ABSTRACT: A prominent pathological feature of osteoarthritis (OA) and rheumatoid arthritis (RA) is degradation of articular cartilage and the release of molecular fragments of the extracellular matrix into synovial fluid. These entities are antigenic and can stimulate an autoimmune response and synovitis. Oral tolerization of arthritic animals or RA patients with type II collagen (Col-II), the most abundant protein of cartilage, has been shown to attenuate disease activity but similar studies on other cartilage-derived antigens has not been previously described. In this review we report our animal and human clinical studies with the divalent salts (Ca, Mg, Zn) of Peptacan that are novel glycosaminoglycan (GAG)-polypeptide complexes isolated from bovine hyaline cartilage. The divalent metal Peptacan derivatives were evaluated for their oral dose dependent (10-300mg/kg) anti-inflammatory and antiarthritic activities in the rat collagen induced (CIA) and adjuvant induced (AIA) arthritis models using tolerogenic and prophylactic/therapeutic protocols. The topical anti-inflammatory property of Peptacan was also investigated under double blinded controlled conditions in human subjects using a standard skin erythema assay. Ca, Mg and Zn Peptacans were orally active at 15 mg/kg in suppressing the onset and severity of arthritis in the rat CIA model using the tolerogenic protocol. In the rat CIA model calcium Peptacan (CaP) at 20 mg/kg was active using tolerogenic or prophylactic protocols. Histologically, joints from CaP treated animals showed a reduction of white cell infiltration and extent of cartilage/bone destruction. Similar findings were observed using a rabbit arthropathy model. The topical antiinflammatory of a Peptacan cream formulation was demonstrated using human subjects confirming the pharmacological activity of these nutraceuticals in the human species.

KEY WORDS: Peptacans, Oral Tolerization, Arthritis, Inflammation.

Abbreviations Used: CaP = Calcium Peptacan; CDA = Cartilage derived antigens; CPC = Cetyl pyridinium chloride; ChS = Chondroitin sulfate; GAG = Glycosaminoglycan; GALT = gut associated lymphoid tissue; HA = hyaluronan, hyaluronic acid; iNOS = inducible Nitric oxide synthase; MgP = Magnesium Peptacan; NCP = non collagenous proteins; NSAIDs = Non steroidal antiinflammatory drugs; OA = Osteoarthritis; PC = Peptacan Cream; PG = Proteoglycan; RA = Rheumatoid arthritis; SC = Sorboline Cream; SLS = Sodium lauryl sulfate; ZnP = Zinc Peptacan.

INTRODUCTION

Diseases and disorders of the musculoskeletal system are a major cause of morbidity and financial burden to public health systems worldwide. However, therapeutic options for their management are limited, particularly for the most common musculoskeletal problem, osteoarthritis (OA). Treatments for this disease mainly rely on analgesics, corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs). Apart from their palliative activity, NSAIDs down-regulate cartilage and bone repair processes (anabolism) (Brandt 1993; Huskisson 1995). They can also cause a variety of serious adverse systemic effects, the most common being gastro-intestinal bleeding and kidney and liver toxicities (Lichtenstein 1995; Aithal 2007). They can also cause a variety of serious adverse systemic effects, the most common being gastro-intestinal bleeding and kidney and liver toxicities (Lichtenstein 1995; Aithal 2007). In recent years a new range of NSAIDs that selectively inhibited the cyclo-oxygenase-2 enzymes (Coxibs) have become available (eg, Celebrex®, Valdecoxib®, Vioxx®, Prexige®). Although the Coxibs certainly reduced gastro-intestinal irritation and bleeding the incidence of other adverse effects remained or were increased (Graham et al., 2005; Moore et al., 2007). In the case of rofecoxib (Vioxx®), thrombogenesis was enhanced causing premature death or precipitating cardiovascular disorders in some OA patients, leading to its withdrawal from worldwide sale (Graham et al., 2005; Moore et al., 2007). In August 2007 Australia’s medicines regulator (Therapeutic Goods
Hyaline cartilage is a quite remarkable connective tissue that provides a flexible support, protects and nourishes its constituent cells but is capable of recovering its shape after physical deformation. Structurally it is composed of a three-dimensional fibrillar network of type II collagen fibrils co-polymerised with other minor collagens (types V, VI, IX and XI collagens) embedded in a hydrated proteoglycan (PG) rich matrix. Type II collagen accounts for over 90% of the collagen content of adult cartilage, the other collagens (types V, VI, IX and XI collagens) embedded in a hydrated proteoglycan (PG) rich matrix. Type II collagen fibrils are covalently cross-linked the type II collagen fibrils, the type IX collagen acts as a link protein, and the type XI collagen is covalently cross-linked to the type II collagen fibrils (Pimentel et al., 1992). The other dominant components of hyaline cartilage are the proteoglycans. Aggrecans are macromolecular aggregates of 20–50 chondroitin sulfate-rich proteoglycan monomers (PG-subunits) covalently bound to a hyaluronan (HA) backbone. The PG subunits of aggrecan consist of a protein core to which up to 100 sulfated glycosaminoglycan (GAG) chains are covalently attached (Figure 1). The major GAG constituents of the PG subunits of cartilage are chondroitin-4-sulfate (Ch-4-S) and chondroitin-6-sulfate (Ch-6-S). Another, but less abundant sulfated-GAG, keratan sulfate, is largely located adjacent to the G2 region of the PG core protein (Figure 1) (Hardingham et al., 1992). Cartilage also contains a significant number of non-collagenous proteins (NCP) other than those that are constituents of aggrecans. Some of these proteins, such as the cartilage oligomeric protein (COMP), the cartilage matrix protein (CMP) and thrombospondin are present in appreciable quantities in cartilage but our knowledge of their functional roles is still limited (Heinegård and Pimentel 1992).

