure 3.4).

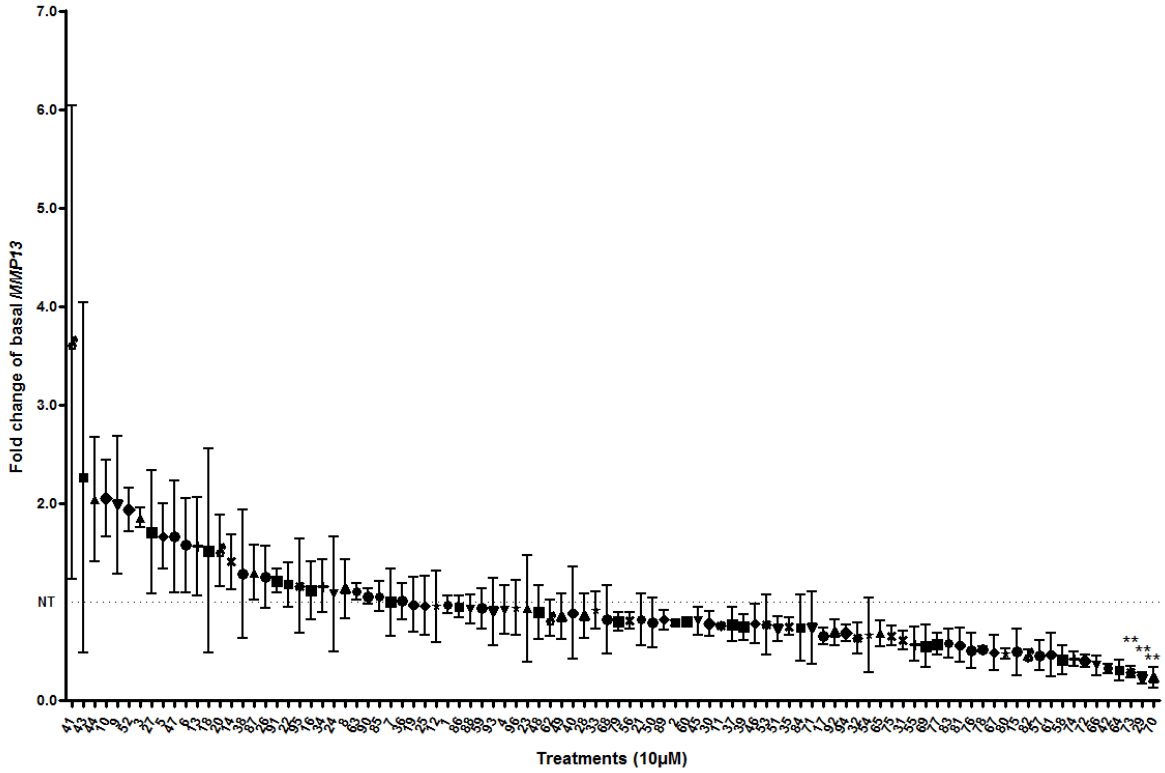


Figure 3.4. Fold change in *MMP13* gene expression normalised to 18s relative to untreated control samples in SW1353 cells. SW1353 cell line pre treated with 10µM of one of 96 diet derived compounds (see Appendix for compound list). Compounds effect on basal *MMP13* levels was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean ± SEM, n=3. Dotted line represents no treatment (NT) control samples. ** $p < 0.01$.

3.2.4 Bioactive compound screen in C28/I2 cells

3.2.4.1 C28/I2 IL1 induced *MMP13* expression screen

C28/I2 chondrocyte cells were cultured and plated as described in section 2.2.1.1. C28/I2 cells were treated with one of 96 dietary-derived compounds described in section 2.1.2.1 at 10µM for 6 hours. Compounds of interest were added an hour before stimulation with 5ng/ml IL1. Apigenin, troxerutin, luteolin, aloe-emodin, methyl-hesperidin, ursolic acid, emodin, fisetin, genistin and polydatin significantly inhibited IL1 induced *MMP13* expression in C28/I2 cells ($p<0.05$-0.0001) (Figure 3.5).

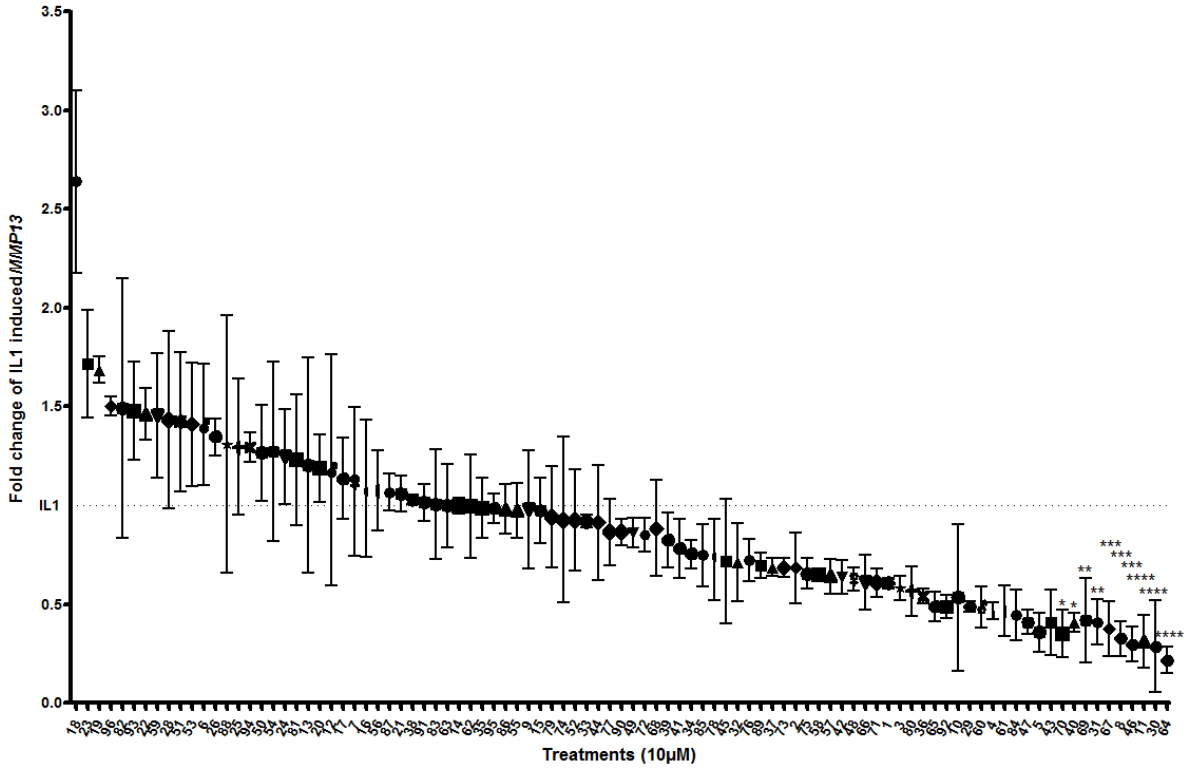


Figure 3.5. Fold change in *MMP13* gene expression normalised to 18s relative to IL1 treated control samples in C28/I2 cells. C28/I2 cell line pre treated with 10µM of one of 96 diet derived compounds (see Appendix for compound list). Compounds effect on IL1 induced *MMP13* levels was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean ± SEM, n=3. Dotted line represents IL1 treatment alone. * $p<0.05$, ** $p < 0.01$, *** $p<0.001$, **** $P<0.0001$.

3.2.4.2 C28/I2 Basal *MMP13* expression screen

C28/I2 chondrocyte cells were culture and plated as described in section 2.2.1.1. C28/I2 cells were treated with one of 96 dietary-derived compounds described in section 2.1.2.1 at 10µM for 6 hours without IL1 stimulation. Apigenin, myricetin, genistin, hesperetin, biochanin A significantly inhibited basal *MMP13* expression in C28/I2 cells (p<0.01-0.0001) (Figure 3.6).

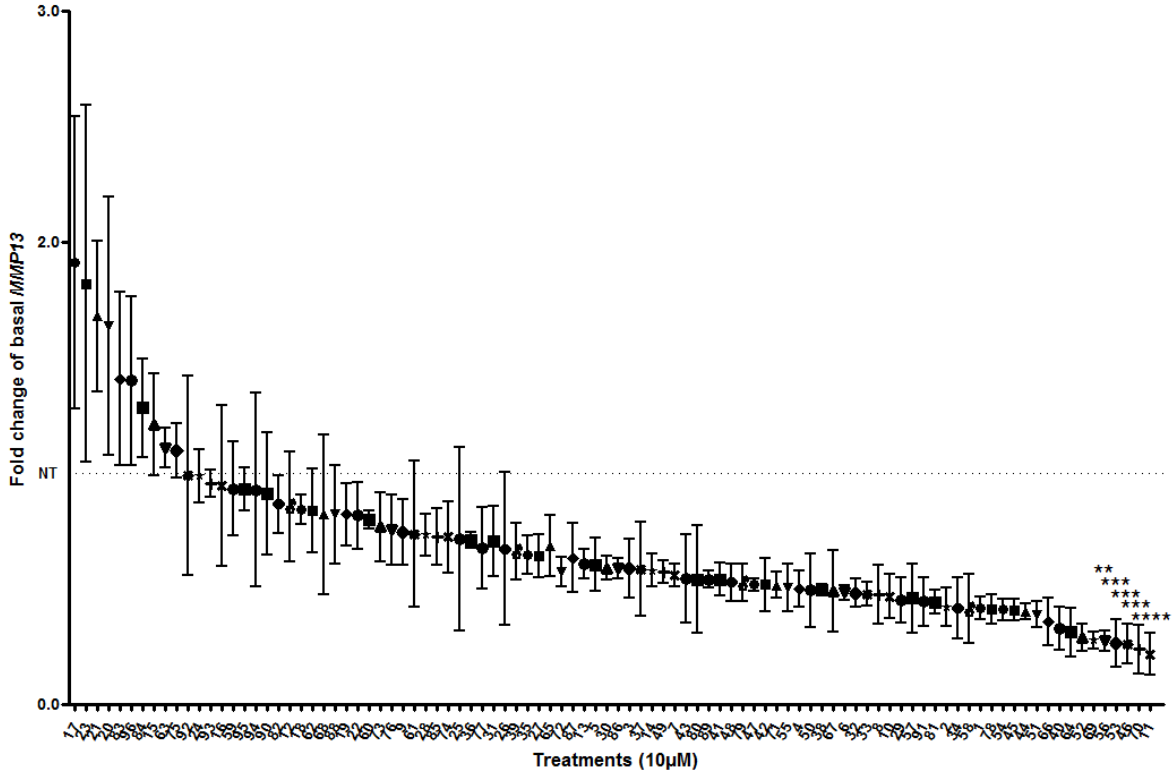


Figure 3.6. Fold change in *MMP13* gene expression relative to untreated control samples in C28/I2 cells. C28/I2 cell line pre treated with 10µM of one of 96 diet derived compounds (see Appendix for compound list). Compounds effect on basal *MMP13* levels was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean ± SEM, n=3. Dotted line represents no treatment (NT) control samples. * p<0.05, ** p < 0.01, *** p<0.001, **** p<0.0001.

3.2.4 Selecting compounds of interest

Compounds showing either a statistically significant effect on *MMP13* expression or a trend for inhibition of *MMP13* expression were selected from each screen and entered into a four set Venn diagram (Figure 3.7). Compounds inhibiting *MMP13* in a single screen included:

- Oleonolic acid, naringin dihydrochalcone, rhein, salacin, sitosterol and bilobalide inhibited IL1 induced *MMP13* in the SW1353 screen alone.
- Troxerutin, gastrodin, usniacin, shikmic acid, fisetin, glycyrrhizic acid, coenzyme Q10, and indole butyric acid inhibited IL1 induced *MMP13* in the C28/I2 screen alone.
- Sesamin, palmatinechloride, osthole, ergosterol, salidroside, icariin and artesunate inhibited basal *MMP13* in the SW1353 screen alone.
- Sclareol, ammonium glycyrrhizinate, gynostemma extract, hesperidin, formonetin, biochanin A, fumalic acid, dihydroartemisinin, capsaicin and hesperetin inhibited basal *MMP13* in the C28/I2 screen alone.

Compounds inhibiting *MMP13* in screens included:

- Aloe emodin, ursolic acid and isoliquiritigenin inhibited IL1 induced *MMP13* in the SW1353 and C28/I2 screens.
- Kinetin, methyl-hesperidin and theobromine inhibited IL1 induced *MMP13* in the C28/I2 screen and inhibited basal *MMP13* in the SW1353 screen.
- Indole-3-carbinol and orotic acid inhibited basal *MMP13* in the SW1353 and C28/I2 screens.
- Myricetin inhibited IL1 induced and basal *MMP13* in the C28/I2 screen and inhibited basal *MMP13* in the SW1353 screen.
- Luteolin inhibited IL1 induced and basal *MMP13* in the SW1353 and C28/I2 screens.
- Quercetin, neohesperidin dihydrochalcone, rheochrysidin and naringin inhibited IL1 induced and basal *MMP13* in the SW1353 screen.
- L-carnitine and nobiletin inhibited IL1 induced and basal *MMP13* in the SW1353 screen and inhibited basal *MMP13* in the C28/I2 screen.

- Apigenin, genistin, polydatin and emodin inhibited IL1 induced *MMP13* in the SW1353 screen and inhibited IL1 induced and basal *MMP13* in the C28/I2 screen.

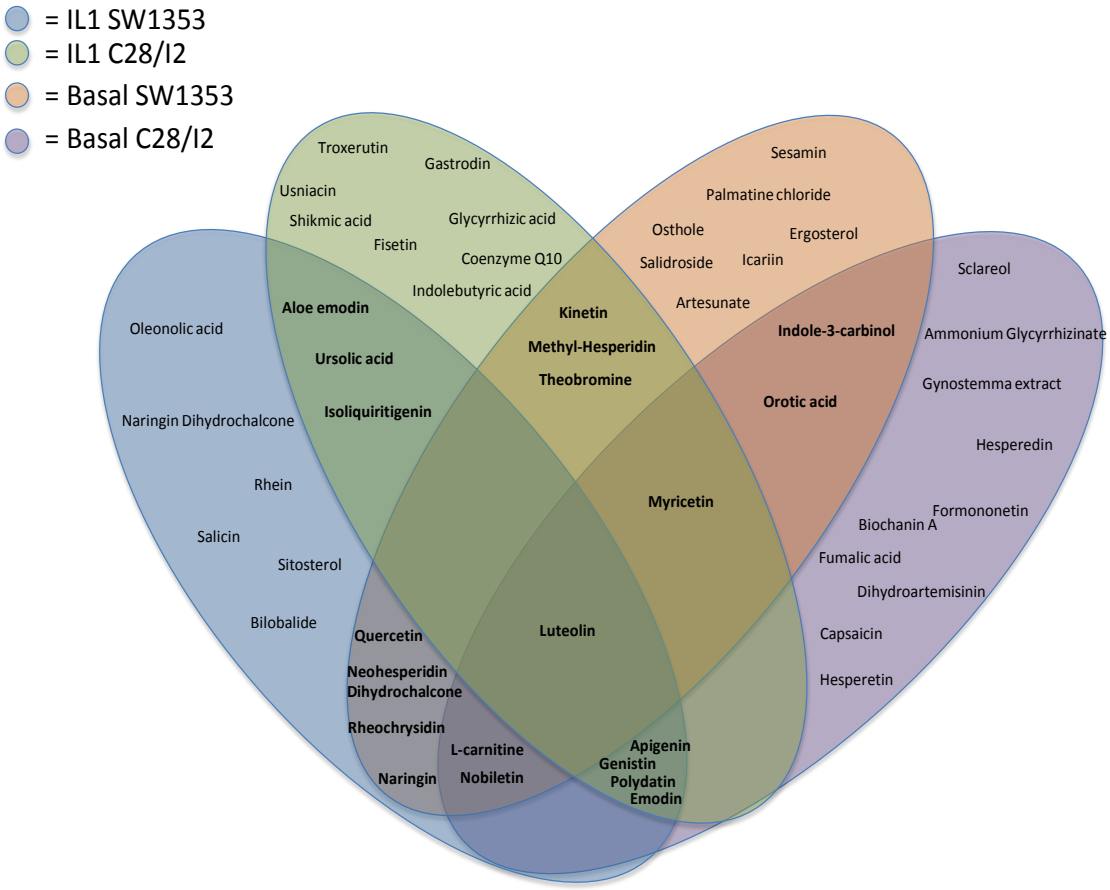


Figure 3.7. Four set Venn diagram. Ellipses displaying compounds that reduced IL1 and/or basal levels of *MMP13* in SW1353 and C28/I2 cell lines.

3.3 Discussion

This project aims to identify novel compounds found in the habitual diet that could offer protection or slow the progression of OA. A custom-built library of 96 compounds was designed from a selection of compounds from a Bioactive Compound Library (Figure 3.1). Ninety six compounds were selected and compiled into a custom-built library in a 96-well plate format. This format was selected to ensure accurate comparisons between compounds, all compounds were at 10µM and dissolved in DMSO. The advantages of using a custom-built library in this format was the standardisation of compounds in terms of purity, concentration and form. Purchasing compounds individually often requires a minimum quantity and can quickly become expensive narrowing the range of bioactive compounds that can be explored. The library was designed to contain bioactive compounds with a range of chemical structures including flavonoids, anthocyanins, terpenoids, carotenoids and other polyphenols as well as precursors of dietary compounds such as polydatin (a natural precursor of resveratrol).

Compounds were screened at an initial concentration of 10µM for IL1 induced and basal *MMP13* inhibition in SW1353 cells. A 0.1-10µM dose range of anthocyanin metabolites has been proposed to be physiologically relevant with phenolic metabolites reported ranging from 0.1-2µM with cumulative concentrations of metabolites reaching 10µM in plasma after oral consumption of a bolus containing 500mg/day of anthocyanins (Ferrars et al., 2014), (Ferrars et al., 2014), (Amin et al., 2015). Cells outside of the gut may not see parent compounds as these are often metabolised during digestion, possibly resulting in metabolites of the parent compounds reaching chondrocytes. However chondrocytes have been shown to metabolise parent compounds such as SFN and accumulate metabolites of the parent compound *in vitro* (Davidson et al., 2013).

For this study 10µM was selected as an initial concentration to represent a ‘high’ but physiologically relevant concentration that may be obtained via diet or supplementation of bioactive compounds. Compounds were screened at an initial concentration of 10µM for IL1 induced and basal *MMP13* inhibition in SW1353 cells. Inhibition of the expression of *MMP13* was selected as an initial readout for a bioactive compounds potential as a chondro-protective agent, as discussed in chapter 1 MMP-13 is the collagenase with the

highest specific activity for type 2 collagen i.e. has the most efficient cleavage of the protein (Minond et al., 2006) and it's thought to be the predominant collagenase involved in OA (Knäuper et al., 1996), (Billinghurst et al., 1997), (Flannelly et al., 2002) compared to other collagenases such as MMP1. A qRT-PCR approach to analysing *MMP13* was selected as a strategy to give a medium-high throughput screen using relatively low cell numbers compared to other techniques such as northern blots. *MMP13* mRNA levels have also been shown to be comparable to MMP-13 protein levels (Ahmad et al., 2009), this allows us to predict compound effects on the MMP-13 protein levels using the medium-high throughput screen, whilst using a western blot method to measure the effect of each compound on actual MMP-13 protein levels would be prohibitively expensive and time consuming.

The SW1353 chondrosarcoma cell line was used as an *in vitro* model for primary human adult articular chondrocytes (PHC). In both SW1353 and PHC cell systems *MMP1, MMP3* and *MMP13* are strongly induced by IL1 making SW1353 cells a useful model for primary chondrocytes at the transcriptional level with respect to catabolic effects after IL1 treatment (Gebauer et al., 2005a). The high throughput nature of the dietary derived bioactive screen would be limited by the lack of sufficient PHCs from operative procedures (Goldring et al., 1994), (Finger et al., 2003), rapid dedifferention of PHC cells after proliferation and the screens would need to be repeated with many patient samples due to gene expression variability (Bonaventure et al., 1994). The SW1353 cells also display a consistent response to phenotype modulating stimuli and have adequate proliferative activities (Gebauer et al., 2005a).

SW1353 cells were treated each compound at 10µM for 6 hours. A 6 hour treatment was selected as a preliminary timepoint, as 5ng/ml of IL1 has been shown to significantly upregulate *MMP13* at 6 hours and chondrocytes treated with SFN for 6 hours have shown significant inhibition of IL1 induced *MMP13* in our lab (Davidson et al., 2013).

The one-way analysis of variance (ANOVA) with Dunnett's post test was used to measure the statistical significance of a compounds effect on IL1 stimulated *MMP13.* While multiple t-tests could have been used to measure significance the chances of a Type 1 error occurring increases with each t-test performed. With 96 compounds being screened 96 t-

tests would need to be completed and the chance of a Type 1 error occurring would be unacceptably high. Using an ANOVA controls for these errors so that the chance of a Type 1 error never exceeds 5%.

Cell cytoxicity was analysed by measuring cell necrosis after compound treatment. Cell necrosis was measured using the CytoTox 96® Non-Radioactive Cytotoxicity as described by the manufacturer's instructions (see Chapter 2 section 2.2.1.2 for more details). For compounds to be considered as being protective to cartilage they must have no significant effect on cell necrosis. All dietary derived compounds had no significant effect on cell necrosis in SW1353 cells (Figure 3.2).

Emodin, ursolic acid, oleanolic acid, luteolin, polydatin, apigenin, neohesperidin dihydrochalcone, l-carnitine and isoliquiritigenin significantly inhibited IL1 induced *MMP13* expression and DL-carnitine hydrochloride significantly increased IL1 induction of *MMP13* in SW1353 cells (Figure 3.3). DL-carnitine is found in mutton and beef (Yonei et al., 2008), while this compound would not be a useful bioactive for prevention of OA its potential role in *MMP13* induction is interesting, however researching compounds that induce MMPs is beyond the scope of this project. Myricetin, theobromine and sesamin significantly inhibited basal *MMP13* expression (Figure 3.4).

The C28/I2 cell line are immortalised transformed human costal chondrocytes (provided by Dr. Mary Goldring, Hospital for Special Surgery, New York). The C28/I2 cells were established by transfection of primary cultures of juvenile costal chondrocytes with vectors encoding simian virus 40 large T antigen (Goldring et al., 1994). This cell line was used as a comparison to the SW1353 cell line to eliminate compounds with cell line specific effects before moving candidate compounds into PHC cells. C28/I2 cells were treated with each of the compounds from the 96 dietary derived bioactive compound library for 6 hours. Apigenin, troxerutin, luteolin, aloe-emodin, methyl-hesperidin, ursolic acid, emodin, fisetin, genistin and polydatin significantly inhibited IL1 induced *MMP13* expression in C28/I2 cells (Figure 3.5). Apigenin, myricetin, genistin, hesperetin, biochanin A significantly inhibited basal *MMP13* expression in C28/I2 cells (Figure 3.6).

As some of these compounds had either IL1/basal or SW1353/C28/I2 specific effects the top twenty compounds showing either a statistically significant effect on *MMP13*

expression or a trend for inhibition of *MMP13* expression were selected from each screen and entered into a four set Venn diagram (Figure 3.7). Compounds were selected from the Venn diagram and compiled into a top twenty compound list for further study in KOA chondrocyte cells if the compound effected more than one of the *MMP13* expression screens. This was done to eliminate compounds that only had an effect in a single experiment and to include compounds that showed a trend of inhibition of *MMP13* expression in multiple experiments that may not have reached statistical significance in individual screens. List of selected compounds can be seen in Table 5.

Isoliquiritigenin is a strong nod gene and glyceollin resistance-inducing flavonoid (Kape et al., 1992) from liquorice. Nod genes encode for proteins that modify nodulation factors which act as signalling molecules between symbiotic bacteria and plants in response to flavonoids (Peck et al., 2006). Theobromine is a xanthine alkaloid found in cocoa beans that has been described to have a bronchodilator and a vasodilator effect in asthma patients comparable to theophylline an anti-asthma medication with no reported side effects (Simons et al., 1985). Kinetin is a cytokinin (class of plant hormone that promotes cell division) found in coconut (Ge et al., 2005) which is used in plant tissue culture for inducing formation of callus (Gavinlertvatana & Li, 1980).

Myricetin is a naturally occurring flavonol, a flavonoid found in many grapes, berries, fruits, vegetables, herbs, as well as other plants. Myricetin has been shown to significantly inhibit *MMP1* at 1 and 10μM in SW982 chondrosarcoma cells (Lee & Choi, 2010). Emodin is a purgative resin trihydroxyanthraquinone from rhubarb and is a commonly used traditional herbal treatment in China (Cai et al., 2008). Polydatin (resveratrol-3-O-β-mono-D-glucoside) is a natural precursor to resveratrol which is much more abundant than resveratrol in grape juices (Romero-Pérez et al., 1999), (Falchetti et al., 2001), polydatin is likely to be the most abundant form of resveratrol in nature (Regev-Shoshani et al., 2003). Ursolic acid is a triterpenoid that exists widely in food (J. Liu, 1995), medicinal herbs and is often used in cosmetics which has been shown to inhibit activation of NFκB in a leukaemia cell line by preventing phosphorylation of Iκb (Shishodia et al., 2003).

Methyl-Hesperidin is a flavonoid found abundantly in citrus fruits, its aglycone form is called hesperetin which has been shown to improve endothelial function and reduce

inflammatory markers in patients with metabolic syndrome (Rizza et al., 2011). Aloe emodin is a hydroxy anthraquinone known to have laxative, anti-fungal, antibacterial, antiviral and hepatoprotective activities. Aloe emodin has also been shown to reduce the DNA binding activities of NFκB (Suboj et al., 2012). Genistin is a isoflavone from soy that attenuates growth factor and cytokine-stimulated proliferation of both normal and cancer cells potentially via TGFbeta1-signaling (Kim et al., 1998). Apigenin has already been shown to suppress *MMP13* expression in IL1 treated SW1353 cells by suppressing the c-fos/AP-1 and JAK2/STAT1/2 pathways (Lim et al., 2011). Luteolin is a flavone found in celery that has been shown to inhibit IL1 induced *MMP13* mRNA levels measured by qRT-PCR, via inhibition of IL1-induced ERK activation in a dose-dependent manner in osteoblasts (Yang et al., 2012).

Orotic acid is a heterocyclic compound and an acid found in carrots and dairy products (Salerno & Crifò, 2002) and is the first pyrimidine formed in the de novo pathway of nucleic acid synthesis, becoming elevated whenever the ammonia load exceeds the capacity of the urea cycle (Visek & Shoemaker, 1986). Indole-3-carbinol is produced by the breakdown of the glucosinolate glucobrassicin, which can be found at relatively high levels in cruciferous vegetables (Fahey et al., 2001), it can act as a negative regulator of estrogen potentially preventing the development of estrogen-enhanced cancers including breast, endometrial and cervical cancers (Auborn et al., 2003).

Nobiletin is a polymethoxyflavone found in citrus fruit peel (Guo et al., 2012). Nobiletin has been shown to inhibit gene expression of IL1 TNF-α and IL-6 in mouse J774A.1 macrophages (Lin et al., 2003). Naringin is a flavanone glycoside and a major flavonoid in grapefruit giving the grapefruit juice its bitter taste, its supplementation has been suggested to be used as a treatment for obesity, diabetes, hypertension and metabolic syndrome (Alam et al., 2014). L-carnitine is constituent of striated muscle and liver, in the diet it can be found in animal products. L-carnitine is used therapeutically to stimulate gastric and pancreatic secretions and in the treatment of hyperlipoproteinemias (Stefanutti et al., 1998). Neohesperidin dihydrochalcone is an artificial sweetener derived from citrus (Kroeze, 2000). The flavonol quercetin has been shown to prevent cartilage degradation in tissue explants, quercetin has also been shown to suppress IL1 stimulated expression of *ADAMTS4* and basal expression of *ADAMTS5* (Lay et al., 2012). Rheochrysidin is one of the

major components of Da-Cheng-Qi decoction used in the treatment of inflammation (Q. Yu et al., 2009).

Compound	Dietary Source
Isoliquiritigenin	Liquorice
Theobromine	Cocoa bean
Kinetin	Coconut
Myricetin	Grapes
Emodin	Rhubarb
Polydatin	Grapes
Ursolic acid	Apple
Methyl Hesperidin	Citrus fruits
Aloe emodin	Aloe vera
Genistin	Soy
Apigenin	Parsley, celery, coffee
Luteolin	Celery
Orotic acid	Carrot
Indole-3-carbinol	Broccoli
Nobiletin	Orange
Naringin	Grapefruit
L-carnitine	Beef
Neohesperidin Dihydrochalcone	Citrus fruit
Quercetin	Black elder berry
Rheochrysidin	Curly dock

Table 5. Twenty selected compounds. Table highlighting the selected compounds for study in primary chondrocytes and examples of sources they can be found in.

3.3.1 Conclusion

In conclusion a custom-built dietary derived library was designed, all compounds from this library were analysed for toxicity via lactate dehydrogenase assay. All compounds analysed had no significant effect on cell necrosis when compared to untreated control. Several compounds were able to inhibit *MMP13* at 10µM in SW1353 and C28/I2 cell lines. From this original list of 96 dietary derived compounds twenty compounds were selected and their effect on primary chondrocytes will be described in the next chapter.

3.3.2 Strengths of Chapter 3

- Shortlisted several compounds that have been shown to inhibit *MMP13* gene expression in two cell lines in a medium-high throughput manner using a qrt-PCR based approach.
- Shown that all compounds have no necrotic effect in SW1353 cells at 10µM.

3.3.3 Weaknesses of Chapter 3

- Lack of data for compound effect on MMP-1 or MMP-13 protein levels.

Chapter 4: Short-listing dietary-derived bioactives in human primary knee osteoarthritis chondrocytes

4.1 Introduction

In the previous chapter, 96 dietary-derived bioactive compounds were shortlisted to 20 compounds for further study. While SW1353 cells are a useful model for protease expression in chondrocytes, a gene array comparing 312 genes has shown that the overlap between gene expression is small between SW1353 cells and PHC cells (Gebauer et al., 2005). Gaubauer et al, concluded that the SW1353 cell line cannot be used as a general prediction for the behaviour of PHCs. The molecular phenotype of the C28/I2, T/C-28a4 and T/C-28a2 human chondrocyte cell lines have also been investigated (Finger et al., 2003). These cell lines showed less expression of genes involved in matrix synthesis and turnover in comparison to PHCs. While the C28/I2 cell line displayed the highest levels of matrix-anabolic and matrix-catabolic genes, the cell line was described as not being a direct substitute for PHCs and that experiments should be validated in PHCs or cartilage-tissue cultures (Finger et al., 2003). Twenty compounds selected in chapter 3 (Table 5) were tested in KOA chondrocytes. KOA cells were isolated from the cartilage of patients with knee OA as described in section 2.1.1.3. The qRT-PCR *MMP13* screens from (Chapter 3 Figure 3.3-3.6) were repeated in the 20 selected compounds in KOA cells. While MMP-13 plays a pivotal role in collagen cleavage in OA (Knauper et al., 1996) MMP-1 is also able to cleave native fibrillar collagen and contributes to the pathological cleavage of collagen fibrils in OA (Burrage et al., 2006). Using the qRT-PCR screen format from (Chapter 3 Figure 3.3-3.6) the effect of the selected 20 compounds (Table 5) on *MMP1* expression in KOA cells was measured. As before, 10µM was selected as an initial concentration for the qRT-PCR metalloproteinase screens to represent a 'high' but physiologically relevant concentration that may be obtained via diet or supplementation.

As discussed in chapter 1 ADAMTSs also play a role in the development of OA. *ADAMTS5* knock out mice had a significant reduction in the severity of cartilage destruction after surgically induced instability when compared with wild type mice (Glasson et al., 2005).

ADAMTS5 is constitutively expressed whereas *ADAMTS4* is induced following IL1 treatment (Tortorella et al., 2001), for this reason only the unstimulated expression of *ADAMTS5* was measured. *ADAMTS4* has been shown to be selectively overexpressed in human osteoarthritic cartilage, with a direct correlation with the degree of cartilage destruction suggesting that *ADAMTS4* may play an important role in the degradation of aggrecan in human osteoarthritic cartilage (Naito et al., 2007). Compounds from the dose response were screened for effects on *ADAMTS4* and *ADAMTS5* in KOA cells. Heme oxygenase-1 (HO-1) is the limiting enzyme in heme catabolism (Rousset et al., 2013), cleaving heme to form biliverdin, this prevents production of ROS giving HO-1 anti-inflammatory properties. HO-1 induction in PHCs cultured in hypoxic conditions prevented IL1 stimulated loss of type 2 collagen (Guillén et al., 2012). HO-1 was hypothesised to be a physiologically important chondro-protective factor.

Transgenic OA mouse models have indicated that upregulation of β-catenin is responsible for the transition of normal chondrocytes into an arthritic phenotype (Wu et al., 2010). β-catenin levels are up-regulated by Wnt ligands, in the absence of Wnt ligands β-catenin is degraded in a glycogen synthase kinase 3 β (GSK3-β) destruction complex brought together by Axin/Axin2 and the adenomatous polyposis coli tumour suppressor protein (APC). *AXIN2* is a Wnt/β-catenin responsive gene which has been shown to be upregulated consistently with net activation of canonical Wnt signalling (Lustig et al., 2002). Wnt signalling can induce the expression of MMPs in articular chondrocytes which promotes cartilage degradation and cartilage catabolism (Yuasa et al., 2008). As the upregulation of the Wnt pathway may be deleterious for the joint cartilage the compounds from the dose response assays were screened for an effect on the expression of *AXIN2*.

We hypothesise that bioactives showing significant inhibition of *MMP13* and/or *MMP1* expression at 10μM may have an inhibitory effects on other chondro-destructive metalloproteinase genes such as *ADAMTS4* and *ADAMTS5.* Potential chondro-protective compounds may also up-regulate chondro-protective genes such as *HO-1* in a dose dependent manner. The aims of this chapter are:

- To use qRT-PCR to screen 20 selected compounds (see Chapter 3 Table 5 for selected compounds) for inhibition of IL1-induced and basal *MMP13* and *MMP1* expression in KOA chondrocytes.

- Complete dose response curves for two candidate compounds from KOA cell experiments looking at effects on chondro-destructive genes, *MMP13, MMP1, ADAMTS4* and *ADAMTS5*.

- Complete dose response curves for two candidate compounds from KOA cell experiments looking at effects on the chondro-protective gene *HO-1*.

- Complete dose response curves for two candidate compounds from KOA cell experiments looking for effects on *AXIN2* as a measurement of Wnt pathway activity.

- Screen the two candidate compounds for potential apoptotic or necrotic effects in KOA cells.

4.2 Results

4.2.1 The effect of the twenty shortlisted compounds on genes of interest in KOA chondrocyte cells

4.2.1.1 Effect on IL1 induced *MMP13* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with one of the selected twenty dietary-derived compounds (described in Chapter 3 Table 5) at 10µM for 6 hours. Compounds of interest were added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *MMP13* using methods described in section 2.2.3.1. Luteolin, aloe-emodin, emodin, isoliquiritigenin and apigenin significantly inhibited IL1 induced *MMP13* expression in KOA cells ($p<0.05$-0.0001) (Figure 4.1).

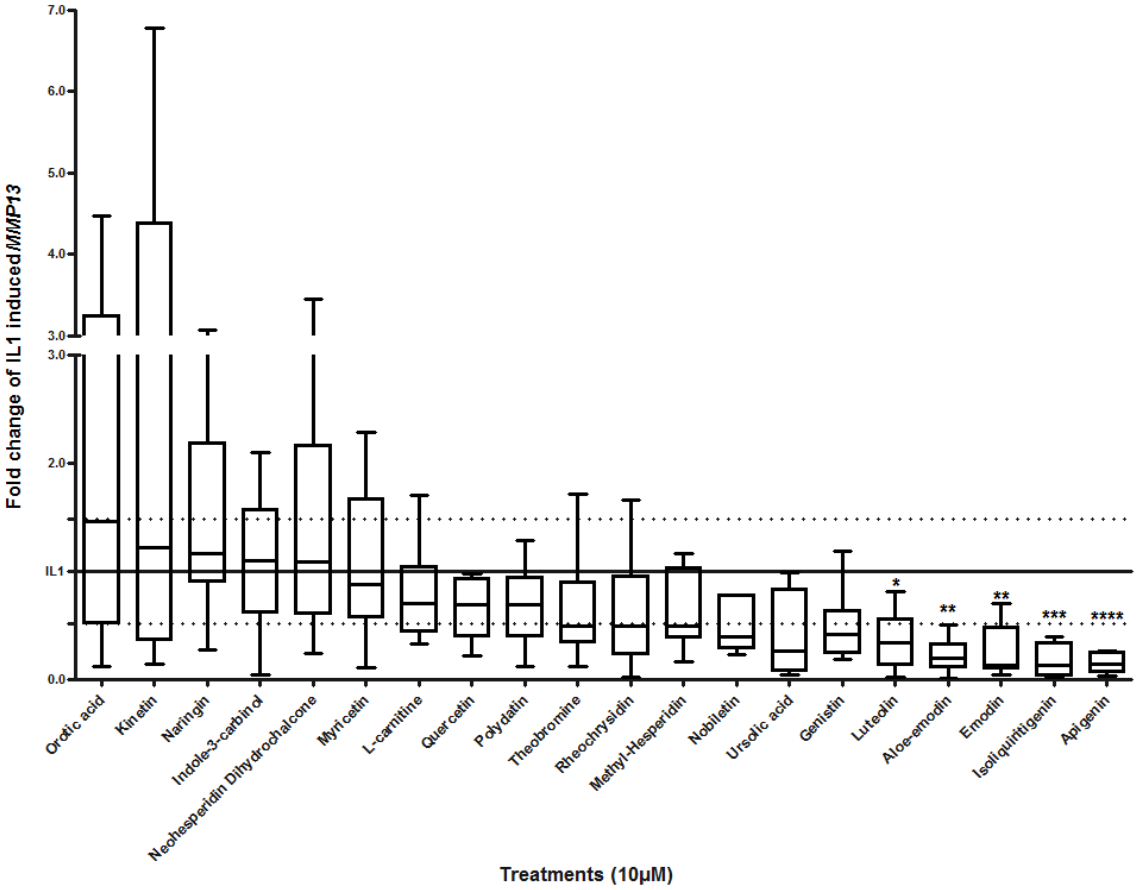


Figure 4.1 Dietary derived compounds effect on IL1 stimulated *MMP13* gene expression relative to 18s in KOA cells. Tukey Box and whisker plot showing fold change in *MMP13* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with 10µM of one of the top 20 diet derived compounds (see Chapter 3 Table 5). Compounds effect on IL1 induced *MMP13* levels was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. *p<0.05, ** p < 0.01, *** p<0.001, **** P<0.0001.

