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BASIC CALCIUM PHOSPHATE CRYSTALS AS A UNIQUE THERAPEUTIC TARGET IN OSTEOARTHRITIS

Linda C. Whelan [1], Maria P. Morgan [1] and Geraldine M. McCarthy [1,2,3]

[1] Clinical Pharmacology, Royal College of Surgeons in Ireland, Dublin 2, [2] Mater Misericordiae Hospital, Eccles St, Dublin 7, [3] Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

TABLE OF CONTENTS

1. ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis that occurs in humans. Despite its prevalence, the pathogenesis of OA is not fully understood. Intraarticular basic calcium phosphate (BCP) (an inclusive term for partially carbonate-substituted hydroxyapatite, octacalcium phosphate and tricalcium phosphate) crystals are implicated in OA and are associated with severe degenerative arthritis characterized by marked synovial hyperplasia, aggravated joint degeneration and large joint effusions. Their pathogenicity relates, at least in part, to their ability to stimulate cellular mitogenesis in a number of cell types including macrophages, porcine articular chondrocytes (PAC) and human fibroblasts (HF) and induce prostaglandin, cytokine and matrix metalloproteinase synthesis and secretion in HF and PAC. Identification of BCP crystals in OA joints remains problematic because of the lack of a simple and reliable analytic procedure. There is currently no drug available that prevents the formation or modifies the biological effects of BCP crystals. This review highlights the recent advances in our knowledge of BCP crystal deposition diseases and discusses the potential therapeutic strategies for BCP crystal-associated OA.

2. INTRODUCTION

Deposition of basic calcium phosphate (BCP) crystals is associated with a variety of age-related pathologies, including osteoarthritis (OA), calcific periarthritis and Milwaukee shoulder syndrome (MSS) (1). OA is characterized by severe cartilage degeneration as well as synovial lining hypertrophy, joint space narrowing and reactive bony sclerosis/osteophyte formation. Intra-articular BCP crystals are uniquely associated with OA, as they are not found in any other forms of arthritis including rheumatoid arthritis. Occurring in up to 70% of OA joints, the presence of BCP crystals correlates strongly with radiographic evidence of cartilage degeneration in knee joints and is associated with larger joint effusions when compared with joint fluid from OA knees without crystals (2, 3). BCP crystals induce a number of biological responses *in vitro*, which help explain the pathologic findings associated with their presence *in vivo*. These include their ability to induce mitogenesis and prostaglandin (PG), cytokine and matrix metalloproteinase (MMP) synthesis in human synovial fibroblasts (HSF), porcine articular chondrocytes (PAC) and human foreskin fibroblasts (HFF). In this report, we explore published evidence of the role of BCP crystals in OA with particular focus on the molecular mechanisms involved in BCP crystal-induced cell activation. We also highlight current potential therapeutic strategies for BCP crystal- associated OA.

3. STRUCTURE, LOCATION, SIZE, IDENTIFICATION AND ORIGIN OF BCP CRYSTALS

BCP crystals include partially carbonate substituted hydroxyapatite (HA), octacalcium phosphate and tricalcium phosphate. They occur in human synovial fluid (4, 5), synovium and hyaline cartilage (5) and are associated with a number of clinical manifestations

including Milwaukee shoulder/knee syndrome (MSS) (6), acute calcific periarthritis (ACP) and OA (3). Secondary deposition of BCP crystals has also been observed in a number of other clinical situations including chronic renal failure, following neurologic injury and after local corticosteroid injection (7). BCP crystals are ultramicroscopic in size and occur as spheroidal microaggregates, which range in size from 1 to 50 microns in diameter. Identification of BCP crystals in synovial fluids and joint tissues remains problematic because of the lack of a simple, reliable analytic procedure. Some methods that have been used to identify and characterize BCP crystals include ^{14}C ethane-1-hydroxy 1, -1-diphosphonate (^{14}C-EHDP) binding, alizarin red S staining, X-ray diffraction, atomic force microscopy, scanning and transmission electron (S/TEM), and Fourier transform infrared spectroscopy (FTIR).

^{14}C-EHDP binding has been used to detect crystals in synovial fluid. However, there are a number of pitfalls associated with this method such as limit of sensitivity (approximately 2 microgram/ml standard HA) (7). EHDP does not enter living cells readily and therefore it cannot detect intracellular apatite. In addition, the binding technique is semi-quantitative and the concentration of HA responsible for binding can be estimated only by comparison of observed binding to that obtained using standard HA. This is at best a rough approximation since the crystal surface area available for binding might be quite different depending on crystal size (8). Alizarin red S staining (ARS) is a highly sensitive technique used to detect calcium-containing crystals in synovial fluid. ARS has the greatest sensitivity for detection of CPPD because crystals are stained regardless of how weakly or strongly birefringent they may be. ARS cannot distinguish between amorphous types of calcium compounds; therefore, the different types of calcium compounds can be distinguished only when typical morphological features are present (9-11). Alizarin red stain does not stain corticosteroid crystals or mono sodium urate crystals (9). Frequent false positive results due to staining of calcium-containing compounds suggest that it lacks sufficient specificity to qualify for routine identification of BCP crystals (7).