**FIGURE 1.** A schematic representation of the structure of the large ChS rich proteoglycan monomer of cartilage aggrecan. Figure modified from Hardingham et al., 1992.

Osteoarthritis, Rheumatoid Arthritis and the role of Cartilage-derived Antigens (CDA)

The aetiology of OA is clearly multi-factorial with such factors as ageing, mechanical, hormonal, genetic and even environmental determinants all contributing to varying degrees (Felson et al., 2000). OA emerges as a clinical syndrome when these etiological determinants provoke sufficient cartilage damage and synovial inflammation to cause pain and impair joint function. Excessive ‘turnover’ of the cartilage extracellular matrix in OA joints is manifested by the release of fragments of PGs, minor collagens and NCP into synovial fluid. This is a consequence of chondrolysis, mediated by endogenous proteinases (Pelletier et al., 2001; Hedbom and Hauselmann 2002).

Many of these chondrolytic products are of sufficient size, yet clearly distinct from normal ‘self’ constituents, to behave as antigens. Therefore, when these cartilage-derived antigens (CDA)
pass into/through the adjacent synovial tissues and enter the draining lymphatics they can trigger (or sustain) a systemic auto-allergic response. This is indicated by the appearance of autoreactive T lymphocytes within the synovial membrane (Yuan et al., 2003; Sakkas and Platsoucas 2007). Furthermore, the presence of CD3-positive and some CD4- and CD8-positive T cell aggregates within the intima and sub-intima of the OA joint capsule implicates a Th1 cell-mediated type of immunity (Yuan et al., 2003; Sakkas and Platsoucas 2007). This has been formerly considered to be the characteristic feature of synovial tissues removed from joints of patients with rheumatoid arthritis (RA).

RA is an autoimmune disease, the unfortunate consequence of extrinsic and intrinsic triggering of T and B cell-mediated immune responses in genetically pre-disposed individuals. The aggressive synovitis, then established, is accompanied by the release of cytokines, proteinases and very reactive free radicals that can all promote the degradation of cartilage and other joint tissues. The ensuing breakdown products pass into the joint synovial fluid and may be sequestered within the synovial membrane (Pelletier et al., 2001; Hedbom and Hauselmann 2002; Yuan et al., 2003; Sakkas and Platsoucas 2007; Holmdahl et al., 1993). Thus, although the original causes of OA and RA may be quite distinct, both arthropathies show the common pathological events of cartilage destruction and synovial inflammation, albeit of different intensities. Nevertheless, the release of the CDA into the synovial cavity and the subsequent systemic response to these potential auto-antigens are significant in maintaining the progression of both diseases.

Type II collagen (Coll-II) is the most potent and abundant CDA of articular cartilage and has been widely studied (Holmdahl et al., 1993; Cremer et al., 1993). The minor cartilage collagens (Type IX and Type XI) (21,22), cartilage oligomeric protein (COMP) (Carlsen et al., 1998), chondrocyte matrix proteins (Anchorin CII) (Alsalameh et al., 1990), aggrecan proteoglycans (PG) (Glant et al., 1998; Wang and Roehrl 2002), PG core and link proteins (Glant et al., 1998; Banerjee and Poole 1992) and chondrocyte heat shock proteins (Yuan et al., 2003) are also strongly immunogenic and can also elicit antibodies in patients with OA and RA. Furthermore, the ability of Coll-II and PG core protein to provoke vigorous immune responses when administered systemically has facilitated their use as experimental arthriogens in animal models of RA (Cremer et al., 1993; Glant et al., 1993; Myers et al., 1997; ‘t Hart et al., 1993).

