The effect of drugs commonly used in the treatment of equine articular disorders on the activity of equine matrix metalloproteinase-2 and 9

P. D. CLEGG*
M. D. JONES* &
S. D. CARTER*†

**Department of Veterinary Clinical Science and Animal Husbandry, †Department of Veterinary Pathology, University Veterinary Teaching Hospital, University of Liverpool, Leahurst, Neston, S.Wirral L64 7TE, UK*

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Loss of articular cartilage, which is the most important pathological lesion occurring in osteoarthritis, has been shown to be enzymatically mediated. The matrix metalloproteinases (MMPs) are a group of enzymes which have been implicated in this degradation of articular cartilage matrix. The use of pharmacological agents to inhibit this catabolic process in the joint is a potential route for therapeutic intervention.

The gelatinase MMPs, MMPs-2 and 9, were purified by affinity chromatography from equine cell cultures. The ability of phenylbutazone, flunixin, betamethasone, dexamethasone, methylprednisolone acetate (MPA), hyaluronan, pentosan polysulphate and polysulphated glycosaminoglycan (PSGAG) to inhibit equine MMPs-2 and 9 were assessed by two degradation assays. Whilst some agents did have direct effects on MMP activity, these effects were only obtained at concentrations which were unlikely to be achieved for any length of time *in vivo*. It is improbable that any pharmacological agent, currently used in the horse, has a significant effect on gelatinase MMP activity.

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P. D. Clegg, Department of Veterinary Clinical Science and Animal Husbandry, University Veterinary Teaching Hospital, University of Liverpool, Leahurst, Neston, S. Wirral L64 7TE, UK.

INTRODUCTION

The matrix metalloproteinases (MMPs) are a group of zinc-dependant endopeptidases which are involved in both the normal physiological turnover and the pathological degradation of extracellular matrix. The MMPs are fundamental in the pathogenesis of joint disease as the breakdown of articular cartilage matrix, which occurs in diseases such as osteoarthritis (OA), is thought to occur primarily by enzymatic degradation (Brandt & Mankin, 1986). Much work has been undertaken examining the role of other MMPs in the enzymatic degradation of articular cartilage in joint disease over the last 10 years, but only recently have the gelatinase MMPs, MMPs-2 and 9, been examined for a possible role in joint disease in humans (Hirose*et al.*, 1992; Koolwijk *et al.*, 1995), dogs (Coughlan *et al.*, 1995) and the horse (Clegg *et al.*, 1997a).

Matrix metalloproteinase-2 and 9 possess degradative action against both collagens and proteoglycans in cartilage matrix. Matrix metalloproteinase-2 and 9 are able to degrade aggrecan in a similar manner to the other MMPs (Fosang *et al.*, 1992), as well as cartilage link protein (Nguyen *et al.*, 1993) and decorin (Imai *et al.*, 1997). Experiments indicate that MMP-2 can degrade type I triple helical collagen (Aimes & Quigley, 1995), as well as minor cartilage collagens such as type XI collagen (Smith *et al.*, 1991; Hirose *et al.*, 1992). It has recently been shown in an *in vitro* model of cartilage breakdown that collagen loss from cartilage could be prevented by addition of a specific gelatinase inhibitor (Kozaci *et al.*, 1997). These enzymes may also be important in joint disease as they have a role in activation of other MMPs such as MMP-3 (stromelysin) and MMP-13 (Birkedal-Hansen *et al.*, 1993; Knauper *et al.*, 1996), as well as potentiating the action of interstitial collagenases by a synergism (Murphy & Docherty, 1992). Matrix metalloproteinase-2 and 9 levels are elevated in synovial fluid in equine joint disease (Clegg *et al.*, 1997b). Matrix metalloproteinase-9 is produced by equine chondrocytes, peripheral blood monocytes and polymorphonuclear neutrophils and MMP-2 is produced by equine chondrocytes and synovial fibroblasts (Clegg *et al.*, 1997a). Thus, MMPs-2 and 9 are potential targets for therapeutic intervention in the treatment of joint disease.

If the degradative action of these enzymes can be inhibited, the enzymatic loss of articular cartilage may be halted and the balance within the matrix could be altered from catabolism to anabolism. It is established that loss of articular cartilage is the

limiting factor for complete restoration of athletic function following injury (McIlwraith & Vachon, 1988). The degradative process in cartilage could be inhibited in three ways; either by direct inhibition of the enzymes; by inhibition of enzyme synthesis and release from cells; or by increase in synthesis and release of local natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Vincenti *et al.*, 1994). It has previously been shown that equine MMP caseinase activity can be inhibited by polysulphated glycosaminoglycan (PSGAG), a drug commonly used in equine osteoarthritis therapy (May *et al.*, 1988). In this study, the effect on the degradative actions of purified equine MMPs-2 and 9 of various drugs, commonly used in the treatment of equine articular injury, was assessed to determine if inhibition of these enzymes could be an important mode of their beneficial action in osteoarthritis. The drugs tested were: the non steroidal anti-inflammatory drugs (NSAIDs); flunixin meglumine and phenylbutazone; the corticosteroids: betamethasone, dexamethasone and methylprednisolone acetate (MPA); and the glycosaminoglycans: hyaluronan, PSGAG and pentosan polysulphate.