4.2.1.2 Effect on basal *MMP13* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with one of the selected twenty dietary-derived compounds (described in Chapter 3 Table 5) at 10µM for 6 hours. Real-time qRT-PCR was used to measure *MMP13* using methods described in section 2.2.3.1. Compounds had no significant effect on basal *MMP13* expression (Figure 4.2).

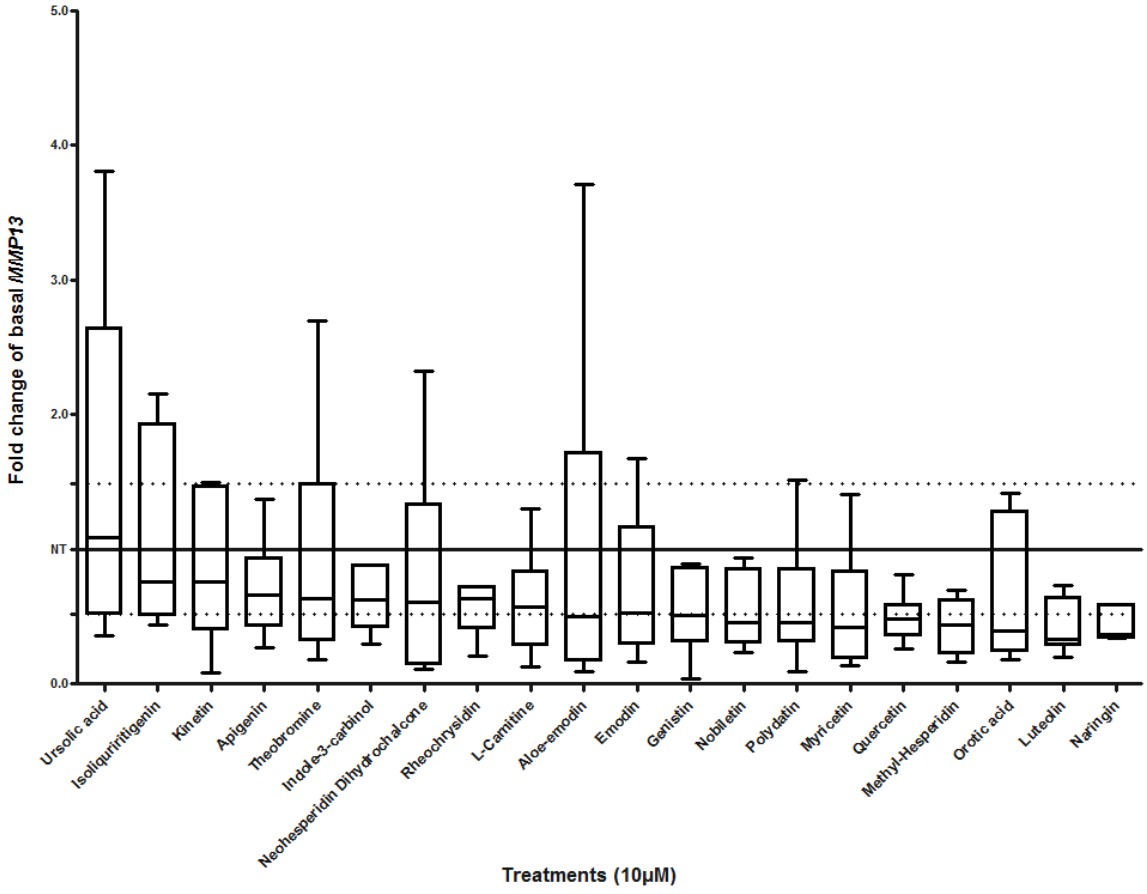


Figure 4.2. Dietary derived compounds effect on unstimulated *MMP13* gene expression relative to 18s in KOA cells. Tukey Box and whisker plot showing fold change in *MMP13* gene expression relative to no treatment in KOA chondrocytes treated with 10µM of one of the top 20 diet derived compounds (see Chapter 3 Table 5). Compounds effect on basal *MMP13* levels was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative to no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation.

4.2.1.3 Effect on IL1 induced *MMP1* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with one of the selected twenty dietary-derived compounds (described in Chapter 3 Table 5) at 10µM for 6 hours. Compounds of interest were added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *MMP1* using methods described in section 2.2.3.1. Emodin, genistin, luteolin, aloe-emodin, ursolic acid, isoliquiritigenin and apigenin significantly inhibited IL1 induced *MMP1* expression in KOA cells ($p<0.05$-0.0001) (Figure 4.3).

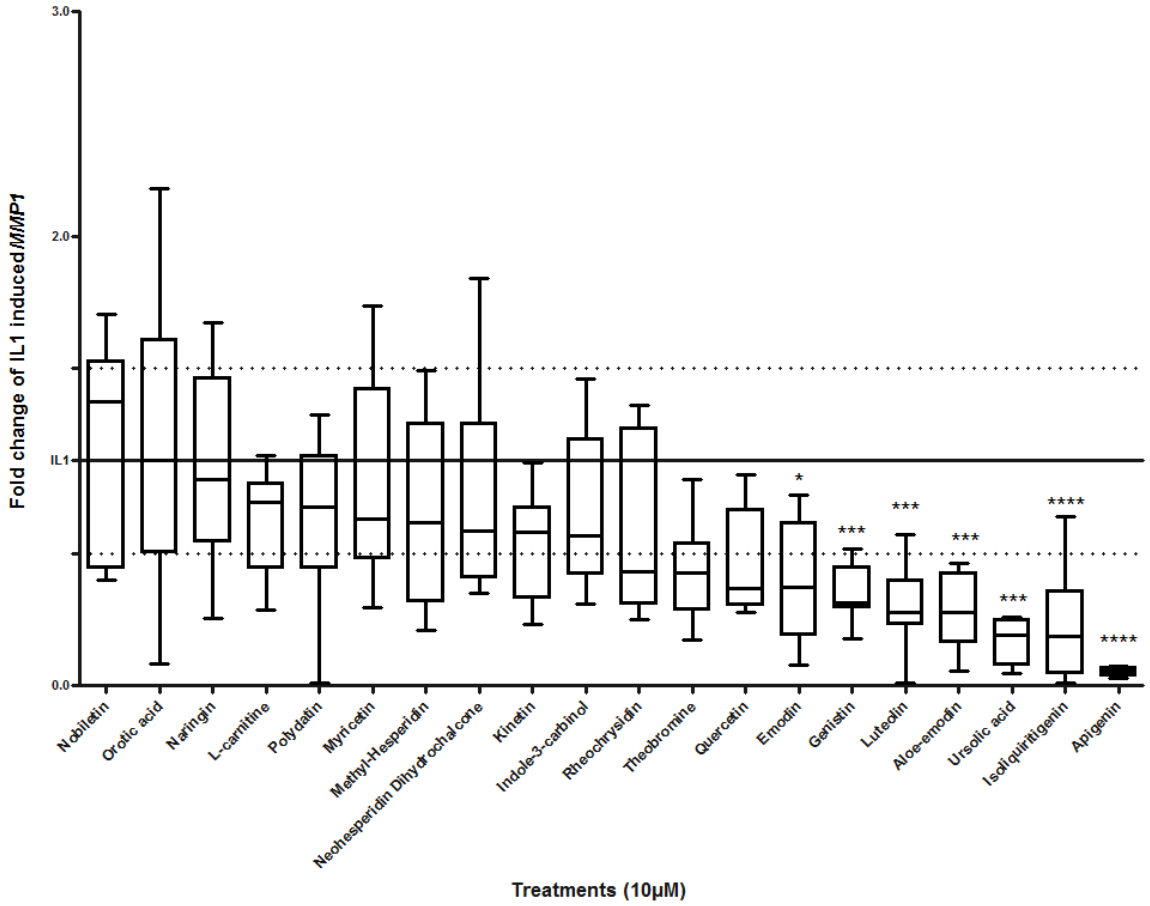


Figure 4.3 Dietary derived compounds effect on IL1 stimulated *MMP1* gene expression relative to 18s in KOA cells. Tukey Box and whisker plot showing fold change in *MMP1* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with 10µM of one of the top 20 diet derived compounds (see Chapter 3 Table 5). Compounds effect on IL1 induced *MMP1* levels was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. *p<0.05, *** p<0.001, **** p<0.0001.

4.2.1.4 Effect on basal *MMP1* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with one of the selected twenty dietary-derived compounds (described in Chapter 3 Table 5) at 10µM for 6 hours. Real-time qRT-PCR was used to measure *MMP1* using methods described in section 2.2.3.1. Compounds had no significant effect on basal *MMP1* expression (Figure 4.4).

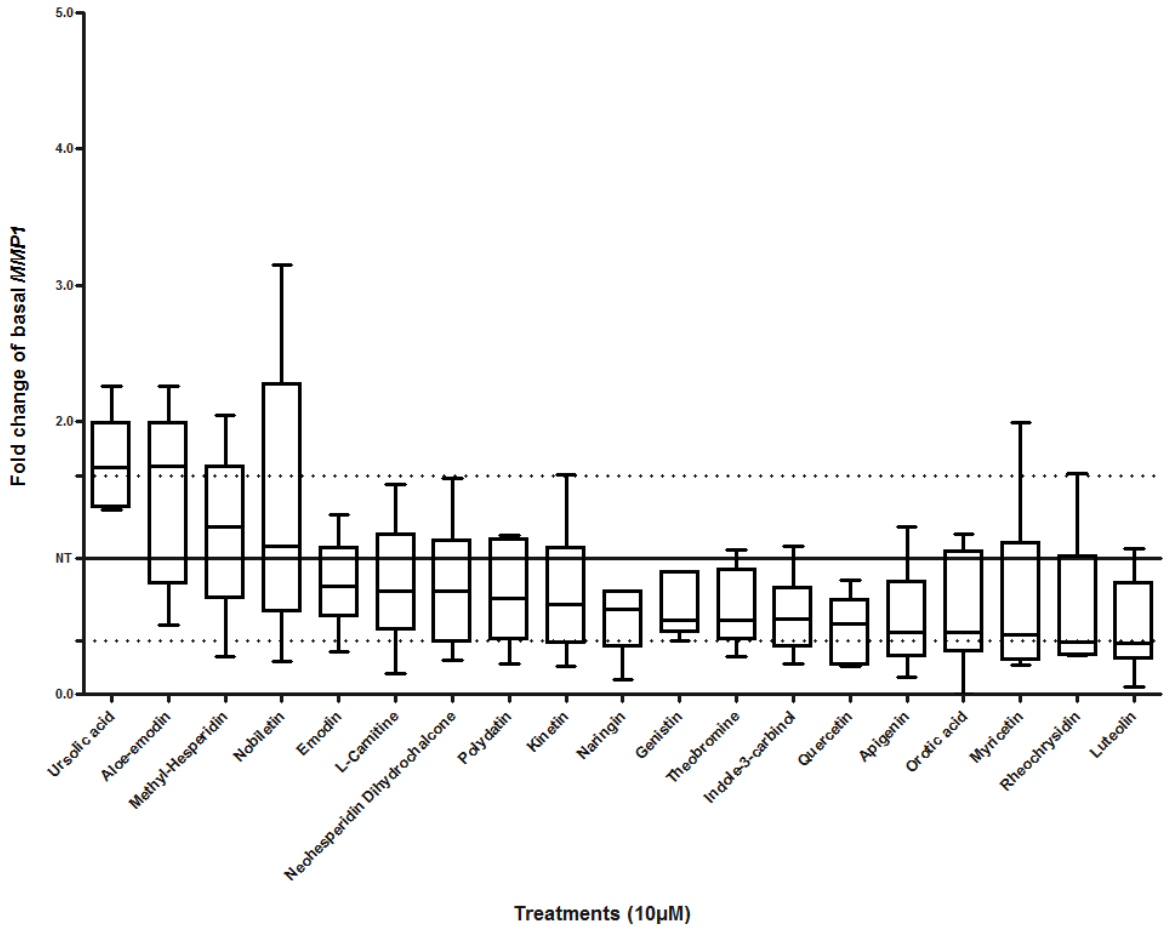


Figure 4.4. Dietary derived compounds effect on unstimulated *MMP1* gene expression relative to 18s in KOA cells. Tukey Box and whisker plot showing fold change in *MMP1* gene expression relative to no treatment in KOA chondrocytes treated with 10µM of one of the top 20 diet derived compounds (see Chapter 3 Table 5). Compounds effect on basal *MMP1* levels was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing -standard deviation.

4.2.1.5 Effect on IL1 induced *ADAMTS4* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with one of the selected twenty dietary-derived compounds (described in Chapter 3 Table 5) at 10µM for 6 hours. Compounds of interest were added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *ADAMTS4* using methods described in section 2.2.3.1. Isoliquiritigenin, apigenin and aloe-emodin significantly inhibited IL1 induced *ADAMTS4* expression in KOA cells ($p<0.05$) (Figure 4.5).

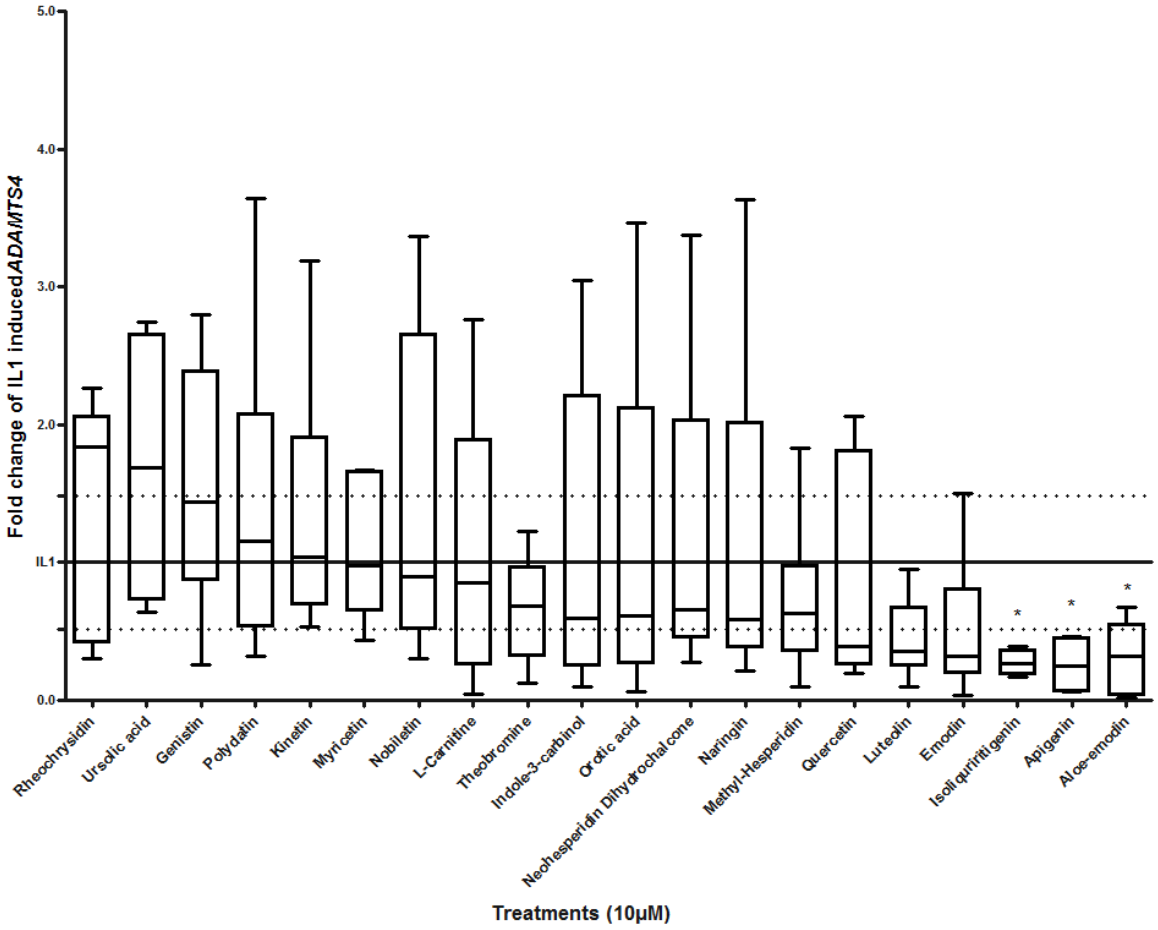


Figure 4.5. Dietary derived compounds effect on IL1 stimulated *ADAMTS4* gene expression relative to 18s in KOA cells. Tukey Box and whisker plot showing fold change in *ADAMTS4* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with 10µM of one of the top 20 diet derived compounds (see Chapter 3 Table 5). Compounds effect on IL1 induced *ADAMTS4* levels was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. *$p<0.05$.

4.2.1.6 Effect on basal *ADAMTS4* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with one of the selected twenty dietary-derived compounds (described in Chapter 3 Table 5) at 10µM for 6 hours. Real-time qRT-PCR was used to measure *ADAMTS4* using methods described in section 2.2.3.1. Genistin, apigenin and isoliquiritigenin significantly inhibited basal ADAMTS4 expression in KOA cells ($p<0.01$-0.001) (Figure 4.6).

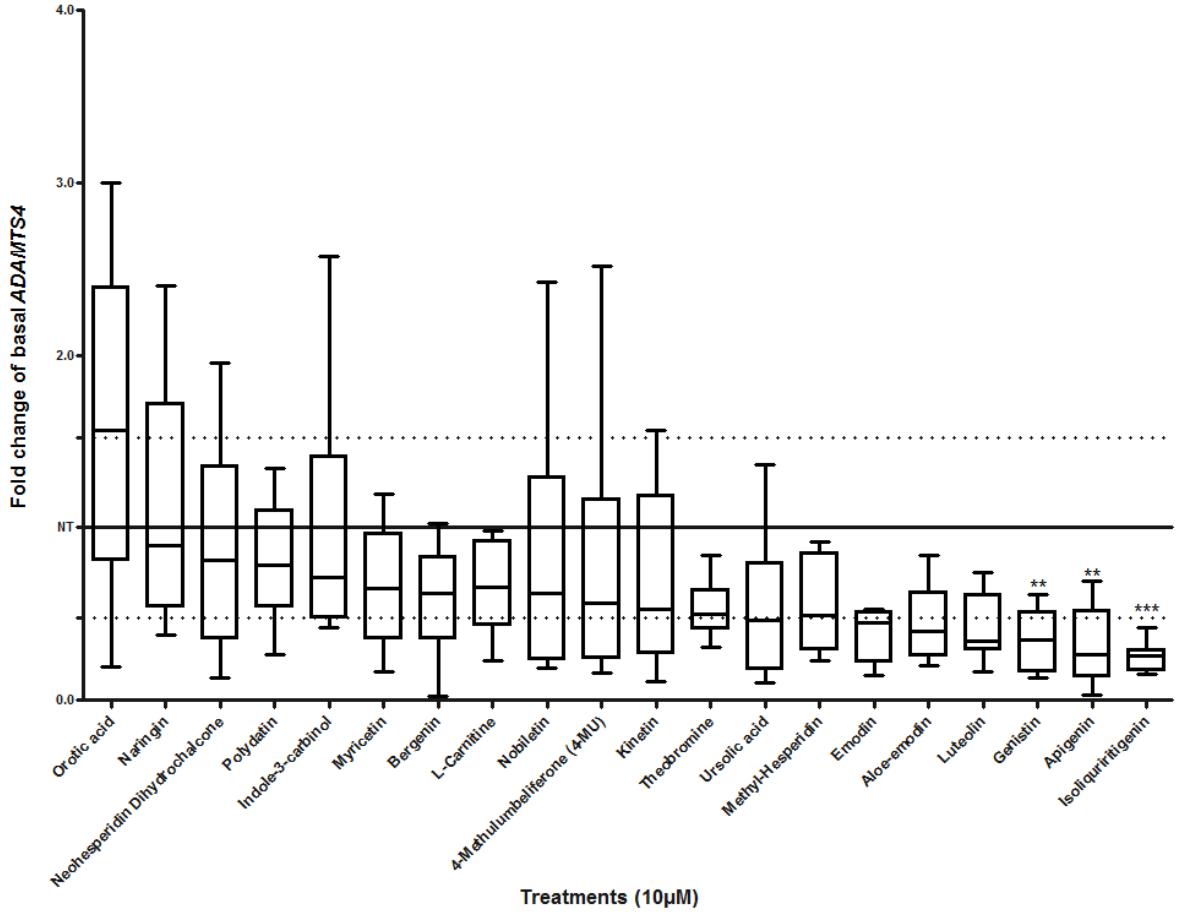


Figure 4.6. Dietary derived compounds effect on unstimulated *ADAMTS4* gene expression relative to 18s in KOA cells. Tukey Box and whisker plot showing fold change in *ADAMTS4* gene expression relative to no treatment in KOA chondrocytes treated with 10µM of one of the top 20 diet derived compounds (see Chapter 3 Table 5). Compounds effect on *ADAMTS4* levels was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation. ** $p<0.01$, *** $p<0.001$.

4.2.1.7 Effect on basal *ADAMTS5* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with one of the selected twenty dietary-derived compounds (described in Chapter 3 Table 5) at 10µM for 6 hours. In these experiments IL1 did not induce *ADAMTS5* expression so basal *ADAMTS5* was measured. Real-time qRT-PCR was used to measure *ADAMTS5* using methods described in section 2.2.3.1. Luteolin and genistin significantly inhibited basal *ADAMTS5* expression in KOA cells ($p<0.01$ - $p<0.001$) (Figure 4.7).

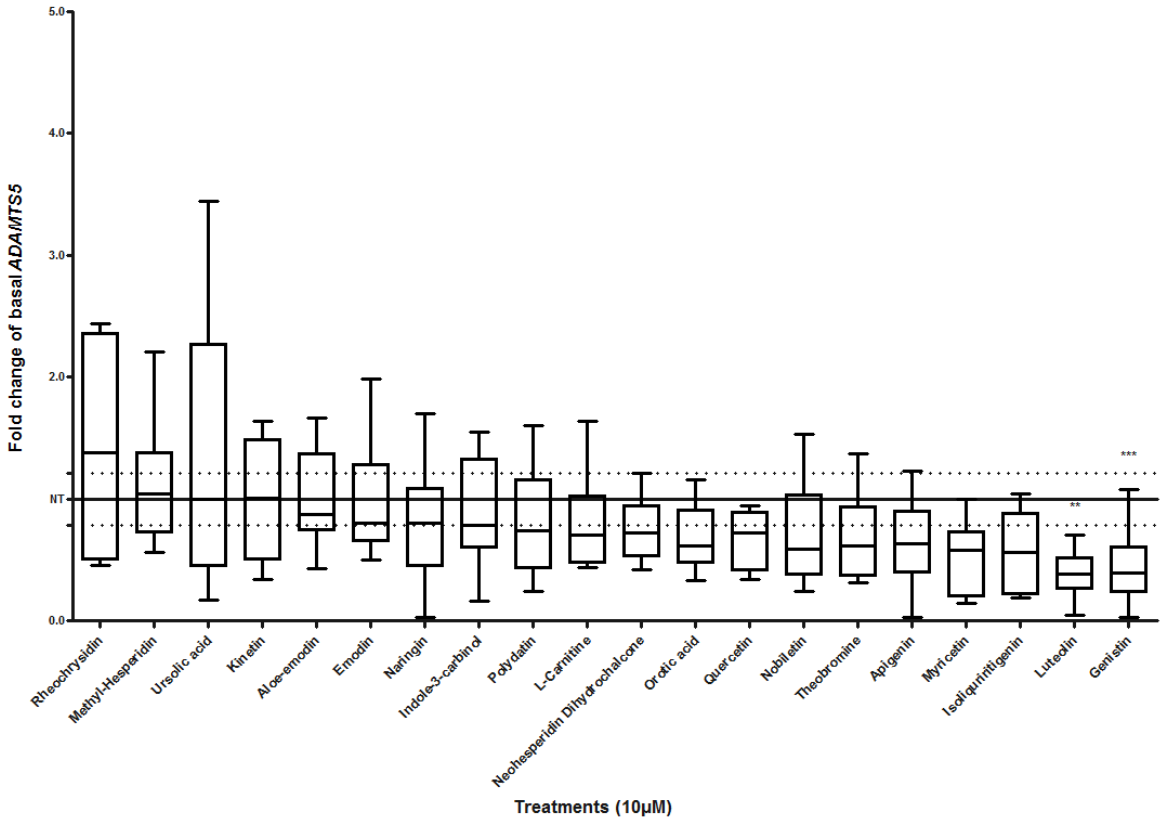


Figure 4.7. Dietary derived compounds effect on unstimulated *ADAMTS5* in KOA cells. Tukey Box and whisker plot showing fold change in *ADAMTS5* gene expression relative to no treatment in KOA chondrocytes treated with 10µM of one of the top 20 diet derived compounds (see Chapter 3 Table 5). Compounds effect on *ADAMTS5* levels was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation. ** $p<0.01$, *** $p<0.001$

4.2.2 Selecting compounds for further study

Compounds that significantly inhibited genes of interest in the KOA cell screens (Figure 4.1-4.7) may be chondro-protective and were entered into Table 6 which was used to select compounds for KOA cell dose response experiments. Compounds were ranked into order based on the number of screens they had a significant effect on. As apigenin and isoliquiritigenin significantly inhibited genes of interest in the most screens they were selected for further study.

Compound	**IL1 induced *MMP13***	**Basal *MMP13***	**IL1 induced *MMP1***	**Basal *MMP1***	**IL1 induced *ADAMTS4***	**Basal *ADAMTS4***	**Basal *ADAMTS 5***
Apigenin	****		****		*	**	
Isoliquiritigenin	***		****		*	***	
Aloe-emodin	**		***		*		
Luteolin	*		***				**
Genistin			***			**	***
Emodin	**		*				
Ursolic acid			***				

Table 6. Selecting compounds for further study. Table highlighting dietary derived bioactive compounds that significantly inhibited IL1 induced and basal expression of genes of interest in KOA cells when using one way ANOVA with a Dunnetts post hoc test ($p<0.05$ - $p<0.0001$). Selected compounds for further analysis in bold. * $p<0.05$, ** $p < 0.01$, *** $p<0.001$, **** $P<0.0001$.

4.2.3 IL1 stimulated *MMP13* Time course

4.2.3.1 Apigenin Time course

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin as before at 10µM and 5ng/ml IL1 for 1, 3, 6, 12, 18 and 24 hours (Figure 4.8). Real-time qRT-PCR was used to measure *MMP13* using methods described in section 2.2.3.1. Apigenin significantly inhibited IL1 induced *MMP13* at 6 and 12 hours ($p<0.05$-0.001).

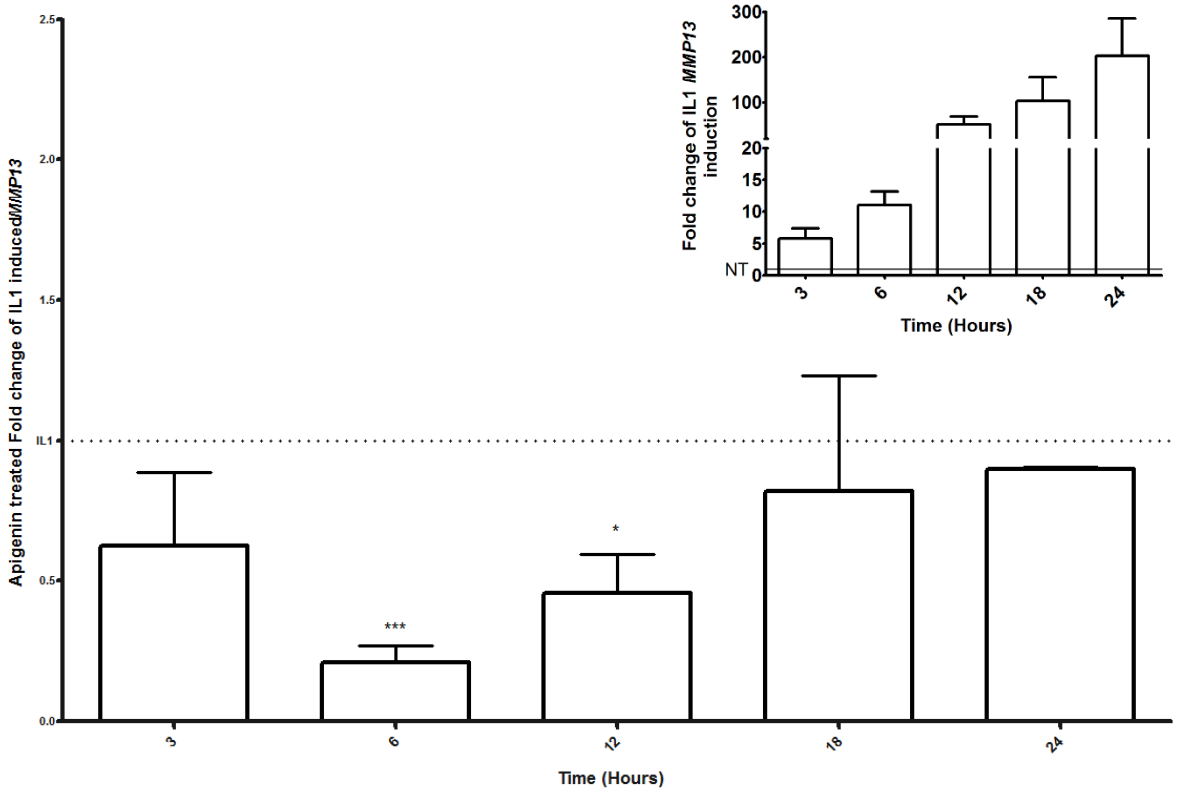


Figure 4.8. Apigenin treated *MMP13* inhibition Time course. Bar chart showing fold change in *MMP13* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with apigenin at 10µM at 1,3,6,12,18 and 24 hours. All samples treated with IL1 (5ng/ml). Data plotted as mean fold change relative to IL1 treatment alone + standard error n=4. The expression of *MMP13* between each timepoint and IL1 control was analysed by unpaired two-tailed t test. Dotted line represents IL1 treatment alone. Insert graph shows IL1 dose dependent induction of *MMP13* at 1,3,6,12,18 and 24 hours relative to no treatment (NT) + standard error n=4. Solid line represents no treatment alone. * $p<0.05$, *** $p<0.001$.

4.2.3.2 Isoliquiritigenin Time course

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin as before at 10µM and 5ng/ml IL1 for 1, 3, 6, 12, 18 and 24 hours (Figure 4.9). Real-time qRT-PCR was used to measure *MMP13* using methods described in section 2.2.3.1. Isoliquiritigenin significantly reduced IL1 induced *MMP13* at 6, 12 and 24 hours ($p<0.01$).

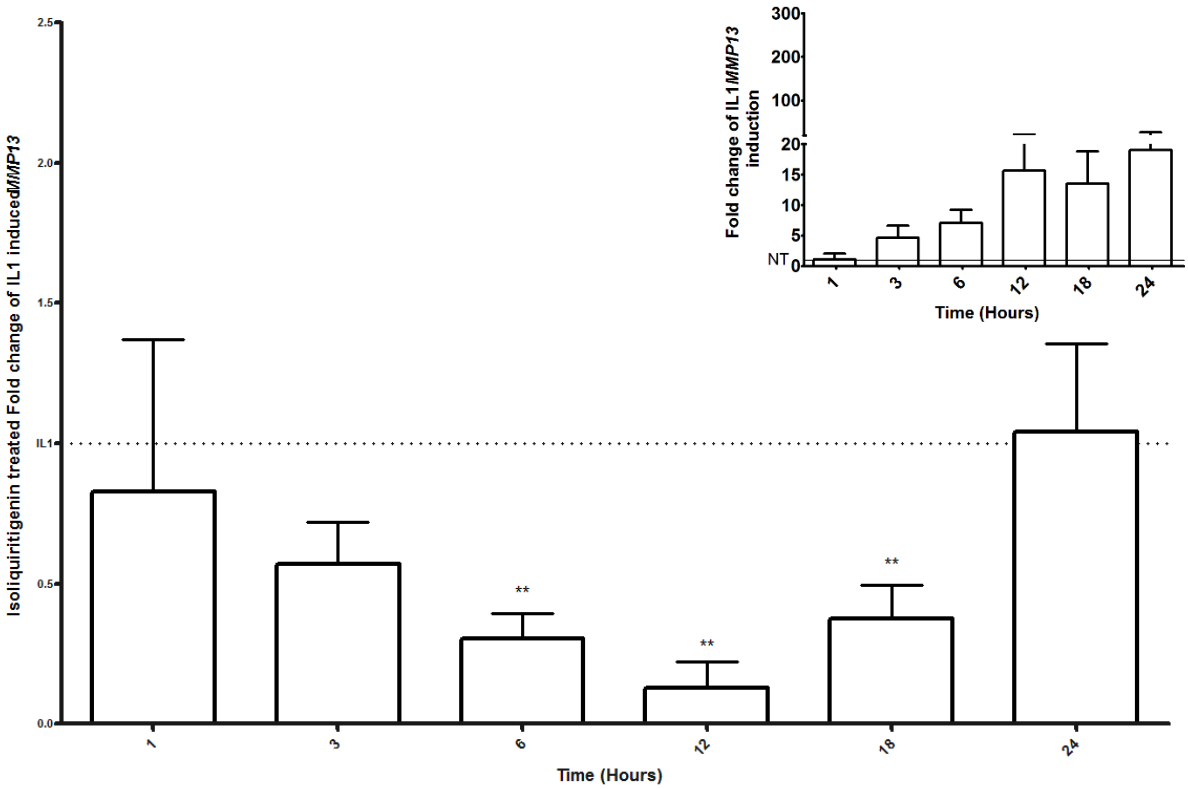


Figure 4.9. Isoliquiritigenin treated *MMP13* inhibition Time course. Bar chart showing fold change in *MMP13* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with isoliquiritigenin at 10µM at 1,3,6,12,18 and 24 hours. All samples treated with IL1 (5ng/ml). Data plotted as mean fold change relative to IL1 treatment alone + standard error n=4. The expression of *MMP13* between each timepoint and IL1 control was analysed by unpaired two-tailed t test. Dotted line represents IL1 treatment alone. Insert graph shows IL1 dose dependent induction of *MMP13* at 1,3,6,12,18 and 24 hours relative to no treatment (NT) + standard error n=4. Solid line represents no treatment alone. ** $p< 0.01$.

4.2.4 Dose response curves - Apigenin

4.2.4.1 Apigenin dose response curve - effect on IL1 induced *MMP13* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin at 0.312-40µM for 6 hours. Apigenin was added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *MMP13* using methods described in section 2.2.3.1. At 1.25µM apigenin appeared to induce *MMP13,* this did not reach statistical significance and there was no significant difference between donor samples. Apigenin significantly inhibited IL1 induced *MMP13* from 2.5-40µM ($p<0.01$-0.0001) in a dose dependent manner (Figure 4.10).

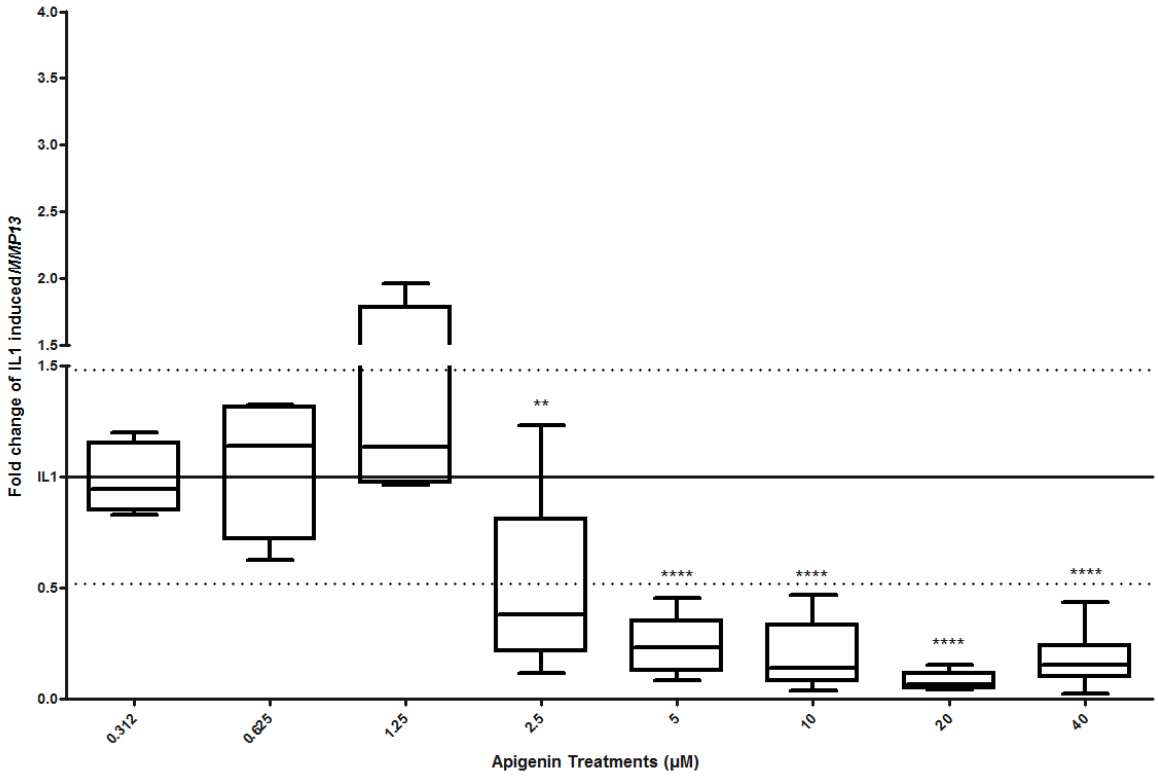


Figure 4.10. Apigenin IL1 induced *MMP13* dose response. Tukey Box and whisker plot showing fold change in *MMP13* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with apigenin at 0.312-40µM for 6 hours. The effect of apigenin on IL1 induced *MMP13* was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. ** $p<0.01$, **** $p<0.0001$.

