Title: Can sulforaphane prevent the onset or slow the progression of osteoarthritis?

Short title: Sulforaphane and osteoarthritis

R. K. Davidson[1], O. Jupp.[1], Y. Bao[2], A. J. MacGregor[2], S. T. Donell[2], A. Cassidy[2], I. M. Clark[1] *

[1]School of Biological Sciences, University of East Anglia, Norwich
[2]Norwich Medical School, University of East Anglia, Norwich

*Corresponding author: Ian M Clark, PhD
                           Professor of Musculoskeletal Biology,
                           School of Biological Sciences,
                           University of East Anglia,
                           Norwich Research Park,
                           Norwich, NR4 7TJ.
                           United Kingdom
                           Email: i.clark@uea.ac.uk

Abstract

Osteoarthritis (OA) is a degenerative joint disease characterised in part by destruction of articular cartilage. There are currently no disease-modifying drugs to treat OA, with joint replacement the only treatment offered to patients at end-stage disease. With age the major risk factor for OA, the number of patients is predicted to double by 2030. An understanding of the role of bioactive molecules from the habitual diet on joint health offers a novel way in which to prevent the onset or slow the progression of OA. Our research has indicated that sulforaphane (SFN), gained from the consumption of cruciferous vegetables, particularly broccoli, could impact upon articular cartilage in laboratory models of OA because (1) it decreased the cytokine-induced expression of cartilage-degrading proteinases from chondrocytes (cartilage cells); (2) it prevented the cytokine-induced degradation of cartilage explants; and (3) it attenuated cartilage destruction in a murine model of OA. The major mechanism of action for SFN in human articular chondrocytes was inhibition of NFκB, not activation of Nrf2 nor inhibition of histone deacetylases. A proof-of-principle human trial was performed to measure uptake of SFN, or its metabolites, in the human knee joint following a broccoli-rich diet, and the expression or levels of several genes and proteins in cartilage, fat and synovial fluid were also measured. Data from this trial are about to be published. Overall, these findings support the utility of SFN in the prevention or treatment of OA. The proof of this requires an appropriately designed clinical trial of pain and function which we are currently pursuing.

Osteoarthritis (OA) is a degenerative joint disease characterised by degradation of articular cartilage as well as thickening of the subchondral bone and the formation of osteophytes at the joint margin (Goldring & Goldring 2006). It is a leading cause of disability in the UK. Approximately 8.5 million people in the UK suffer from moderate to severe OA (Arthritis Care 2012). There is a lack of effective, disease-modifying drugs available to treat OA and the drugs that target symptomatic pain are often inadequate (McHughes & Lipman 2006). Joint replacement is the only treatment offered to patients reaching end-stage OA disease with 66 436 hip and 77 578 knee replacements performed in 2011 (Porter *et al.* 2012).

Two major risk factors for OA are age (most patients are >45 years of age and the greatest morbidity is seen in patients >60 years of age) (Shane Anderson & Loeser 2010) and obesity (Richmond *et al.* 2013). With an ageing population and increasing rates of obesity, OA is a growing public health and economic burden. Though studies show high variability, direct health costs of OA have been estimated at approximately £1 billion per year, with indirect costs at approximately £3.5 billion in the UK (Chen *et al.* 2012). Given the current demographic trend toward an older population, OA, for which age is an important risk factor, will be an increasing burden on society with 17 million OA patients predicted for 2030. The ability to slow or stop progression of the disease would significantly improve both quality of life and the economic burden of OA.

There are many issues which make drug development in OA problematic, including a lack of biomarkers and the need for a low toxicity profile in this non-life threatening disease. An alternative strategy is to focus on compounds in the UK population's diet which may reduce the risk of developing or slow the progression of osteoarthritis. Dietary modification or increased intake of specific dietary constituents is attractive both in terms of risk/benefit ratio and implementation (Green *et al.* 2013).

The function of cartilage and its maintenance

Articular cartilage which lines the ends of long bones, functions to allow low friction movement of the joint, and to distribute force correctly to the underlying bone when movement occurs. Resident cells, the chondrocytes, are sparsely distributed throughout cartilage and must maintain and turnover the cartilage extracellular matrix (ECM) which is crucial to tissue function (Goldring 2006). Cartilage ECM is mainly made up of type II collagen and the proteoglycan, aggrecan. These components can be degraded by proteolytic enzymes. Type II collagen is principally turned over by enzymes from the matrix metalloproteinase family [MMPs, *e.g.* MMP-13 (Cawston & Wilson 2006)], whilst enzymes from the 'a disintegrin and metalloproteinase domain with thrombospondin motifs' [ADAMTS,

e.g. ADAMTS-5 (Fosang *et al.* 2008)] family metabolise the latter. An imbalance between the activity of these enzymes and their inhibitors (the tissue inhibitors of metalloproteinases) is thought to underlie cartilage destruction in OA (Burrage & Brinckerhoff 2007).