MATERIALS AND METHODS

Production of equine MMP-2

MMP-2 was produced as described by Clegg *et al.* (1997a) from cell culture of equine synovial fibroblasts in serum-free media.

Production of equine MMP-9

MMP-9 was produced as described by Clegg *et al.* (1997a) from short-term culture of equine peripheral blood polymorph neutrophils stimulated with phorbol myristate acetate.

Purification of MMP-2 and 9

The enzymes were purified from the cell culture supernatants by affinity chromatography using gelatin-Sepharose (Clegg *et al.*, 1997a). In each case, the proteolytic activity was demonstrated by gelatin zymography, and the purification of the enzyme was assessed with SDS-PAGE and silver staining to demonstrate the protein present within the sample.

Gelatin degradation ELISA

A degradation assay was used to assess the breakdown of gelatin by MMPs (Clegg *et al.*, 1997b; Clegg *et al.*, 1998). This assay measures the amount of gelatin remaining on the plate following the incubation with a test enzyme and the effects of a putative inhibitor. Purified MMPs-2 or 9 was added to each well of a gelatin-coated ELISA plate (1:100) in a volume of 50 μL, combined with 50 μL of test drug solution diluted in the same buffer and the plate incubated at 37°C for 2 h. The concentration of enzyme was chosen which would cause 100% degradation of the bound gelatin during the incubation period. The samples were removed, the plate washed with PBS/Tween (0.05%) and a rabbit anti gelatin antisera added (1:4000) and incubated at 37°C (1 h). This was removed, the plate washed with PBS/Tween, and anti rabbit IgG-alkaline phosphatase conjugate added (1:4000) and incubated for 37°C (1 h). The conjugate was removed, the plate washed with PBS/Tween, and Sigma 104 phosphatase substrate (1 mg/mL) in glycine buffer (pH 10.3) added and the colour change read at 405 nm between 30 and 60 min later. Four replicates of each sample were assayed.

Fluorescein labelled casein degradation assay

This assay was based on the protocol described by Beynon (1991). Briefly, 2 g of casein was dissolved in 100 mL of sodium carbonate buffer, pH 9.5. Fluorescein isothiocyanate (FITC) (100 mg) was added and the solution mixed at room temperature (20°C) for 1 h. The FITC-casein was dialysed several times against 0.05 M Tris/HCl pH 8.5. Residual unbound FITC was removed and the FITC-casein exchanged into the assay buffer of Tris/HCl (0.05 M), $CaCl_2$ (0.005 M), Brij 35 (0.05%) pH 7.6, by passing the FITC-casein through a Sephadex G-25 column (Amersham Pharmacia Biotech, St Albans, Herts) previously equilibrated and eluted with the assay buffer. The elution profile was monitored spectrophotometrically at 280 nm, and the protein peak was collected and stored at −20°C until required. Upon assay, FITC-casein solution (10 μL) was placed in a microfuge tube and mixed with 40 μL of purified MMPs-2 or 9 enzyme solution (1:50 dilution) and 50 μL of drug solution dissolved in assay buffer to make a final reaction volume of 100 μL. The concentration of enzyme was chosen which would cause 100% degradation of casein during the incubation period. Four replicates of each reaction were assayed. The solution was incubated for 24 h at 37°C. The reaction was stopped by mixing 200 μL of 5% (w/v) trichloroacetic acid to each reaction mixture and incubating for 1 h at 4°C to allow any proteins to floculate. Precipitated proteins were then sedimented by centrifugation at 10 000 ***g*** for 10 min. The supernatant (100 μL) was added to 2.9 mL of 0.5 M Tris/HCl, pH 8.6, and the fluorescence of the mixture measured at an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

Drug concentrations

The concentration ranges of phenylbutazone (1.2–150 μg/mL), flunixin meglumine (0.32–40 μg/mL), betamethasone (1–125 μg/mL), sodium hyaluronate (0.375–3.0 mg/mL) and PSGAG (2.5–20 mg/mL) were based on those used *in vitro* by May *et al.* (1988), and were chosen to encompass concentrations which may be achieved following articular or systemic administration in the horse. Further doses of betamethasone (500–1000 μg/mL) were also used to allow direct comparison with the levels of dexamethasone used. The concentrations of MPA and dexamethasone used encompassed a range which may be seen following intra-articular administration in the horse using the same calculations as employed by May *et al.* (1988). Pentosan

polysulphate concentrations were based on concentrations which had previously been used in an *in vitro* experiment (Collier & Ghosh, 1989).

Statistical analysis

Comparisons of the effects of different drugs and the effects of concentrations were analysed by Mann–Whitney *U*-test (non parametric).