4.2.4.2 Apigenin dose response curve - effect on IL1 induced *MMP1* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin at 0.312-40µM for 6 hours. Apigenin was added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *MMP1* using methods described in section 2.2.3.1. At 0.312µM apigenin appeared to induce *MMP1,* this did not reach statistical significance and there was no significance difference between donor samples. Apigenin significantly inhibited IL1 induced *MMP1* from 2.5-40µM ($p<0.01$-0.0001) in a dose dependent manner (Figure 4.11).

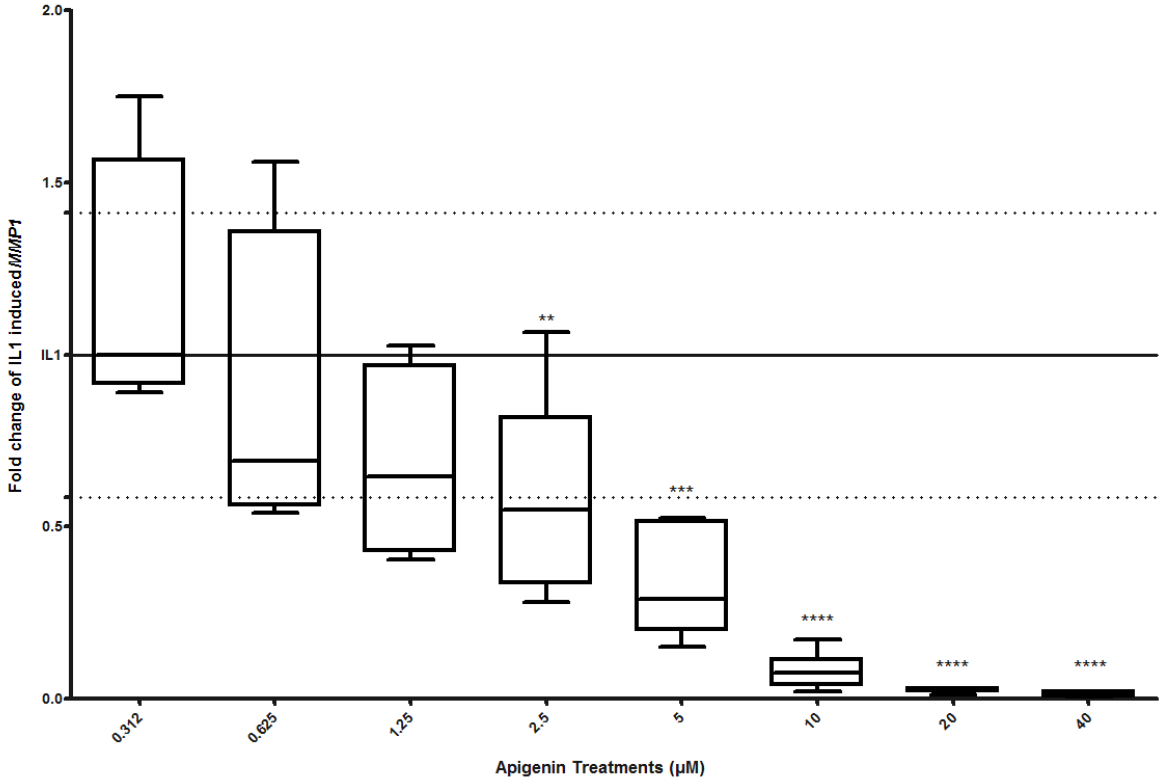


Figure 4.11. Apigenin IL1 induced *MMP1* dose response. Tukey Box and whisker plot showing fold change in *MMP1* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with apigenin at 0.312-40µM for 6 hours. The effect of apigenin on IL1 induced *MMP1* was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. ** $p< 0.01$, *** $p<0.001$, **** $p<0.0001$.

4.2.4.3 Apigenin dose response curve - effect on IL1 induced *ADAMTS4* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin at 0.312-40µM for 6 hours. Apigenin was added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *ADAMTS4* using methods described in section 2.2.3.1. Apigenin significantly inhibited IL1 induced *ADAMTS4* from 5-20µM ($p<0.01$-0.001) in a dose dependent manner (Figure 4.12). At 40µM *ADAMTS4* expression was undetectable.

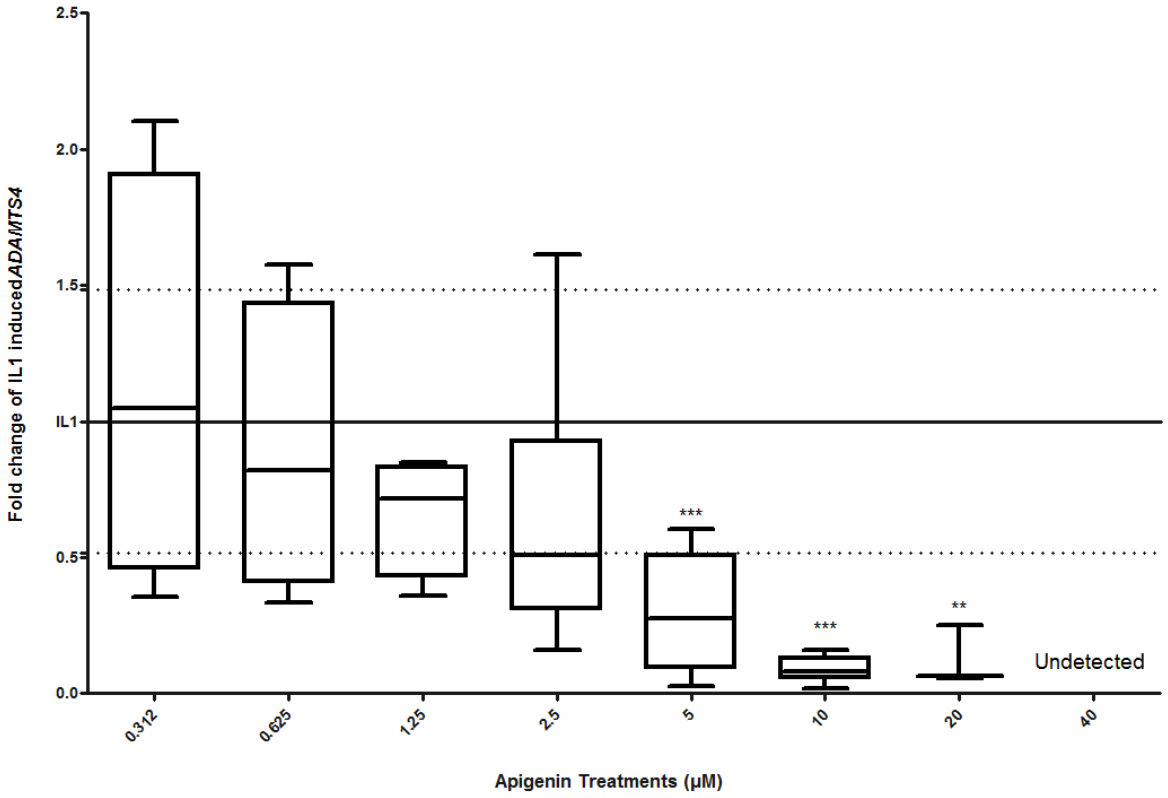


Figure 4.12. Apigenin IL1 induced *ADAMTS4* dose response. Tukey Box and whisker plot showing fold change in *ADAMTS4* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with apigenin at 0.312-40µM for 6 hours. The effect of apigenin on IL1 induced *ADAMTS4* was analysed using one way ANOVA with a Dunnetts post hoc test. At 40µM *ADAMTS4* expression was undetectable. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. ** $p<0.01$, *** $p<0.001$.

4.2.4.4 Apigenin dose response curve - effect on Basal *ADAMTS5* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin at 0.312-40µM for 6 hours. Real-time qRT-PCR was used to measure *ADAMTS5* using methods described in section 2.2.3.1. Apigenin significantly reduced basal *ADAMTS5* expression at 20 and 40µM ($p<0.01$) (Figure 4.13).

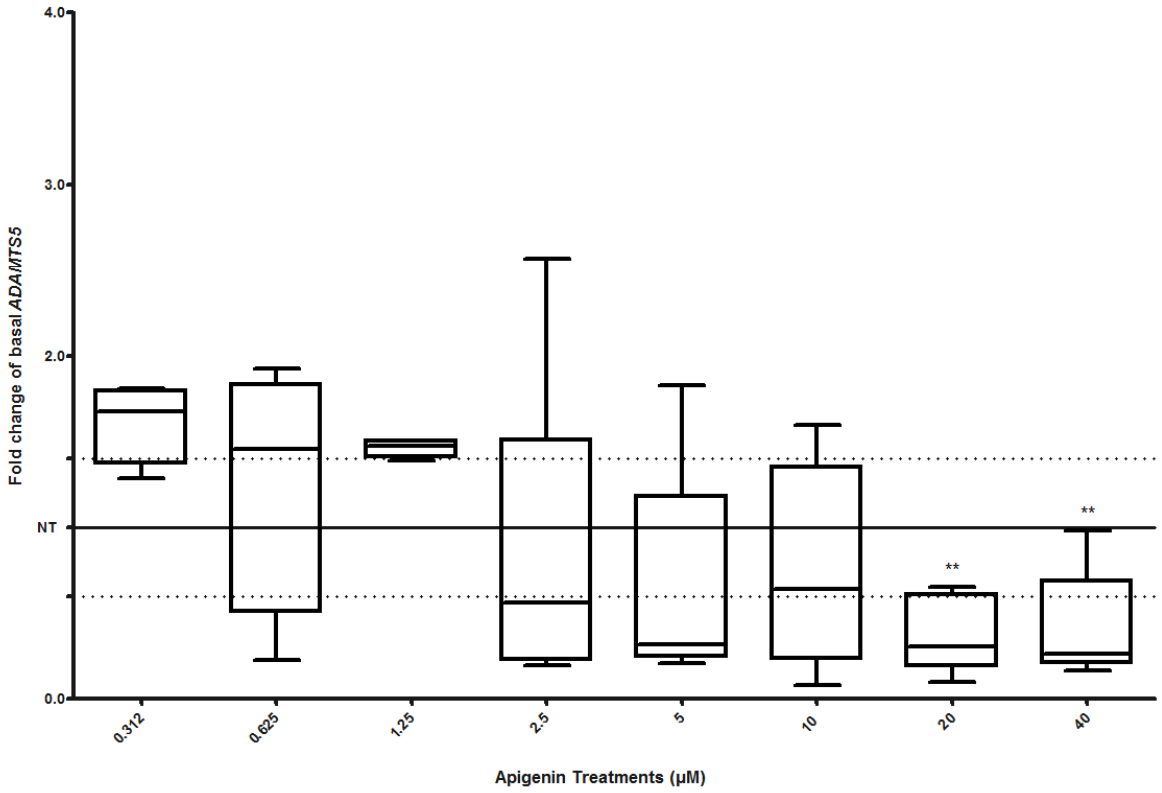


Figure 4.13. Apigenin basal *ADAMTS5* dose response. Tukey Box and whisker plot showing fold change in *ADAMTS5* gene expression relative to no treatment in KOA chondrocytes treated with apigenin at 0.312-40µM for 6 hours. The effect of apigenin on basal *ADAMTS5* expression was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation. ** $p<0.01$.

4.2.4.5 Apigenin dose response curve - effect on Basal *HO-1* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin at 0.312-40µM for 6 hours. Real-time qRT-PCR was used to measure *HO-1* using methods described in section 2.2.3.2. Apigenin significantly induced basal *HO-1* at 2.5-10µM ($p<0.05$-0.001) (Figure 4.14).

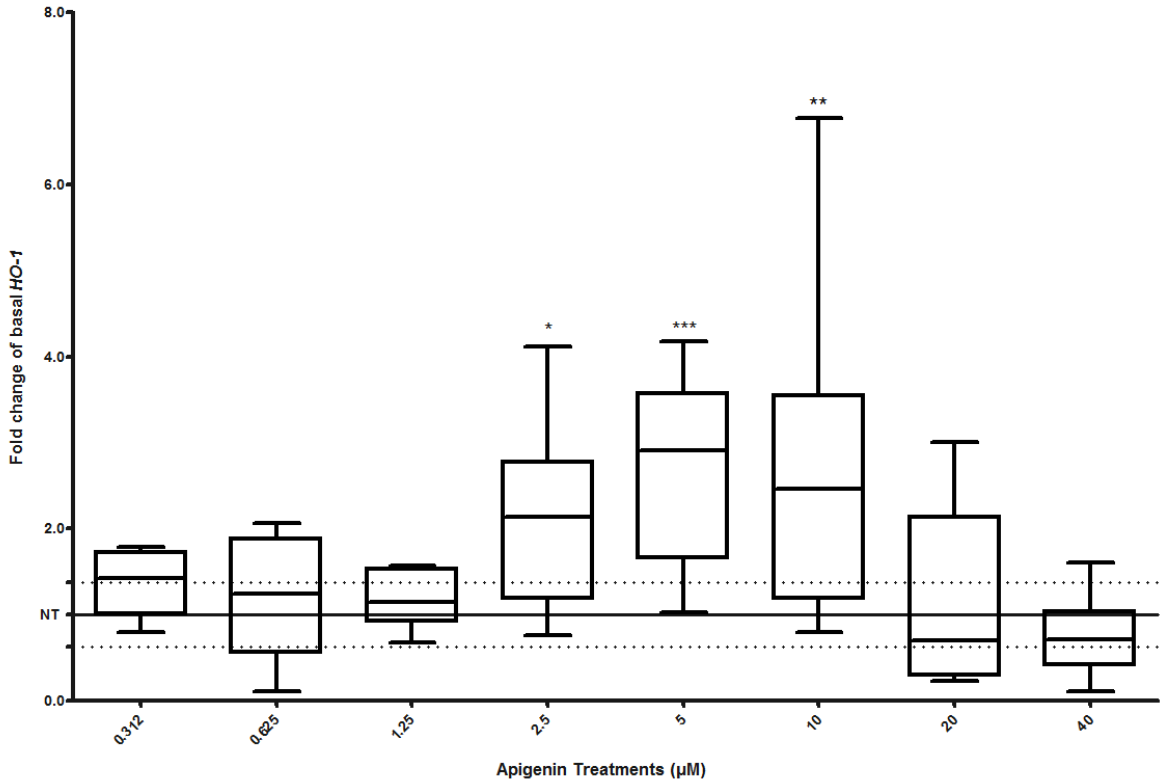


Figure 4.14. Apigenin basal *HO-1* dose response. Tukey Box and whisker plot showing fold change in *HO-1* gene expression relative to no treatment in KOA chondrocytes treated with apigenin at 0.312-40µM for 6 hours. The effect of apigenin on basal *HO-1* expression was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.4.6 Apigenin dose response curve - effect on Basal *AXIN2* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin at 2.5-40µM for 6 hours. Real-time qRT-PCR was used to measure *AXIN2* using methods described in section 2.2.3.2. Apigenin significantly inhibited basal *AXIN2* expression at 10-40µM in a dose dependent manner ($p<0.01$-$p<0.001$) (Figure 4.15).

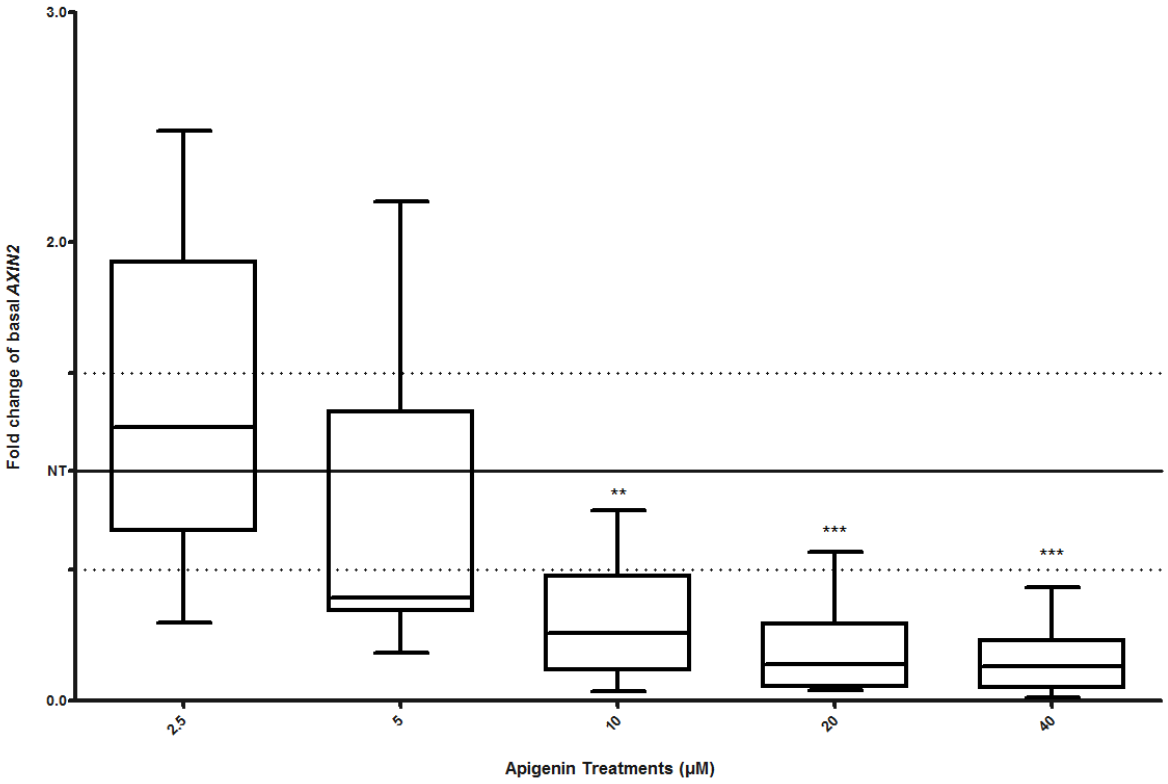


Figure 4.15. Apigenin basal *AXIN2* dose response. Tukey Box and whisker plot showing fold change in *AXIN2* gene expression relative to no treatment in KOA chondrocytes treated with apigenin at 2.5-40µM for 6 hours. The effect of apigenin on basal *AXIN2* expression was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment (NT) ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation. ** $p<0.01$, *** $p<0.001$.

4.2.5 Dose depended response curves - Isoliquiritigenin

4.2.5.1 Isoliquiritigenin dose response curve - effect on IL1 induced *MMP13* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 0.312-40µM for 6 hours. Isoliquiritigenin was added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *MMP13* using methods described in section 2.2.3.1**.** Isoliquiritigenin significantly inhibited IL1 induced *MMP13* from 1.25-40µM ($p<0.01$-0.001) in a dose dependent manner (Figure 4.16).

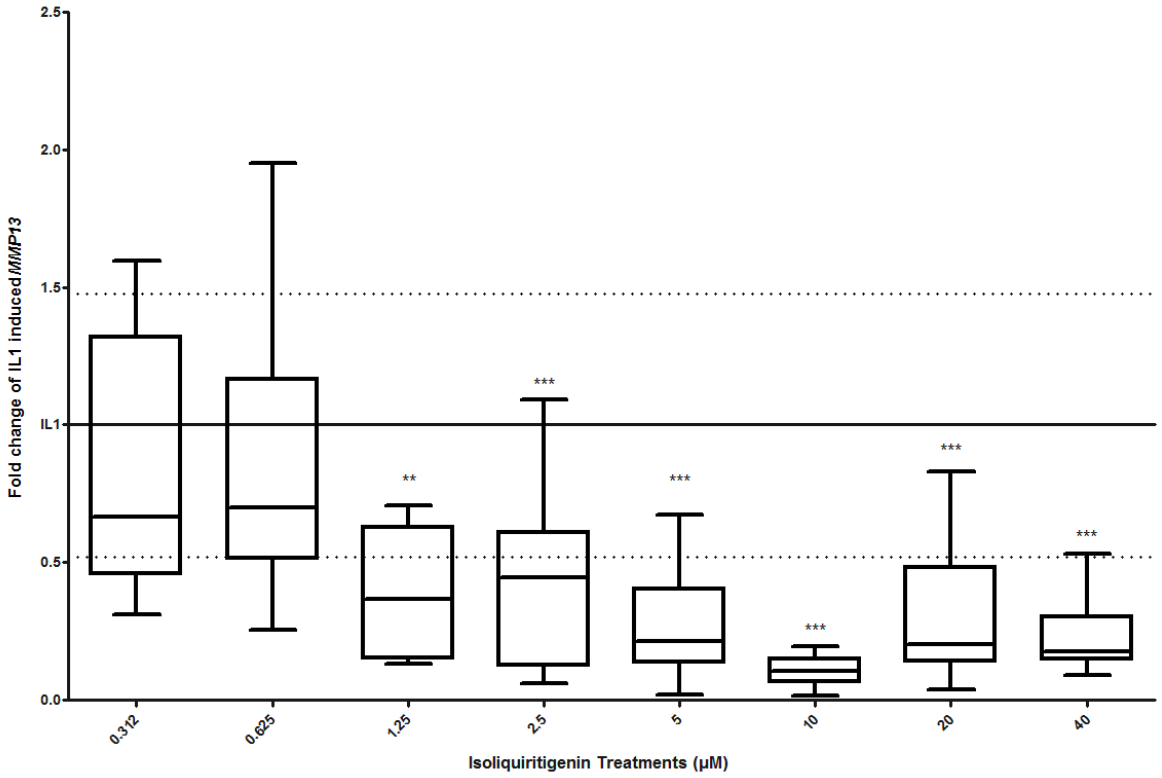


Figure 4.16. Isoliquiritigenin IL1 induced *MMP13* dose response. Tukey Box and whisker plot showing fold change in *MMP13* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with isoliquiritigenin at 0.312-40µM for 6 hours. The effect of isoliquiritigenin on IL1 induced *MMP13* was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. **p<0.01, ***p<0.001.

4.2.5.2 Isoliquiritigenin dose response curve - effect on IL1 induced *MMP1* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 0.312-40µM for 6 hours. Isoliquiritigenin was added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *MMP1* using methods described in section 2.2.3.1. Isoliquiritigenin significantly inhibited IL1 induced *MMP1* from 5-40µM ($p<0.001$) in a dose dependent manner (Figure 4.17).

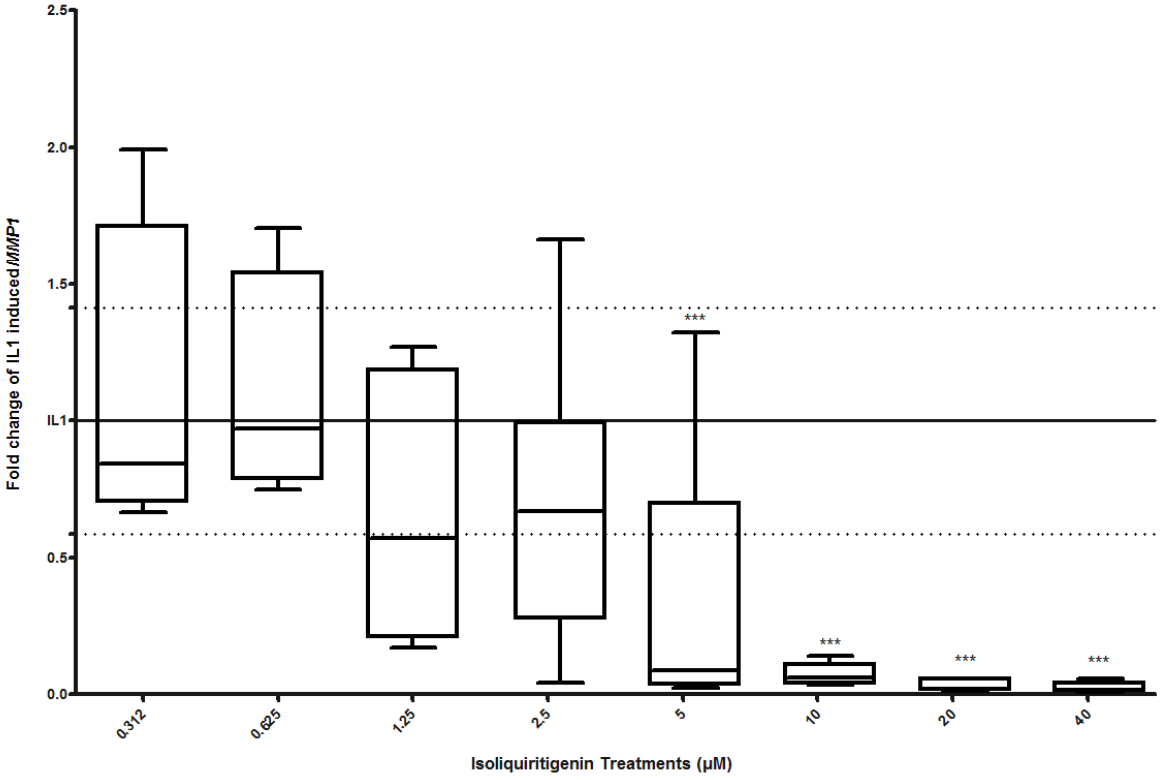


Figure 4.17. Isoliquiritigenin IL1 induced *MMP1* dose response. Tukey Box and whisker plot showing fold change in *MMP1* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with isoliquiritigenin at 0.312-40µM for 6 hours. The effect of isoliquiritigenin on IL1 induced *MMP1* was analysed using one way ANOVA with a Dunnetts post hoc test. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. *** $p<0.001$.

4.2.5.3 Isoliquiritigenin dose response curve - effect on IL1 induced *ADAMTS4* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 0.312-40µM for 6 hours. Isoliquiritigenin was added an hour before stimulation with 5ng/ml IL1. Real-time qRT-PCR was used to measure *ADAMTS4* using methods described in section 2.2.3.1. Isoliquiritigenin significantly inhibited IL1 induced *ADAMTS4* from 10-20µM ($p<0.01$-0.001) in a dose dependent manner (Figure 4.18). At 40µM *ADAMTS4* expression was undetectable.

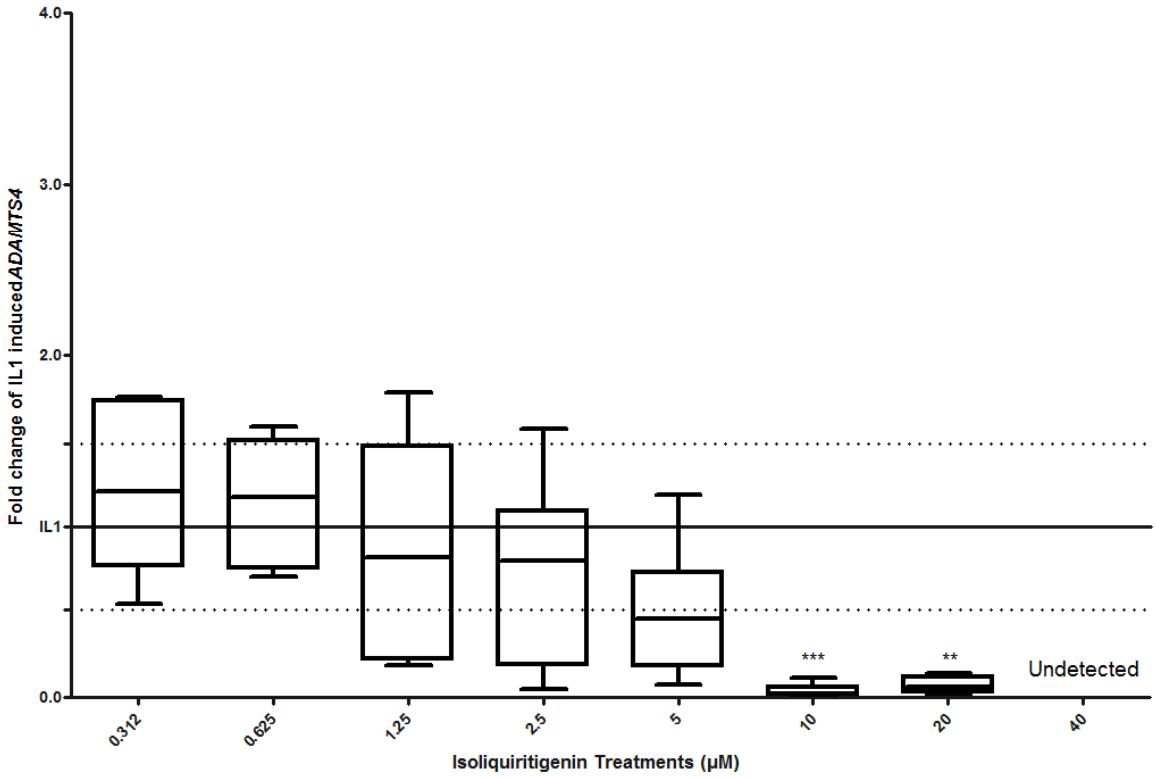


Figure 4.18. Isoliquiritigenin IL1 induced *ADAMTS4* dose response. Tukey Box and whisker plot showing fold change in *ADAMTS4* gene expression relative to IL1 treated control samples in KOA chondrocytes treated with isoliquiritigenin at 0.312-40µM for 6 hours. The effect of isoliquiritigenin on IL1 induced *ADAMTS4* was analysed using one way ANOVA with a Dunnetts post hoc test. At 40µM *ADAMTS4* expression was undetectable. All samples treated with IL1 (5ng/ml) for 6 hours. Data plotted as mean fold change relative to IL1 treatment alone ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents IL1 treatment alone with dotted lines representing IL1 treated controls standard deviation. ** $p<0.01$, *** $p<0.001$.

4.2.5.4 Isoliquiritigenin dose response curve - effect on Basal *ADAMTS5* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 0.312-40µM for 6 hours. Real-time qRT-PCR was used to measure *ADAMTS5* using methods described in section 2.2.3.1. Isoliquiritigenin significantly induced basal *ADAMTS5* expression at 40µM ($p<0.05$) (Figure 4.19).

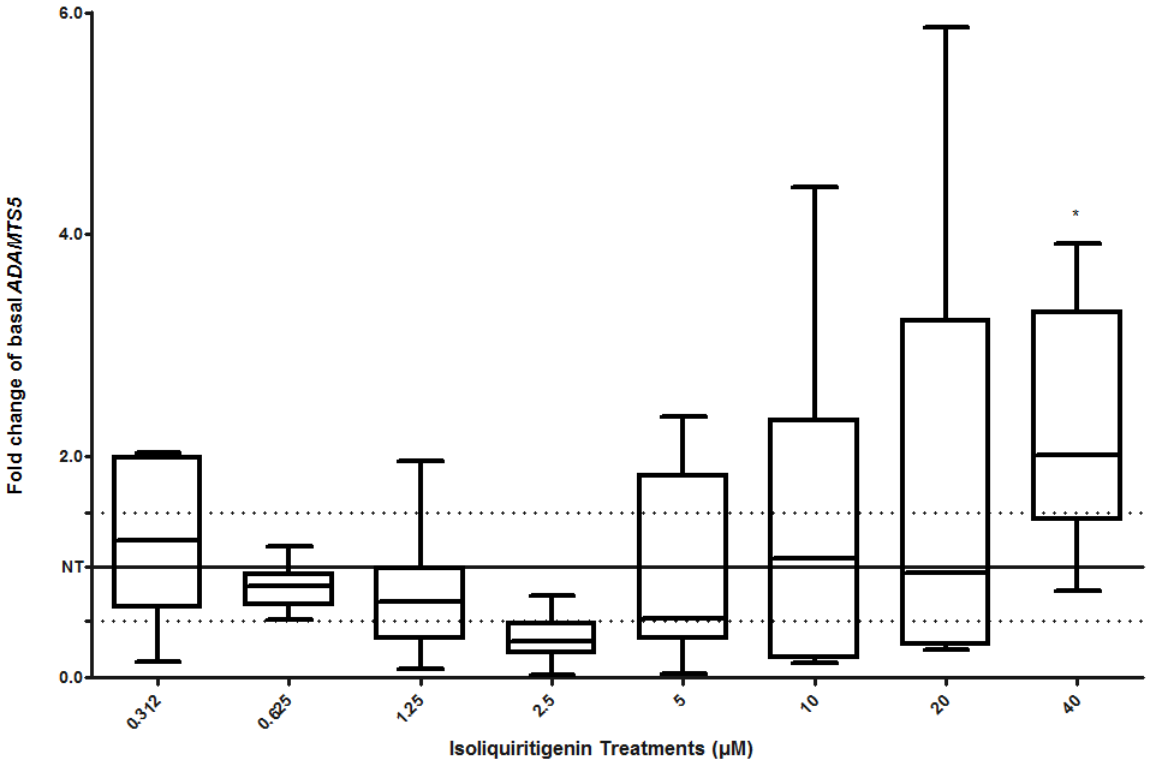


Figure 4.19. Isoliquiritigenin basal *ADAMTS5* dose response. Tukey Tukey Box and whisker plot showing fold change in *ADAMTS5* gene expression relative to no treatment in KOA chondrocytes treated with isoliquiritigenin at 0.312-40µM for 6 hours. The effect of isoliquiritigenin on basal *ADAMTS5* expression was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation. * $p<0.05$.

4.2.5.5 Isoliquiritigenin dose response curve - effect on Basal *HO-1* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 0.312-40µM for 6 hours. Real-time qRT-PCR was used to measure *HO-1* using methods described in section 2.2.3.2. Isoliquiritigenin significantly induced basal *HO-1* at 2.5-20µM ($p<0.01$-0.001) (Figure 4.20).

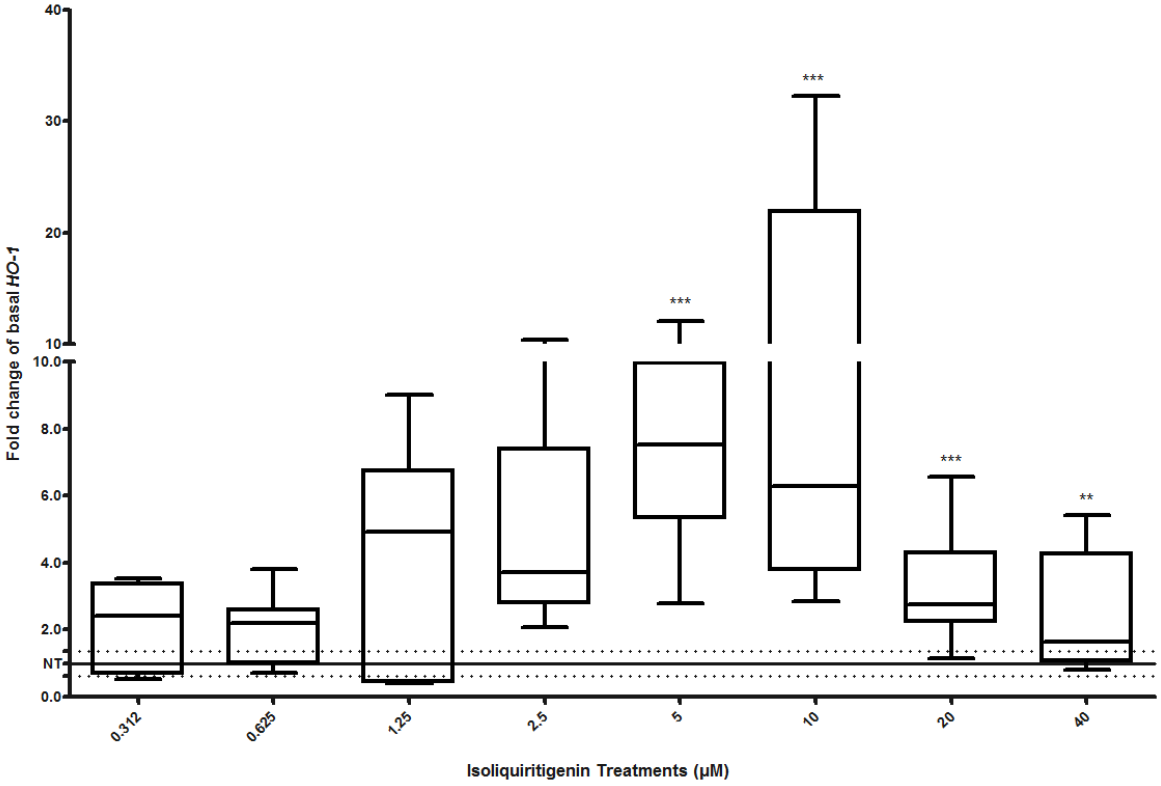


Figure 4.20. Isoliquiritigenin basal *HO-1* dose response. Tukey Box and whisker plot showing fold change in *HO-1* gene expression relative to no treatment in KOA chondrocytes treated with isoliquiritigenin at 0.312-40µM for 6 hours. The effect of isoliquiritigenin on induced basal *HO-1* was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation. ** $p<0.01$, *** $p<0.001$.