Oral Tolerance

The body’s ability to become unresponsive to antigens derived from either the host (self-antigens) or from the environment (allergens) has been known for a very long time. The immune system selectively acquires this state of tolerance during growth and development. After exposure to the antigen, a state of systemic immunological hypo-responsiveness or non-responsiveness is induced in clones of immunoregulatory T cells (Weiner et al., 1994; Weiner and Komagata 1998). The most common method of inducing immuno-tolerance is to administer the antigen orally so that it gains access to the mucosal immune system within the Gut-Associated Lymphoid Tissue (GALT) (Weiner et al., 1994; Weiner and Komagata 1998). The precise mechanisms by which oral tolerance is first established and then sustained are still the subject of much debate. There is however a general consensus that at least three major pathways are activated in the GALT (Weiner et al., 1994; Weiner and Komagata 1998; Schulze-Koops and Kalden 2001). Administering a high dose of antigen can lead to clonal deletion and/or anergy of T helper cell sub-sets (Th1 and Th2 cells). With low levels of the antigen the T suppressor cells (Th2 and Th3 cells) may be preferentially selected to secrete anti-inflammatory cytokines including IL-4, IL-5, IL-10 and TGF-beta that also protect articular cartilage (Weiner et al., 1994; Weiner and Komagata 1998; Schulze-Koops and Kalden 2001). Thus, orally administering small amounts of CDA to a host could generate clones of specific T suppressor cells. After migrating to the joint these cells release anti-inflammatory cytokines that non-specifically suppress lymphoid cell-mediated synovial inflammation thereby reducing cartilage and bone destruction (Weiner et al., 1994; Weiner and Komagata 1998; Schulze-Koops and Kalden 2001). This down-regulation of joint inflammation by CDA-specific T suppressor cells is known as ‘antigen driven bystander suppression’ (Kagnoff 1996). This antigen-targeted i.e. tissue-specific mechanism has the potential for generating new therapeutic strategies to treat arthritic diseases.

Such a therapeutic approach has been validated in laboratory animals by the suppression of the onset and severity of experimental arthritis after oral tolerization with Coll-II (Myers et al., 1997; ‘t Hart et al., 1993; Kagnoff 1996). Human clinical studies in which RA patients were administered small oral doses of either bovine or chicken type II collagen has produced conflicting outcomes (Trentham et al., 1993; Sieper 1996; Barnett et al., 1996; Choy et al., 2001).

The differences between the responses by arthritic animals and RA patients, after oral administration of Coll-II, may be partly explained by the difficulty of identifying the correct oral dose to achieve bystander suppression, particularly in groups of patients with heterogeneous disease status and different rates of enteric absorption/digestion. The problem is probably further compounded by ethical requirements that the clinical investigators co-administer, throughout the duration of a clinical trial, appropriate doses of analgesics and/or disease-modifying medications to continue to control the pain and other debilitating symptoms experienced by RA patients enrolled in such experimental studies. For example, corticosteroids and some NSAIDs are generally used for this purpose and it is known that these agents, apart from exerting an independent anti-arthritis effect, may break collagen-induced immuno-tolerance (McKown et al., 1999; Whitehouse 2005).

Many non-collagenous components of cartilage are also immunogenic (Carlson et al., 1998; Alsalameh et al., 1990; Glant et al., 1998; Wang and Roehrl 2002; Banerjee and Poole 1992), being released in appreciable amounts during the breakdown of cartilage in both RA and OA (Pelletier et al., 2001; Hedbom and Hauselmann 2002; Yuan et al., 2003;
Sakkas and Platsoucas 2007). Therefore it is rather surprising that no studies have been reported examining their potential as oral tolerogens for the treatment of rheumatic diseases. These putative toleragens would essentially de-sensitize the patient to cartilage auto-antigens released from their arthritic joints. To address this question we had first to devise methods for isolating those breakdown products from the cartilage extracellular matrix that would be generated by the action of the endogenous proteinases. Once this goal was achieved we could then determine the oral anti-arthritic/anti-inflammatory/anti-aging activities of these fragments in animal models of arthritis ultimately to be followed by human clinical studies. These investigations eventually led to the development of the Peptacans, which are the subject of this review.

Isolation and Chemistry of Peptacans

It has been known for more than 30 years that exhaustively extracting hyaline cartilage with concentrated solutions of chaotropic solvents, such as 4M guanidine hydrochloride, separated the bulk of the aggrecans and non-covalently bound NCP from the extracellular matrix (Mayaes et al., 1973). However these native macromolecules were not the components normally released from cartilage into the synovial fluid of arthritic joints by the action of endogenous proteinases (Ilic et al., 1992; Lohmander et al., 1995). Moreover, using high molarity chaotropic solvents also disrupted the cartilage cells (chondrocytes) with the simultaneous co-extraction of DNA and other intracellular components. We therefore examined methods to activate the endogenous proteinases of cartilage so they might selectively degrade the extracellular matrix producing molecular fragments analogous to the (putative) antigens normally released from cartilage in arthritic joints.

Maintaining diced hyaline cartilage tissues at 37ºC in aqueous buffers at pH 4.5 generally released into solution more than 60% of their content of sulfated glycosaminoglycans (S-GAG) and 30% of the NCPs after 16-24 hours incubation (Shen and Ghosh 2005). The efficiency of this auto-chondrolytic process depended on both the anatomical location and the animal species from which the cartilage was taken. This procedure did not disrupt the chondrocytes, as indicated by the absence of DNA in the autolysis media (Shen and Ghosh 2005). The type II collagen ultra-structure of the cartilage was also preserved, as shown by histological examination of the tissue before and after autolysis (Figure 2).