X-ray diffraction identifies crystals. However, the quantity of crystals necessary for analysis exceeds that required for other techniques. Atomic force microscopy achieves subnanometer resolution of crystal surface topology and measurement of lattice unit cell dimension. S/TEM with high energy dispersive X-ray (EDX) and FTIR has identified BCP crystals deposits consisting of mixtures of HA, octacalcium and rarely tricalcium phosphate crystals (7). These methods, however, are generally only available in research setting, are expensive, and are unable to identify the site of origin of BCP crystals. Moreover, the methods of detection are operator dependent and therefore can lack accuracy and reproducibility. BCP crystals cannot be identified using conventional light or polarizing light microscopy, the two most common clinical methods for analysing synovial fluid samples for crystals, as they are either too small or too few in number to be identified by conventional techniques (12, 13). Therefore, there is still no simple, accurate method for their detection clinically (7, 14).

Histological examination of OA tissue revealed the deposition of HA in superficial regions of articular cartilage and synovium (15). HA deposits in the synovium resemble bone fragments or crystalline aggregates of HA found in the synovial fluid and cartilage (15). Electron microscopy identifies early stages of deposition as aggregates of HA crystals lying between the collagen bundles around the chondrocytes (15). The origin and mechanism of BCP production is only partially understood, but data suggest that articular cartilage-derived matrix vesicles and chondrocyte-derived apoptotic bodies are at least partly responsible for their formation. Matrix vesicles are cell derived, membrane-enclosed units that are found in articular cartilage and are associated with HA deposition (16). Apoptotic bodies (AB) are defined as membrane-enclosed structures of varying size that arise during apoptosis. During programmed cell death, AB are recognised by specific cell surface receptors and phagocytosed to prevent exposure of intact tissue to cell constituents that can cause inflammation. Articular cartilage, however, which is not vascularised and contains chondrocytes as its only cellular constituent, does not contain phagocytes that can ingest AB. Therefore, AB remain within the articular cartilage unless extracellular matrix is degraded and release into the joint space occurs (16).

Studies suggest that apoptotic bodies derived from chondrocytes treated with the nitric oxide donor, sodium nitroprusside, and cartilage-derived matrix vesicles precipitate calcium. ^{45}Calcium precipitation studies revealed that chondrocyte-derived matrix vesicles and chondrocyte-derived apoptotic bodies precipitate calcium in the presence of ATP (16, 17). *In vitro* studies indicate that MV derived from collagenase-digested normal PAC generates CPPD crystals (17). Human OA cartilage contains similar enzyme-rich vesicles capable of ATP-dependent calcification (18). Ultrastructural analysis of human OA cartilage has demonstrated apatite and apatite-like crystals in association with matrix vesicles (17, 19). PAC MV produce BCP crystals *in vitro* in the absence of ATP. Functional analysis studies revealed that both MV and AB produce similar levels of inorganic pyrophosphate (PPi), alkaline phosphatase activity, and pyrophosphate-generating nucleoside triphosphate pyrophosphohydrolase (NTPPH) (16).These findings suggest that chondrocyte-derived apoptotic bodies may contribute to the pathologic cartilage calcification in OA (16).

4. BCP CRYSTALS AND THE MITOGENIC RESPONSE

Synovial lining hypertrophy is commonly associated with BCP crystal deposition disease. BCP crystals can control the transition of cells from the G_0/G_1 to S-phase of the cell cycle and initiate proliferation by rendering fibroblasts competent to respond to progression growth factors such as insulin-like growth factor (20). BCP crystals at concentrations found in human pathological joint fluids stimulate mitogenesis in quiescent cultured HFF,

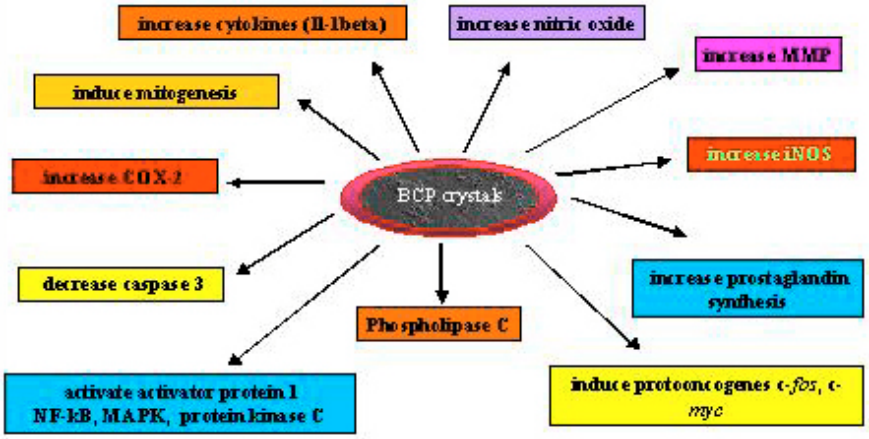

Figure 1: Some biological effects of BCP crystals.