4.2.5.6 Isoliquiritigenin dose response curve - effect on Basal *AXIN2* in KOA chondrocytes

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 2.5-40µM for 6 hours. Real-time qRT-PCR was used to measure *AXIN2* using methods described in section 2.2.3.2. Isoliquiritigenin had no significant effect on *AXIN2* expression (Figure 4.21).

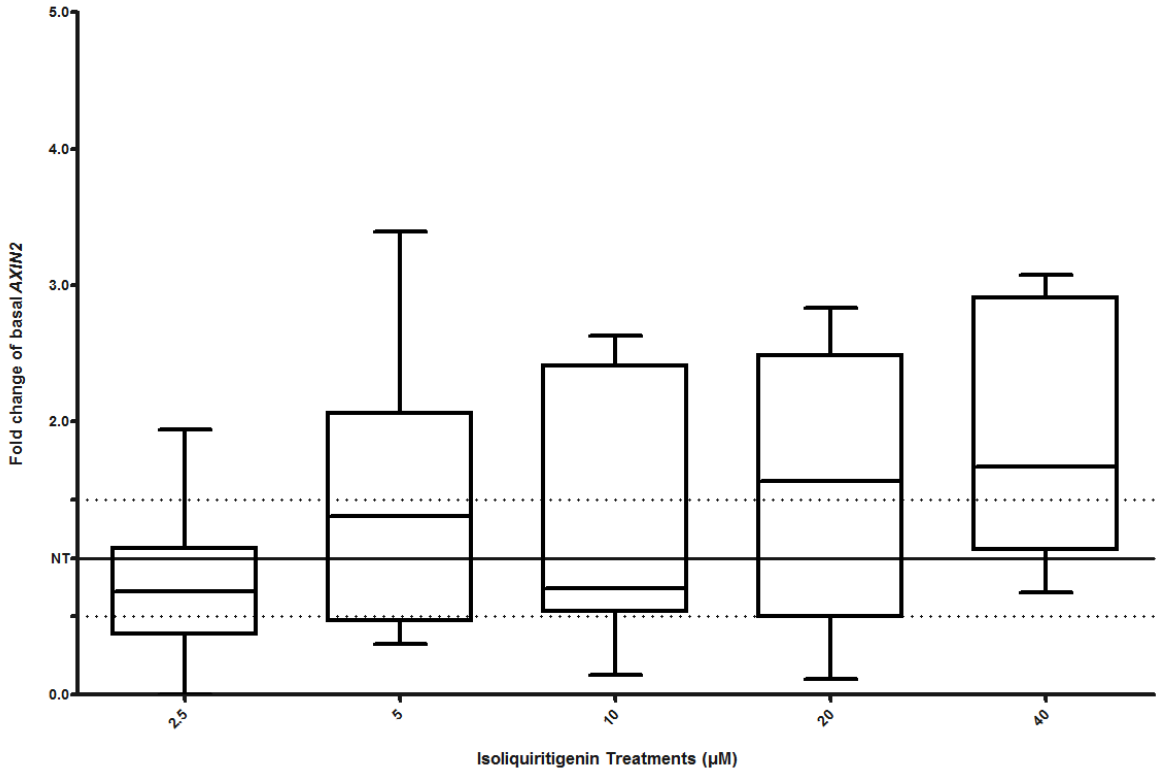


Figure 4.21. Tukey Box and whisker plot showing fold change in *AXIN2* gene expression relative to no treatment in KOA chondrocytes. KOA cells were treated with isoliquirtigenin at 2.5-40µM for 6 hours. The effect of isoliquiritigenin on *AXIN2* was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean fold change relative no treatment ± Tukey whiskers (Tukey whiskers are 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter), n=4. Solid line represents no treatment (NT) with dotted lines representing standard deviation.

4.2.6 Cell cytoxicity screens

4.2.6.1 Apigenin effect on cell necrosis - Lactate dehydrogenase assay

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with apigenin at 2.5-40µM for 6 hours and apigenin at 2.5-40µM with IL1 (5ng/ml). The CytoTox 96® Non-Radioactive Cytotoxicity assay was used as described by the manufacturer's instructions see section 2.2.1.2 for more details. LDH was detected in harvested supernates as a measure of compound toxicity. Apigenin had no significant effect on cell necrosis compared to no treatment control (Figure 4.22).

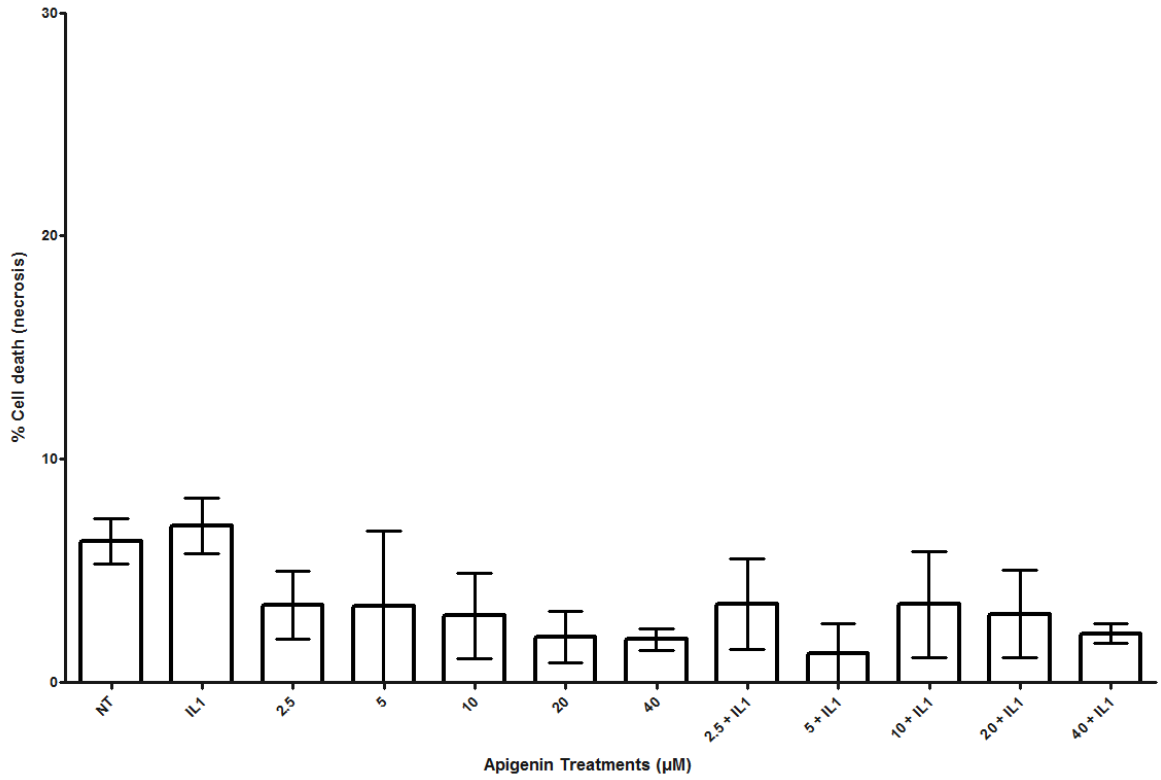


Figure 4.22. Apigenin has no effect on cell necrosis. Bar chart showing percentage of cell death (necrosis) in KOA chondrocytes treated with apigenin at 2.5-40µM for 6 hours and apigenin at 2.5-40µM with IL1 (5ng/ml). The effect of apigenin on cell necrosis was compared to no treatment (NT) control and was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean percentage of cell death ± standard deviation n=3.

4.2.6.2 Isoliquiritigenin effect on cell necrosis - Lactate dehydrogenase assay

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 2.5-40µM for 6 hours and isoliquiritigenin at 2.5-40µM with IL1 (5ng/ml). The CytoTox 96® Non-Radioactive Cytotoxicity assay was used as described by the manufacturer's instructions see section 2.2.1.2 for more details. LDH was detected in harvested supernates as a measure of compound toxicity. Isoliquiritigenin had no significant effect on cell necrosis compared to no treatment control (Figure 4.23).

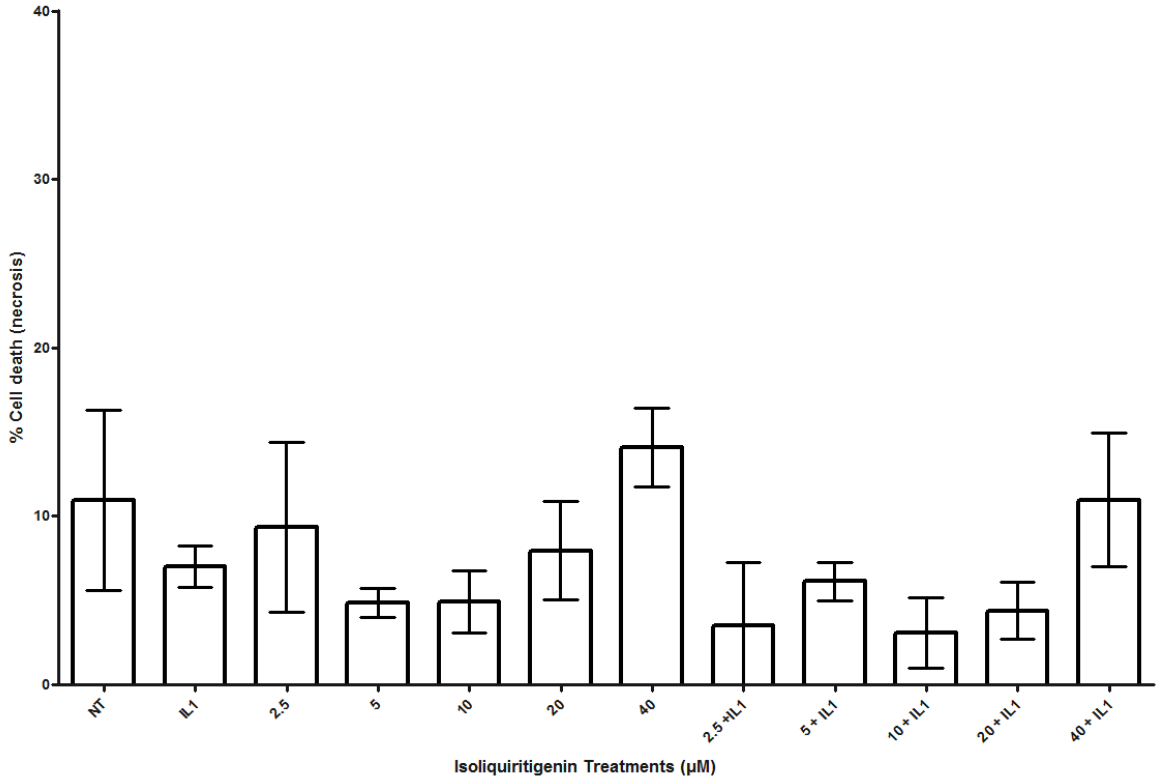


Figure 4.23. Isoliquiritigenin has no effect on cell necrosis. Bar chart showing percentage of cell death (necrosis) in KOA chondrocytes treated with isoliquiritigenin at 2.5-40µM for 6 hours and isoliquiritigenin at 2.5-40µM with IL1 (5ng/ml). The effect of isoliquiritienin on cell necrosis compared to no treatment (NT) control was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean percentage of cell death ± standard deviation n=3.

4.2.6.3 Apigenin effect on cell apoptosis - Caspase-Glo® 3/7 Assay

KOA chondrocytes were cultured and plated as described in section 2.2.3.1. KOA cells were treated with apigenin at 2.5-40µM for 6 hours. Cytotoxicity of compounds were assessed using the Caspase-Glo® 3/7 Assay as described by the manufacturer's instructions see section 2.2.1.3 for more details. The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in cultures of adherent cells. Total cell death control (100% cell death) calculated by treating cells with 1µM stuarosporine for 6 hours. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. Apigenin had no significant effect on cell apoptosis compared to no treatment control (Figure 4.24).

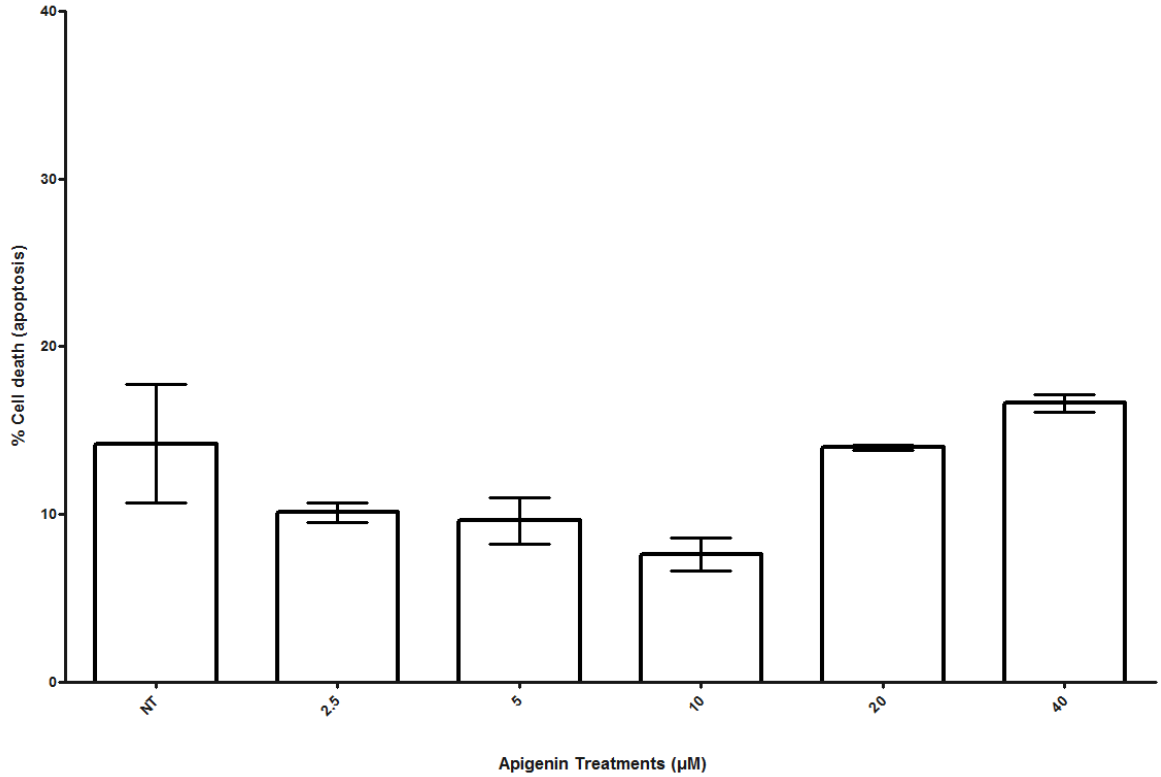


Figure 4.24. Apigenin has no effect on cell apoptosis. Bar chart showing percentage of cell death (apoptosis) in KOA chondrocytes treated with apigenin at 2.5-40µM for 6 hours. The effect of apigenin on cell necrosis compared to no treatment (NT) control was analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean percentage of cell death ± standard deviation n=3.

4.2.6.4 Isoliquiritigenin effect on cell apoptosis - Caspase-Glo® 3/7 Assay

KOA chondrocytes were cultured and plated as described in section 2.2.1.1. KOA cells were treated with isoliquiritigenin at 2.5-40µM for 6 hours. Cytotoxicity of compounds were assessed using the Caspase-Glo® 3/7 Assay as described by the manufacturer's instructions see section 2.2.1.3 for more details. Total cell death control (100% cell death) calculated by treating cells with 1µM stuarosporine for 6 hours. Isoliquiritigenin had no significant effect on cell apoptosis compared to no treatment control (Figure 4.25).

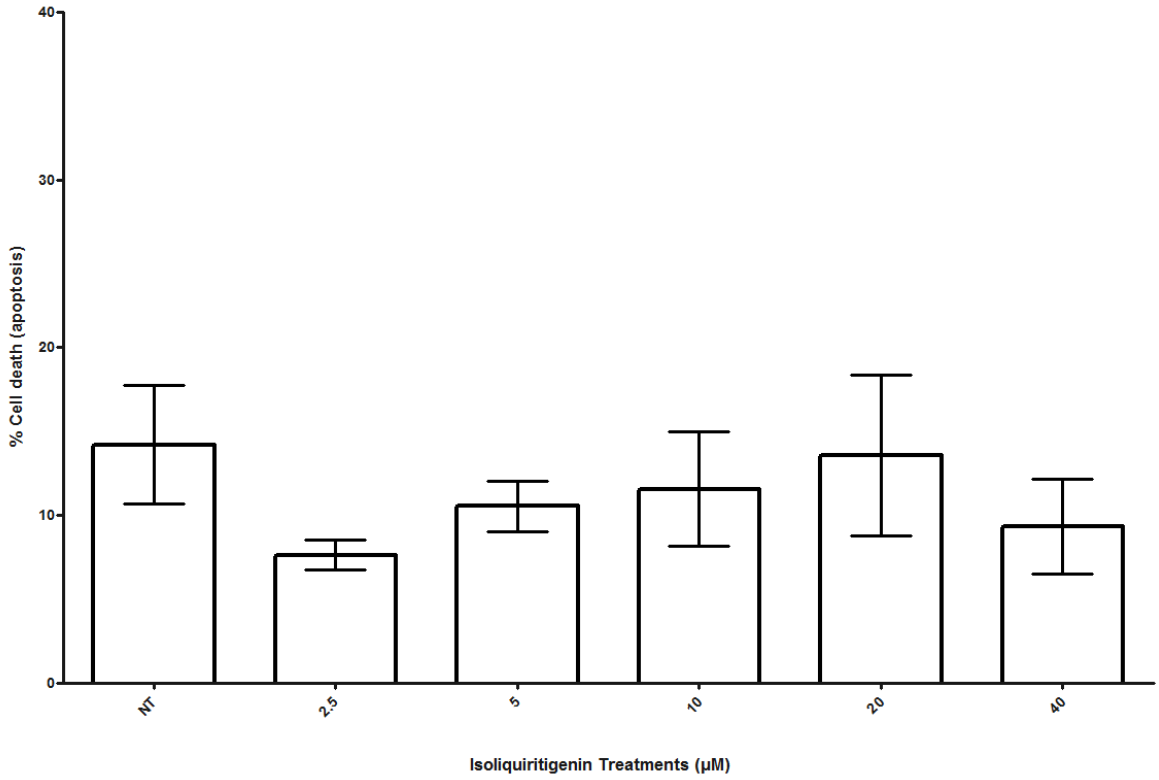


Figure 4.25. Isoliquiritigenin has no effect on cell apoptosis. Bar chart showing percentage of cell death (apoptosis) in KOA chondrocytes treated with isoliquiritigenin at 2.5-40µM for 6 hours. The effect of isoliquiritigenin on cell necrosis was compared to no treatment (NT) control and analysed using one way ANOVA with a Dunnetts post hoc test. Data plotted as mean percentage of cell death ± standard deviation n=3.

4.3 Discussion

In the previous chapter 20 compounds were selected for further study (see Chapter 3 Table 5). Here, these compounds were screened for inhibition of cartilage degrading genes *MMP13, MMP1, ADAMTS4* and *ADAMTS5* in primary KOA chondrocytes.

At 10µM apigenin, isoliquiritigenin, aloe-emodin, luteolin, genistin and ursolic acid significantly inhibited IL1 induced *MMP13* expression ($p<0.05$-$p<0.0001$) (Figure 4.1). These results support data shown in Chapter 3 (Figure 3.3) where at 10µM apigenin, isoliquiritigenin, emodin, luteolin and ursolic acid significantly inhibited *MMP13* expression in SW1353 cells ($p<0.05$-$p<0.0001$). Similarly apigenin, isoliquiritigenin, aloe-emodin, lutelin, genistin and ursolic acid significantly inhibited *MMP13* expression in C28/I2 cells at 10µM ($p<0.05$-$p<0.0001$) (Chapter 3 Figure 3.5). These data show that for IL1 induced *MMP13* expression SW153 and C28/I2 cells are useful models for primary chondrocytes. Compounds that significantly inhibited *MMP13* in KOA cells (Figure 4.1) were entered into Table 6 which was used to select compounds for dose response experiments.

Compounds screened in KOA chondrocytes had no significant effect on basal *MMP13* at 10µM. Quercetin, methyl-hesperidin, luteolin and naringen showed a trend towards inhibition of basal *MMP13* which didn't reach significance (Figure 4.2). Hesperetin is the alglycone form of methyl-hesperedin which did show a trend towards inhibition of basal *MMP13* in KOA chondrocytes (Figure 4.2). While myricetin and theobromine significantly reduced basal *MMP13* in SW1353 cells (Chapter 3 Figure 3.4) and apigenin, myricetin and gensitin significantly reduced basal *MMP13* in C28/I2 cells (Chapter 3 Figure 3.6) these compounds had no significant effect on basal *MMP13* in KOA chondrocytes (Figure 4.2). The difference in basal *MMP13* expression results seen between the cell models and KOA chondrocytes may be due to basal *MMP13* inter-patient variability in the KOA chondrocyte experiments. As compounds screened in KOA chondrocytes had no significant effect on basal *MMP13* at 10µM basal *MMP13* was not used as a gene on interest in the dose response assays.

Compounds that significantly inhibited IL1 induced *MMP1* in KOA cells (Figure 4.3) may be chondro-protective and were entered into Table 6 which was used to select compounds for dose response experiments. Compounds screened in KOA chondrocytes had no significant

effect on basal *MMP1* at 10µM. Luteolin showed a trend towards inhibition of basal *MMP1* which didn't reach significance (Figure 4.4) similar to the trend of inhibition seen with basal *MMP13* when KOA cells were treated with luteolin (Figure 4.2). As compounds screened in KOA chondrocytes had no significant effect on basal *MMP1* at 10µM basal *MMP1* was not used as a gene on interest in the dose response assays.

As aloe-emodin, apigenin and isoliquiritigenin significantly reduced IL1 induced *ADAMTS4* expression (Figure 4.5) they may be chondro-protective and were entered into Table 6. Genistin, isoliquiritigenin and apigenin significantly reduced basal *ADAMTS4* expression and emodin, aloe-emodin and luteolin showed a trend towards IL1 induced *ADAMTS4* inhibition which did not reach significance in KOA cells (Figure 4.6). Compounds that significantly inhibited basal *ADAMTS4* may be chondro-protective and were entered into Table 6.

In these experiments IL1 did not induce *ADAMTS5* expression so basal *ADAMTS5* was measured. Luteolin and genistin significantly inhibited basal *ADAMTS5* at 10µM ($p<0.01$-$p<0.001$) (Figure 4.7). Compounds that significantly inhibited basal *ADAMTS5* may be chondro-protective and were entered into Table 5.

Table 6 shows seven compounds from the 20 selected compounds (Chapter 3 Table 5) that significantly inhibited one or more of the cartilage chondro-destructive genes screened at 10µM. Each of these compounds could be chondro-protective and future studies looking at their mechanisms of action and potential synergies between them may be beneficial when trying to identify bioactives that protect cartilage from destruction in OA. In the scope of this project only two compounds could be selected from Table 6 for further analysis due to lack of sufficient KOAs from operative procedures and time constraints. As apigenin and isoliquiritigenin significantly inhibited genes of interest in the most screens they were selected for further study. Inhibition of *MMP13* was required in a candidate compound as *MMP13* has been considered critical for OA progression. Pharmacologic inhibition of *MMP13* has been shown to be an effective strategy to decelerate articular cartilage loss in a murine model of injury-induced knee OA (Wang et al., 2013).

Ursolic acid is a triterpenoid that exists widely in food (Liu, 1995), medicinal herbs and is often used in cosmetics (Figure 4.26). Ursolic acid is present in a wide variety of fruits such

as apples and cranberries (Arnold & Hsia, 1957). Ursolic acid significantly reduced IL1 induced *MMP1* ($p<0.001$) (Figure 4.3) but had no significant effect on *MMP13, ADAMTS4* or *ADAMTS5* in KOA cells at 10µM (Figure 4.1-4.2, 4.4-4.7). In the previous chapter (Figure 3.3) ursolic acid significantly inhibited IL1 induced *MMP13* ($p<0.0001$) at 10µM, while ursolic acid did not significantly inhibit IL1 induced *MMP13* in KOA cells (Figure 4.1) there was a trend towards inhibition that may have become significant with more cartilage samples. Ursolic acid has been shown to inhibit activation of the transcription factor NFκB in a leukaemia cell line by preventing phosphorylation of IKb (Shishodia et al., 2003). Ursolic acid could potentially work through a similar mechanism in primary chondrocytes as *MMP1* and *MMP13* are NFκB responsive genes. Ursolic acid was not selected for further study as at 10µM ursolic acid only inhibited IL1 induced *MMP1* in KOA cells (Figure 4.3).

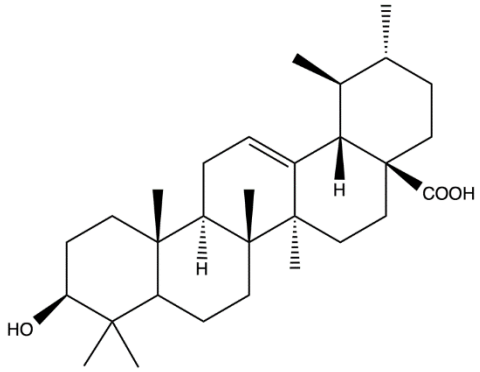


Figure 4.26. The chemical structure of ursolic acid. Ursolic acid is a triterpenoid it is also called prunol, malol, β-ursolic acid, NSC4060, CCRIS 7123, TOS-BB-0966, 3-β-hydroxyurs-12-en-28-oic acid with a molecular formula of $C_{30}H_{48}O_3$ and a molar mass of 456.7 g/mol.

Genistin is an isoflavone from soy that attenuates growth factor and cytokine stimulated proliferation of both normal and cancer cells potentially via TGFβ1 signaling (Kim et al., 1998). Genistin significantly reduced IL1 induced *MMP1* ($p<0.05$) (Figure 4.3) basal *ADAMTS4* and *ADAMTS5* (Figure 4.6-4.7) but had no significant effect on *MMP13* (Figure 4.1). In the previous chapter (Figure 3.6) genistin significantly inhibited IL1 induced *MMP13* ($p<0.0001$) at 10μM in C28/I2 cells, while genistin did not significantly inhibit IL1 induced *MMP13* in KOA cells (Figure 4.1) there was a trend towards inhibition that may have become significant with more cartilage samples. Digestive enzymes in the digestive system convert genistin into genestein. Genistein has been shown to inhibit MMP-1 synthesis in aged normal human dermal fibroblasts and was hypothesised to be a useful agent for preventing intrinsic aging (Yang et al., 2007). In KOA cells genistin shows a similar inhibition of *MMP1* (Figure 4.3), basal *ADAMTS4* and *ADAMTS5* are also inhibited (Figure 4.6-4.7). These results suggest that genistin may be chondro-protective. Genistin was not selected for further study as it did not significantly inhibit IL1 induced *MMP13* (Figure 4.1).

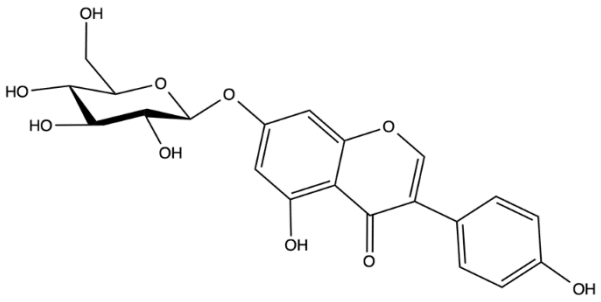


Figure 4.27. The chemical structure of genistin. Genistin is an isoflavone from soy it is also called genistoside, genistine, genistein 7-glucoside, genistein glucoside, genistein-7-glucoside, genisteol 7-monoglucoside and glucosyl-7-genistein with a molecular formula of $C_{21}H_{20}O_{10}$ and a molar mass of 432.37 g/mol.

Luteolin is a flavone found in celery that has been shown to inhibit IL1 induced *MMP13* via inhibition of IL1 induced ERK activation in a dose-dependent manner in osteoblasts (Yang et al., 2012). Luteolin significantly reduced IL1 induced *MMP13* ($p<0.05$) (Figure 4.1), IL1 induced *MMP1* ($p<0.001$) (Figure 4.3) and basal *ADAMTS5* (Figure 4.7) but had no

significant effect on *ADAMTS4* (Figure 4.5, 4.6). In the previous chapter (Figure 3.3) luteolin significantly inhibited IL1 induced *MMP13* ($p<0.0001$) at 10µM in SW1353 cells and C28/I2 cells (Figure 3.6). These results suggest that luteolin may be chondro-protective and may possibly inhibit *MMP13* and *MMP1* via inhibition of IL1 induced ERK activation. Luteolin was not selected for further study as it did not have a significant effect on as many genes of interest as apigenin or isoliquiritigenin.

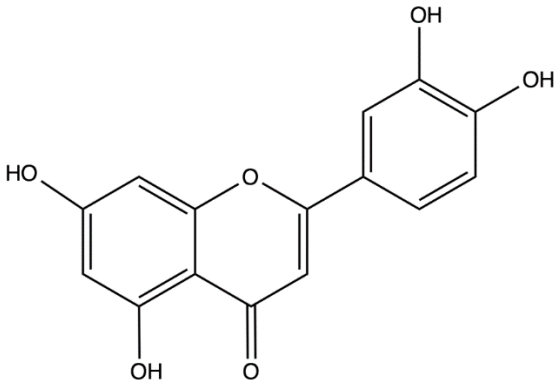


Figure 4.28. The chemical structure of luteolin. Luteolin is a flavone found in celery it is also called luteolol, digitoflavone, flacitran and luteoline with a molecular formula of $C_{15}H_{10}O_6$ and a molar mass of 286.24 $g \cdot mol^{-1}$.

Emodin is a purgative resin trihydroxyanthraquinone from rhubarb (Figure 4.29) and is a commonly used traditional herbal treatment in China (Xiao et al., 2014). Emodin significantly reduced IL1 induced *MMP13* ($p<0.01$) (Figure 4.1), *MMP1* ($p<0.001$) (Figure 4.3) and basal *ADAMTS4* ($p<0.05$) (Figure 4.6) but had no significant effect on *ADAMTS5* (Figure 4.7). In the previous chapter (Figure 3.3, 3.6) emodin significantly inhibited IL1 induced *MMP13* at 10µM in SW1353 ($p<0.001$) and C28/I2 cells ($p<0.05$), supporting the results seen in KOA cells (Figure 4.1). The expression of cartilage-degrading metalloproteinases is regulated in part by changes in acetylation via histone acetyltransferases and HDACs (Higashiyama et al., 2010). At 10µM emodin has been shown to inhibit the expression of *MMP1* and *MMP13* in IL1 stimulated synoviocytes under hypoxia, it was hypothesised that this was due to emodin ability to inhibit HDAC activity (Ha et al., 2011). These results suggest that emodin may be chondro-protective and may

possibly inhibit *MMP13*, *MMP1* and *ADAMTS4* via a reduction in HDAC activity. Emodin has been reported to have laxative properties, at 20μM emodin significantly increased aquaporin 3 (AQP3) protein and mRNA expression in human intestinal epithelial cells. AQP3 plays an important role in regulating water transfer in the colon, it was hypothesised that emodins laxative effects are associated with the increased expression of AQP3 (Zheng et al., 2014). At the concentrations used in the KOA cell experiments emodin may exhibit unwanted laxative effects in patients, for this reason emodin was not selected for further study.

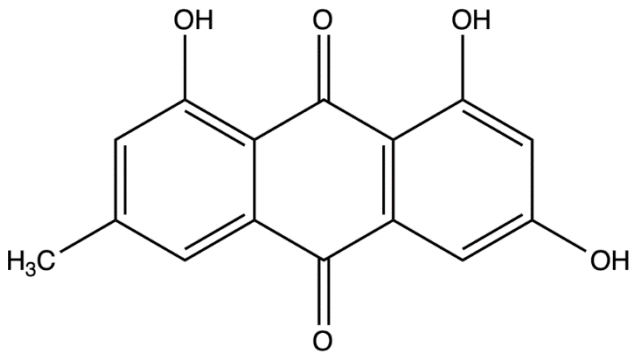


Figure 4.29. The chemical structure of emodin. Emodin is an anthraquinone found in rhubarb it is also called 6-methyl-1,3,8-trihydroxyanthraquinone with a molecular formula of $C_{15}H_{10}O_5$ and a molar mass of 270.24 $g \cdot mol^{-1}$.

Aloe-emodin is a hydroxy anthraquinone (Figure 4.30) known to have anti fungal, antibacterial, antiviral and hepatoprotective activities. Aloe-emodin significantly reduced IL1 induced *MMP13* ($p<0.01$) (Figure 4.1), *MMP1* ($P<0.001$) (Figure 4.3) and *ADAMTS4* ($p<0.05$) (Figure 4.5) but had no significant effect on *ADAMTS5* (Figure 4.7). In the previous chapter (Figure 3.6) aloe-emodin significantly inhibited IL1 induced *MMP13* at 10μM in C28/I2 cells ($p<0.05$), supporting the results seen in KOA cells (Figure 4.1). Aloe-emodin has also been shown to reduce the DNA binding activities of NFκB (Suboj et al., 2012). Aloe-emodin could potentially work through a similar mechanism in primary chondrocytes as *MMP1* and *MMP13* are NFκB responsive genes. Aloe-emodin may also inhibit HDAC activity similar to emodin due to the structural similarities between the two compounds (Figure

4.29, 4.30). These results suggest that emodin may be chondro-protective and may possibly inhibit *MMP13, MMP1* and *ADAMTS4* via a reduction of the DNA binding activities of NFκB and/or the inhibition of HDAC activity. Aloe-emodin has also been reported to have laxative effects (Müller et al., 1996) similar to those seen with emodin treatment (Zheng et al., 2014), for this reason aloe-emodin was not selected for further study.

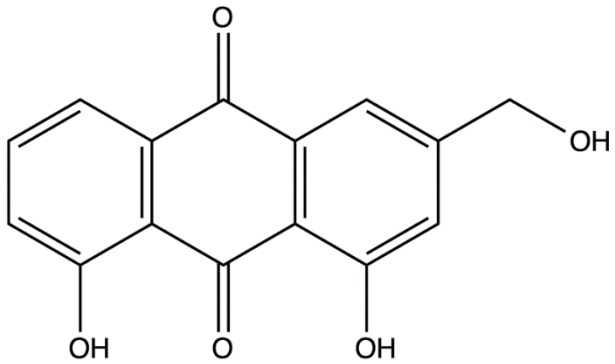


Figure 4.30. The chemical structure of aloe-emodin. Aloe-emodin is an anthraquinone found in aloe vera. Aloe-emodin is also called 1,8-Dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione with a molecular formula of $C_{15}H_{10}O_5$ and a molar mass of 270.24 $g \cdot mol^{-1}$.

Isoliquiritigenin is a chalcone (Figure 4.31) found in liquorice root and other plants and has been shown to have potent antitumor, antioxidant, and phytoestrogenic activity in vitro (Guo et al., 2008). Isoliquiritigenin significantly reduced IL1 induced *MMP13* ($p<0.001$) (Figure 4.1), *MMP1* ($p<0.0001$) (Figure 4.3) *ADAMTS4* (Figure 4.4) and basal *ADAMTS4* ($p<0.01$) (Figure 4.6). In the previous chapter (Figure 3.3) isoliquiritigenin significantly inhibited IL1 induced *MMP13* at 10μM in SW1353 cells ($p<0.05$), supporting the results seen in KOA cells (Figure 4.1). Isoliquiritigenin has also been shown to dose dependently (1-10μM) inhibit LPS stimulated (200ng/ml for 16 hours) IL1 and TNFα production in RAW 264.7 cells. Isoliquiritigenin was also shown to induce heme oxygenase-1 at the mRNA and protein level (S. Lee et al., 2009). These anti-inflammatory properties of Isoliquiritigenin could be beneficial for patients suffering with OA. These results suggest that isoliquiritigenin may be chondro-protective. Isoliquiritigenin was one of the two

compounds selected for further study (Table 6), as it inhibited the most chondro-degenerative genes out of the compounds screened.

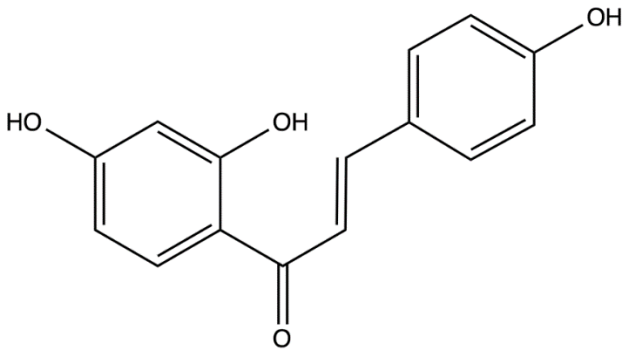


Figure 4.31. The chemical structure of isoliquiritigenin. Isoliquiritigenin is a flavonoid found in liquorice. Isoliquiritigenin is also called 6'-deoxychalcone, 2',4,4'-Trihydroxychalcone, 4,2',4'-Trihydroxychalcone, 4'2'4'-trihydroxychalcone 2',4',4-Trihydroxychalcone with a molecular formula of $C_{15}H_{12}O_4$ and a molar mass of 256.25 g/mol.