Preparations obtained by this method contained molecular fragments from the cartilage extracellular matrix of diverse composition and structure. Therefore we introduced the term ‘Peptacans’ to avoid using more cumbersome chemical nomenclatures to describe them. The calcium, magnesium and zinc salts of Peptacan were preferred as end products being non-hygroscopic but readily soluble in water. They offered the added advantage of providing a nutritional source of Ca, Mg or Zn, essential metal ions known to be beneficial for treating OA and related musculoskeletal diseases (Whang 1987; Campbell 2001; Fox et al., 2001; Gums 2004; Moe 2005). These particular salts of Peptacans are hereafter abbreviated as: CaP for calcium Peptacan, MgP for magnesium Peptacan and ZnP for zinc Peptacan.
Peptacans and rheumatic diseases. 87

Agarose gel electrophoresis of CaP, MgP and ZnP from tracheal cartilage confirmed the similar molecular size and charge densities of the CaP and MgP preparations noted previously (Shen and Ghosh 2005). They were both of smaller size than ZnP, as indicated by their respective Rf values (Figure 3). A commercial sample of chondroitin sulfate with a molecular weight of 17.5 kDa provided a molecular weight marker for this comparison (Figure 3). ChS as normally prepared by exhaustive proteolytic digestion of tracheal cartilage with the plant proteinase papain consists of a single ChS chain covalently attached to a short peptide stub. Chromatographic studies of Peptacan salts using a gel exclusion column (calibrated with sulfated polyanions of known molecular weight) had earlier shown that CaP and MgP contained, on average, two ChS chains attached to a polypeptide core. By contrast, the larger ZnP contained molecular fragments with 3 CS chains attached to a polypeptide core (Shen and Ghosh 2005).

ZnP was confirmed as containing a larger GAG-polypeptide complex than the corresponding GAG complexes in CaP and MgP by chromatography on a Superdex S-200 gel exclusion column. However, chromatography of the ZnP also revealed the presence of single ChS chains eluting after the larger GAG-peptide complex (Figure 4). These single ChS components were also detected as a weaker staining band ahead of the main band for ZnP on the agarose electrophoretic gel (Figure 3). The sulfated-GAGs present in CaP and MgP eluted in similar fractions from the Superdex S-200 column ahead of the reference ChS (Figure 4), supporting their earlier designation as sulfated GAG complexes composed of at least 2 ChS chains covalently attached to a polypeptide (Shen & Ghosh 2005). The absence of detectable amounts of keratan sulfate in these Peptacan preparations suggests that the GAG-peptide complexes in CaP and MgP were derived by proteolysis of the core protein of the PG subunit within the CS1–CS2 domain (Figure I).
in CaP (or MgP) compared to the ZnP show that the nature of the buffer used to activate the endogenous proteinases could significantly alter the protein/polypeptides released into solution. Protein sub-fractions separated from the GAG-P complexes by the CPC procedure described are now being investigated using proteomic techniques to establish their molecular identity.

**FIGURE 5.** Molecular size distribution of the protein/polypeptide fractions in Calcium Peptacan (CaP), Magnesium peptacan (MgP) and Zinc Peptacan (ZnP) relative to Cytochrome-C (mw = 13kDa) and bovine Testicular hyaluronidase (MW = 43kDa). Samples were eluted from a Superdex S-200 in a 0.5 NaCl buffer and fractions monitored for protein using the Bicinchoninic acid (BCA) assay (Shen and Ghosh 2005). $V_0$ = column void volume, $V_t$ = column total volume.

Our previous studies, using a range of different class-specific protease inhibitors, had shown that the Cathepsin proteinases were active in the chondrolytic process used to prepare Peptacans (Shen and Ghosh 2005). However, the matrix metallo proteinases Birkedal-Hansen et al, 1993 and aggrecanases (ADAMTS-4 and ADAMTS-5) (Sandy et al., 2006), recently recognised as prominent mediators of cartilage matrix turnover, may also be implicated since the addition of specific inhibitors of the cathepsin to the autolysis media did not completely abolish the release of Peptacans (Shen and Ghosh 2005).

**Pharmacological studies of Peptacans in animal models of arthritis**

The anti-arthritic activity of the Peptacans has now been evaluated in some well-established rodent models of arthritis i.e. with chronic inflammation. Most of the studies were undertaken using the rat collagen-induced arthritis (CIA) model (Ghosh et al., 2006) and also the rat mycobacterial (adjuvant)-induced arthritis (AIA) model (Ghosh et al., 2006; Prakken et al., 2002). An acute inflammation/arthritis model, induced by a single intra-articular injection of a polycation (PC) (polylysine) hyaluronan complex into rabbit joints, (Page-Thomas 1977; Cambray et al., 1981; Smith et al., 1994) was also used to study the pharmacological activity of CaP.