murine 3T3 cells, PAC and canine synovial fibroblasts (21-24). Two distinct events appear necessary for BCP crystal-induced mitogenesis (Figure 1). Firstly, a rapid membrane-associated event in which activated phospholipase C leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 releases calcium from the endoplasmic reticulum, modulating the activities of protein kinases (PK) and proteases. DAG phosphorylates intracellular proteins such as growth factor receptors and activates the plasma membrane Na+/H+ antiporter, leading to cellular alkalinization, which has been correlated with the stimulation of DNA synthesis (25). A second event involves endocytosis and intracellular dissolution of BCP crystals. BCP crystal dissolution raises the intracellular calcium level, which is required for the full mitogenic response. Using the photoactive dye Fura-2AM as an indicator of intracellular calcium levels, BCP crystal treatment caused an immediate 10-fold increase in intracellular calcium over baseline level within 1min in HFF. This increase was derived from extracellular calcium, as it did not occur when calcium-free medium containing BCP crystals were added to fibroblasts. A further increase in intracellular calcium started within 60 min after stimulation and continued to rise up to at least 3h due to intracellular dissolution of phagocytosed crystals (25). In a similar set of experiments, the specific vacuolar pump inhibitor, bafilomycin A_1 (which raises intracellular lysosomal pH) inhibited intracellular crystal dissolution in HF and caused a dose-dependent inhibition of BCP crystal-induced mitogenesis (26). Current data suggests that endocytosis precedes dissolution of BCP crystals. HF incubated with ^{45}Ca-labeled BCP crystals in the presence or absence of ammonium chloride were pulsed with [^{3}H]-thymidine. Following trypinisation and fractionation on preformed Percoll density gradient, fractions were analyzed for incorporation of ^{45}Ca, [^{3}H]-thymidine and cell number. Cells containing ^{45}Ca-labeled crystals were heavily labeled with [^{3}H]-thymidine. Ammonium chloride decreased the amount of crystal endocytosis by 20% and inhibited mitogenesis by 90%. These findings suggest that BCP crystal-induced mitogenesis is preceded by endocytosis and dissolution in the acidic environment of phagolysosomes (27, 28).

Cytochalasin B (CB) inhibits phagocytosis in murine peritoneal macrophages. Murine macrophages were incubated with ^{45}Ca-labeled BCP crystals in the presence or absence of CB. In the absence of CB, endocytosis of crystals occurred continuously throughout incubation. Dissolution began within 3h and was linear up to 24h, when 30-50% of the added crystals had been solubilised. Addition of CB to cultured macrophages did not prevent BCP crystals' adherence to cells but inhibited their solubilization in a dose-dependent manner. When CB-treated cells were washed with EDTA, all adherent BCP crystals were removed, suggesting that BCP crystals were bound to the surface. In contrast, when untreated cells were washed with EDTA, only 10-20% of the adherent BCP crystals were removed suggesting that endocytosis of BCP crystals had occurred. These findings suggest that cell-association of BCP crystals is not sufficient for their dissolution and that endocytosis precedes solubilisation of BCP crystals by macrophages (29). Hamilton and others reported increased macrophage survival and DNA synthesis in response to BCP crystals, the latter response being potentiated in the presence of low concentrations of colony stimulating factor (CSF-1). Since CSF-1 is an important regulator in the development and function of macrophage lineages throughout the body, the presence of BCP crystals in OA joints may prolong the lifespan of macrophages. Such enhanced macrophage survival or proliferation may contribute to the synovial hyperplasia noted in crystal-associated arthropathies (3, 24, 28).

5. MATRIX METALLOPROTEINASE AND BCP CRYSTALS

Matrix metalloproteinase (MMP) are members of the family of zinc-dependent endopeptidases, which cleave one or several extracellular matrix proteins. MMP play an important role during embryo development, morphogenesis, connective tissue remodeling, tumour progression and arthritis. MMP are implicated in the degradation of articular cartilage matrix in OA. Collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) are at least partly responsible for the destruction of the extracellular matrix. Under normal conditions, most connective tissue cells produce low or undetectable levels