Apigenin is a plant-derived flavone (Figure 4.32). Apigenin has been shown to suppress *MMP13* expression in IL1 treated SW1353 cells by suppressing the c-fos/AP-1 and JAK2/STAT1/2 pathways (Lim et al., 2011). SW1353 cells were treated with IL1 and apigenin (5, 25µM) for 24 hours. Western blot detected lower MMP-13 levels in cell media when SW1353 cells were treated with apigenin, an enzyme-linked immunosorbent assay showed that when SW1353 cells were treated with IL1 and apigenin at 10µM for 24 hours there was a significant reduction in MMP-13 levels in the media (when compared to IL1 control group). In the previous chapter (Figure 3.3) apigenin significantly inhibited IL1 induced *MMP13* at 10µM in SW1353 cells ($p<0.05$) and C28/I2 cells (Figure 3.6) ($p<0.0001$). These results support the data seen in KOA cells (Figure 4.1). Apigenin significantly reduced IL1 induced *MMP13* ($p<0.001$) (Figure 4.1), *MMP1* ($p<0.0001$) (Figure 4.3) *ADAMTS4* (Figure 4.5) ($p<0.05$) and basal *ADAMTS4* ($p<0.0001$) (Figure 4.6). These results suggest that apigenin may be chondro-protective and may possibly inhibit *MMP13, MMP1* and *ADAMTS4* via suppressing the c-fos/AP-1 and JAK2/STAT1/2 pathways. Apigenin was one

of the two compounds selected for further study (Table 6) as it inhibited the most chondro-degenerative genes out of the compounds screened.

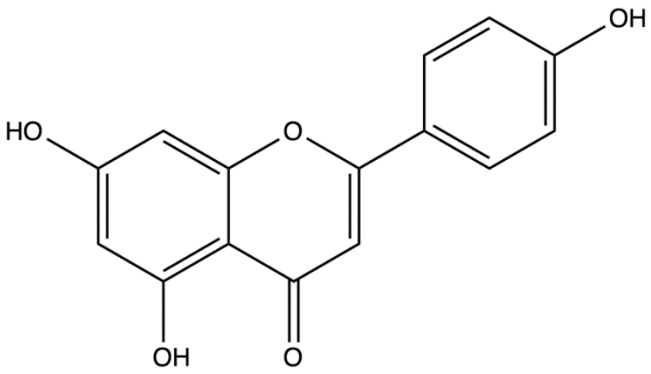


Figure 4.32. The chemical structure of apigenin. Apigenin is a flavone found in various plants. Apigenin is also called Apigenine, Chamomile, Apigenol, Spigenin, Versulin; 4',5,7-Trihydroxyflavone and C.I. Natural Yellow 1 with a molecular formula of $C_{15}H_{10}O_5$ and a molar mass of 270.24 $g \cdot mol^{-1}$.

The time course of the expression of *MMP13* was used to analyse the effects of the two selected compounds apigenin (Figure 4.8) and isoliquiritigenin (Figure 4.9) over time. KOA cells were treated with apigenin or isoliquiritigenin at 10µM and 5ng/ml IL1 for 1, 3, 6, 12, 18 and 24 hours. IL1 stimulated *MMP13* reached maximal inhibition at 6 hours when treated with 10µM of apigenin. IL1 stimulated *MMP13* reached maximal inhibition at 12 hours when treated with 10µM of isoliquiritigenin. These results were used to select a 6 hour time point for the apigenin and isoliquiritigenin dose response experiments (Figures 4.10-4.21). Both compounds inhibited *MMP13* at 6 hours (Figure 4.8, 4.9) and while isoliquiritigenin was more potent at 12 hours, 6 hours was selected to make comparisons of *MMP13* inhibition between the two compounds more accurate. 0.312-40µM was used as the dose range for the dose response assays, as doses between 0.1-10µM have been suggested as physiologically relevant concentration for dietary derived bioactive compounds (Ferrars et al., 2014), (Amin et al., 2015). Concentrations higher than 10µM

were considered 'high' concentrations likely to only be achieved by supplementation of these compounds as opposed to obtaining them from the diet. As apigenin and isoliquiritigenin had no effect on basal *MMP13* or basal *MMP1* at 10µM in KOA cells (Table 6), they were not analysed in the dose response assays. Basal *ADAMTS4* was not analysed in the dose response experiments because unstimulated *ADAMTS4* expression levels were very low in KOA cells. As discussed in the introduction, *HO-1* was analysed in the dose response assay as a measure of a compounds anti-inflammatory potential and *AXIN2* was analysed to measure a compounds effect on Wnt signalling.

Cell cytoxicity was analysed by measuring cell necrosis and cell apoptosis after compound treatment. Cell necrosis was measured using the CytoTox 96® Non-Radioactive Cytotoxicity as described by the manufacturer's instructions (see Chapter 2 section 2.2.1.2 for more details). Cell apoptosis was assessed using the Caspase-Glo® 3/7 Assay as described by the manufacturer's instructions (see Chapter 2 section 2.2.1.3 for more details). For compounds to be considered as being protective to cartilage they must not significantly increase cell necrosis or apoptosis.

While apigenin showed a trend towards inducing IL1 induced *MMP13* at 1.25µM (Figure 4.10) and *MMP1* at 0.312µM (Figure 4.11) these did not reach statistical significance. Future experiments should explore the effects of apigenin on *MMP13* and *MMP1* at these lower doses to ensure that apigenin treatment does not result in there upregulation. Apigenin significantly inhibited IL1 induced *MMP13* from 2.5-40µM ($p<0.01$-0.0001) in a dose dependent manner (Figure 4.10). IL1 induced *MMP1* was significantly inhibited by apigenin at 2.5-40µM ($p<0.01$-0.0001) in a dose dependent manner (Figure 4.11). Apigenin significantly inhibited IL1 induced *ADAMTS4* from 5-20µM ($p<0.01$-0.001) in a dose dependent manner (Figure 4.12.) Apigenin significantly reduced basal *ADAMTS5* expression at 20 and 40µM ($p<0.01$) (Figure 4.13). Apigenin significantly induced basal *HO-1* at 2.5-10µM ($p<0.05$-0.001) (Figure 4.14). The bell shaped curve of induction of *HO-1* suggests that apigenin may have the most effective anti-inflammatory effects around 5-10µM, interestingly this could mean that over supplementation of apigenin may not be as protective to cartilage as finding a correct dose for maximal anti-inflammatory and chondro-protective effects. Concentrations of apigenin in human subjects have been hypothesised to be around 1.7µM after consumption of 10g of dried parsley per day

(Shibata et al., 2014), and while it would not be impossible to reach plasma concentrations of apigenin used in this project through normal dietary intake, supplementation could potentially be used to obtain higher doses.

Basal *AXIN2* expression was inhibited at 10-40μM ($p<0.01$-$p<0.001$) (Figure 4.15) by apigenin. While inhibition of chondro-destructive genes becomes more pronounced at higher concentrations (Figures 4.10-4.12) apigenins potentially anti-inflammatory upregulation of *HO-1* is lost at these higher concentrations (Figure 4.14). Apigenin dose dependently inhibits *AXIN2* expression (Figure 4.15) and as discussed in the introduction *AXIN2* is a Wnt/β-catenin responsive gene which has been shown to be upregulated consistently with net activation of canonical Wnt signalling (Lustig et al., 2002). Wnt signalling can induce the expression of MMPs in articular chondrocytes which promotes cartilage degradation and cartilage catabolism (Yuasa et al., 2008). Apigenin may be downregulating MMPs via Wnt signalling disruption. Apigenin was non-cytotoxic at all concentrations analysed in the dose response assays (Figures 4.22, 4.24). Apigenin had no statistically significant effect on cell necrosis (Figure 4.22) showing instead a trend towards preventing necrosis. Apigenin had no statistically significant effect on cell apoptosis (Figure 4.22). At lower concentrations 0.312-1.25μM apigenin appeared to show a trend towards inducing *MMP13* and *MMP1* (Figure 4.10-4.11), these trends could be further explored by increasing the range of concentrations between lower concentrations of apigenin and including higher patient numbers in future experiments. These results suggest that at concentrations between 2.5-10μM apigenin may be of most benefit to chondrocytes, having both anti-catabolic and anti-inflammatory effects in chondrocytes.

Isoliquiritigenin significantly inhibited IL1 induced *MMP13* from 1.25-40μM ($p<0.01$-0.001) in a dose dependent manner (Figure 4.16). IL1 induced *MMP1* was significantly inhibited by isoliquiritigenin at 5-40μM ($p<0.001$) in a dose dependent manner (Figure 4.17). Isoliquiritigenin significantly inhibited IL1 induced *ADAMTS4* from 10-20μM ($p<0.01$-0.001) in a dose dependent manner (Figure 4.18). Isoliquiritigenin significantly induced basal *ADAMTS5* expression at 40μM ($p<0.05$) (Figure 4.19). Isoliquiritigenin significantly induced basal *HO-1* at 2.5-20μM ($p<0.01$-0.001) (Figure 4.20) and basal *AXIN2* expression at 40μM ($p<0.05$) (Figure 4.21). These results show that isoliquiritigenin may have chondro-protective properties at potentially physiologically relevant concentrations of 1.25μM.

While inhibition of chondro-destructive genes becomes more pronounced at higher concentrations (20-40µM) basal *ADAMTS5* (Figure 4.19) was induced at the very high concentration of 40µM which may cause aggrecan destruction in the joint, isoliquiritigenins anti-inflammatory upregulation of *HO-1* was lost (Figure 4.20) and isoliquiritigenin showed a trend towards *AXIN2* upregulation, potentially increasing Wnt activity (Figure 4.21) at these higher concentrations. Oral doses of isoliquiritigenin (100mg/kg) in mice have achieved these high concentrations in blood plasma, the highest concentration of isoliquiritigenin found in plasma was 55.8µM (Wang et al., 2014), (Qiao et al., 2014). At these high doses isoliquiritigenin posed little toxicity to the organs, including heart, liver, spleen, lung and kidney, when mice were fed 100mg/kg/day for 3 months (Wang et al., 2014). While these concentrations of isoliquiritigenin are unlikely consumed in the normal human diet they could potentially be reached by supplementation so it could be important not to over supplement isoliquiritigenin so as not to reach concentrations that could potentially increase basal *ADAMTS5* (Figure 4.19). Isoliquiritigenin was non-cytotoxic at all concentrations analysed in the dose response assays (Figures 4.23, 4.25). Isoliquiritigenin had no statistically significant effect on cell necrosis (Figure 4.23) or cell apoptosis (Figure 4.25). These results suggest that at concentrations between 1.25-20µM isoliquiritigenin may be of most benefit to chondrocytes, having both anti-catabolic and anti-inflammatory effects in chondrocytes.

4.3.1 Conclusion

In conclusion several of the compounds selected in the previous chapter from the SW1353 and C28/I2 screens were able to inhibit *MMP13, MMP1 ADAMTS4* and *ADAMTS5* at 10µM in KOA chondrocytes. Of these compounds apigenin and isoliquiritigenin showed the greatest statistically significant inhibition of *MMP13* while also inhibiting *MMP1, ADAMTS4* and *ADAMTS5*. These two selected candidate compounds inhibited *MMP13, MMP1* and *ADAMTS4* and upregulated the anti-inflammatory gene *HO-1* in KOA cells. Apigenin may be having these effects via regulation of the Wnt pathway as it dose-dependently inhibited *AXIN2,* a Wnt/β-catenin responsive gene. Apigenin and isoliquiritigenin appear to have chondro-protective properties and show no cytotoxic effects in chondrocytes. In the next chapter we will begin to explore apigenin's and isoliquiritigenin's mechanisms of action.

4.3.2 Strengths of Chapter 4

- This chapter has shown that several compounds are able to inhibit genes that are involved in OA in KOA cell islets from four different patient donors.
- While only apigenin and isoliquiritigenin have been selected for further study this chapter has shown that aloe-emodin, luteolin, genistin, emodin and ursolic acid could still have chondroprotective activity as they inhibited chondro-destructive genes at 10µM.
- Apigenin and isoliquiritigenin have been shown to have no necrotic or apoptotic effects in KOA cells when used in concentrations up to 40µM.

4.3.3 Weaknesses of Chapter 4

- Dose responses could be extended to look at effects of concentrations below 0.312µM or above 40µM.
- The inhibition of *MMP13* by Isoliquiritigenin was stronger at 12 hours than at 6 hours (Figure 4.9), while isoliquiritigenin was more potent at 12 hours, 6 hours was selected to make comparisons of *MMP13* inhibition between isoliquiritigenin and apigenin more accurate. Experiments could be repeated to see if a 12 hour timepoint increases isoliquiritigenin inhibition of genes of interests.

Chapter 5: Mechanism of action of apigenin and isoliquiritigenin

5.1 Introduction

In the previous chapter, I identified two dietary-derived bioactive compounds that significantly reduced expression of chondro-destructive genes *MMP13, MMP1, ADAMTS4* and *ADAMTS5* while inducing the anti-inflammatory gene *HO-1*. These compounds included, apigenin a plant-derived flavone and isoliquiritigenin a chalcone found in liquorice root. In this chapter, I will explore the mechanism of action of these compounds by analysing their effect on cell signalling pathways and transcription factors. Lipid-based transfection protocols show transfection efficiencies ranging from 1.5 to 27% of the total number of PHCs (Morrey et al., 2008), (Gresch et al., 2004), (Madry & Trippel, 2000). In our lab we have been unable to transfect PHC cells. For this reason the easily transfected SW1353 cell line was selected as a transfection model for primary chondrocytes. We used cell based luciferase assays to measure NFκB, TGFβ and Wnt pathways in SW1353 cells. As discussed in the introduction the NFκB, TGFβ and Wnt pathways are known to be implicated in cartilage homeostasis and OA (Chen et al., 2008), (Moulharat et al., 2004), (Lories et al., 2007). The effects of apigenin and isoliquiritigenin seen in the previous chapter may be dependent on these pathways.

To select a time point to study compounds mechanism of action total and phosphorylated extracellular signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinases (JNK) protein levels were analysed. The phosphorylation of the MAPK family of serine/threonine kinases, ERK, p38 and JNK were selected for analysis as they are known to be involved in the regulation of metalloproteinase gene expression and were included in the 43 kinases of the Human Phospho Kinase screen. Inflammatory cytokines, osmostic stress and apoptotic signals activate the JNKs and p38 kinases (Davis, 2000). ERKs are activated by cytokines, growth factors and phorbol esters (Garrington & Johnson, 1999) (Kolch, 2000). Stimulation activates the MAPK kinase kinases (MAPKKKs) which phosphorylate and activate MAPK (Vincenti & Brinckerhoff, 2002). Activated MAPKs translocate to the nucleus where they

phosphorylate and activate various transcription factors. ERKs and JNKs phosphorylate and activate activating protein-1 (AP-1) family member c-JUN which dimerises with c-Fos during MMP gene transcription (Karin, 1995), (Leppä et al., 1998). The p38 kinases phosphorylates activating transcription factor-2 , driving the *c-jun* promoter and the ternary complex factor Elk-1 which activates the *c-fos* promoter*,* indirectly contributing to MMP transcription by promoting expression of AP-1 genes (Davis, 2000).

As discussed in chapter 1, OA is a multifactorial disease and bioactive compounds were considered an attractive alternative treatment for OA as they can simultaneously interact with multiple cellular pathways (Ameye & Chee, 2006). The Proteome Profiler Array Human Phospho-Kinase Array Kit is able to simultaneously detect the relative levels of phosphorylation of 43 kinase phosphorylation sites and 2 related total proteins (see Chapter 2 section 2.2.6 for more details) making it an attractive strategy for measuring compounds effects across multiple cell pathways. While IL1 does not induce the phosphorylation of all the kinases in the Human Phospho Kinase screen it was selected to stimulate the assay as the effect of apigenin and isoliquiritigenin on IL1 stimulated gene expressions from chapter 4 was under investigation.

I hypothesise that the chondro-protective gene changes seen in KOA cells treated with apigenin or isoliquiritigenin in the last chapter may be the result of changes to the NFκB, TGFβ and/or Wnt pathways. Also, apigenin's and isoliquiritigenin's patterns of kinase activity may be different from each other, providing an insight into the variations seen between the compounds in the chondro-protective gene screens seen in the last chapter. The aims of this chapter are:

- To use cell based luciferase assays to analyse the effect of apigenin and isoliquiritigenin pretreatment on the NFκB, TGFβ and Wnt pathways in IL1 stimulated and unstimulated SW1353 cells.

- Measure phosphorylated and total ERK, p38 and JNK levels in IL1 stimulated KOA cells after treatment with apigenin or isoliquiritigenin and to find a timepoint at which compounds inhibit kinase phosphorylation for use in the Human Phospho-Kinase Array.

- Ascertain the effects apigenin and isoliquiritigenin have on 43 kinases and 2 related total proteins in IL1 stimulated and unstimulated KOA cells at the time point selected in the previous aim using Human Phospho-Kinase Array.

5.2 Results

5.2.1 NFκB, TGFβ and Wnt pathways in SW1353 cells

SW1353 cells were cultured and plated as described in section 2.2.4.3. SW1353 cells were transfected with either 100ng NFκB signalling reporter (κB) vector (Figure 5.1a) and 10ng of Renilla expression vector, or 100ng of the Smad2/3/4 signalling reporter ($CAGA_{12}$-luc) (Figure 5.1b) and 10ng of Renilla expression vector, or 100ng of the canonical Wnt signalling reporter (TOPFLASH) vector (Figure 5.1c) and 10ng of Renilla expression vector. (For more details on the reporters see Chapter 2 section 2.2.4).

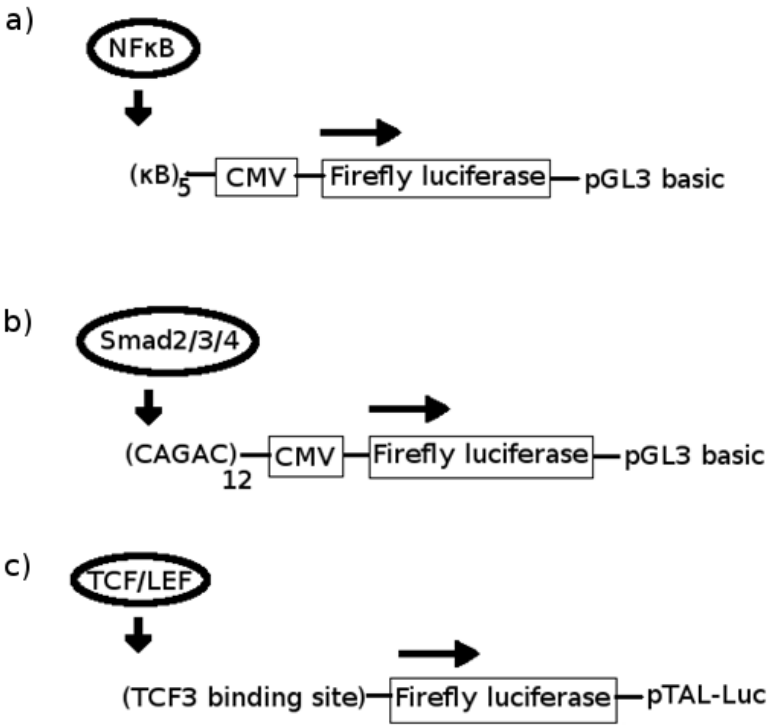


Figure 5.1. The NFκB, Smad2/3/4 and Wnt signalling reporters. (a) The NFκB signalling reporter (κB) vector contains 5 binding sites of NFκB upstream of the firefly luciferase-encoding gene pGL3. (b) The Smad2/3/4 signalling reporter ($CAGA_{12}$-luc) contains 12 binding sites of the Smad2/3/4 binding consensus upstream of the pGL3 gene. (c) The canonical Wnt signalling reporter (TOPFLASH) vector contains 7 binding sites of TCF/LEF upstream of the firefly luciferase encoding gene in the pTAL-Luc vector. (For more details on the reporters see Chapter 2 section 2.2.4).

5.2.1.1 Cytokine stimulated luciferase assay Time course

κB vector transfected SW1353 cells were stimulated with IL1 5ng/ml at 1,3,6,12 and 24 hours (Figure 5.2a). IL1 significantly increased NFκB relative luciferase activity at 3, 6, 12 and 24 hours (p<0.05-0.01). Smad2/3/4 signalling reporter (CAGA12-luc) transfected cells were stimulated with TGFβ1 5ng/ml at 1,3,6,12 and 24 hours (Figure 5.2b). TGFβ1 significantly increased Smad relative luciferase activity at 3,6,12 and 24 hours (p<0.01-0.001). Wnt signalling reporter (TOPFLASH) vector transfected cells were stimulated with Wnt3a 50ng/ml at 1,3,6,12 and 24 hours (Figure 5.2c). Wnt3a significantly increased Wnt relative luciferase activity at 6,12 and 24 hours (p<0.05-0.01).

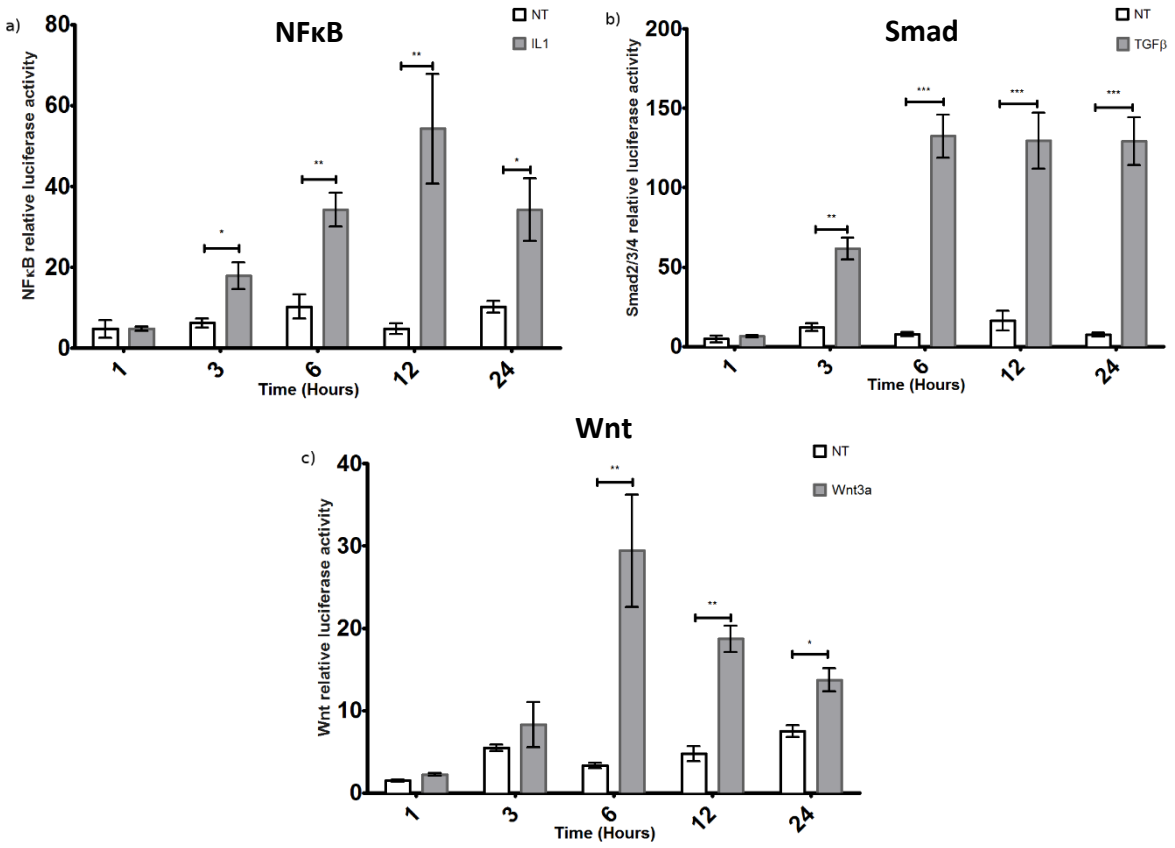


Figure 5.2. The regulation of the NFκB, TGFβ and Wnt pathways by IL1. (a) SW1353 cells were transfected with the NFκB signalling reporter (κB) vector, IL1 (5ng/ml) was added for the time points shown and luciferase activity measured and normalised to Renilla activity as a loading control. (b) SW1353 cells were transfected with the Smad2/3/4 signalling reporter (CAGA12-luc), TGFβ (5ng/ml) was added for the time points shown and luciferase activity measured and normalised to Renilla activity as a loading control. (c) SW1353 cells were transfected with Wnt signalling reporter (TOPFLASH) vector, Wnt3a 50ng/ml was added for the time points shown and luciferase activity measured and normalised to Renilla activity as a loading control. Means ± standard errors are presented, n=3. The difference of luciferase activity was analysed by Student's unpaired two-tailed t test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

5.2.1.2 Effect of apigenin on the NFκB pathway in SW1353 cells

SW1353 cells (Figure 5.3a,b) were transfected with 100ng of κB vector (Figure 5.1a) and 10ng of Renilla expression vector as described above. SW1353 cells were treated with apigenin at 1.25-40μM for 6 hours. In Figure 5.3a apigenin was added an hour before stimulation with 5ng/ml IL1. Figure 5.3b samples were left unstimulated. As compounds were suspended in DMSO, DMSO controls were used to show that DMSO had no significant effect on luciferase activity. Luciferase assay data shows that apigenin significantly reduced IL1 induced NFκB activity at 20 and 40μM ($p<0.001$) in a dose dependent manner (Figure 5.3a) and significantly reduced unstimulated NFκB activity at 40μM ($p<0.05$) in a dose dependent manner (Figure 5.3b).

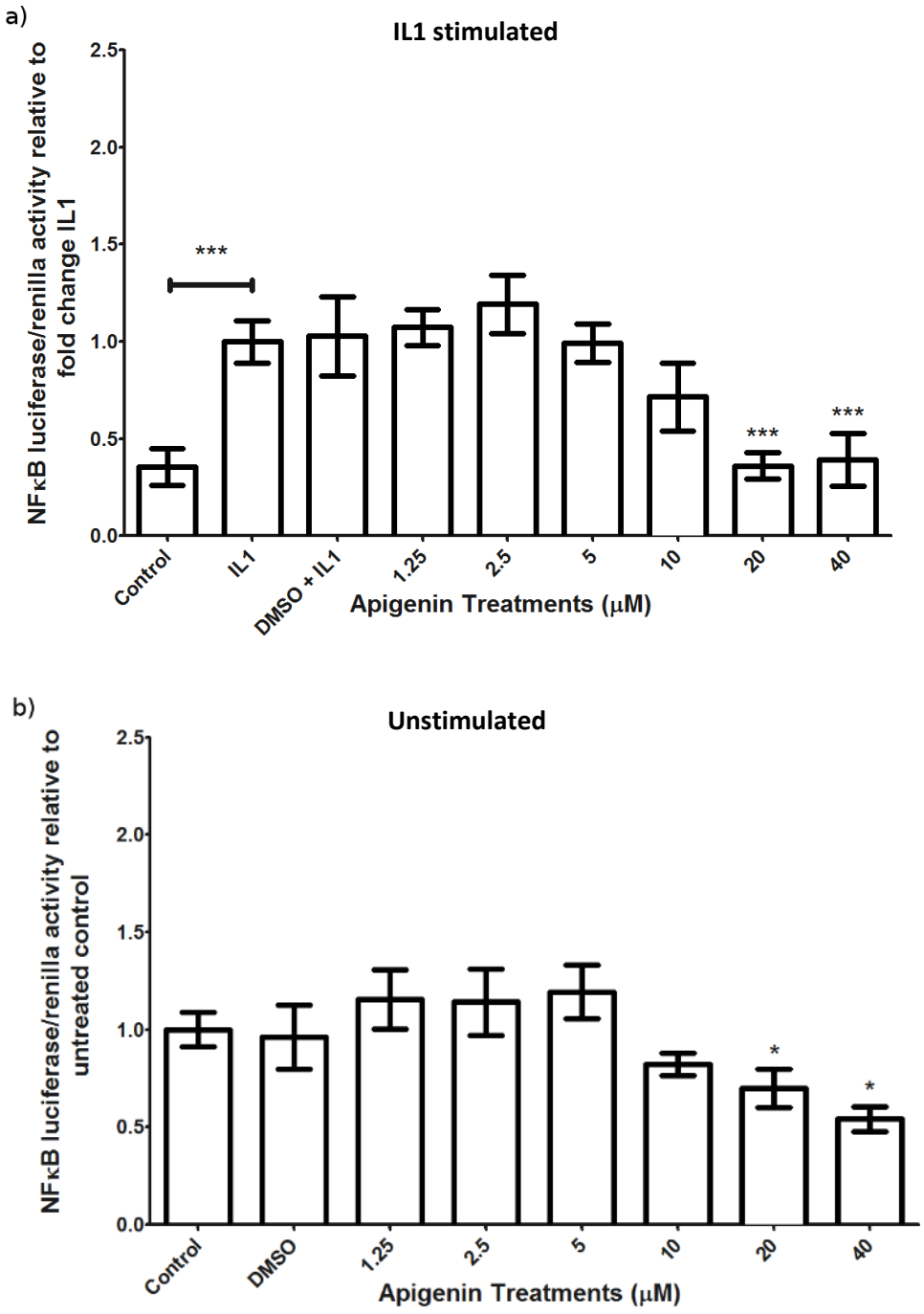


Figure 5.3. The effect of apigenin on the NFκB pathway. (a) Bar chart showing NFκB luciferase/renilla activity relative to fold change of IL1 luciferase/renilla activity in SW1353 cells treated with apigenin at 1.25-40μM for 6 hours ± standard deviation n=3. (b) Bar chart showing NFκB luciferase/renilla activity relative to fold change of untreated control luciferase/renilla activity in SW1353 cells treated with apigenin at 1.25 - 40μM for 6 hours ± standard deviation n=3. The effect of apigenin on luciferase/renilla activity was analysed using one way ANOVA with a Dunnetts post hoc test. * $p<0.05$, *** $p<0.001$.

5.2.1.3 Effect of apigenin on the Smad2/3/4 pathway in SW1353 cells

SW1353 cells (Figure 5.4a,b) were transfected with 100ng of the Smad2/3/4 signalling reporter (CAGA12-luc) (Figure 5.1b) and 10ng of Renilla expression vector as described above. SW1353 cells were treated with apigenin at 1.25-40μM for 6 hours. In Figure 5.4a apigenin was added an hour before stimulation with TGFβ1 5ng/ml. Figure 5.4b samples were left unstimulated. As compounds were suspended in DMSO, DMSO controls were used to show that DMSO had no significant effect on luciferase activity. Luciferase assay data shows that apigenin significantly reduced TGFβ1 stimulated Smad2/3/4 activity at 10, 20 and 40μM ($p<0.001$) in a dose dependent manner (Figure 5.4a) and significantly reduced unstimulated Smad2/3/4 activity at 5, 10, 20 and 40μM ($p<0.01$-0.001) in a dose dependent manner (Figure 5.4b).

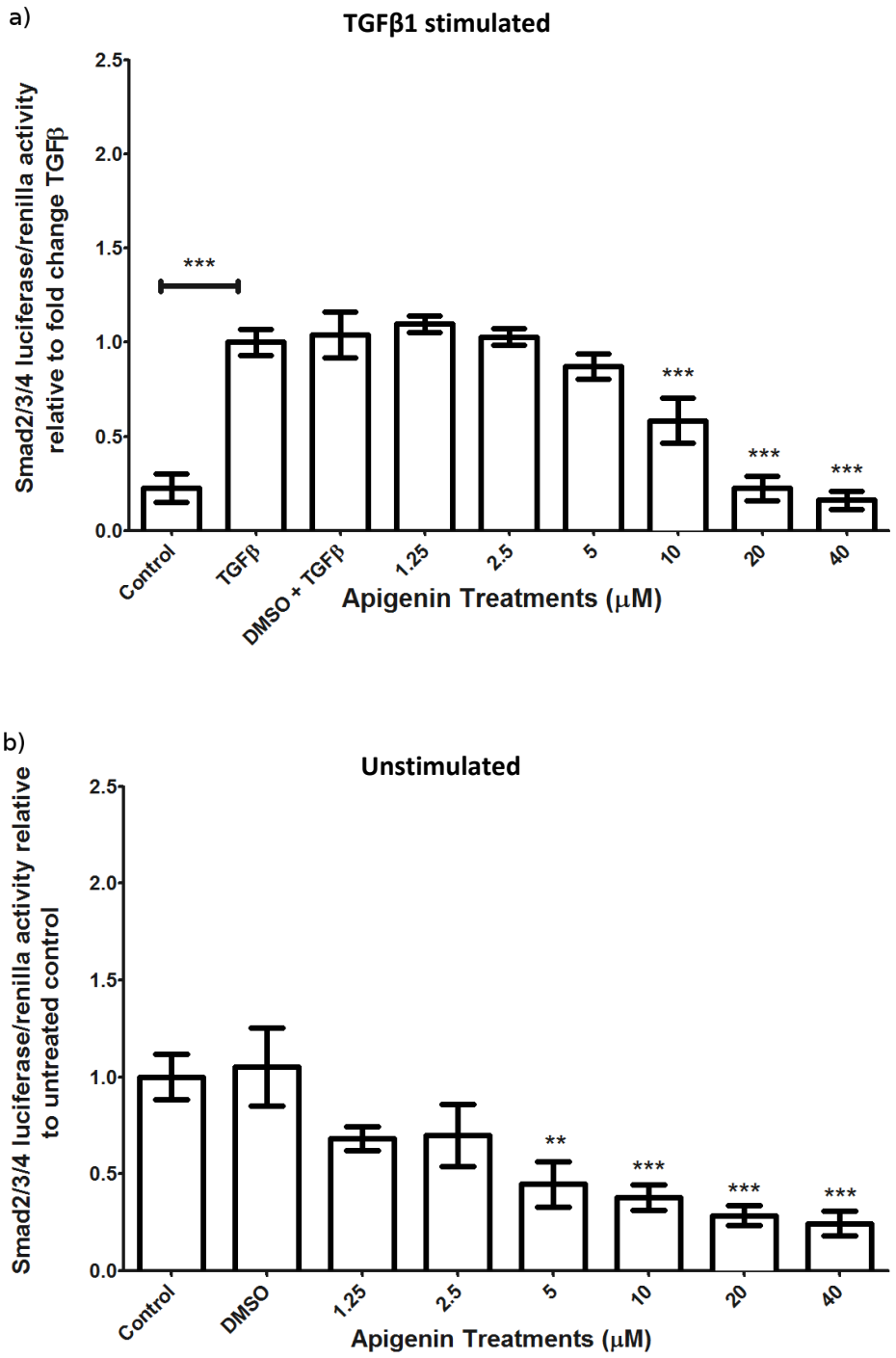


Figure 5.4. The effect of apigenin on the Smad2/3/4 pathway. (a) Bar chart showing Smad2/3/4 luciferase/renilla activity relative to fold change of TGFβ1 stimulated luciferase/renilla activity in SW1353 cells treated with apigenin at 1.25-40μM for 6 hours ± standard deviation n=3. (b) Bar chart showing Smad2/3/4 luciferase/renilla activity relative to fold change of untreated control luciferase/renilla activity in SW1353 cells treated with apigenin at 1.25 - 40μM for 6 hours ± standard deviation n=3. The effect of apigenin on luciferase/renilla activity was analysed using one way ANOVA with a Dunnetts post hoc test. ** $p<0.01$, *** $p<0.001$.

5.2.1.4 Effect of apigenin on the Wnt pathway in SW1353 cells

SW1353 cells (Figure 5.5a,b) were transfected with 100ng of the canonical Wnt signalling reporter (TOPFLASH) vector (Figure 5.1c) and 10ng of Renilla expression vector as described above. SW1353 cells were treated with apigenin at 1.25 - 40µM for 6 hours. In Figure 5.5a apigenin was added an hour before stimulation with Wnt3a 50ng/ml. Figure 5.5b samples were left unstimulated. As compounds were suspended in DMSO, DMSO controls were used to show that DMSO had no significant effect on luciferase activity. Luciferase assay data shows that apigenin significantly reduced Wnt3a stimulated Wnt activity at 5, 10, 20 and 40µM ($p<0.05$-0.01) in a dose dependent manner (Figure 5.5a) and had no significant effect on unstimulated Wnt activity (Figure 5.5b).