In the rat arthritis models both toleragenic and prophylactic/therapeutic protocols were used. For the toleragenic protocol Peptacan(s) were administered orally to the animals for 6 or 7 days before injecting an arthritogen. For the prophylactic/therapeutic protocols, Peptacans were given daily immediately following the induction of arthritis and up to the time of sacrifice, normally 15-21 days after inoculating the arthritogen. In several of these animal studies the anti-arthritic effects of ChS or D-glucosamine were also evaluated for direct comparison with Peptacans. Female albino Wistar and Hooded Wistar rats were used for these experiments; all animal studies being carried out under protocols approved and supervised by the Animal Ethics Committee, Griffith University, Brisbane, Queensland.

Peptacans, dissolved in deionised water were administered daily for 6 days before inducing the CIA (on day zero). The CIA was induced by a single intradermal inoculation around the tail base of bovine collagen type-II in acetic acid emulsified with incomplete Freund’s adjuvant (Ghosh et al., 2006). For some of these experiments arthritis expression was amplified (Whitehouse 2003) by adding sodium thiocyanate to the drinking water (1 mg/ml) throughout the study.

Animals were observed daily for possible adverse effects or change in body weights. On days 15-21 after inoculating the arthritigen, signs of arthritis were measured by the increase in rear paw thickness (determined with a micrometer) relative to non-arthritic controls. Indices of the forepaw inflammation and for an overall arthritis score (on scales of 0→4+) were also assigned. The arthritis score included clinical impressions of wellbeing or otherwise e.g. mobility, grooming, behaviour, curiosity, piloerection, low grade fever, etc. The mean scores and SD of the mean were determined on days 15 and 21 for each Peptacan-treated group - the results being compared

**FIGURE 6.** Coomassie blue stained polyacrylamide (4 – 12%) gel electrophoretogram of the Peptacan proteins/polypeptides. Lane A shows the molecular weight markers (SeeBlue), Lane B, 20 microL of Peptacan proteins at 100microL/mL, Lane C 10 microL of Peptacan proteins at 100microL/mL.
statistically with signs of arthritis in the untreated (control) group using the null hypothesis.

Induction of tolerance

Figure 7 summarises the results obtained using this tolerogenic protocol for evaluating MgP, ZnP and CaP when given prior to administering the arthritigen at doses of 15mg/kg. In the untreated control group, the joint swelling and arthritis scores were comparable to those reported in previous studies using this rat model (Ghosh et al., 2006; Ghosh et al., 2005). An interesting finding was that the mean signs of arthritis on day 15 after the MgP treatment were less than those after treatments with the corresponding ZnP and CaP suggesting the magnesium derivative was more potent. However, these differences were not statistically significant and no longer apparent by day 21 when the CIA activity was more advanced (Figure 7). At this time all three Peptacan salts significantly reduced the arthritis scores. Rear paw inflammation was significantly less than the non-treated control group (Figure 7) only after MgP and CaP treatments.

Musculoskeletal disorders are most frequent in the elderly who are often taking medications for other associated age-related problems e.g. type II diabetes or cardiovascular diseases. For this population there would be inherent further benefits in using CaP or MgP for the management of their arthritis. A regular intake of calcium ions is essential to maintain bone integrity but clinical studies have also shown that bone mineral density is determined by the dietary magnesium intake: deficiencies in this metal being associated with a higher incidence of bone fractures in the elderly (Lech 2001; Saito et al., 2004). Low magnesium levels (hypomagnesaemia) are also associated with other serious disorders including atherosclerosis, cardiovascular disorders, depression, allergies, inflammation and arthritis (Whang 1987; Campbell 2001; Fox et al., 2001; Gums 2004; Moe 2005; Seelig 2006; Lech 2001). Hypomagnesaemia in the general population is not uncommon, particularly in individuals with gastrointestinal and renal problems. Calcium, magnesium and zinc may also be depleted by ingestion of aluminium and certain drugs, particularly diuretics. A low dietary intake of these elements may be related to the increased consumption of highly processed fatty foods in modern societies (Whang 1987; Campbell 2001).

Used as a toleragen, CaP induced a dose-dependent suppression of CIA development over the range of 3.3-200 mg/kg when animals were assessed on Day 15 (Ghosh 2006). At the lowest dose of 3.3 mg/kg CaP had no significant effect on the arthritis score or front paw inflammation (Figure 8) but nevertheless there was significant improvement for all parameters relative to the ChS group administered 20 mg/kg.

Figure 8. Suppression of arthritic disease activity in the rat collagen induced arthritis (CIA) model by oral administration of Chondroitin sulfate (ChS)(20 mg/kg) or Calcium Peptacan (CaP) at 3.3, 10, or 20 mg/kg for 7 days prior to immunisation with the arthritigen (tolerogenic protocol). * P < 0.05, ** P < 0.01. P < 0.05 was considered to be statistically significant relative to saline treated controls.

FIGURE 7. Effects of Calcium Peptacan (CaP), Magnesium Peptacan (MgP) and Zinc Peptacan (ZnP) when administered orally at 15 mg/kg for 6 days prior to initiating collagen induced arthritis (CIA) in female Wistars (tolerogenic protocol). Panel A: assessment on day 15 post CIA induction. Panel B: assessment on day 21 post CIA induction. P < 0.05 was considered to be statistically significant relative to saline treated controls.