of MMP-1. In the presence of pro-inflammatory cytokines such as interleukin-1 (IL-1) and/or tumour necrosis factor alpha (TNF-alpha), phorbol esters or growth factors, fibroblasts synthesize high levels of MMP-1 (30-32). MMP-1 and MMP activity has been demonstrated in synovial fluid from the shoulder joints of some but not all MSS patients (33). BCP crystals induce MMP-1, -3, -8 and 92kDa gelatinase (MMP-9) production in HF and MMP-1 and collagenase-3 (MMP-13) production in PAC (22, 34-38). BCP crystal-induced mitogenesis is accompanied by induction and secretion of MMP-1 in HF (39). Since intracellular crystal dissolution is important in BCP crystal-induced mitogenesis, the role of crystal dissolution in BCP crystal-induced MMP production has been examined. Increasing lysosomal pH with bafilomycin A_1 (30nM and 50nM) to inhibit intracellular BCP crystal dissolution attenuated the mitogenic response of HF to BCP crystals by 66% but had no effect on BCP crystal-induced MMP-1 synthesis and secretion. These findings suggest that BCP crystal dissolution, which is important for mitogenesis, does not play a role in BCP crystal-induced MMP production and that lysosomotropic agents such as hydroxychloroquine would not dramatically alter the secretion of chondrolytic MMP *in vivo* (40). Therapeutically targeting lysosomes would only partially ameliorate the synovial proliferation seen in joints containing BCP crystals (40).

The activity of MMP can be inhibited by tissue inhibitor of matrix metalloproteinase (TIMP) including TIMP-1 and -2. Expressed in both normal tissue and tumour cells, TIMP-1 and-2 play a pivotal role in maintaining the balance between the extracellular matrix deposition and degradation in different physiological processes (41, 42). TIMP and MMP are differentially regulated by cytokines and/or growth factors in rabbit aorta smooth muscle cells and human bronchial epithelial cells (HBECs) (43-45). Numerous studies suggest that excess production of MMP compared with TIMP contribute to cartilage degeneration in OA and rheumatoid arthritis (RA) (46). Consistent with these findings, Bai and co-workers demonstrated increased MMP production and decreased TIMP-1 and -2 synthesis in HF following treatment with BCP crystals (34). The ability of BCP crystals to induce MMP synthesis and decrease TIMP synthesis, could, at least partly, lead to a net increase in MMP activity and explain matrix degradation associated with BCP crystal deposition (23).

6. PROSTAGLANDIN, CYCLOXYGENASES AND BCP CRYSTALS

Prostaglandins (PG), produced by most human tissues including human blood mononuclear cells, synovial cells, chondrocytes and fibroblasts (35, 37, 47-49) are involved in bone turnover and in modulating bone resorption in inflammatory arthritis (50-52). Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to PGH_2, the first committed step in the biosynthesis of prostanoids. Two COX isoforms have been identified in mammalian cells. COX-1 is constitutively expressed in most tissues and is responsible for supporting the level of prostanoid biosynthesis required for maintaining organ and tissue homeostasis but can be regulated by certain agonists, including acidic fibroblast growth factor, stem cell factor and dexamethasone (53, 54). COX-2 is the predominant COX isoform induced by pro-inflammatory agents including cytokines, endotoxins, lipid mediators and mitogens in a number of cell and tissue types including OA, and is involved in inflammation (55).

Recent studies demonstrate spontaneous and IL-1beta-induced COX-2 expression and PGE_2 production in OA articular chondrocytes and synoviocytes (55, 56). PG modulates glucocorticoid receptor expression in articular chondrocytes via the cyclic adenosine monophosphate (cAMP) pathway (57). Both PGE_2 and E_1 inhibit cytokine-induced MMP expression in human synovial fibroblasts, up-regulate the expression of insulin-like growth factor binding protein -3 and -4 via cAMP signaling pathway and stimulate aggregan synthesis in chondrocytes (58-60). Previous studies suggest that PGE_2 mediates the anti-proliferative effects of IL-1-induced nitric oxide on chondrocytes and suppresses cellular proliferation in RA synovial fibroblasts (61, 62). BCP crystals stimulate PGE_2 production via the phospholipidase A2/cyclooxygenase pathway in mammalian cells (35, 37, 63, 64). PGE_1 and E_2 inhibit BCP crystal-induced mitogenesis in HF. Unlike PGE_2, PGE_1 also inhibits BCP crystal-induced MMP-1 mRNA accumulation. Misoprostol, a PGE_1 analogue inhibits BCP crystal-induced mitogenesis and collagenase accumulation in HF (65). It is possible that BCP crystal-induced PGE production may modulate mitogenesis and MMP production by a feedback mechanism.

Morgan and co-workers demonstrated increased COX-2, COX-1, and IL-1beta mRNA levels in HF exposed to BCP crystals, leading to an increase in PGE_2 production. In this study, COX-2 was the primary isoform up regulated by BCP crystals with COX-1 mRNA occurring as a secondary effect. The induced COX-1 may contribute to the total pool of PG at sites of inflammation. Additionally, treatment of HF with PKC inhibitor bisinbolymaleimide I, phosphoinositide 3-kinase (PI3-K) inhibitor LY294002 and staurosporine inhibited BCP crystal-induced COX-2 expression in HF (64). In contrast, treatment with either the MEK1 inhibitor (PD98059) or the p38 mitogen-activated protein kinases (MAPK) inhibitor, SB203580, did not attenuate BCP crystal-induced COX-2 expression in HF suggesting that BCP crystal-mediated up-regulation of COX-2 expression does not require activation of either p42/p44 MAPK or p38 MAPK in this cell line (64).