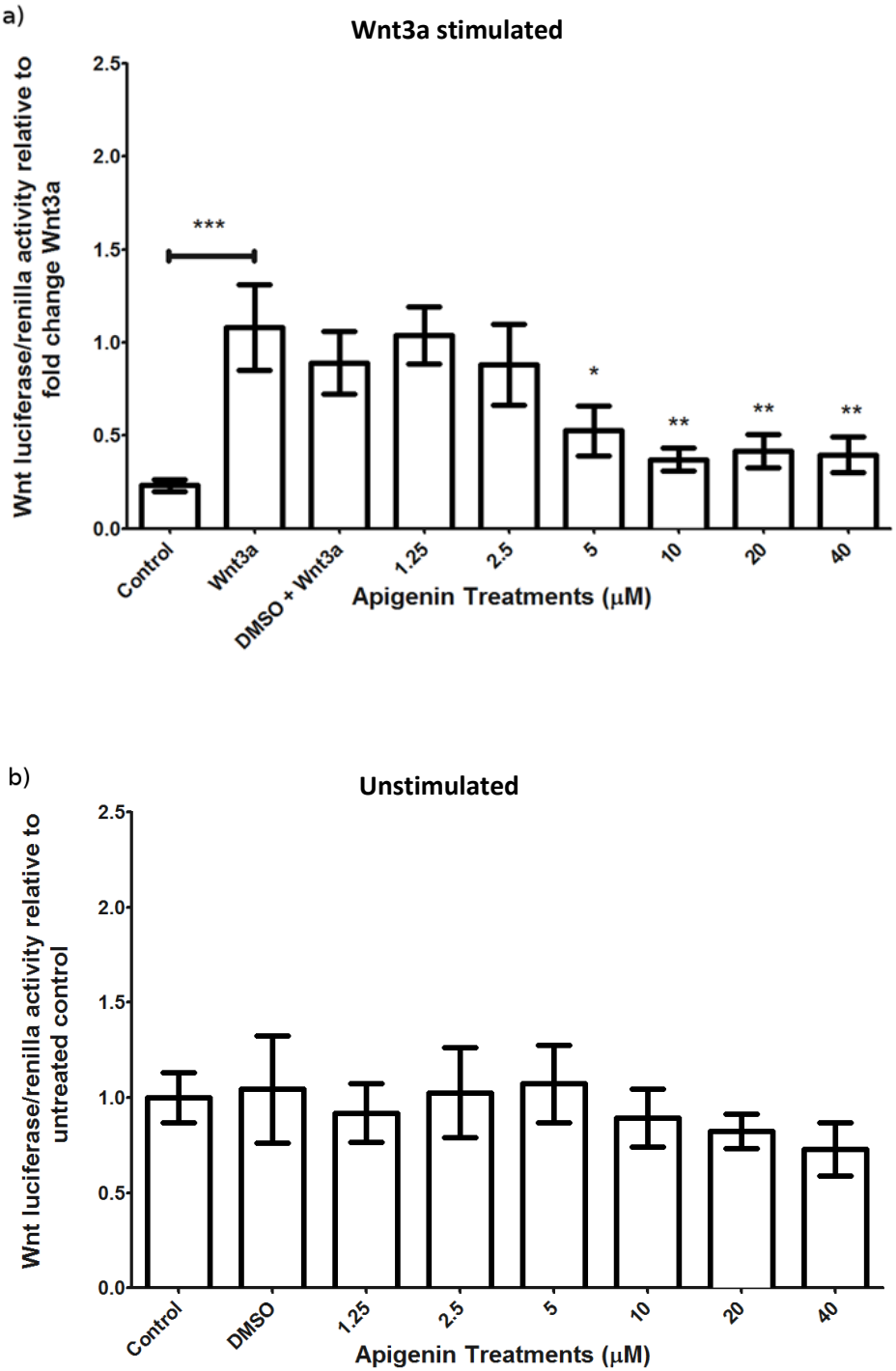


Figure 5.5. The effect of apigenin on the Wnt pathway. (a) Bar chart showing Wnt luciferase/renilla activity relative to fold change of Wnt3a stimulated luciferase/renilla activity in SW1353 cells treated with apigenin at 1.25-40μM for 6 hours ± standard deviation n=3. (b) Bar chart showing Wnt luciferase/renilla activity relative to fold change of untreated control luciferase/renilla activity in SW1353 cells treated with apigenin at 1.25-40μM for 6 hours ± standard deviation n=3. The effect of apigenin on luciferase/renilla activity was analysed using one way ANOVA with a Dunnetts post hoc test. * $p<0.05$, ** $p<0.01$.

5.2.1.5 Effect of isoliquiritigenin on the NFκB pathway in SW1353 cells

SW1353 cells (Figure 5.6a,b) were transfected with 100ng of κB vector (Figure 5.1a) and 10ng of Renilla expression vector as described above. SW1353 cells were treated with isoliquiritigenin at 1.25 - 40μM for 6 hours. In Figure 5.6a isoliquiritigenin was added an hour before stimulation with 5ng/ml IL1. Figure 5.6b samples were left unstimulated. As compounds were suspended in DMSO, DMSO controls were used to show that DMSO had no significant effect on luciferase activity. Luciferase assay data shows that shows that isoliquiritigenin significantly reduced NFκB activity at 10, 20 and 40μM ($p<0.05$-0.001) in a dose dependent manner (Figure 5.6a) and had no significant effect on unstimulated NFκB activity in a dose dependent manner (Figure 5.6b).

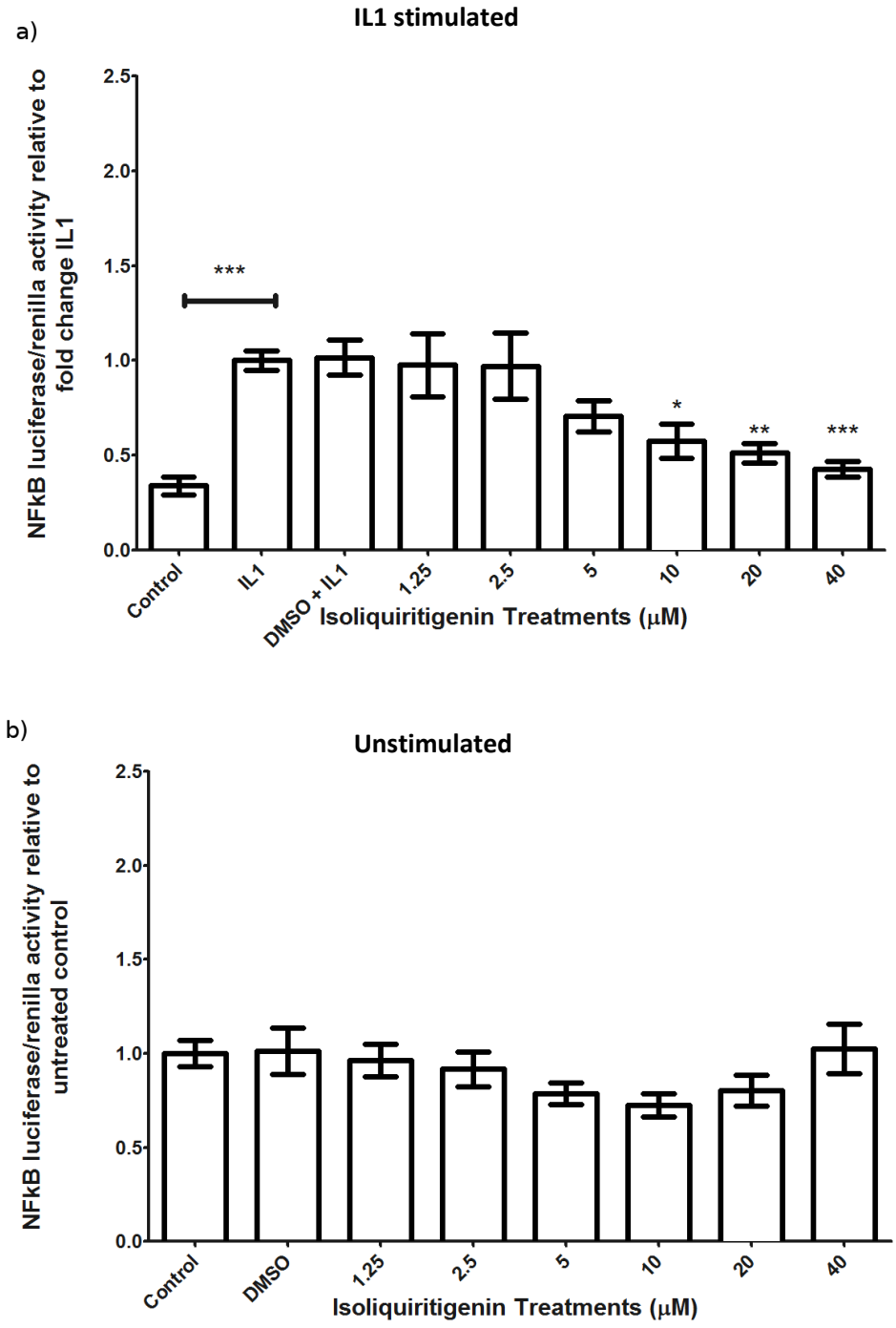


Figure 5.6. The effect of isoliquiritigenin on the NFκB pathway. (a) Bar chart showing NFκB luciferase/renilla activity relative to fold change of IL1 luciferase/renilla activity in SW1353 cells treated with isoliquiritigenin at 1.25-40μM for 6 hours ± standard deviation n=3. (b) Bar chart showing NFκB luciferase/renilla activity relative to fold change of untreated control luciferase/renilla activity in SW1353 cells treated with isoliquiritigenin at 1.25-40μM for 6 hours ± standard deviation n=3. The effect of apigenin on luciferase/renilla activity was analysed using one way ANOVA with a Dunnetts post hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.2.1.6 Effect of isoliquiritigenin on the Smad2/3/4 pathway in SW1353 cells

SW1353 cells (Figure 5.7a,b) were transfected with 100ng of the Smad2/3/4 signalling reporter (CAGA12-luc) (Figure 5.1b) and 10ng of Renilla expression vector as described above. SW1353 cells were treated with isoliquiritigenin at 1.25-40μM for 6 hours. In Figure 5.7a isoliquiritigenin was added an hour before stimulation with TGFβ1 5ng/ml. Figure 5.7b samples were left unstimulated. As compounds were suspended in DMSO, DMSO controls were used to show that DMSO had no significant effect on luciferase activity. Luciferase assay data shows that isoliquiritigenin significantly reduced TGFβ1 stimulated Smad2/3/4 activity at 10, 20 and 40μM ($p<0.05$-0.001) in a dose dependent manner (Figure 5.7a) and significantly reduced unstimulated Smad2/3/4 activity at 40μM ($p<0.05$) (Figure 5.7b).

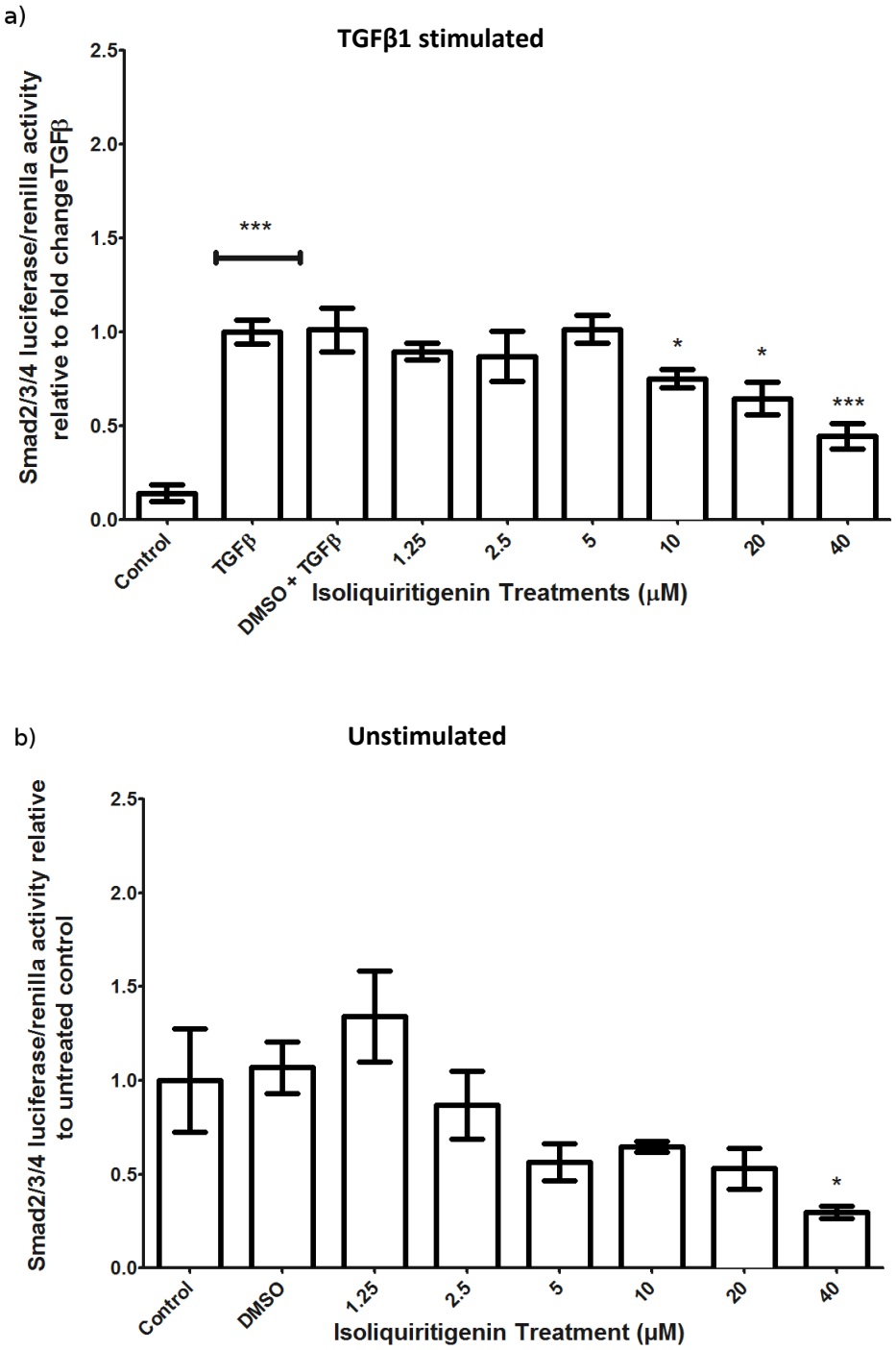


Figure 5.7. The effect of isoliquiritigenin on the Smad2/3/4 pathway. (a) Bar chart showing Smad2/3/4 luciferase/renilla activity relative to fold change of TGFβ1 stimulated luciferase/renilla activity in SW1353 cells treated with isoliquiritigenin at 1.25-40μM for 6 hours ± standard deviation n=3. (b) Bar chart showing Smad2/3/4 luciferase/renilla activity relative to fold change of untreated control luciferase/renilla activity in SW1353 cells treated with isoliquiritigenin at 1.25-40μM for 6 hours ± standard deviation n=3. The effect of apigenin on luciferase/renilla activity was analysed using one way ANOVA with a Dunnetts post hoc test. * $p<0.05$, *** $p<0.001$.

5.2.1.7 Effect of isoliquiritigenin on the Wnt pathway in SW1353 cells

SW1353 cells (Figure 5.8a,b) were transfected with 100ng of the canonical Wnt signalling reporter (TOPFLASH) vector (Figure 5.1c) and 10ng of Renilla expression vector as described above. SW1353 cells were treated with isoliquiritigenin at 1.25-40μM for 6 hours. In Figure 5.7a apigenin was added an hour before stimulation with Wnt3a 50ng/ml. Figure 5.7b samples were left unstimulated. As compounds were suspended in DMSO, DMSO controls were used to show that DMSO had no significant effect on luciferase activity. Luciferase assay data shows that isoliquiritigenin significantly reduced Wnt activity at 10, 20 and 40μM ($p<0.05$-0.001) in a dose dependent manner (Figure 5.8a) and had no significant effect on unstimulated Wnt activity (Figure 5.8b).

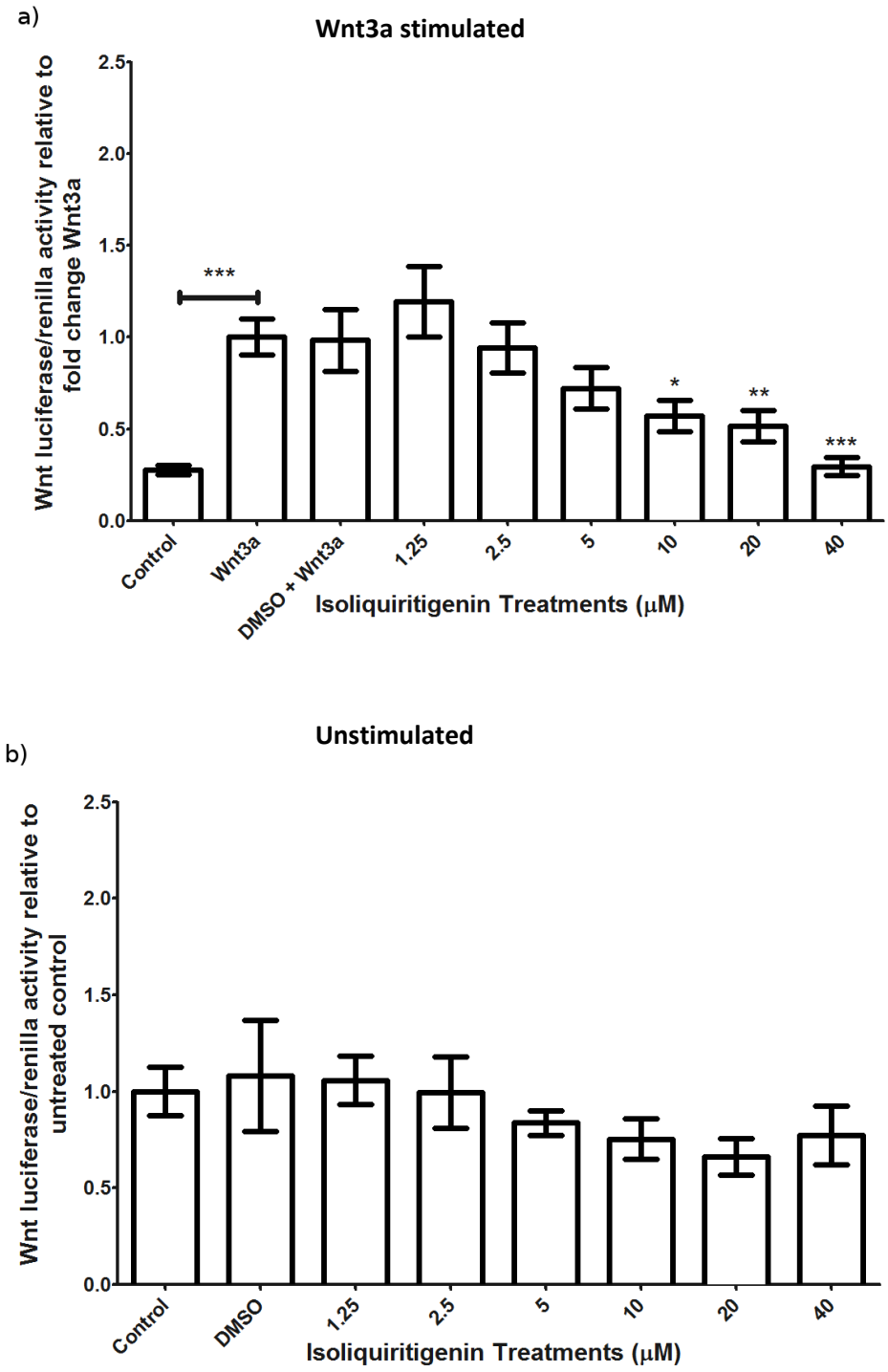


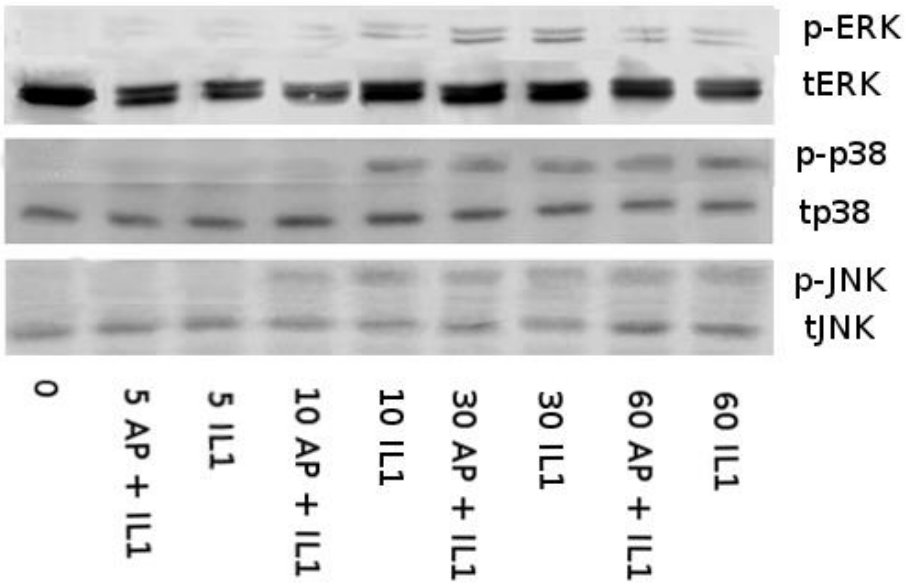
Figure 5.8. The effect of isoliquiritigenin on the Wnt pathway. (a) Bar chart showing Wnt luciferase/renilla activity relative to fold change of Wnt3a stimulated luciferase/renilla activity in SW1353 cells treated with isoliquiritigenin at 1.25-40μM for 6 hours ± standard deviation n=3. (b) Bar chart showing Wnt luciferase/renilla activity relative to fold change of untreated control luciferase/renilla activity in SW1353 cells treated with isoliquiritigenin at 1.25-40μM for 6 hours ± standard deviation n=3. The effect of isoliquiritigenin on luciferase/renilla activity was analysed using one way ANOVA with a Dunnetts post hoc test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

5.2.2 The effect of apigenin on MAPK kinase expression and activation

KOA chondrocytes were cultured and plated as described in section 2.2.5. KOA cells were treated with apigenin at 10µM for 5, 10, 30 and 60 minutes and stimulated with 5ng/ml IL1. 10µM was selected as a concentration to screen for effects on MAPK kinase expression and activation as at this concentration apigenin and isoliquiritigenin inhibited many chondro-degenerative genes (Figure 4.10-4.15), apigenin inhibited the Smad2/3/4 and Wnt pathways (Figure 5.4-5.5), 10µM represents a 'high' but physiologically relevant concentration that may be obtained via diet or supplementation of bioactive compounds. A 5-60 minute time course was selected as time points of interest as previous research has shown dietary compounds such as SFN effect MAPK activation in KOA cells across these time points (Davidson et al., 2013).

Total protein was extracted and resolved by SDS-PAGE described in section 2.2.5.2-2.2.5.3. Total ERK (tERK) and p-ERK, total p38 (tp38) and p-p38, total JNK (tJNK) and p-JNK were assessed by immunoblotting with specific antibodies described in section 2.1.4 (Figure 5.9).

a)



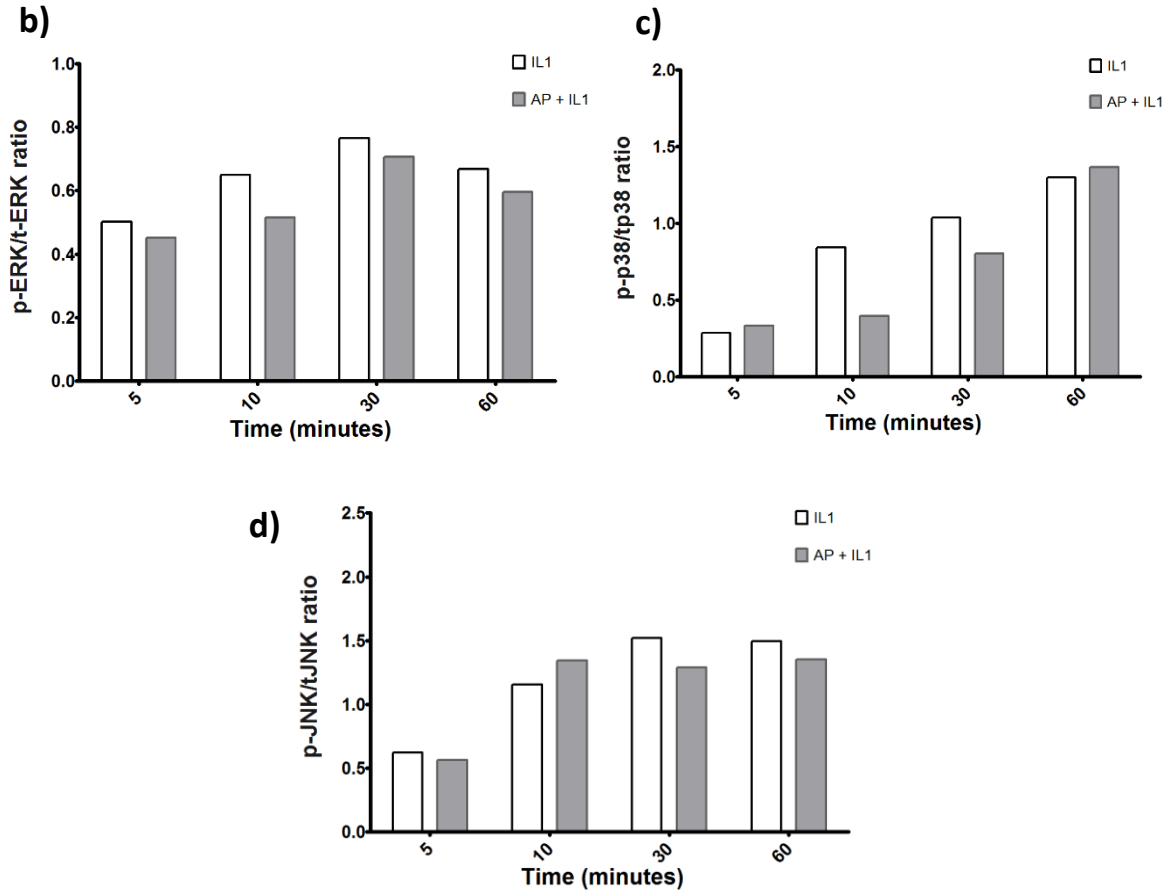


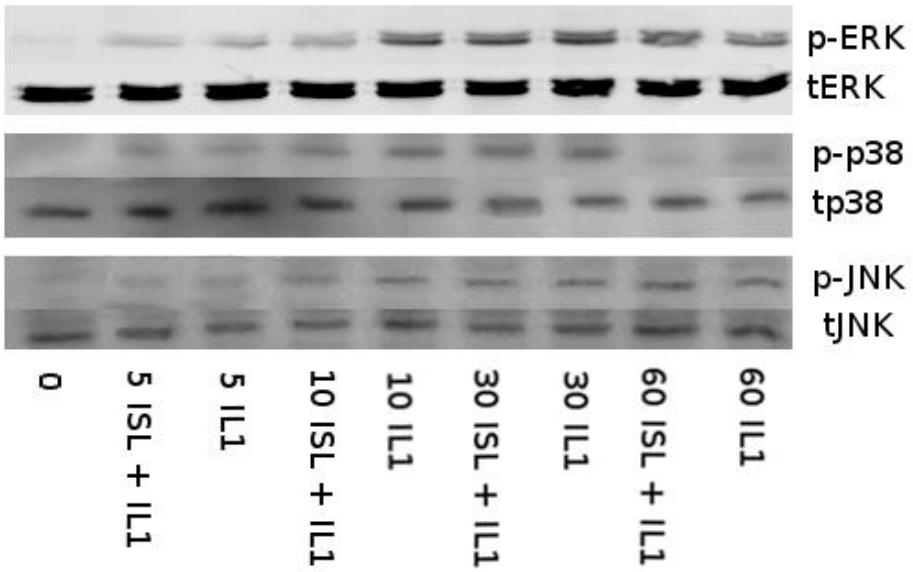
Figure 5.9. The effect of apigenin on MAPKs. Immunoblot analysis of apigenin effect on ERK, p38 and JNK in KOA cells. (a) Representative immunoblot of ERK (t-ERK), p-ERK, total p38 (tp38) and p-p38, total JNK (tJNK) and p-JNK showing effects of 10µM apigenin treatment over 0-60 minute timecourse in KOA cells. n =2. (b) Analysis of ERK activity (pERK/tERK ratio), bar chart showing p-ERK/tERK ratio showing effects of 10µM apigenin treatment over timecourse in KOA cells. (c) Analysis of p38 activity (p-p38/tp38 ratio), bar chart showing p-p38/tp38 ratio showing effects of 10µM apigenin treatment over timecourse in KOA cells. (d) Analysis of JNK activity (p-JNK/tJNK ratio), bar chart showing p-JNK/tJNK ratio showing effects of 10µM apigenin treatment over timecourse in KOA cells. n =2 ± mean.

5.2.3 The effect of isoliquiritigenin on MAPK kinase expression and activation

KOA chondrocytes were cultured and plated as described in section 2.2.5. KOA cells were treated with apigenin at 10µM for 5, 10, 30 and 60 minutes and stimulated with 5ng/ml IL1. 10µM was selected as a concentration to screen for effects on MAPK kinase expression and activation as at this concentration isoliquiritigenin inhibited many chondro-degenerative genes (Figure 4.16-4.21), isoliquiritigenin inhibited the NFκB, TGFβ and Wnt pathways (Figure 5.6-5.8) and 10µM represents a 'high' but physiologically relevant concentration that may be obtained via diet or supplementation of bioactive compounds. A 5-60 minute time course was selected as time points of interest as previous research has shown dietary compounds such as SFN effect MAPK activation in KOA cells across these time points (Davidson et al., 2013).

Total protein was extracted and resolved by SDS-PAGE described in section 2.2.5.2-2.2.5.3. Total ERK (tERK) and p-ERK, total p38 (tp38) and p-p38, total JNK (tJNK) and p-JNK were assessed by immunoblotting with specific antibodies described in section 2.1.4 (Figure 5.10).

a)



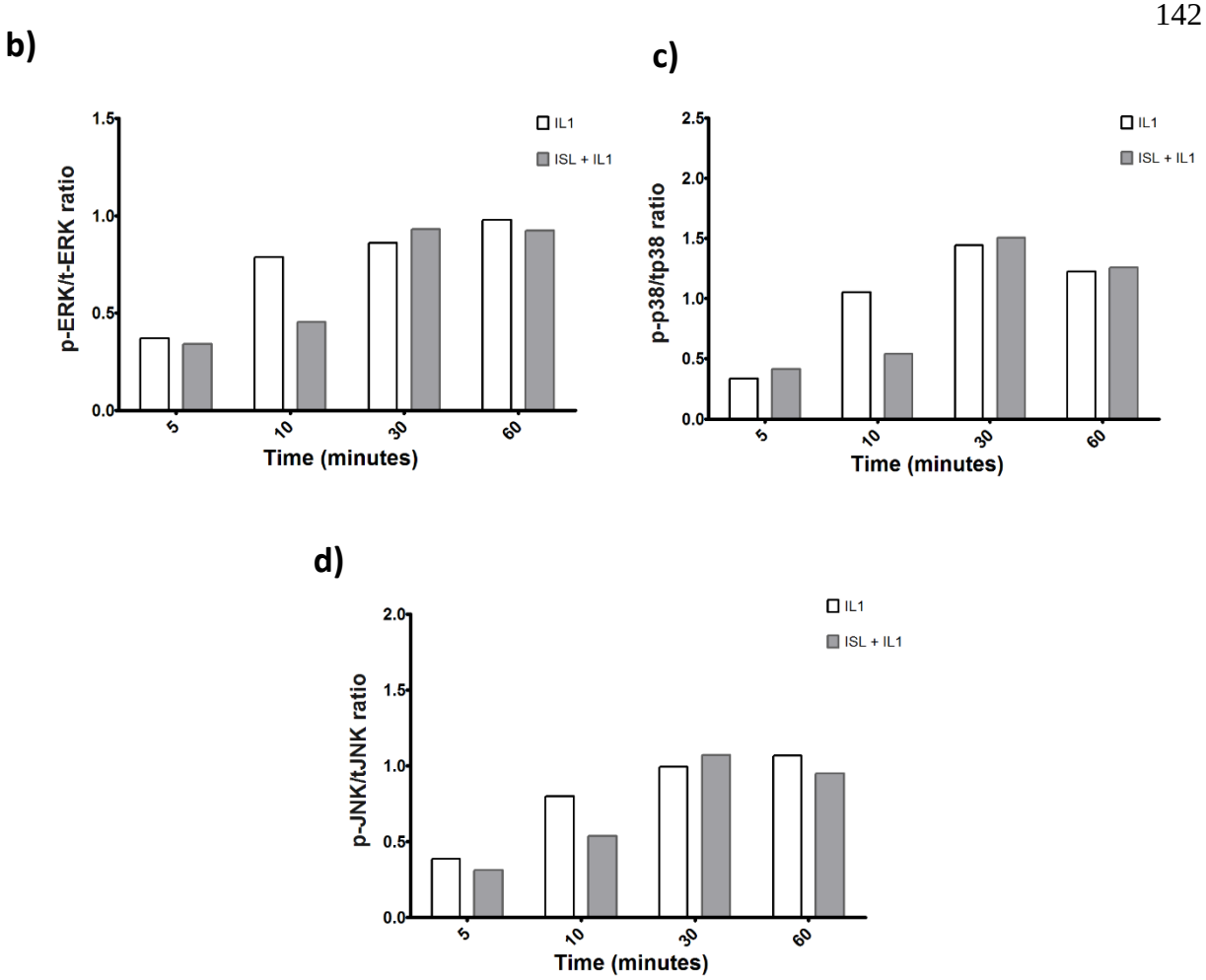


Figure 5.10. The effect of isoliquiritigenin on MAPKs. Immunoblot analysis of apigenin effect on ERK, p38 and JNK in KOA cells. (a) Representative immunoblot of ERK (t-ERK), p-ERK, total p38 (tp38) and p-p38, total JNK (tJNK) and p-JNK showing effects of 10µM isoliquiritigenin treatment over 0-60 minute timecourse in KOA cells. n =2. (b) Analysis of ERK activity (pERK/tERK ratio), bar chart showing p-ERK/tERK ratio showing effects of 10µM isoliquiritigenin treatment over timecourse in KOA cells. (c) Analysis of p38 activity (p-p38/tp38 ratio), bar chart showing p-p38/tp38 ratio showing effects of 10µM isoliquiritigenin treatment over timecourse in KOA cells. (d) Analysis of JNK activity (p-JNK/tJNK ratio), bar chart showing p-JNK/tJNK ratio showing effects of 10µM apigenin treatment over timecourse in KOA cells. n =2 mean.

5.2.4 Human Phospho-Kinase Array

KOA chondrocytes were cultured and plated as described in section 2.13. KOA cells were treated with isoliquiritigenin or apigenin at 10μM for 10 minutes and stimulated with 5ng/ml IL1. The analysis of phosphorylation profiles of 43 kinases and their protein substrates was completed with the Human Phospho-Kinase Array as described by the manufacturer's instructions described in section 2.14 (Figure 5.11). Cell lysates were analysed using the Human Phospho-kinase array kit and compounds were considered to show a weak reduction on a kinase phosphorylation event if the compound treated samples signal fold change was below 0.75-fold the IL1-induced change and a strong reduction below 0.5-fold change of the IL1 treated control (Figure 5.11). Figure 5.12 shows quantified results from Figure 5.11 relative to IL1 control. Compounds were considered to induce a weak kinase phosphorylation event if the compound-treated samples signal fold change was above 1.25-fold the IL1 induced change and a strong induction above 1.5-fold the IL1 induced change (Figure 5.12a). The effects of isoliquiritigenin on the Human Phospho Kinase Array from Figure 5.12 were compiled into Table 7. The effects of apigenin on the Human Phospho Kinase Array from Figure 5.12 were compiled into Table 8. Apigenin showed an induction of the phosphorylation of GSK-3α/β. The phosphorylated levels of p53, p70 S6 kinase, eNOS, Fgr, STAT3, p27, PLC-γ1, STAT3 and PYK2 were undetected and so were not analysed in Figure 5.12. Figure 5.12b shows quantified results from Figure 5.11 relative to unstimulated control (C).