Sulforaphane and osteoarthritis

Sulforaphane (1-isothiocyanato-4-methylsulphinylbutane; SFN) is an isothiocyanate (ITC) obtained in the diet through consumption of cruciferous vegetables, particularly broccoli (Juge *et al.* 2007). SFN (and other ITCs) are synthesised in plant cells as glucosinolates (glucoraphanin in the case of SFN). Upon disruption (*e.g.* by chewing or chopping) glucosinolates are converted to ITCs by an enzyme myrosinase (which is normally physically separated from the glucosinolate). Myrosinase is also present in enteric microflora and a significant proportion of ingested glucoraphanin (up to 20%) in humans can be converted to SFN in the intestine (Conaway *et al.* 2000). In humans, 2 hours after consumption of 100 g of broccoli, prepared as a soup, an average peak plasma concentration of 2.2 µM SFN was measured, with 7.3 µM achieved with a high-glucosinolate broccoli (Gasper *et al.* 2005).

SFN is a potent inducer of phase II (detoxification) metabolism via activation of the Nrf2 transcription factor, which binds to an antioxidant response element (ARE) in cognate genes including a variety of 'indirect' antioxidants (Dinkova-Kostova & Talalay 2008; Juge *et al.* 2007). Thus the action of SFN is protective from oxidative stress (Dinkova-Kostova & Talalay 2008). SFN has also been shown to modulate other signalling pathways including the MAP kinase (ERK, p38 and JNK), Akt-PI3K and NFκB pathways (Heiss *et al.* 2001; Jakubikova *et al.* 2005) in a cell-type dependent manner. There is also a literature suggesting that SFN and related compounds are inhibitors of HDACs both *in vitro* and *in vivo* (Dashwood & Ho 2007). In chondrocytes, high shear stress is pro-apoptotic and promotes ECM degradation. Shear stress (via JNK2 and c-Jun) induces cyclooxygenase-2 expression and activity and suppresses PI3K activity leading to repression of Nrf2-mediated transcription. This reduces the antioxidant capacity of chondrocytes under shear stress and contributes to their apoptosis. Addition of SFN, which induces Nrf2-mediated transcription, negates shear-mediated apoptosis (Healy *et al.* 2005). SFN has been shown to abrogate the IL-1 mediated induction of MMP-1, -3 and -13 in human articular chondroyctes. SFN was able to inhibit the activation of JNK and NFκB in these cells (Kim *et al.* 2009). In a mouse model of rheumatoid arthritis, SFN induced apoptosis in the proliferating synovium, albeit at high dose (peak plasma concentration of 200 µM following intraperitoneal administration) and showed efficacy in this model of inflammatory arthritis (Kong *et al.*2010). Dosing at this level every other day for 5 weeks showed no overt toxicity in this model.

These data encouraged the collaboration of our team at the University of East Anglia to investigate the role of SFN in OA under funding from the Biotechnology and Biological Sciences Research Council (BBSRC) Diet and Health Research Industry Club (DRINC), with data from animal models supported by Arthritis Research UK.

Results of the research

The effect of SFN was investigated in three laboratory models of OA involving cells, tissue explants and animals. This was followed by a proof-of-principle human study (Davidson *et al.* 2013).

The first model investigated was the expression and production of cartilage-degrading metalloproteinases by cartilage cells (chondrocytes). Primary human articular chondrocytes (HACs) were isolated from cartilage taken at joint replacement surgery and used at early passage in monolayer culture to maintain phenotype. The expression of cartilage-degrading metalloproteinases, *MMP1* and *MMP13* (collagen-degrading enzymes), *ADAMTS4* and *ADAMTS5* (aggrecan-degrading enzymes) acted as a surrogate marker of cartilage destruction. The expression of these genes was induced using a combination of IL-1 and oncostatin M and this was dose-dependently reduced by the addition of SFN across a 2.5 – 10 µM dose-range (measured by qRT-PCR). The levels of protein for MMP-1 and MMP-13 followed the same pattern. SFN could also inhibit IL-1-induced *MMP1* and *MMP13* expression in primary human synovial fibroblasts too (with no measurable expression of the *ADAMTS* genes). We investigated the role of the transcription factor Nrf2 in the repression of the metalloproteinase genes as a possible mechanism-of-action for SFN. Sulforaphane was able to induce expression of the haemoxygenase-1 gene in HACs, known to be dependent on Nrf2 and this was confirmed by siRNA knockdown. However, the repression of IL-1/OSM-induced *MMP1* and *MMP13* expression was not Nrf2 dependent. Interestingly in HACs, we could find no evidence of SFN acting as a histone deacetylase inhibitor. We measured the impact of SFN with or without IL-1 on the activation of mitogen-activated protein kinase signalling: ERK, JNK and p38 MAP kinases. The IL-1-induced phosphorylation of JNK and p38 MAPK was sustained for a longer time in the presence of SFN.