7. SIGNALING PATHWAYS AND BCP CRYSTALS

One of the major signal transduction mechanisms involved in BCP crystal-induced mitogenesis includes inositol phosphate turnover (28). Rothenberg reported enhanced phospholipase C degradation of inositol phospholipid in rabbit synoviocytes by BCP crystals (66). Proto-oncogenes (such as c-fos and c-myc), MAPK, protein kinase C (PKC) and nuclear factor kappa B (NF-kB) play an important role in cell proliferation. BCP crystal-induced cell activation is associated with NF-kB induction in

balb/c/3T3 and HF and PKC activation and activator protein 1 (AP-1) induction in HF. BCP crystals activate p42 and p44 MAPK (67), increase PKC activity in PAC (22) and enhance phospholipase C activity in synovial cells (28). MAPK, in particular p42/p44 MAPK and PKC have also been shown to regulate cell proliferation by a mechanism involving c-fos and c-myc. Stimulation of quiescent Balb/c3T3 cells with BCP crystals resulted in the expression of c-fos within minutes and was maximum at 30min. Similarly, the induction of c-myc transcription by BCP occurred within 1h and was maximal within 3h (28). Mitchell and colleagues reported that depletion of PKC with tetradecanoyl phorbol acetate (TPA), a known analogue of DAG, blocks the induction of proto-oncogene activation and DNA synthesis (23). Since DAG or PKC are critical for BCP crystal-induced mitogenesis, depletion of PKC should waver the effects of BCP crystals. In PKC-deficient Balb/3T3 cells, BCP crystals inhibited c-fos and c-myc expression by over 80%. McCarthy and others demonstrated that inhibition of BCP crystal-induced mitogenesis by the PKC inhibitor staurosporine was accompanied by inhibition of BCP crystal-induced NF-kB and c-fos, but not c-jun mRNA (26). BCP crystal treatment induces phosphorylation of p42/44 MAPK, an effect that is inhibited by phosphocitrate (PC). Blocking of p42/44 MAPK signal transduction with an inhibitor of MEK1 (PD98059) reduces BCP crystal-induced cell proliferation.

Activated c-fos, c-jun and p42/44 MAPK are involved in MMP expression (1) (68). BCP crystal-induction of the MMP-1 promoter activation is associated with AP-1, polyomavirus enhancer activator-3 (PEA-3) and serum response factor (SRF) transcription factors and follows the Ras/Raf/MAPK kinase/c-fos/AP-1/MMP-1 signaling pathway (69). BCP crystals enhance the binding of proteins to an oligonucleotide containing the consensus binding sequence for AP-1 transcription factor (26). BCP crystals also activate early growth response gene Egr2 through a calcium-dependent protein kinase C-independent p42/p44 MAPK pathway. By RT-PCR BCP crystals caused a 8-fold increase in Egr2 transcription, which peaked at 24h. The induction was confirmed by transient transfection assays and could be inhibited by p44/p42 MAPK-specific inhibitor U0126 or calcium chelator TMB-8. Using the Mercury Pathway Profiling System (Clontech) to assess the activation of signal transduction pathways, induced Egr2 stimulated cell proliferation transcription factors c-fos, SRF and c-myc (70). The induction of Egr2 by BCP crystals may stimulate the activities of several transcription factors that are associated with cell proliferation further contributing to the inflammatory process associated with OA.

8. CYTOKINES AND BCP CRYSTALS

Pro-inflammatory cytokines such as TNF-alpha and IL-1, -6, -17 have been implicated in the pathogenesis of OA (71). Maintaining matrix homeostasis in the normal adult cartilage phenotype requires normal turnover of matrix components, primarily collagen and proteoglycan (72). IL-1beta has been implicated in the regulation of PG synthesis, MMP, nitric oxide production and COX-2. Current literature suggests that COX-2 regulation by IL-1beta involves activation of NF-kB, p42/p44 and PKC signaling pathway, all of which are induced by BCP crystals. Morgan and co-workers demonstrated that BCP crystals can directly induce COX-2 independent of IL-1beta and that BCP crystal-induced IL-1beta could contribute to the total induction of PGE_2, possibly through induction of both COX-1 and -2 (64).

TNF-alpha mRNA has been detected in OA-affected cartilage but not normal cartilage. OA-affected cartilage (in explant assays) have been shown to spontaneously release TNF-alpha and IL-8 in *ex vivo* conditions (73). Both TNF-alpha and IL-1 promote MMP release and inhibit the synthesis of proteoglycans and collagen synthesis by synovial cells and chondrocytes (72). TNF-alpha induces MMP-1 and MMP-3 production in adult PAC (74). Recent studies suggest that BCP crystals induce IL-1beta in HF and co-incubation of human osteoarthritic synoviocytes with BCP and TNF-alpha and/or IL-1 augment the expression of MMP-1 (75, 76). BCP crystals appear to induce fibroblast MMP-1 independent of an autocrine route via IL-1beta (76) .