Similar dose-related findings for CaP were observed using the CIA rat model when CaP was given daily after the arthritigen, doses of 10 and 20 mg/kg significantly reducing arthritis expression compared to both the untreated controls and the ChS-treated group (Figure 9). The failure of ChS to suppress arthritis development or reduce its severity in the CIA model was consistent. 

Prophylactic suppression of arthritis development

FIGURE 8. Effects of various oral doses of CaP and ChS on the severity of arthritis on day 15 using the rat CIA tolerogenic protocol! * P < 0.05, ** P < 0.01. P < 0.05 was considered to be statistically significant relative to saline treated controls.
with other reports that could only demonstrate a therapeutic effect of this nutraceutical in animals at oral doses exceeding 1000mg/kg (Omata et al., 2000). In the more aggressive rat AIA model, CaP only showed prophylactic anti-arthritic activity at the high oral dose of 200mg/kg (Figure 10). Both glucosamine hydrochloride (GmHCl) and glucosamine sulfate (GmSO4) were inactive at this high dose. Failure to observe any response to glucosamine in the AIA model could be related to the quantity and frequency of dosing in this study; since it has been reported that glucosamine administered at 300 mg/kg for 22 days was effective when tested in the AIA model (Hua et al., 2005). The suppressive effects of CaP of inhibiting arthritis development in both the CIA and AIA rat models at doses between 10-200 mg/kg was also reflected by the preservation of articular cartilage and bone in the joints of treated animals (Ghosh et al., 2006). Furthermore, leukocyte infiltration of the synovial cavity of CaP treated animals by white cells was much reduced - a finding suggesting that the Peptacans could indeed moderate, even prevent, inflammatory cell mediated joint damage by the process of oral tolerization. Collectively, these findings highlight the beneficial effects of CaP in the context of chronic inflammation.

Effects of orally administered Calcium Peptacan (CaP) and Chondroitin Sulfate (ChS) in a rabbit polycation (PC) model of arthritis

The rationale, methodology and arthropathology of the PC rabbit model of proliferative arthritis has been described in detail elsewhere (Page-Thomas 1977; Cambray et al., 1981; Smith et al., 1994). We used this model to determine the prophylactic effects of oral CaP on the development of synovitis, cartilage breakdown and peripheral blood white cell populations (Shen and Ghosh 2005). We also monitored hyaluronan biosynthesis by synovial fibroblasts isolated from the pre-inflamed joints of the rabbits. The severity of synovial inflammation and cartilage injury was assessed using both histological and immuno-staining techniques. Chondroitin sulfate was used as a comparator treatment for this study. The ex vivo biosynthesis of HA by the joint synovial cells was of particular interest as previous in vitro studies, using synovial cells taken from joints of patients with OA, indicated that CaP might up-regulate HA biosynthesis (Shen and Ghosh 2005).

The principal non-protein component of the joint synovial fluid is HA, synthesized mainly by the type-B lining cells (synovial fibroblasts). Inflammatory mediators released from activated synovial macrophages, infiltrating leukocytes and lymphocytes residing in inflamed joints can all modify HA biosynthesis (Dahl and Husby 1985; Konttinen et al., 2000). These same mediators may also increase vascular permeability within the synovium, facilitating the infusion of plasma proteins and extra fluid into the joint cavity effectively decreasing the concentration of HA within...
the synovial fluid (SF). Such dilution, together with impaired HA biosynthesis, reduces the rheological functions of SF including the lubrication and protection of articular cartilage, particularly during weight-bearing activities (Ghosh and Guidolin 2002).

For the CaP study male New Zealand White rabbits, approximately 4 months of age, were used and randomly divided into 5 experimental groups. The acute joint inflammation was induced in all groups by the intra-articular injection of a sterile poly lysine-HA complex (PC) (1.0 mL) prepared as described previously Smith, Ghosh, Numata, Bansal 1994. An equal volume of sterile isotonic saline was injected into the contralateral knee joints as the internal control (on day 7).

Group A served as non-drug-treated controls while the remaining groups received orally, for 14 days commencing on day –7, either ChS (300 mg/kg) (Group B), CaP (300 mg/kg) (Group C), CaP (200 mg/kg) (Group D) or CaP (100 mg/kg) (Group E).

On day 15 rabbits were first anaesthetised, their blood collected and subjected to a Percoll gradient centrifugation to separate the mononuclear and polymorphonuclear (neutrophil) leukocytes. Animals were then sacrificed and synovial membrane from all knee joints harvested, synovial fibroblasts isolated and established in cell culture to determine the ex-vivo biosynthesis of HA, using the protocol shown in Figure 11 (Ghosh et al., 2005). The whole patellae and suprapatella mound of attached synovium were also collected and processed for immunohistological studies to characterise the extent of pannus formation, the levels of inducible nitric oxide synthase (iNOS) and presence of the stable marker for NO production, nitro-tyrosine, produced by the chondrocytes.