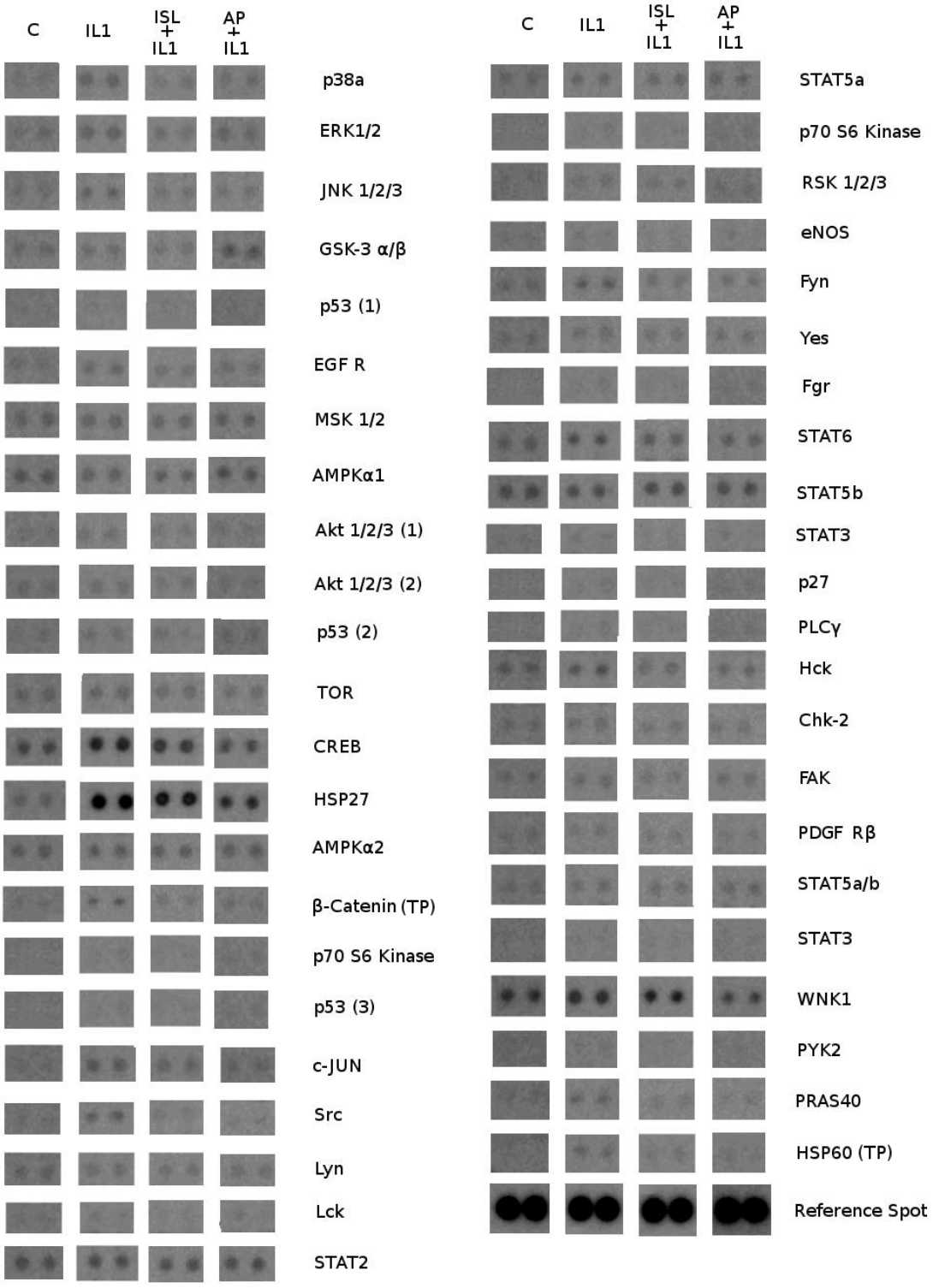


Figure 5.11. Alteration of kinase signalling in KOA cells after isoliquiritigenin or apigenin treatment. KOA cells were either untreated = (C), treated with 5ng/ml IL1 = (IL1) or with isoliquiritigenin (ISL + IL1) or apigenin (AP + IL1) at 10μM with 5ng/ml IL1 for 10 minutes. The diluted cell lysates were analysed using a human phosphokinase array kit (R&D Systems). Mean values (n=2 in each blot). p53, p70 S6 kinase, eNOS, Fgr, STAT3, p27, PLC-γ1, STAT3 and PYK2 were undetected and so were not analysed in Figure 5.12. (TP) = total protein samples.

WEAK REDUCTION WITH ISOLIQUIRITIGENIN TREATMENT	STRONG REDUCTION WITH ISOLIQUIRITIGENIN TREATMENT
HCK	HSP27
JNK 1/2/3	Src
CREB	FAK
FYN	PRAS40
C-JUN	p38a
ERK 1/2	**HSP60**
	β-catenin

Table 7. The effect of isoliquiritigenin treatment on the Human Phospho Kinase Array. Table showing the IL1 induced kinase phosphorylation events reduced in KOA cells with 10μM isoliquiritigenin treatment for 10 minutes. HSP60 and β-catenin highlighted in bold were total protein values.

WEAK INHIBITION WITH APIGENIN TREATMENT	STRONG INHIBITION WITH APIGENIN TREATMENT
HCK	HSP27
PDGF RB	Src
WNK1	c-JUN
STAT6	Lck
P38A	CREB
FAK	Akt 1/2/3
JNK 1/2/3	PRAS40
	Fyn
	HSP60
	β-catenin

Table 8. The effect of apigenin treatment on the Human Phospho Kinase Array. Table showing the IL1 induced kinase phosphorylation events reduced in KOA cells with 10μM apigenin treatment for 10 minutes. HSP60 and β-catenin highlighted in bold were total protein values.

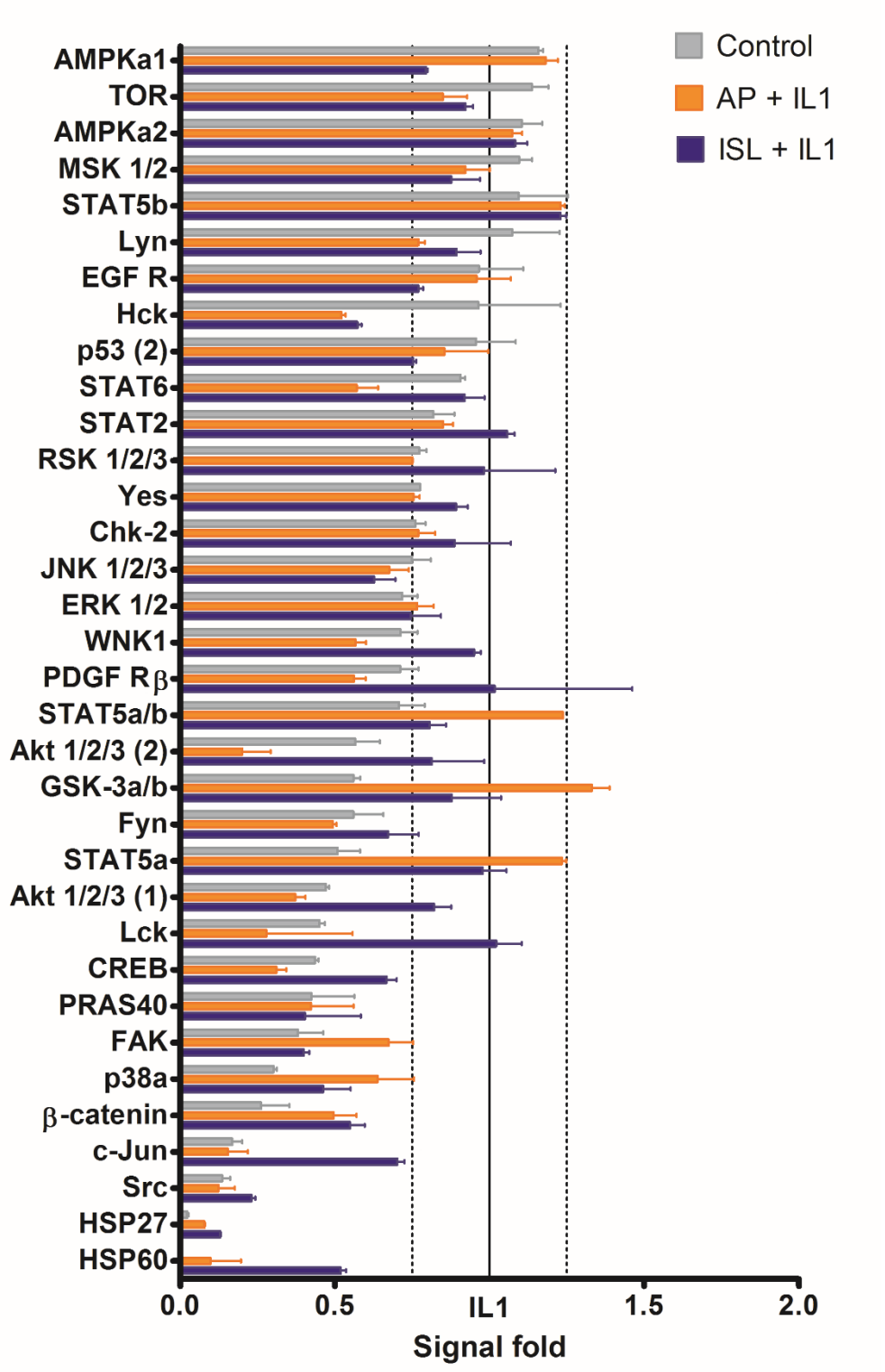


Figure 5.12a. Analysis of IL1 stimulated kinase signalling in KOA cells after isoliquiritigenin or apigenin treatment relative to IL1 fold change. Bar chart analysing data from Figure 5.11. Cell lysates were analysed using a human phosphokinase array kit and compounds were considered to reduce a kinase phosphorylation event if the compound treated samples signal fold change was below 0.75 fold change of the IL1 treated control. Compounds were considered to induce a kinase phosphorylation event if the compound treated samples signal fold change was above 1.25 signal fold change of the IL1 treated control. Mean values (n=2 in each blot) ± mean range of phosphoproteins quantified relative to IL1 treated control.

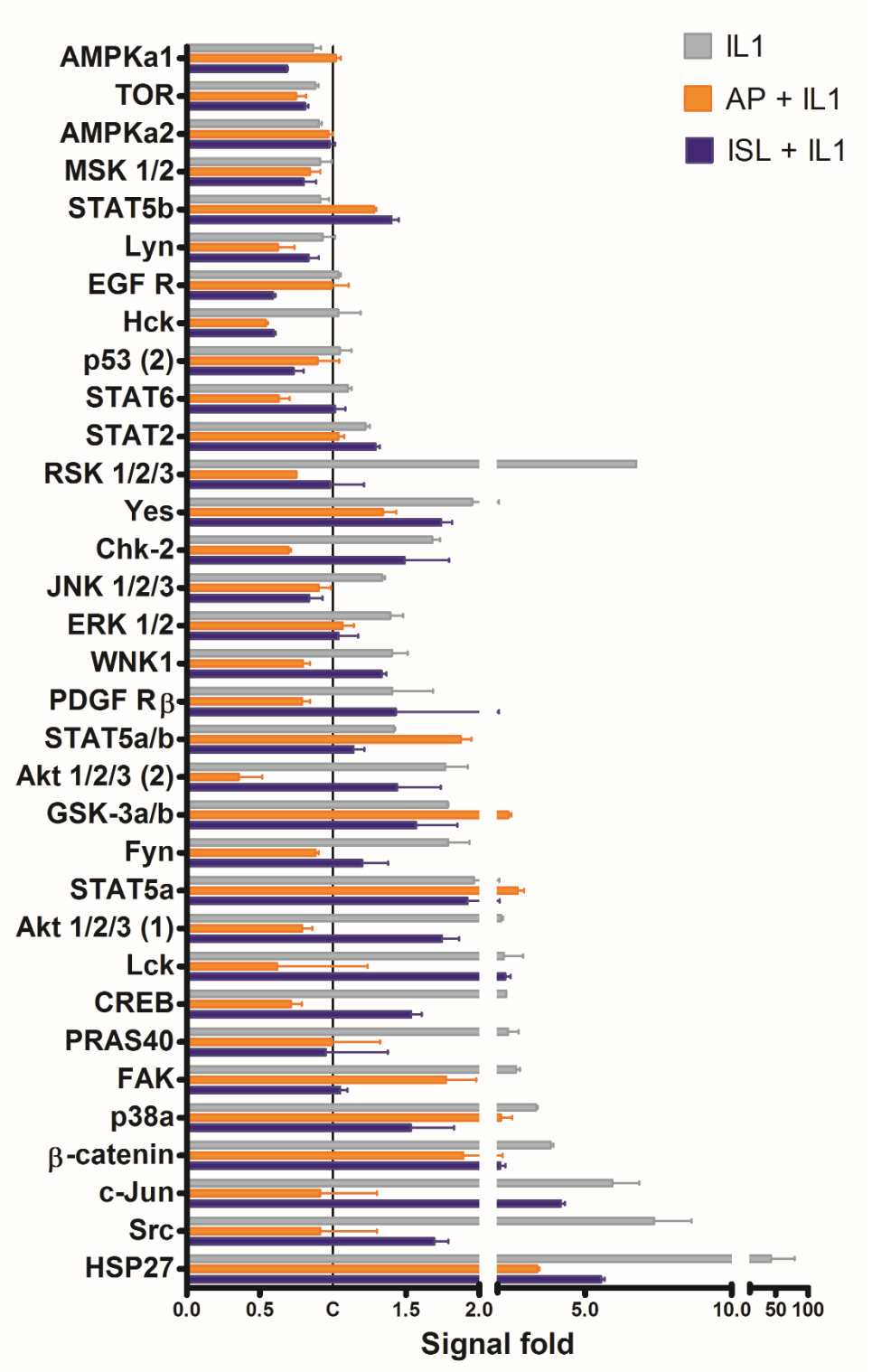


Figure 5.12b. Analysis of IL1 stimulated kinase signalling in KOA cells after isoliquiritigenin or apigenin treatment relative to unstimulated control fold change. Bar chart analysing data from Figure 5.11. Mean values (n=2 in each blot) ± mean range of phosphoproteins quantified relative to unstimulated control (C) treated control.

5.3 Discussion

In the previous chapter apigenin and isoliquiritigenin dose-dependently inhibited *MMP13*, *MMP1*, *ADAMTS4* and *ADAMTS5* and up-regulated the anti-inflammatory gene *HO-1*. We hypothesised that as apigenin dose-dependently inhibited *AXIN2* (a Wnt/β-catenin responsive gene) the chondro-protective properties of apigenin may in part be due to alterations to the Wnt pathway. In this chapter, I explored the mechanism of action of apigenin and isoliquiritigenin.

Cell-based luciferase assays were used to measure the effect of apigenin or isoliquiritigenin pretreatment on the NFκB, TGFβ and Wnt pathways in SW1353 cells. Initially these assays were used to measure the effect of cytokines on the NFκB, TGFβ and Wnt pathways over 1,3,6,12 and 24 hours (Figure 5.2). As all cytokines significantly increased their corresponding pathway at 6 hours (Figure 5.2a-c) and apigenin and isoliquiritigenin have been shown to significantly inhibit chondro-destructive genes in the previous chapter at 6 hours, a 6 hour time point was selected for subsequent luciferase experiments.

Both apigenin and isoliquiritigenin significantly reduced IL1 stimulated NFκB activity in dose dependent manners (Figure 5.3a,5.6a) however isoliquiritigenin showed inhibition at 10μM whereas apigenin only inhibited NFκB activity at concentrations of 20μM and above. While isoliquiritigenin was the more potent IL1 stimulated NFκB inhibitor apigenin was able to inhibit unstimulated NFκB activity whereas isoliquiritigenin did not (Figure 5.3b, 5.6b). As discussed in the introduction NFκB plays a central role in the development of OA. Overexpression of IκB has been shown to decrease *MMP1, MMP13* and *ADAMTS4* in OA synovial cells (Amos et al., 2006), (Bondeson et al., 2007). This data suggests that apigenin and isoliquiritigenin may be acting as NFκB inhibitors (Figure 5.3, 5.6) which may contribute to the inhibition of *MMP1, MMP13* and *ADAMTS4* by apigenin and isoliquiritigenin in KOA cells seen in the previous chapter. By using siRNA knock down and rescue experiments on the p65/p50 subunits of NFκB we could measure whether apigenin or isoliquiritgenin still had effects on *MMP1, MMP13, ADAMTS4* and *ADAMTS5* when the NFκB pathway is knocked down.

While apigenin and isoliquiritigenin appeared to show the same patterns of TGFβ1 stimulated Smad2/3/4 inhibition (Figure 5.4a, 5.7a) apigenin showed a strong inhibition of

unstimulated Smad 2/3/4 (Figure 5.4b) whereas isoliquiritigenin did not (Figure 5.7b). These results suggest that apigenin may over inhibit basal levels of TGFβ and could potentially prevent the anabolic role TGFβ plays in the joint as TGFβ is known to stimulate proteoglycan and collagen synthesis (Redini et al., 1988). As discussed in the introduction the role of TGFβ in the joint is complicated as it has been shown to have anabolic and catabolic effects in OA models. It has been hypothesised that TGFβ could stimulate aggrecan synthesis and degradation (TGFβ has been shown to stimulate *ADAMTS4* expression in human articular chondrocytes (Moulharat et al., 2004) resulting in increased aggrecan turnover. The resulting proteoglycan fragments produced by TGFβ stimulated aggrecanase could be an inflammatory trigger for cytokine production by chondrocytes (Pelletier et al., 2001) leading to an increase in metalloproteinase production and aggrecan and collagen degradation seen in OA. This data suggests that the inhibition of *ADAMTS4* in KOA cells treated with apigenin seen in the previous chapter may be due to apigenin inhibiting the TGFβ/Smad2/3/4 pathway (Figure 5.4). By using siRNA targeting Smad2 and Smad3 we could measure whether apigenin or isoliquiritigenin still have effects on *MMP1, MMP13, ADAMTS4* and *ADAMTS5* expression when the TGFβ/Smad2/3/4 pathway is knocked down.

Both apigenin significantly reduced Wnt3a stimulated Wnt activity in dose dependent manners (Figure 5.5a, 5.8a) and had no significant effect on unstimulated Wnt activity (Figure 5.5b, 5.8b). As discussed in the introduction Wnt signalling can induce the expression of MMPs in articular chondrocytes, promoting cartilage degradation and cartilage catabolism (Yuasa et al., 2008). This data suggests that apigenin and isoliquiritigenin may be inhibiting the Wnt/β-catenin potentially contributing to the inhibition of *MMP1, MMP13* and *ADAMTS4* by apigenin in KOA cells seen in the previous chapter. These results also support the data seen in the previous chapter where we hypothesised that apigenin may be inhibiting the Wnt pathway as apigenin inhibited *AXIN2,* a Wnt/β-catenin responsive gene. Over inhibition of the Wnt/β-catenin signalling pathway has been shown to increase cell apoptosis and articular cartilage destruction (Zhu et al., 2008). As both compounds had no effect on unstimulated Wnt activity (Figure 5.5b 5.8b) they would be unlikely to over inhibit the Wnt/β-catenin pathway as seen in the *Col2a1*-

ICAT-transgenic mice (Zhu et al., 2008) and instead the compounds may rebalance the Wnt/β-catenin pathway to the activity present in a healthy joint.

Apigenin and isoliquiritigenin may have different mechanisms of actions and could be interacting with the pathways in different ways. These data are similar to the effects curcumin and resveratrol have on the NFκB pathway. Curcumin had previously been shown to suppress the activation of NFκB (Singh & Aggarwal, 1995) as had resveratrol (Meng et al., 2005). Inhibition of NFκB by curcumin was shown to be mainly through inhibition of IKK activation, in contrast the inhibition of NFκB activation by resveratrol mainly occurred through accumulation of phosphorylated IKβα and ubiquitinated IKβα (Csaki et al., 2009). If apigenin and isoliquiritigenin inhibited the NFκB and the other pathways in different ways they may have stronger inhibitory effects when used together, allowing us to use each compound at lower, potentially more easily obtained, concentrations.

As discussed in chapter 1, OA is a multifactorial disease and pharmacological intervention for OA has had a lack of clinical efficacy. This may be due to pharmacological treatments typically only having a single mode of action and OA being a multifactoral disease. Bioactive compounds were considered an attractive alternative treatment for OA as bioactive compounds can simultaneously interact with multiple cellular pathways (Ameye & Chee, 2006). This chapter has shown that apigenin and isoliquiritigenin are capable of interacting with multiple cell pathways including the NFκB, TGFβ and Wnt pathways. The Proteome Profiler Array Human Phospho-Kinase Array Kit was used to perform an unbiased dissection of the kinase pathways affected by apigenin and isoliquiritigenin. This assay was used to detect simultaneously the relative levels of phosphorylation of 43 kinase phosphorylation sites and 2 related total proteins measuring the effects of apigenin and isoliquiritigenin across multiple cell pathways. To select and validate a timepoint for this assay the phosphorylation of the MAPK family of serine/threonine kinases, ERK, p38 and JNK were selected for analysis as they were known to be involved in the regulation of metalloproteinase gene expression and were included in the 43 kinases of the Human Phospho Kinase screen. While IL1 does not induce the phosphorylation of all the kinases in the Human Phosphor Kinase screen it was selected to stimulate the assay and the phosphorylation of the MAPK family as the effects on IL1 stimulated genes inhibited by apigenin and isoliquiritigenin in chapter 4 were under investigation. Over the 60 minute

timecourse apigenin and isoliquiritigenin showed the strongest inhibition of the IL1 stimulated phosphorylation of ERK and p38 at 10 minutes (Figure 5.9b,c 5.10b,c) while isoliquiritigenin also showed a slight inhibition of JNK phosphorylation (Figure 5.10d). These results were used to select a 10 minute timepoint for the Human Phospho-Kinase array.

Isoliquiritigenin showed a reduction of the phosphorylation of HSP27, Src, FAK, PRAS40, p38a and showed a weak reduction of HSP60, β-catenin, Hck, JNK 1/2/3, CREB, Fyn, c-JUN and ERK 1/2 (Table 7). Apigenin showed a strong reduction of the phosphorylation of HSP27, HSP60, Src, c-Jun, Lck, CREB, Akt 1/2/3 (at phosphorylation sites S427 and T308), PRAS40, Fyn and β-catenin and showed a weak reduction of Hck, PDGF Rβ, WNK1, STAT6, p38a, FAK and JNK 1/2/3 (Table 8). Apigenin also showed an induction of the phosphorylation of GSK-3α/β (Figure 5.12a).

Heat shock protein 27 (HSP27) is a protein chaperone and an antioxidant that plays a role in cytoskeletal remodelling and the inhibition of apoptosis (Vidyasagar et al., 2012). Changes to the expression, phosphorylation and subcellular location of HSP27 are induced by environmental stress (Wong et al., 2000). Chondrocytes expression of HSP27 has been knocked down with the use of siRNA. This knock down resulted in a reduced secretion of IL6 in response to IL1 (Lambrecht et al., 2010). The role of IL6 in OA remains controversial. IL6 is elevated in the synovial fluid of OA joints compared to healthy donors, but reports regarding its effects on cartilage matrix metabolism are conflicting. IL6 decreases the production of type 2 collagen and increases the production of enzymes from the MMPs group in cartilage (Porée et al., 2008), (Rowan et al., 2001). However IL6 deficient (IL6(-/-)) mice show a tendency to develop much more advanced degenerative changes than healthy mice (de Hooge et al., 2005). C6 glioma cells that were transfected with phosphorylated HSP27 had suppressed IL1 induced IL6 release and mRNA expression level but cells that were transfected with unphosphorylated HSP27 had an enhanced level of IL1 induced IL6 release and mRNA expression. These results suggest that the phosphorylated status of HSP27 plays a role in IL1 induced IL6 synthesis (Tanabe et al., 2010). In our results both apigenin and isoliquiritigenin decreased phosphorylated HSP27 levels compared to IL1 induced control (Figure 5.12a), these lower levels of phosphorylated HSP27 levels may be due to the chondrocytes undergoing less oxidative stress after apigenin or isoliquiritigenin treatment. Future experiments could look at the protein levels and mRNA expression of IL6

to see if the decrease in HSP27 phosphorylation results in changes to IL6. Interestingly apigenin and isoliquiritigenin also reduced the total level of heat shock protein 60 (HSP60) which may be due to the chondrocytes undergoing less cell stress. HSP60 was one of the two total protein levels detected by the Human Phospho-Kinase assay, the other being β-catenin. HSP60 is expressed in the synovial membrane of patients with rheumatoid arthritis and OA, shear stress has been identified as an inducer of HSP60 in endothelial cells (S. Watanabe et al., 2014). HSP60 has been shown to promote the TNF-α-mediated activation of the NFκB pathway via direct interaction with Inhibitor of Kappa B Kinase alpha/beta (IKKα/β) in the cytoplasm (Chun et al., 2010). The lower levels of HSP60 seen with apigenin and isoliquiritigenin treatment in the Human Phospho-Kinase assay (Figure 5.12) may result in the lower NFκB activity levels seen in the cell based luciferase assays (Figures 5.3, 5.6). We could investigate this by knocking down HSP60 using siRNA and measure the effects on the NFκB pathway after apigenin or isoliquiritigenin treatment using cell based luciferase assays (Ghosh et al., 2010).

Proto-oncogene tyrosine-protein kinase Src (Src) is a proto-oncogene involved in the regulation of embryonic development and cell growth (Meyn et al., 2005). Src can trigger the activation of downstream signalling pathways including MAPK and NFκB, (Lee et al., 2007), (Lei & Ingbar, 2011). Inhibition of Src kinase activity using the pharmacological compound PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine) in primary murine chondrocytes reduced the number of primary chondrocytes but also increased the expression of both early markers (such as Sox9, collagen type 2, aggrecan and xylosyltransferases) and late markers (collagen type X, Indian hedgehog and p57) of chondrocyte differentiation (Bursell et al., 2007). It was hypothesised that PP2 promotes chondrogenic gene expression and morphology in monolayer culture and that strategies to block Src activity might therefore be useful both in tissue engineering of cartilage and in the maintenance of the chondrocyte phenotype in OA. Apigenin and isoliquiritigenin strongly reduced the phosphorylation of Src (Figure 5.12) and the phosphorylation of Src at Y419 is important for activating the kinase activity towards substrates (Boerner et al., 1996). IL1 stimulated Hck phosphorylation levels were reduced in KOA cells treated with apigenin or isoliquiritigenin. Hck is a tyrosine-protein kinase and a member of the Src family of tyrosine kinases. PP2 the Src kinase inhibitor has been shown to repress the expression

of the Src kinases Hck, Lyn and Frk reversing morphological dedifferentiation of chondrocytes in monolayer culture (Bursell et al., 2007). Phosphorylation of Hck at T511 increases kinase activity (Khan et al., 2010). These results suggest that Hck plays a role in dedifferentiation in chondrocytes and apigenin and isoliquiritigenin may prevent this dedifferentiation by inhibiting Hck activity. The reduction in the level of phosphorylated Src caused by apigenin and isoliquiritigenin may contribute to changes seen in the ERK and NFκB pathways seen in this chapter. The PP2 inhibitor could be used to inhibit Src kinases in KOA cells treated with apigenin or isoliquiritigenin and the effects of apigenin and isoliquiritigenin on *MMP1, MMP13, ADAMTS4* and *ADAMTS5* expression could be measured.

Focal Adhesion Kinase (FAK) is a cytoplasmic protein tyrosine kinase found in the focal adhesions that form between cells growing in the presence of ECM constituents. FAK is a marker for integrin activation and decreased FAK levels alters cell-matrix interactions and response to mechanical load (Svoboda, 1998). Osteochondral explants treated with a FAK inhibitor (FAKi) before a cell death-inducing impact load increased cell viability (Jang et al., 2014). Targeting of FAK by siRNAs has been shown to reduce chondrocyte redifferentiation capacity in alginate beads culture with type 2 collagen (Y. Kim & Lee, 2009). Apigenin and isoliquiritigenin apparently reduced FAK phosphorylation in KOA chondrocytes (Figure 5.12), these lower phosphorylated levels of FAK may be closer to those found in normal healthy joints as FAK phosphorylation has been reported to be significantly higher in rabbit OA chondrocytes (Cheng et al., 2014). Proline-rich AKT1 substrate 40 (PRAS40) is a protein that regulates Target of rapamycin (TOR) by acting as a direct inhibitor of substrate binding (L. Wang et al., 2007). The phosphorylation of PRAS40 by Akt relives PRAS40 inhibition of TOR (Vander Haar et al., 2007). Akt 1/2/3 phosphorylation at S473 was reduced by apigenin which is required for its activation (Figure 5.12) less activated Akt 1/2/3 may result in the lower levels of phosphorylated PRAS40 seen with apigenin and isoliquiritigenin treatment and suggests that the PI3K/Akt pathway is being inhibited by the compounds. PI3K/Akt regulates a cascade of changes through its broad target proteins such as NFκB and p53, (Dan et al., 2008) (Chen & Huang, 2009). Activated Akt induces NFκB transcription activity by stimulating the NFκB trans-activation domain. The reduction of activated Akt 1/2/3 by apigenin may result in decrease in the PI3K/Akt and could result in the lower NFκB activity

levels seen in the apigenin treated cell based luciferase assays (Figures 5.3). Curcumin (a curcuminoid of turmeric) has been suggested to inhibit IL1-stimulated MMP secretion by inhibiting pAkt and NFκB (Schulze-Tanzil et al., 2004) and apigenin may have a similar mechanism of action.

Apigenin also reduced IL1 stimulated phosphorylated Beta-type platelet-derived growth factor receptor (PDGF Rβ) levels (Figure 5.12a). PDGF Rβ coprecipitates with PI3K following stimulation of 3T3 fibroblast cells when stimulated with platelet derived growth factor (PDGF), mutagenesis of PDGF Rβ Y751 phosphorylation site results in reduced PI3K activity (Kazlauskas & Cooper, 1990). Apigenin effects on NFκB in the cell based luciferase assays (Figure 5.3) may be due to the reduction in PI3K activity from a reduction in phosphorylated PDGF Rβ (at Y751).

Apigenin and isoliquiritigenin reduced the phosphorylation of p38a in the Human Phospho-Kinase screen (Figure 5.12) which supports the timecourse western blot data (Figures 5.9c, 5.10c). While normal and OA chondrocytes express p38 OA chondrocytes show much higher phosphorylated p38 compared to normal chondrocytes (Takebe et al., 2011) and heat stress or mechanical stress induces apoptosis and increases phosphorylated p38 in normal chondrocytes. NO induction of NFκB, p53 and caspase-3 activation has been shown to be via phosphorylation of p38 MAPK in rabbit articular chondrocytes inducing chondrocyte apoptosis (H. Wang et al., 2007). As discussed in previous chapters elevated levels of markers of NO production are found in OA joints suggesting that NO is involved in the pathogenesis of osteoarthritis (OA) (Vuolteenaho et al., 2007). These results suggest that the decrease in the phosphorylated p38 seen with apigenin and isoliquiritigenin treatment could result in the lower NFκB activity levels seen in the cell based luciferase assays (Figures 5.3, 5.6).

Apigenin and isoliquiritigenin reduced IL1 total β-catenin protein levels (Figure 5.12a). β-catenin was one of the total protein levels detected by the Human Phospho-Kinase assay, the other being HSP60. As discussed in the introduction both over and under activation of the Wnt/β-catenin can result in OA and a joint must have the correct homeostasis of the pathway to remain healthy. The reduced total protein level of β-catenin seen in the Human Phospho-Kinase results may contribute to the reduced Wnt pathway activity seen in the

cell based luciferase assays (Figure 5.5, 5.8) potentially rebalancing the Wnt/β-catenin pathway to the activity present in a healthy joint. The lysine deficient protein kinase 1 (WNK1) is a submember of the serine/threonine protein kinases. WNK1 has been shown to upregulate β-catenin in endothelial cells (Dbouk et al., 2014) and siRNA mediated knockdown of WNK1 in human embryonic Kidney 293 cells had a significantly reduced expression of a Wnt-signalling reporter (Serysheva et al., 2013). These results suggest that WNK kinases are involved in the regulation of the β-catenin/Wnt pathway. While WNK1s role in OA is unknown, if the regulation of the β-catenin/Wnt pathway is in part controlled by the phosphorylation of WNK1 in chondrocytes apigenin may inhibit the Wnt pathway in the cell based luciferase assays (Figure 5.5) by reducing the phosphorylation level of WNK1 (Figure 5.12) potentially resulting in the dose dependent repression of *AXIN2* seen in chondrocytes treated with apigenin (Figure 4.15).

Interestingly apigenin increased GSK-3α/β phosphorylation levels (Figure 5.12). GSK3 comprises of 2 serine/threonine protein kinases GSK-3α and GSK-3β. GSK-3 is constitutively active and is negatively regulated via phosphorylation at S21 (GSK-3α) and S9 (GSK-3β). GSK-3 inhibition has been shown to reduce collagen induced arthritis and collagen antibody induced arthritis in mice (Cuzzocrea et al., 2006), (Hu et al., 2006) suggesting that the inhibition of GSK-3 could have anti inflammatory effects during chronic inflammation. By increasing the phosphorylation of GSK-3α/β apigenin may be inactivating the constitutively active form of GSK-3, however levels of total GSK-3 need to be measured in future experiments to clarify these results. GSK-3 is a part of the β-catenin degradation complex and increased levels of GSK-3 could result in higher levels of β-catenin degradation. Isoliquiritgenin had no effect on GSK-3α/β phosphorylation levels.

As discussed in the introduction inflammatory cytokines, osmostic stress and apoptotic signals activate JNKs (Davis, 2000). JNKs phosphorylate and activate AP-1 family member c-Jun which dimerises with c-Fos during MMP gene transcription (Karin, 1995), (Leppä et al., 1998). Apigenin and isoliquiritigenin weakly inhibited the phosphorylation of JNK1/2/3 in the Human Phospho-Kinase assay (Figure 5.12) which supports the timecourse western blot data where isoliquiritigenin showed a slight inhibition of JNK phosphorylation at the 10 minute timepoint (Figure 5.10d). Phosphorylation of c-JUN is associated with increased transcriptional activation capacity of the protein (Pulverer et al., 1991). c-JUN can also

transactivate p53-upregulated modulator of apoptosis (PUMA) gene expression to promote OA. c-JUN and PUMA have been shown to be upregulated in chondrocytes from the articular cartilage of OA patients. PUMA is a pro-apoptotic member of the BH3-only subgroup of the Bcl-2 family. IL1 stimulated mouse chondrocytes had an increase in PUMA protein and mRNA levels (H. Lu et al., 2014). PUMA was hypothesised to play an important role in chondrocyte apoptosis as siRNA knock down of PUMA reduced apoptosis. When the JNK/c-Jun pathway was pharmacologically inhibited PUMA expression was supressed and apoptosis levels were decreased in IL1 treated chondrocytes (H. Lu et al., 2014). Apigenin and isoliquiritigenin reduced the phosphorylation levels of c-JUN in the Human Phospho-Kinase assay (Figure 5.12) this may be due to chondrocytes lower protein levels of JNK after apigenin or isoliquiritigenin treatment (Figure 5.9d, 5.10d). These results suggest that the inhibition of IL1 stimulated MMPs by apigenin and isoliquiritigenin seen in previous chapters may be due to the inhibition of the JNK/c-JUN pathway.These results suggest that the inhibition of IL1 induced MMPs seen in previous chapters may be in part due to inhibition of the IL1 induced JNK pathway, future experiments could explore JNK phosphorylation levels at different timepoints.

As discussed in the introduction ERK is a member of the MAPK family of serine/threonine kinases. ERKs phosphorylate and activate c-JUN. An ERK specific inhibitor U0126 has been shown to significantly inhibit IL1 induced ERK activation resulting in inhibition of IL1 induced upregulation *of iNOS, IL-6, Cox-2, MMP3,* and *MMP13* in rat annulus fibrosus cells (Wei et al., 2013). Apigenin and isoliquiritigenin inhibited ERK protein levels in the western blot timecourse assays (Figure 5.9b, 5.10b), isoliquiritigenin also reduced ERK phosphorylation levels in the Human Phospho Kinase assay and apigenin showed a trend towards ERK phosphorylation reduction (Figure 5.12). These results suggest that the inhibition of IL1 stimulated MMPs by apigenin and isoliquiritigenin seen in previous chapters may be due to the inhibition of the ERK pathway.

The cAMP response element-binding protein (CREB) is a transcription factor and phosphorylation at S133 increases CREB-mediated gene transcription (De Falco et al., 2012). Human articular chondrocytes having undergone CpG demethylation of the *MMP13* promoter showed CREB recruitement leading to the elevated expression of *MMP13*, this CpG is demethylated in OA cartilage, indicating a role for DNA demethylation and thus CREB

in the increased expression of *MMP13* in OA (Bui et al., 2012). CREB phosphorylation was inhibited by apigenin and isoliquiritigenin in the Human Phospho-Kinase assay (Figure 5.12) potentially decreasing CREBs transcriptional activity which may contribute to the inhibition of *MMP13* seen in KOA treated chondrocytes treated with apigenin and isoliquiritigenin in the previous chapter.

Fyn is a proto-oncogene tyrosine kinase that has been implicated in the control of cell growth (Berwanger et al., 2002). While the role of Fyn in OA is unknown apigenin and isoliquiritigenin lowered IL1 stimulated Fyn phosphorylation levels closer to unstimulated levels (Figure 5.12b).

The lymphocyte-specific protein tyrosine kinase (Lck) is a key signalling molecule in the selection and maturation of developing T-cells (Isakov & Biesinger, 2000). During rheumatoid arthritis T cells infiltrate the synovium and produce cytokines, chemokines, and degradative enzymes promoting inflammation and joint destruction. Inhibition of Lck could result in immunosuppression and has been suggested as a potential therapeutic for patients with rheumatoid arthritis (Meyn & Smithgall, 2008). Whether Lck play a role in OA is unknown. Lck levels were low in KOA cells and apigenin reduced IL1 Lck phosphorylation in the Human Phospho-Kinase array below levels of the unstimulated control (Figure 5.12b). Isoliquiritigenin had no effect on Lck phosphorylation.

The signal transducer and activator of transcription 6 (STAT6) is a member of the STAT family of transcription factors. CD1a positive dendritic cells express STAT6 in seropositive rheumatoid tissue and STAT6 has been suggested to be an alternative marker for identifying RA at the level of the synovium. IL4 signalling through STAT6 has been reported to have anti-inflammatory effects. A proteoglycan induced murine arthritis model has shown that IL4 signalling through STAT6 is required to attenuate the severity of joint inflammation (Finnegan et al., 2002). The role of STAT6 in OA is unknown. Apigenin decreased IL1 stimulated STAT6 phosphorylation levels to below unstimulated KOA cells STAT6 phosphorylation levels (Figure 5.12b).

5.3.1 Conclusion

In conclusion apigenin and isoliquiritigenin inhibited IL1 stimulated and NFκB, TGFβ and Wnt pathways and apigenin inhibited the unstimulated NFκB and TGFβ in cell based luciferase assays in a dose dependent manner and with distinct differences in the patterns of inhibition suggesting that these compounds may have different mechanisms of actions. Over 60 minute timecourses 10μM of apigenin and isoliquiritigenin showed inhibition of the IL1 stimulated phosphorylation of ERK, p38 and JNK at 10 minutes and this timepoint was selected for use with the Human Phospho-Kinase array experiments. The Human Phospho-Kinase Array ascertained the effects apigenin and isoliquiritigenin had on 43 kinases and 2 related total proteins in IL1 stimulated and unstimulated KOA cells. Apigenin and isoliquiritigenin had similar effects on many phosphorylation events, however there were kinase phosphorylation events that were specific to either apigenin or isoliquiritigenin and these may explain some of the variations seen between the compounds qRT-PCR experiments seen in previous chapters and the cell based luciferase assays.