9. POTENTIAL THERAPEUTIC AGENTS

The presence of BCP crystals in up to 70% of OA joints is well established. Our present understanding of the mechanisms of intra-articular calcification is incomplete. An important factor in the control of mineral growth is the presence of inhibitors or accelerators at the mineralization sites. Inhibitors of hydroxyapatite formation include Mg^{2+}, CO_3^{2-} (carbonate ion), pyrophosphates, adenosine triphosphate (ATP), cartilage proteoglycans and phospholipids (77). Two keystones are required for targeting by a drug. Firstly, inhibition of nucleation formation and the growth of the primary crystals and secondly if the growth of crystals cannot be controlled then formulating compounds that can target crystal-stimulated cellular responses (78).

9.1. Phosphocitrate (PC)

PC is a natural occurring compound found in mammalian mitochondria, human urine and crab hepatopancreas (79-82). PC prevents calcium phosphate precipitation in cells or cellular compartment maintaining high concentrations of calcium and phosphate by restricting transformations involved in the nucleation, growth and aggregation of many calcium salts including phosphate, oxalate and carbonate (83, 84). PC interferes with the biological effects of BCP crystals. PC inhibits BCP crystal-induced MAPK (85, 86), blocks NO-induced calcification of cartilage and chondrocyte-derived apoptotic bodies (87), inhibits adenosine triphosphate-induced calcinosis, inhibits MMP-1,-3 and -8 in HF (88) and delays the progression of ankylosis in murine progressive ankylosis (MPA) (89). PC also inhibits BCP crystal-induced c-fos and c-jun expression, and mitogenesis in HF *in vitro*. Recent studies suggest that PC inhibits BCP crystal stimulated endocytotic activity of cells. In this study, various cell lines were treated with BCP crystal-pCMV-luciferase plasmid aggregates or with pCMV-luciferase plasmid followed by

BCP crystals. Analysis of luciferase activities demonstrated that BCP crystals stimulated the endocytosis of DNA plasmid by cells. Since endocytosis of DNA plasmid was stimulated by BCP crystals, increasing amounts of PC were added to the cells prior to the addition of BCP crystals-pCMV-luciferase plasmid mixtures/aggregates. As expected, PC inhibited the BCP crystal-stimulated endocytosis of pCMV-luciferase plasmid (90). PC inhibits mitochondrial and cytosolic accumulation of calcium in kidney cells *in vivo* (79) and does not produce any significant toxicity in rats or mice when given in doses up to 150 μmol/kg/day. PC also prevents aortic calcification at 1 μmole/day/rat (83). PC (10-1000 μM) blocked both ATP-dependent and -independent mineralization in articular cartilage vesicles (91).

Insight into the mechanism of action of PC has been achieved by exploring its interaction with crystal faces of calcium oxalate monohydrate (COM) crystals, struvite crystals, hydroxyapatite (HA) and CPPD using both experimental evidence and molecular modeling. Binding of PC to COM crystals, struvite crystals, HA and CPPD changes the zeta potential of the crystal surface, leading to total cessation of crystal growth and thus interfering with the crystal-plasma membrane interactions that lead to cellular responses (81, 92-95). Although PC has proven to be effective in inhibiting the biological effects of BCP crystals, PC is clinically unavailable at present.

9.2. n-sulfo-2-amino-tricarballylate (SAT) and citrate versus PC

SAT, a PC analogue and citrate have been examined as possible modulators of the biological effects of BCP crystals. Like PC, SAT and citrate suppress BCP crystal-induced CREB serine 133 phosphorylation and activation of p42/p44 MAP kinases, block BCP mineralization in articular cartilage vesicles in a dose dependent fashion (91) and inhibit aortic calcification in rats (83). Neither SAT nor citrate are as efficient inhibitors as PC for calcium containing crystals. PC suppresses signal transduction pathways at lower concentrations (10^{-3}-10^{-5} mM as opposed to 1mM for SAT and citrate) and inhibits not only BCP mineralization but also CPPD mineralization. PC prevents aortic calcification at 1 μmole/day/rat compared to SAT at 10 μmole/day/rat (83). Recent studies suggest that because of its similarity to citrate, PC may bind to and use the same transport protein as citrate for passage through the phospholipid bilayer, thus making it readily available at the site of undesirable formation of calcium-containing crystals (81).