The results of these studies confirmed that the in vitro stimulation by CaP of the biosynthesis of HA, noted previously in OA human synovial fibroblasts (Shen and Ghosh 2005) also occurred when CaP was administered orally to rabbits. The effects of CaP or ChS on the biosynthesis of HA were monitored by the incorporation of radioactivity into newly synthesised HA, elaborated into the media by the cultured synovial fibroblasts from the PC-injected joints. The magnitude of radioactivity incorporated into HA was assessed from the difference in areas under the void volume elution profiles obtained on Superose-6 chromatography before and after digestion with Streptomyces Hyaluronidase, an enzyme that specifically degrades macromolecular HA into disaccharide fragments. This is exemplified by the chromatographic profiles for media obtained from cultures of fibroblasts obtained from CaP (300 mg/kg)-treated and untreated control (Group A) animals shown in Figure 12. The mean±SEM area under the V₀ elution profiles obtained for the radioactively labelled HA produced ex vivo from the untreated animals was 46±25 units, compared to 103±17 units for the CaP (300 mg/kg)-treated animals. This higher incorporation of radioactivity into HA for the CaP group cells was statistically significant relative to the non-treated control group (P = 0.017). Animals treated with lower doses of CaP showed higher HA synthesis than the untreated controls but the differences were not statistically significant.
The extensive synovial inflammation provoked in the rabbit joints by the intra-articular injection of the HA-PC complex was very apparent from histological examination of the synovium. Sections showed massive leukocyte infiltration, connective tissue proliferation, hyperplasia and neovascularization. In the non-drug treated control group these inflammatory events were further characterised by the presence of an extensive pannus originating at the joint margins, invading and degrading the adjacent articular cartilage (Figure 13). In the high dose CaP treated group synovitis was not abrogated but pannus formation was reduced and the structural integrity of cartilage largely preserved (Figure 13). Furthermore, the expression by chondrocytes in the patella cartilage of inducible nitric oxide synthase (iNOS) was particularly mitigated in the high dose CaP-treated animals (Figure 13). This finding was reinforced by the low level of immunohistochemical staining for nitrotyrosine in chondrocytes from joints of the CaP-treated animals (Figure 14). Nitrotyrosine is formed by the chemical interaction of nitric oxide free radicals with intra-cellular proteins (Kobayashi et al., 2001). Interleukin-1 is a major initiator of nitric oxide production (and other noxious oxy-radicals), synovial inflammation and tissue destruction within arthritic joints (Taskiran et al., 1994). Earlier in vitro experiments had shown...
that CaP abrogated the inhibitory effects of IL-1 on PG biosynthesis (52). This could indicate that the observed reduction in chondrocyte expression of iNOS in cartilages of animals treated with CaP might be due to its modulating effects on interleukin-1 activation of chondrocytes (Taskiran et al., 1994). However, without direct evidence to support this view it must remain speculative.

CaP also modulated blood levels of both neutrophils and mononuclear leukocytes in these joint-inflamed rabbits in a dose dependant fashion (Figures 15 and 16). In contrast, ChS at 300g/kg had minimal effect on these leukocytes populations (Figures 15 and 16). Once activated, both mononuclear and polymorphonuclear leukocytes provide an abundant source of destructive proteinases, oxygen-derived free radicals and pro-inflammatory cytokines; all of which could contribute to the synovial inflammation and cartilage destruction seen in joints of these animals. The question whether the reduction of pannus formation and down-regulation of nitric oxide synthase within chondrocytes by oral administration of CaP was due to systemic immunosuppression or a direct effect on the cartilage cells was not addressed in the present study but it is clearly an important issue requiring further research.

FIGURE 14. Photomicrograph of histological sections of patella cartilage and adjacent synovium from joints of the rabbit PC arthritis model showing the extensive synovial inflammation and pannus formation accompanied by brown antibody staining in chondrocytes for the nitric oxide synthase oxidation product, nitrotyrosine. The section shown in Panel A is from a PC injected non-drug treated rabbit joint processed and stained with the primary and secondary bodies for nitrotyrosine as described in reference (Kobayashi et al., 2001). Note as in FIGURE 13 the strong brown non-specific staining of synovial tissue but specific staining for the antigen in chondrocytes, particularly in the cartilage superficial zone. Magnification X400. Panel B shows a representative section from a PC injected joint of a ChS treated animal where staining is reduced in intensity relative to Panel A but still present. Magnification X400. Panel C is a section from a PC injected joint of an animal given CaP (300mg/kg) showing reduced staining for nitrotyrosine in chondrocytes of the superficial cartilage zone relative to the non-drug treated control (Panel A). Panel D is a negative control section from a PC injected joint that confirms the non specific binding of the nitrotyrosine antibody to synovial tissues (brown) but light staining of cartilage and chondrocytes.

FIGURE 15. Histogram showing the mean values (+ SEM) for neutrophils in the blood of animals from the ChS (hatched column) and CaP treated rabbit groups. Note the reduced level of neutrophils in blood from the 200 and 300mg/kg CaP treated groups (solid columns).