Apigenin and isoliquiritigenin reduced β-catenin/Wnt transcriptional activity (Figures 5.5,5.8) these effects could potentially be due to reduced levels of total β-catenin (Figure 5.12a), apigenin also reduced levels of WNK1 which has been shown to be important in regulation of the β-catenin/Wnt pathway (Serysheva et al., 2013) (Figure 5.13).

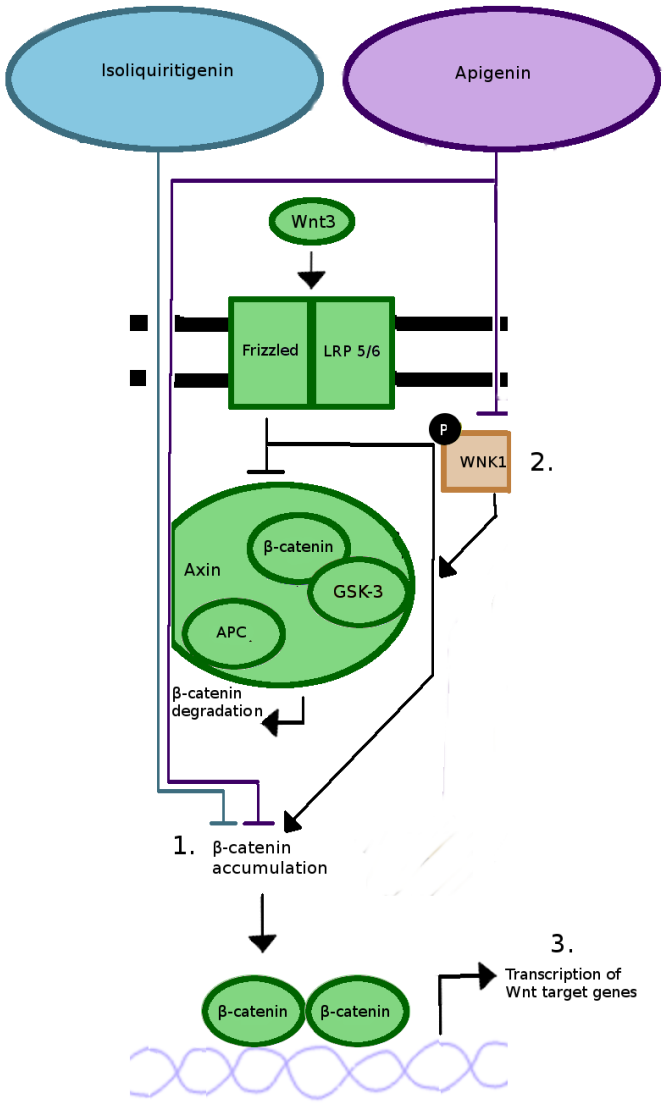


Figure 5.13. Summary of the effects of apigenin or isoliquiritigenin treatment on the Wnt pathway. (1) Total β-catenin levels were reduced in the human phosphor kinase screen by apigenin and isoliquiritigenin treatment. (2) Phosphorylated levels of WNK1 were reduced by apigenin. (3) Transcriptional activity of the Wnt pathway was reduced by apigenin and isoliquiritigenin treatment as shown by luciferase reporter assays.

NFκB transcriptional activity was reduced with apigenin and isoliquiritigenin treatment (Figures 5.3, 5.6) these effects could potentially be due to reduced total levels of HSP60 (Figure 5.12a), apigenin also reduced levels of phosphorylated AKT 1/2/3 which could potentially result in less NFκB transcriptional activation (Figure 5.14).

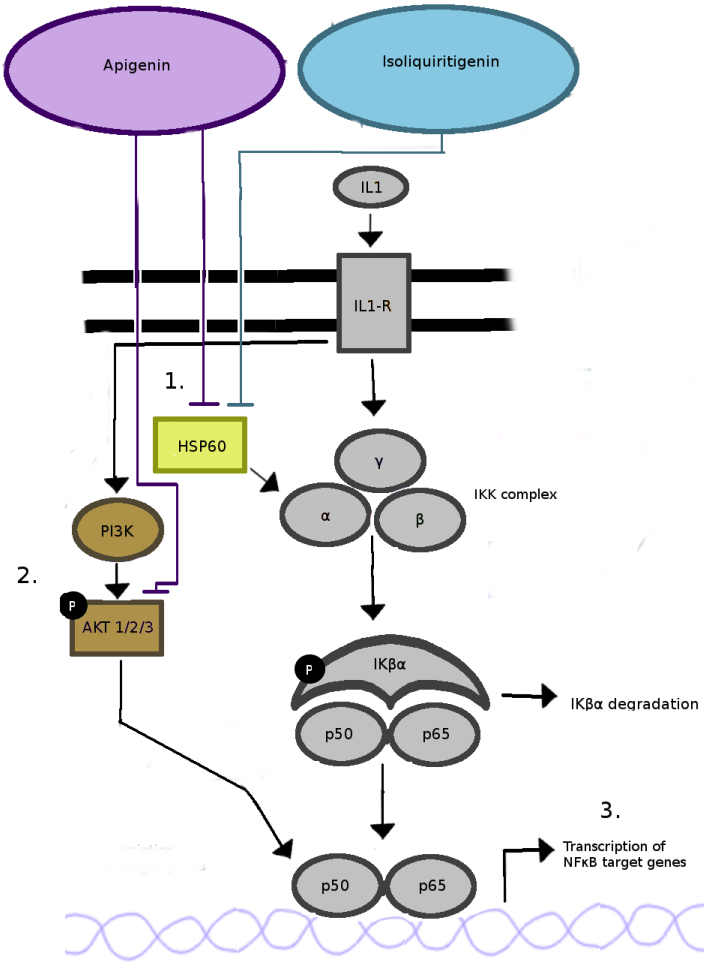


Figure 5.14. Summary of the effects of apigenin or isoliquiritigenin treatment on the NFκB pathway. (1) Total HSP60 levels were reduced in the human phosphor kinase screen by apigenin and isoliquiritigenin treatment. (2) Phosphorylated levels of AKT 1/2/3 were reduced by apigenin. (3) Transcriptional activity of the NFκB pathway was reduced by apigenin and isoliquiritigenin treatment as shown by luciferase reporter assays.

Apigenin has previously been shown to suppress *MMP13* expression in IL1 treated SW1353 cells by suppressing the c-fos/AP-1 pathways (Lim et al., 2011). The Human Phospho Kinase array (Figure 5.12a) reported that several phosphorylation events involved in the regulation of the c-fos-AP-1 pathway were reduced after apigenin and isoliquiritigenin treatment (Figure 5.15). These results could be further validated in future experiments by measuring the transcriptional activity of AP-1 by using a luciferase reporter plasmid.

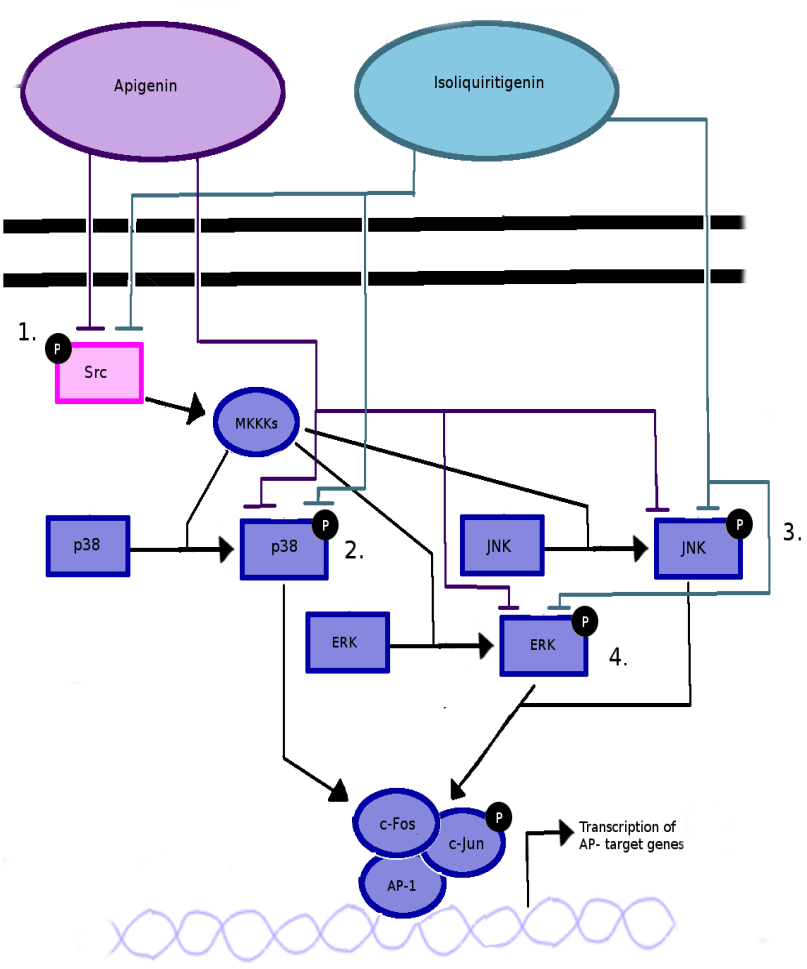


Figure 5.15. Summary of the effects of apigenin or isoliquiritigenin treatment on the AP-1 pathway. (1) Phosphorylated levels of Src were reduced in the human phosphor kinase screen by apigenin and isoliquiritigenin treatment. (2) Phosphorylayed p38 levels were reduced by apigenin and isoliquiritigenin. (3) Phosphorylated JNK levels were reduced by apigenin and isoliquiritigenin. (4) Phosphorylated ERK levels were reduced by apigenin and isoliquiritigenin.

While future experiments are still required to determine the mechanisms of actions of apigenin and isoliquiritigenin this data suggests that apigenin and isoliquiritigenin effect different pathways and could potentially have synergistic effects when used together.

Chapter 6: General discussion

Pharmacological intervention for OA has had a lack of clinical efficacy. OA is a poorly defined disease with a lack of good outcome measures and biomarkers. Current treatments for OA have focused on anti-inflammatories and pain relief rather than addressing the degradation of cartilage. This lack of clinical efficacy for pharmacological intervention may, in part, be due to pharmacological treatments typically only having a single mode of action and OA being a multifactoral disease. For this reason nutrition was seen as an attractive alternative treatment as foods typically contain multiple bioactives that can interact with multiple cellular pathways (Ameye & Chee, 2006). Current treatments for OA such as painkillers and anti-inflammatories provide symptomatic relief and are unable to prevent the progression of the disease. Joint replacement is offered to patients at end-stage disease, at large financial burden to the NHS, and with increasing patient numbers, will likely not remain practicable. MMP-13, MMP-1, ADAMTS4 and ADAMTS5 have been shown to be a key collagenases and aggrecanases in cartilage degradation. This project aimed to identify novel compounds found in the human diet that could offer protection or slow the progression of OA via collagenase and/or aggrecanase inhibition.

6.1 Summary of findings

6.1.1 Dietary Derived Bioactive Screen

Compounds were selected from a Bioactive Compound Library which contained 1853 bioactive chemical compounds from synthetic and natural sources. Only compounds that were produced by living organisms were selected from this list creating the Natural Product Library containing 143 compounds. Ninety-six dietary derived compounds were selected from the Natural Product Library and compiled into a custom-built library in a 96-well plate format. This format was selected to ensure accurate comparisons between compounds, all compounds were at 10µM and dissolved in DMSO. The advantages of using a custom-built library in this format was the standardisation of compounds in terms of purity, concentration and form. Purchasing compounds individually often requires a minimum quantity and can quickly become expensive narrowing the range of bioactive compounds that can be explored. In order to be selected for the 96-well plate custom library the natural

products had to be dietary derived. The library was designed to contain bioactive compounds with a range of chemical structures including flavonoids, anthocyanins, terpenoids, carotenoids and other polyphenols as well as precursors of parent compounds such as polydatin (a natural precursor of resveratrol).

Compounds were screened at an initial concentration of 10µM for IL1 induced and basal *MMP13* inhibition in SW1353 cells. 10µM was selected as a pragmatic choice based on past literature on dietary derived compounds and what concentrations of bioactives are often achievable through the diet. Inhibiton of the expression of *MMP13* was selected as an initial readout for a bioactive compounds potential as a chondro-protective agent, as MMP-13 is the collagenase with the highest specific activity for type 2 collagen i.e. the most efficient cleavage of the protein (Minond et al., 2006) and it is thought to be the predominant collagenase involved in OA (Lopez-Otin, 1996), (Billinghurst et al., 1997), (Flannelly et al., 2002) compared to other collagenases such as MMP-1. A qRT-PCR approach to analysing *MMP13* was selected as a strategy to give a medium-high throughput screen using relatively low cell numbers compared to other techniques such as northern blots. *MMP13* mRNA levels have also been shown to be comparable to MMP-13 protein levels (Ahmad et al., 2009), this allows us to predict compounds effects on the MMP-13 protein levels using the medium-high throughput screen, whilst using a western blot method to measure the effect of each compound on actual MMP-13 protein levels would be prohibitively expensive and time consuming.

The one-way analysis of variance (ANOVA) with Dunnett's post test was used to measure the statistical significance of a compounds effect on IL1 stimulated *MMP13.* While multiple t-tests could have been used to measure significance the chances of a Type 1 error occurring increases with each t-test performed. With 96 compounds being screened 96 t-tests would need to be completed and the chance of a Type 1 error occurring would be unacceptably high. Using an ANOVA controls for these errors so that the chance of a Type 1 error never exceeds 5%.

Emodin, ursolic acid, oleanolic acid, luteolin, polydatin, apigenin, neohesperidin dihydrochalcone, l-carnitine, isoliquiritigenin, myricetin, theobromine, sesamin, troxerutin, aloe-emodin, methyl-hesperidin, fisetin, genistin, hesperetin and biochanin A significantly

reduced either IL1 stimulated or unstimulated *MMP13* expression in either SW1353 or C28/I2 cells. As some of these compounds had either IL1/basal or SW1353/C28/I2 specific effects the top twenty compounds showing either a statistically significant effect on *MMP13* expresssion or a trend for inhibition of *MMP13* expression were selected from each screen and entered into a four set Venn diagram. Compounds were selected from the Venn diagram and compiled into a top twenty compound list for further study in KOA cells if the compound effected more than one of the *MMP13* expression screens. This was done to eliminate compounds that only had an effect in a single experiment and to include compounds that showed a trend of inhibiton of *MMP13* expression in multiple experiments that may not have reached statistical significance in individual screens. The top twenty compounds selected included: emodin, ursolic acid, luteolin, polydatin, apigenin, neohesperidin dihydrochalcone, l-carnitine, Isoliquiritigenin, myricetin, theobromine, aloe emodin, methyl hesperidin, genistin, orotic acid, indole-3-carbinol, nobiletin, naringin, kinetin, quercetin and rheochrysidin.

6.1.2 Short-listing dietary-derived bioactives in human primary knee osteoarthritis chondrocytes

In chapter 3, twenty compounds were selected for further study (see Chapter 3 Table 5). In chapter 4, compounds were screened for inhibition of genes encoding cartilage cartilage-degrading enzymes *MMP13, MMP1, ADAMTS4* and *ADAMTS5.* Since the two cell lines were used to shortlist compounds, the 20 remaining compounds (Table 5) were tested in in primary KOA chondrocytes. KOA cells were isolated from the cartilage of patients with knee OA. The qRT-PCR *MMP13* screen from chapter 3 was repeated in the 20 selected compounds in KOA cells. While MMP-13 plays a pivotal role in collagen cleavage in OA (Knauper, Lopez-Otin, Smith, Knight, & Murphy, 1996) MMP-1 is also able to cleave native fibrillar collagen and contributes to the pathological cleavage of collagen fibrils in OA (Burrage, Mix, & Brinckerhoff, 2006). Using the qRT-PCR screen format from chapter 3 the effect of the selected 20 compounds on *MMP1* expression in KOA cells was measured. As discussed in chapter 1 ADAMTSs also play a role in the development of OA. ADAMTS-5 knock out mice had a significant reduction in the severity of cartilage destruction after surgically induced instability when compared with wild type mice (Glasson et al., 2005).

ADAMTS4 has been shown to be selectively overexpressed in human osteoarthritic cartilage, with a direct correlation with the degree of cartilage destruction suggesting that *ADAMTS4* may play an important role in the degradation of aggrecan in human osteoarthritic cartilage (Naito et al., 2007). *ADAMTS5* is constitutively expressed whereas *ADAMTS4* is induced following IL1 treatment (Tortorella et al., 2001), for this reason only the unstimulated expression of *ADAMTS5* was measured. Compounds from the dose response were screened for effects on *ADAMTS4* and *ADAMTS5* in KOA cells.

Heme oxygenase-1 (HO-1) is the limiting enzyme in heme catabolism (Rousset et al., 2013), cleaving heme to form biliverdin, this prevents production of ROS giving HO-1 anti-inflammatory properties. HO-1 induction in PHCs cultured in hypoxic conditions prevented IL1 stimulated loss of type 2 collagen (Guillén et al., 2012). HO-1 was hypothesised to be a physiologically important chondro-protective factor. Compounds from the dose response assays were screened for effects on *HO-1* expression.

AXIN2 is a Wnt/β-catenin responsive gene which has been shown to be upregulated consistently with net activation of canonical Wnt signalling (Lustig et al., 2002). Wnt signalling can induce the expression of MMPs in articular chondrocytes which promotes cartilage degradation and cartilage catabolism (Yuasa et al., 2008). As the upregulation of the Wnt pathway may be deleterious for the joint cartilage the compounds from the dose response assays were screened for an effect on the expression of *AXIN2*.

Several of the compounds selected in chapter 3 from the SW1353 and C28/I2 screens were able to inhibit *MMP13, MMP1 ADAMTS4* and *ADAMTS5* at 10µM in KOA chondrocytes. Of these compounds apigenin and isoliquiritigenin showed the greatest statistically significant inhibition of *MMP13* while also inhibiting *MMP1, ADAMTS4* and *ADAMTS5*. These two selected candidate compounds dose dependently inhibited *MMP13, MMP1, ADAMTS4* and *ADAMTS5* and upregulated the anti-inflammatory gene *HO-1* in KOA cells. It was hypothesised that apigenin may be having these effects via regulation of the Wnt pathway as it dose- dependently inhibited *AXIN2*, a Wnt/β-catenin responsive gene. Apigenin and isoliquiritigenin were hypothesised to have chondro-protective properties and showed no cytotoxic effects in chondrocytes.

Oral doses of isoliquiritigenin (100mg/kg) in mice have achieved the higher concentrations of isoliquiritigenin used in the experiments in this project. In blood plasma, the highest concentration of isoliquiritigenin found in plasma was 55.8μM (Wang et al., 2014), (Qiao et al., 2014). While absorption of apigenin is much lower, concentrations of apigenin in human subjects have been hypothesised to be around 1.7μM after consumption of 10g of dried parsley per day (Shibata et al., 2014), and while it would not be impossible to reach plasma concentrations of apigenin used in this project through normal dietary intake, supplementation could potentially be used to obtain higher doses.

6.1.3 Ascertaining mechanism of action

In chapter 4, I identified two potentially chondro-protective dietary-derived bioactive compounds, apigenin a plant-derived flavone and isoliquiritigenin a chalcone found in liquorice root. In chapter 5, I explored the mechanism of action of these compounds by analysing their effect on cell signalling pathways and transcription factors implicated in cartilage homeostasis and OA. Cell based luciferase assays were used to measure the effect of apigenin or isoliquiritigenin pretreatment on the NFκB, TGFβ and Wnt pathways in SW1353 cells. Apigenin and isoliquiritigenin inhibited the NFκB, TGFβ and Wnt pathways in SW1353 cells. While both compounds affected the same pathways there was distinct differences in the patterns of inhibition. Isoliquiritigenin inhibited NFκB activity at a lower dose than apigenin when the pathway was stimulated with IL1, however isoliquiritigenin had no effect on the unstimulated NFκB pathway, whereas apigenin significantly inhibited the unstimulated NFκB pathway at 40μM. Both compounds inhibited the TGFβ stimulated Smad2/3/4 pathway at 10μM, but only apigenin inhibited the unstimulated Smad2/3/4 pathway and inhibiting the unstimulated Smad2/3/4 pathway in concentrations as low as 5μM. While what is driving the basal expression of luciferase in these experiments is unknown it could potentially be due to epigenetic changes in OA cells, possibly in the form of methylation on the promoters of these pathways. Both compounds significantly inhibited the Wnt3a stimulated Wnt/ β-catenin with apigenin showing significant inhibition at 5μM and isoliquiritigenin at 10μM. Apigenin and isoliquiritigenin had no significant effect on the unstimulated Wnt/ β-catenin pathway. It was hypothesised that the distinct differences in the patterns of inhibition in the NFκB and TGFβ pathways suggested that apigenin and isoliquiritigenin may have different mechanisms of actions and could be

interacting with the pathways in different ways. These data are similar to the effects curcumin and resveratrol have on the NFκB pathway. Curcumin had previously been shown to suppress the activation of NFκB (Singh & Aggarwal, 1995) as had resveratrol (Meng et al., 2005). Inhibition of NFκB by curcumin was shown to be mainly through inhibition of IKK activation, in contrast the inhibition of NFκB activation by resveratrol mainly occurred through accumulation of phosphorylated IKβα and ubiquinated IKβα (Csaki et al., 2009). If apigenin and isoliquiritigenin inhibited the NFκB and the other pathways in different ways they may have stronger inhibitory effects when used together, allowing us to use each compound at lower, potentially more easily obtained, concentrations.

The Proteome Profiler Array Human Phospho-Kinase Array Kit was used to perform an unbiased dissection of the kinase pathways affected by apigenin and isoliquiritigenin. This assay was used to detect simultaneously the relative levels of phosphorylation of 43 kinase phosphorylation sites and 2 related total proteins measuring the effects of apigenin and isoliquiritigenin across multiple cell pathways. To select and validate a timepoint for this assay the phosphorylation of the MAPK family of serine/threonine kinases, ERK, p38 and JNK were selected for analysis as they were known to be involved in the regulation of metalloproteinase gene expression and were included in the 43 kinases of the Human Phospho Kinase screen. Over a 60 minute time course apigenin and isoliquiritigenin showed the strongest inhibition of the IL1 stimulated phosphorylation of ERK and p38 at 10 minutes while also showing a slight inhibition of JNK phosphorylation. These results were used to select a 10 minute time point for the Human Phospho-Kinase array. For the Human Phospho-Kinase assay KOA cells were treated with isoliquiritigenin or apigenin at 10μM for 10 minutes and stimulated with 5ng/ml IL1. The Human Phospho-Kinase Array ascertained the effects apigenin and isoliquiritigenin had on 43 kinases and 2 related total proteins in IL1 stimulated and unstimulated KOA cells (Figure 7.1). Apigenin and isoliquiritigenin had similar effects on many phosphorylation events, however there were kinase phosphorylation events that were specific to either apigenin or isoliquiritigenin and these could explain some of the variations seen between the compounds qRT-PCR experiments seen in chapter 3 and 4. Apigenin increased phosphorylation levels of GSK-3 possibly via the inhibition of phosphorylated Akt 1/2/3 this could result in the lower levels of total β-catenin levels seen after apigenin treatment as GSK-3 is a part of the β-catenin degradation

complex. Both apigenin and isoliquiritigenin inhibited total HSP60 levels, together these results suggest mechanisms as to how these compounds reduce NFκB activity. The reduced Wnt/β-catenin pathway activity seen with apigenin and isoliquiritigenin treatment may be due to the reduced levels of total β-catenin levels seen after compound treatment. Apigenin also reduced phosphorylated WNK1 levels which could result in reduced Wnt/β-catenin pathway activity and may result in the dose dependent repression of *AXIN2* seen in chondrocytes treated with apigenin. While apigenin and isoliquiritigenin reduced TGFβ/Smad activity, further experiments need to be done to clarify the compounds mechanism of action. It was hypothesised that apigenin and isoliquiritigenin could be used at varying concentrations to effect different pathways and could potentially have synergistic effects when used together.

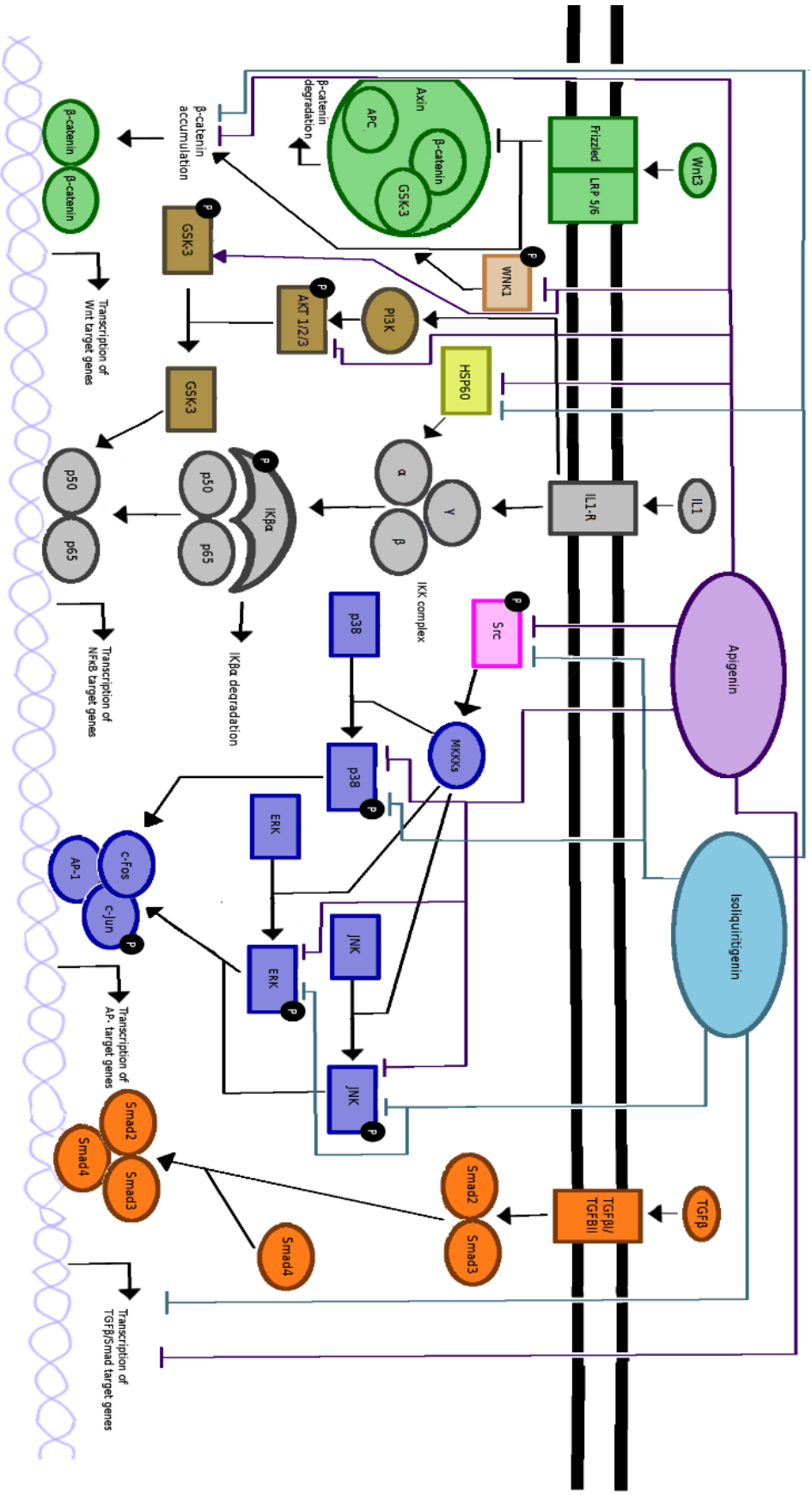


Figure 7.1 Summary of the effects of apigenin and isoliquiritigenin treatment in chondrocytes

6.2 Future directions

Future work could involve taking compounds shown to inhibit expression of *MMP1* and *MMP13* from chapter 4 that were not selected for further study and complete dose response qRT-PCR experiments for each compound, looking at *MMP1, MMP13, ADAMTS4, ADAMTS5, HO-1* and *AXIN2* expression. While these compounds did not inhibit *MMP1* and *MMP13* to the extent seen with apigenin and isoliquiritigenin treatment, many of them could potentially be inhibiting *MMP1* and *MMP13* via different pathways or be more potent at different timepoints/doses. These other compounds would be worth studying as they may have synergistic effects when used with apigenin and/or isoliquiritigenin. KOA cell experiments from this project could be repeated in normal primary chondrocytes to see if apigenin and/or isoliquiritigenin effect gene expression or kinase activity in healthy cell models.

Synergy between compounds could also be assayed. Compounds could be tested for synergistic interaction by using the Chou-Talalay method for drug combination. This method is based on the median-effect equation, which is derived from the mass-action law principle. This is the unified theory that provides the common link between single entity and multiple entities, and first order and higher order dynamics. The Chou-Talalay method provides a combination index (CI) that gives a quantitative definition for; additive effect (CI=1), synergism (CI<1) and antagonism (CI>1) of bioactive combinations (Chou, 2010).

Future work would also involve using siRNA to knock down individual kinases that were hypothesised to be involved in the compounds mechanism of action. For example inhibition of total HSP60 was hypothesised to be involved with the reduction of NFκB activity seen with apigenin and isoliquiritigenin treatment. By using siRNA to knockdown HSP60 we can see if apigenin or isoliquiritigenin treatment reduces NFκB activity further than HSP60 knockdown alone. It is also possible to introduce protein using cell-penetrating peptides. For kinases of interest western blot time courses could be performed to see if apigenin or isoliquiritigenin are more or less potent at different time points and at different concentrations.

Limitations of the Human Phospho Kinase assay included the low number of replicates and the use of only one cytokine to stimulate the assay. Repeating the Human Phospho Kinase

array will help validate the results seen in this project. Many kinases from the kit that were not stimulated with IL1 could potentially be involved in the reduced TGFβ/Smad activity seen in the cell luciferase experiments. As the TGFβ/Smad luciferase assays were stimulated with TGFβ1 it would be useful to repeat the Human Phospho Kinase array and use TGFβ1 to stimulate the chondrocytes. This same method could be repeated using Wnt3a to stimulate cells to explore the repression of the Wnt/β-catenin pathway in more detail. Western blots could be used to measure the phosphorylation of individual kinases that are included in the Human Phospho Kinase assay to provide validation of the results seen in the kinase assays.

Combinations of compounds could be tested in assays of cartilage destruction *in vitro*. These techniques have been developed in our lab and bovine nasal cartilage and murine femoral head cartilage can be used to measure the effects compounds have on proteoglycan and collagen breakdown. A three week old mouse femoral head cartilage assay based on methods developed for larger animal species (Stanton et al., 2011) has been developed in our lab to monitor GAG and hydroxyproline release to analyse whether dietary compounds can prevent aggrecanolysis. During this project we attempted to measure the effects of apigenin and isoliquiritigenin on GAG release in bovine cartilage samples, however due to a lack of cartilage tissue samples data was incomplete and could not be analysed. With more bovine cartilage tissue samples, future experiments could measure GAG release and the effects of apigenin and isoliquiritigenin treatments on proteoglycan breakdown could be elucidated.

Blood, plasma and cartilage samples could be taken from mice fed diets designed to contain high concentrations of apigenin or isoliquiritigenin and High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) could be used to measure levels of apigenin and isoliquiritigenin absorbed by the mice. High apigenin or high isoliquiritigenin diets could also be fed to mice to measure the change in arthritis score in the destabilisation of the medial meniscus (DMM) murine model of OA. Gene changes induced by this model (Such as *MMPs*, *TIMPs*, *ADAMTSs*, *COX2* etc.) could then be measured and high apigenin or high isoliquiritigenin diets can be compared to control chow diet. These murine models could also be used to predict physiologically achievable concentrations of apigenin and isoliquiritigenin in man.

6.3 Conclusions

Dietary derived bioactive compounds have huge potential as new treatments for OA. Diet offers a route by which the health of the joint can be protected and OA incidence or progression decreased. With escalating rates of obesity and increase in life expectancy, incidence of OA will rise over the next decades. Since the unmet need in OA is huge and the pharmaceutical industry has not made progress in this area, prevention or slowing of OA via dietary intervention or supplementation is an attractive alternative.

This work has focused on 96 dietary derived bioactive compounds and their effect on IL1 stimulated and basal *MMP1* and *MMP13* levels in SW1353 and C28/I2 cell lines. Several dietary derived compounds were able to repress *MMP1* and *MMP13* in these cell lines with a sub-group showing significant inhibition of these genes in primary chondrocytes. While apigenin and isoliquiritigenin were selected for further study many of the compounds that were not taken forward still have potential as OA therapeutics and future studies will examine these compounds mechanisms of action and whether they will have synergistic effects when used together. Apigenin and isoliquiritigenin likely inhibit metalloproteinase gene expression via the NFκB, TGFβ and Wnt pathways and the Human Phospho Kinase assay was used to begin to explore the compounds mechanisms of actions. Taken together these data suggest that the chondro-protective gene expression changes seen with apigenin and isoliquiritigenin treatment involves a complex network of signalling pathways that require further characterisation. If these compounds are able to get to the site of OA damage and at high enough concentrations they may prevent progression of OA offering a safe alternative treatment.

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Appendix

Compound No.	Compounds	Food types
1	Capsaicin	Chilli peppers
2	Sclareol	Salvia sclarea
3	(-)-Hupersine A	Huperzia serreta
4	(+)-Usniacin (D-Usnic acid)	Lichen
5	3-Indolebutyric acid (IBA)	Maize
6	4-Methylumbeliferone (4-MU)	Rice
7	Aesculin	Conkers (horse chestnut)
8	Aloe-emodin	Aloe vera
9	Sclareolide	Salvia sclarea
10	shikimic acid	American sweetgum
11	Apigenin	Parsley, celery, coffee
12	Silibinin	Milk thistle
13	Arbutin	Bear berries
14	Artemether	Sweet worm wood
15	Artesunate	Sweet wormwood
16	Asiatic acid	Gotu kola
17	Silymarin	Milk thistle
18	Baicalein	Blue and common skullcap

19	Sinomenine	Sinomenium Actum
20	Bergenin	Bergenia ciliata
21	Berberine Hydrochloride	Berberis
22	Sitosterol	Pecan
23	Bilobalide	Ginkgo biloba
24	Stigmasterol	Soy
25	Caffeic acid	Cucumber
26	Chlorogenic acid	Coffee
27	Chrysin	Honeycomb and passion flower
28	Tangeretin	Tangerine
29	Theobromine	Cocoa bean
30	Troxerutin	Styphnolobium japonicum
31	Ursolic acid	Apples
32	Cytisine	Gymnocladus dioica
33	Daidzin	Soy
34	Dihydroartemisinin	Sweet wormwood (tea)
35	Diosgenin	White yam
36	Vanillylacetone	Ginger
37	Diosmin	Peppermint
38	DL-Carnitine hydrochloride	Beef
39	Ecdysone	Cordyceps, sinensis

40	Emodin	Rhubarb
41	Enoxolone	Liquorice
42	Ergosterol	Maize
43	Fisetin	Strawberries
44	Formononetin	Red clover
45	Fumalic acid	Asafoetida
46	Genistin	Soy
47	Glycyrrhizic acid	Liquorice
48	Hydroxytryptophan	Turkey
49	Aloin (Barbaloin)	Aloe vera
50	Gramine	Arundo donax
51	Ammonium Glycyrrhizinate	Liquorice
52	Gynostemma Extract	Gynostemma pentaphyllum
53	Hesperetin	Black peppermint
54	Hesperedin	Orange
55	Honokiol	Magnolia grandiflora (tea)
56	Biochanin A (4-Methylgenistein	Soy
57	Icariin	Epimedium grandiflorum (Horny goat weed)
58	Indole-3-carbinol	Broccoli
59	Kaempferol	Broccoli

60	Gastrodin (Gastrodine)	Gastrodia elata
61	Kinetin	Coconut
62	Hordenine	Barley
63	Limonin	Orange/Lemons
64	Luteolin	Celery
65	Indirubin	Woad
66	L-carnitine	Beef
67	Methyl-Hesperidin	Citrus fruits
68	Naringin Dihydrochalcone	Grapefruit
69	Polydatin (Piceid)	Grapes
70	Myricetin	Grapes
71	Myricitrin	Myrica cerifera (Bayberry tree)
72	Quercetin (Sophoretin)	Black elder berry
73	Sesamin (Fagorol)	Sesame seeds
74	Naringin	Grapefruits
75	Sorbitol (Glucitol)	Apples
76	Neohesperidin dihydrochalcone	Citrus fruits
77	Rheochrysidin	Rumex crispus
78	Nobiletin	Orange
79	Oleanolic acid	Garlic
80	Salidroside	Rhodiola rosea (tea)

81	Orotic acid	Carrots
82	Osthole	Shishiudo
83	Palmatine chloride	Phellodendron amurense (Amur cork tree)
84	Coenzyme Q10	Red meat
85	Paeonol	Peony
86	Parthenolide	Feverfew
87	Phloretin	Apple
88	Dihydromyricetin	Ampelopsis grossdentata
89	Rhein	Rhubarb
90	Puerarin	Kudzu roots
91	Quercetin dihydrate	Black elder berry
92	Isoliquiritigenin	Liquorice
93	Rutin	Asparagus
94	Salicin	Willow bark
95	Chrysophanic acid	Rhubarb
96	Curcumol	Tumeric