The strong binding affinity that PC possesses for growing crystals accounts for the superior inhibitory capacity compared with SAT and citrate and is believed to result from both its multinegative charges and natural stereochemistry (93, 96). PC, with both its PO_4 and carboxylate groups contributing, binds more favorably than citrate (possessing carboxylates only) with calcium ions distributed on the (- 1 0 1) and (0 1 0) surfaces of calcium oxalate monohydrate crystals, thus blocking growth. Also, SAT has one less charge than PO_4 and the presence of a nitrogen atom reduces its capacity to position itself correctly for crystal face interaction (67).

9.3. MMP inhibitors

The importance of MMP in arthritides including OA has already been established. The development of compounds that selectively target OA, are orally bioavailable and have a low toxicity profile still continues. Early MMP inhibitors of physiological or chemical origin including SC-44463, batimastat (BB-94) and ilomastat (GM-6001), TIMP (see section 5) and alpha2-macroglobulin (present in the synovial fluid and serum of normal and OA patients) have been identified. However, their usage is limited by low oral bioavailability and inability to penetrate tissue, especially cartilage, due to the size of the protein/molecule (97, 98).

Second-generation compounds capable of targeting the MMP active site, MMP expression and synthesis, which are orally available, are currently being developed. Marimastat and primomastat (AG-3340) represent the first orally available MMP inhibitors to be characterized and have been shown to act on a range of MMP including MMP-1, -2,-3,-7 and -9 (marimastat) and MMP-2, -3, -9 and -13 (primomastat). Bisphosphonates (99) and antibiotic compounds such as doxycycline have shown potential as MMP inhibitors. A bisphosphonate, alendronate inhibits MMP-3 activity in RA (100). Doxycycline inhibits MMP-1, -8 and MMP-13 in TNF-alpha-stimulated OA chondrocytes, MMP-8 activity and synthesis in RA synovial fibroblasts and decreases MMP-1 and -13 in OA chondrocytes (101, 102). Agents inhibiting MMP synthesis include glucocorticoids (dexamethasone) and retinoids. Dexamethasone inhibits MMP-1 and -3 induced by IL-1 in OA cartilage explant cultures and bovine articular chondrocytes (103, 104). PC inhibits BCP crystal-induced MMP production and secretion in HF. The use of MMP inhibitors in OA could, in the presence or absence of BCP crystals, protect against progressive and chronic degeneration of articular tissue.

9.4. Cytokines inhibitors

Natural and /or physiological inhibitors capable of directly counteracting the binding of cytokines to cells or reducing the pro-inflammatory level have been identified such as IL-1 receptor antagonists (IL-1Ra), soluble receptors (type I and type II soluble IL-1 receptor) and anti-inflammatory cytokines i.e. IL-4 and -10. Type II IL-1 receptor inhibits IL-1beta-induced NO and PGE_2 in human OA chondrocytes and synovial cells and inhibits IL-1beta-induced MMP production in human OA chondrocytes (105, 106). Similarly, soluble receptors (type I and type II soluble IL-1 receptor) inhibit NO production in OA chondrocytes. IL-1 inhibited proteoglycan synthesis that could be reversed by type II soluble IL-1 receptor (105). Recent studies suggest that BCP crystals induce IL-1beta in HF and co-incubation of human osteoarthritic synoviocytes with BCP and TNF-alpha and/or IL-1 augment the expression of MMP-1 (75, 76). Since type II IL-1 receptor inhibits IL-1beta-induced MMP production, which is induced by BCP crystals, type II IL-1 receptor may have

potential as a treatment for BCP crystal deposition disease (105, 106).

9.4.1. Diacerein (IL-1 inhibitor)

Diacerein is a anthraquinone compound currently under clinical evaluation as a disease modifying OA drug. Diacerein is well tolerated by most patients and presents few side effects. A randomized, double-blinded, placebo-controlled, 3-year study concluded that diacerein retards the progressive decrease in joint space width in OA patients (107). Diacerein is as effective as non-steroidal anti-inflammatory drugs (NSAIDs) in the relief of joint pain and is effective even after treatment withdrawal (108). *In vitro* studies have shown that diacerein augments hyaluronan synthesis in synovial cells and prevents cartilage breakdown by reducing proinflammatory cytokines in mice. Rhein, an active metabolite of diacerein, suppresses IL-1alpha-induced proteoglycan degradation and down-regulates the gene expression of proMMPs-1 and -3 and the production of proMMP-1, -3, -9 and -13 in cultured articular chondrocytes (109). In human OA chondrocytes, both diacerein and rhein inhibit IL-1beta induction of iNOS synthesis and activity and prevent IL-1beta-induced NF-kB activation and iNOS expression in bovine chondrocytes (110, 111). Diacerein and rhein inhibited IL-1beta in OA chondrocytes and OA synovium and decreased the number of IL-1 receptors (IL-1R) on OA chondrocytes. This effect was mediated through a reduction in the level of the type I IL-1R as shown by experiments using a blocking monoclonal antibody against this receptor type. Both agents also markedly reduced the IL-1 induced synthesis and expression of stromelysin 1 (112). In experimental dog OA cartilage, diacerein inhibits chondrocyte DNA fragmentation, caspase-3 and iNOS (113). Rhein inhibits IL-1beta-induced activation of MEK/ERK pathway and DNA binding of NF-kB and AP-1 transcription factors in bovine articular chondrocytes (114). BCP crystals activate various transcription factors including NF-kB, MAPK and AP-1 and modify signal transduction pathways *in vitro*. Since diacerin and rhein inhibit activation of a number of transcription factors including NF-kB and AP-1, and genes, which are induced by BCP crystals, diacerin and rhein may have potential as disease-modifying drugs for BCP crystal deposition disease.