FIGURE 16. Histogram showing the mean values (+ SEM) for mononuclear cells in the blood of animals from the ChS (hatched column) and CaP treated rabbit groups. Note the reduced level of mononuclear cells in blood from the 200 and 300mg/kg CaP treated groups (solid columns).
Evaluation of Peptacans as a topical anti-inflammatory agent in human subjects

The drugs most frequently used to treat contact dermatitis and other forms of dermal inflammation are the topical corticosteroids. However, chronic use of these agents is very often accompanied by untoward side effects, the most common being pharmacological resistance (i.e. requiring higher doses to achieve a therapeutic effect), skin atrophy and thinning arising from the down regulation of proteoglycan and collagen biosynthesis by dermal fibroblasts (Oikarinen et al., 1998; Schoepe et al., 2006).

It was considered that the Peptacans might offer a safer alternative to corticosteroids for the topical treatment of certain dermatological conditions in which auto-immunity and inflammation were implicated. Accordingly, preliminary studies were undertaken to evaluate the topical anti-inflammatory activity of a Peptacan preparation applied over an 8-day period using sodium lauryl/dodecyl sulfate (SLS/SDS) as the irritant to provoke erythema. The SLS patch test is a validated method for assessing the anti-inflammatory activity of novel topically applied agents, the objective data obtained being amenable to statistical analysis (Fuchs et al., 2005). The Peptacan preparation was formulated as a 5% ‘active’ in a standard cream base containing glycerine [glycerol], diisopropyl adipate, octyl salicylate, isopropyl adipate, isopropyl palmitate, stearic acid, sodium ascorbate, cyclomethicone, xanthan, carbomer, allantoin, with preservatives and water as further excipients (Ghosh 2007).

The same cream base, without the Peptacan, was used as the placebo. The experimental protocol is shown in Figure 17 and was conducted under double blind conditions in 10 healthy volunteers. Neither the test subject nor the assessor of the erythema score were aware which cream formulation (active or placebo) had been applied. The subjects’ left or right arms were randomly assigned to receive either the placebo or the Peptacan cream (active), each subject effectively providing their own control.

Skin inflammation was induced by applying Park-Davis ReadiBandage occlusive patches (2cmx2cm) impregnated with varying concentrations of SLS over the range of 0.25% to 2.0% (w/v). Similar test sites on the inside of both forearms of each subject were selected and covered with a series of the prepared patches to elicit a graded SLS response. All patches were removed 24 hours after application. One hour later all sites were assessed for the severity of erythema (baseline values), using the scoring system (Figure 17).

After this assessment, the coded placebo or the active creams, were then applied by the subjects twice daily, morning and evening, for eight days to the erythemic sites on either the right or left arms. The response to treatments was assessed for the various erythema sites 2, 4, and 8 days later using the same grading system (Figure 17). The magnitude of the mean changes from baseline erythema scores for all sites after the Peptacan or placebo (vehicle) applications is shown Figure 18. Although the mean decrease in inflammation from baseline on days 2, 4 and 8 for the Peptacan treated group was always greater than that obtained after applying the placebo, it was only statistically significant on days 2 and 4 after inducing the erythema (Figure 18).
The data from this preliminary study demonstrated that Peptacans exhibit anti-inflammatory activity in human subjects, when formulated in a suitable transdermal vehicle for topical use. The mechanism(s) of action of CaP in the context of dermal inflammation have yet to be clarified but they might include:

- Presentation of connective tissue antigens to skin Langerhans/dendritic cells to induce bystander suppression of local inflammation;
- Direct blockade of receptor-mediated transduction of pro-inflammatory ligands on dermal fibroblasts and keratinocytes;
- Stimulating connective tissue cell proliferation and biosynthesis of structural components of the extracellular matrix (Shen and Ghosh 2005; Ghosh 2007).

CONCLUSIONS

The studies described herein provide evidence within the context of chronic inflammation and connective tissue degeneration that Ca, Mg and Zn derivatives of Peptacan(s) are potential disease modifying agents. Moreover, the origin of these preparations from hyaline cartilages, which have historically constituted part of the normal human diet, support the contention that they can be considered free of adverse side effects. The Peptacans therefore offer a safer alternative to many of the traditional pharmaceuticals used for the treatment of RA and OA. Indeed, unpublished toxicity studies undertaken with CaP by an independent laboratory have demonstrated an oral LD50 in rats greater than 2 grams/kg with no adverse organ pathology noted in any of the animals at the time of necropsy.

This combination of safety and efficacy, at least as demonstrated in the animal experiments and limited human studies presented here, offers a new paradigm for the future management of chronic musculoskeletal disease/disorders which generally require daily therapeutic intervention spanning several decades.

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CONFLICT-OF-INTEREST STATEMENT

Professor Peter Ghosh is Director and shareholder in the Institute of Nutraceutical Research. Susan Shimmon, Nancy Wilson-Ghosh and Professor Michael Whitehouse have no potential conflict of interest.

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