The clearest mechanism by which SFN acted in HACs was via the inflammatory NFκB pathway. Sulforaphane was able to delay the reaccumulation of IκBα following NFκB activation by IL-1, indicating inhibition of the pathway. This was reinforced by the inhibition by SFN of the IL-1 induction of an NFκB-responsive luciferase reporter. The IL-1-induced expression of a number of known NFκB-responsive genes was also dose-dependently

inhibited by SFN. However, neither phosphorylation of p65, nor the translocation of p65 to the nucleus were inhibited by SFN. Using electrophoretic mobility shift assays, we demonstrated that SFN added to cells at 10 μM could directly block the binding of a p50/p65 complex to its cognate response element, but if this SFN was added exogenously to the binding reaction, it was only effective at high concentrations (>100 μM). When 10μM SFN was added to HACs, this led to the rapid intracellular accumulation of SFN-GSH to a peak concentration of >200 μM by 10-15 minutes. At this concentration, exogenous SFN-GSH was able to block p50/p65 DNA binding and this was redox sensitive (to dithiothreitol), suggesting a direct mode of NFκB inhibition as previously described (Heiss *et al.* 2001).

In a second model, we measured the ability of SFN to block the release of extracellular matrix components from explants of cartilage. This utilised bovine nasal cartilage as a robust source of tissue from young animals which degrades reproducibly in culture in response to IL-1/OSM stimulation. In this tissue, breakdown of proteoglycan occurs over 2 days and that of collagen over 14 days. The addition of SFN into the culture, led to a dose-dependent inhibition in the release of glycosaminoglycan (as a measure of proteoglycan breakdown) at day 2, and hydroxyproline (as a measure of collagen breakdown) at day 14. The effective dose range was higher than in isolated cells, over 5-20 μM.

Funded by Arthritis Research UK, we also explored efficacy of SFN in a third model, the 'destabilisation of the medial meniscus' (DMM) murine model of OA. Mice were fed on a SFN-rich chow which delivered approximately 3 μmoles SFN/day and underwent resection of the medial meniscotibial ligament. This leads to osteoarthritic lesions in the cartilage at 1-3 months post-surgery. In mice fed the SFN-rich chow, there was a significant reduction in cartilage destruction at 12 weeks compared to the control diet. Microarray analysis of the mouse knee joints showed that a number of genes increased expression at day 7 after surgery in the control chow-fed mice, but not in the SFN chow-fed mice. Amongst these were *Col2a1* and *Col10a1*, both known to be involved in cartilage extracellular matrix and, in part, inducible by NFκB.

Within the DRINC-funded study, in order to begin to translate our findings from the laboratory and animal models to human we undertook a proof-of-principle trial in humans. This randomised, parallel-design trial involving 40 patients with knee OA undergoing total knee replacement. After a week-long washout period, where all patients consumed a low glucosinolate diet, participants were randomised to either a low (all known glucosinolates excluded from the diet) or high glucosinolate diet (consisting of 100 g per day of a high glucosinolate broccoli, 1.8 μmols g^{-1}) for 14 days ahead of surgery. Blood samples were taken immediately after the washout period, then after the 14 day dietary intervention prior to

surgery. At surgery, synovial fluid, cartilage and adipose tissue were collected. The primary endpoints of this study were the detection of isothiocyanates in plasma and synovial fluid, with secondary endpoints of synovial fluid proteome analysis, cartilage degradation *rate in vitro* and plasma biomarker analysis. This research has been completed and is currently being written up for publication.

These data support the utility of SFN in the prevention or treatment of OA. The proof of this requires an appropriately designed clinical trial of pain and function which we are currently pursuing. If efficacy is proven, then health professionals will have an evidence base on which to advise patients about dietary intervention for OA. Moreover, our research methodology provides a paradigm for understanding the role of other dietary factors in OA. If our research does show that an increased intake of SFN impacts upon pain and function, and potentially the structural progression of OA, this also highlights the need for a deeper knowledge of mechanism-of-action which would inform therapeutic strategies in OA.

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