9.5. COX-2 inhibitors

Selective COX-2 inhibitors are commonly used to control pain and inflammation in OA. COX-2 inhibitors act by reducing PG synthesis through the inhibition of COX-2 activation (115). NSAIDs inhibit PGE_2 production and thus partially ameliorate some aspects of the inflammatory state. BCP crystals have been shown to induce PGE_2 production in HF. Aspirin, a nonselective COX inhibitor and NS398, a selective COX-2 inhibitor inhibited BCP crystal-induced PGE_2. Morgan demonstrated a 21.4-fold and 8.7-fold increase in PGE_2 production at 4h and 30h respectively in HF following stimulation with BCP crystals. At 4h, the increase in PGE_2 was inhibited by the addition of both NS398 and aspirin. At 30h, NS398 no longer significantly inhibited BCP crystal-induced PGE_2 production, but aspirin continued to inhibit to control levels (64). These findings suggest that BCP crystals are an important amplifier of PGE_2 production through induction of both COX-1 and -2 enzymes and thereby may contribute to the severity of BCP crystal-associated OA. The use of COX inhibitors to reduce inflammation and pain may also protect against cartilage damage in BCP crystal-associated OA.

9.6. Glucosamine sulphate and HCL

Glucosamine (2-amino-2-deoxy-D-glucose), a glycosaminoglycan (GAG) constituent in cartilage matrix and synovial fluid plays an important role in the formation and repair of cartilage. Glucosamine has received attention as a putative disease modifying and chondroprotective agent for OA (116, 117). A 3-year randomised, double-blinded placebo-controlled study demonstrated that oral glucosamine sulphate (GS) retards the radiographic progression of OA (117). GS prevents structural changes in the joints of patients with knee OA with a significant improvement of symptoms (117, 118). *In vitro*, glucosamine stimulates synovial cell production of hyaluronic acid (HA) (119) and stimulates PG synthesis (120), GAG, aggrecan mRNA and protein levels (116) and decreases protein kinase C (PKC) (121) and cellular PLA_2 activity (121) and matrix metalloproteinase (MMP)-3 production in cultured human OA articular chondrocytes (116, 121). Glucosamine suppresses neutrophil function in synovial tissue, inhibits IL-1beta-induced NF-kB activation and inhibits the generation of superoxide radicals *in vitro* (122). Both glucosamine sulfate and HCL prevent experimentally induced cartilage degradation *in vitro* (123). The mechanism of action of glucosamine in OA is unknown, but its activity is currently attributed to stimulation of GAG, collagen synthesis, proteoglycans and hyaluronic acid (122). *In vitro* studies have shown that glucosamine HCL inhibits BCP crystal-induced mitogenesis, IL-1beta, TNF-alpha and COX-2 in HF (124). Glucosamine HCL could retard the progression of OA by modifying the biological effects of BCP crystals and thus supports the potential use of glucosamine as a treatment for BCP crystal deposition diseases.

10. CONCLUSIONS AND PERSPECTIVES

This review highlights the recent advances in our knowledge of BCP crystal deposition diseases and discusses the potential therapeutic strategies for BCP crystal-associated OA. *In vitro* studies reveal that synovial lining cells and chondrocytes can be targeted by pro-inflammatory cytokines, whose actions are further enhanced by BCP crystals. BCP crystals induce mitogenesis, proto-oncogene expression and prostaglandin, cytokine and matrix metalloproteinase (MMP) synthesis and secretion and activate multiple signal transduction pathways, which differ depending on the cell type studied. The lack of a simple, accurate and reliable analytic procedure hinders the identification of BCP crystals in OA joints. Current treatment for OA is predominantly focused on relief of pain and maintenance of quality of life. No known drug prevents or treats BCP crystal deposition. Although potential drugs have being identified, treatment approaches capable of protecting articular tissues from the effects of BCP crystals remain to be developed. An

improved understanding of the molecular mechanisms by which BCP crystals induce joint inflammation and degeneration is essential for the rational prevention or reversal of the consequences of calcium-containing crystal deposition.

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Send correspondence to: Dr Geraldine M. McCarthy, Clinical Pharmacology, Royal College of Surgeons in Ireland, 123 St. Stephens Green, Dublin 2., Ireland, Tel: 353-1- 8858753, Fax: 353-1-8858689, E-mail: gmccarthy@rcsi.ie