RESULTS

Matrix metalloproteinase-2 and 9 were both able to degrade gelatin in the gelatin-degradation ELISA, but MMP-9 alone was shown to have caseinolytic activity in the fluorescein-labelled casein degradation assay. The results for MMP-2 and 9 activity and their inhibition assayed by gelatin-degradation ELISA (Fig. 1a-h), and by fluorescein-labelled casein degradation assay (MMP-9) (Fig. 2a-h) showed similar drug effects. Matrix metalloproteinase-2 and 9 were not inhibited at all by phenylbutazone (Figs 1a and 2a), flunixin (Figs 1b and 2b), betamethasone (Figs 1c and 2c), hyaluronan (Figs 1f and 2f), or pentosan polysulphate (Figs 1g and 2g). There was significant ($P < 0.05$) inhibition of MMPs-2 and 9 by PSGAG at concentrations above 10 mg/mL (Figs 1h and 2h). Matrix metalloproteinase-9 was not inhibited at all by dexamethasone nor MPA, though MMP-2 was significantly ($P < 0.05$) inhibited by dexamethasone at the highest dose tested (1 mg/mL), and by MPA at the higher concentrations tested (1.2 and 5 mg/mL) (Fig. 1d, e).

DISCUSSION

At present there are numerous therapeutic options available for the treatment of joint disease in the horse, though it is often unclear whether these agents are acting in just a symptomatic manner, or whether they are altering the course of the disease process. In OA, net loss of articular cartilage matrix is occurring due to both increased degradation and decreased synthesis of matrix macromolecules. It is proposed that one mechanism by which drugs may alter the course of the disease process is by affecting the degradative process by direct inhibition of matrix degrading enzymes. A drug which alters the progression of the disease could be classified as a disease modifying osteoarthritis drug (DMOAD) (Dieppe, 1995).

There is difficulty in studies such as this, in identification of appropriate drug concentrations. For many drugs there are few studies demonstrating what concentrations of drugs are achieved intra-articularly following therapy, nor what concentrations are required for therapeutic effect. Furthermore some of these drugs, such as the NSAIDs, are highly plasma bound and much of the drug is thus not available therapeutically (May & Lees, 1996). Moreover, other drugs, such as MPA, are converted to a more active form (methyl prednisolone) *in vivo*, which obviously does not occur in such an *in vitro* test system (Trotter, 1996). In this study, drug concentrations were based on those used in previous *in vitro* studies, though obviously caution has to be used prior to extrapolation of these levels to *in vivo* situations and to what is a therapeutically achievable concentration.

It has already been shown that, in the horse, both resident articular cells and inflammatory cells are able to produce MMPs-2 and 9, and also in the horse, that MMPs-2 and 9 levels are elevated in synovial fluid in articular disease (Clegg *et al*., 1997a, b). In humans, elevated expressions of gelatinase MMPs have also been identified in osteoarthritic cartilage (Tsuchiya *et al*., 1997). As MMPs-2 and 9 are able to cleave many of the macromolecules in the matrix of articular cartilage, it is reasonable to suppose that a drug which is able to inhibit the activity of these MMPs could be classified as a DMOAD. The enzymatic degradative process in articular cartilage could be affected at several points; either at the point of synthesis/secretion of the enzymes from cells; or by modulation of the enzyme activity once it has been released by the cell; or alternatively by increase in synthesis of the natural local inhibitors of MMPs, the TIMPs (Clegg *et al*., 1998). This work addresses the question whether it is possible to alter the activity of the free enzyme with certain selected drugs. It has been shown that the gelatin degradation assay can be used to assess MMP inhibitory activity by demonstrating inhibition of purified MMPs by TIMPs (Clegg *et al*., 1998) and EDTA (results not shown). The fluorescein-labelled casein degradation assay can be used similarly, though MMP-9 alone of the gelatinase MMPs was able to degrade this substrate, whilst inhibition of MMP-9 can be shown in this assay using EDTA (results not shown).

Neither phenylbutazone nor flunixin had any inhibitory activity against MMPs-2 or 9. This is in accord with clinical experience where OA often continues to progress despite treatment with NSAIDs (May *et al*., 1988). This finding is in agreement with the data of May *et al*. (1988) except that they demonstrated a small inhibition of MMP activity by 150 μg/mL phenylbutazone, a concentration which is not achieved *in vivo* in the horse. It has been demonstrated that certain NSAIDS (diclofenac and indomethacin) may have an effect to increase MMP-9 production by articular chondrocytes, as MMP-9 synthesis is inhibited by PGE_2, and this negative feedback is abolished by various NSAIDs acting as cyclooxygenase inhibitors (Ito *et al*., 1995). Thus it may be that certain NSAIDs may increase articular cartilage degradation. In contrast, it has been shown that some NSAIDs (piroxicam and tenoxicam) have MMP collagenase and proteoglycanase inhibitory activity, though neither of these are used in the horse nor were they assessed in this study (Vignon *et al*., 1991). The effects of different NSAIDs on articular cartilage matrix is controversial. Further research is required to ascertain whether individual NSAIDs used in the clinical treatment of OA are deleterious or protective to articular cartilage, and as a result may either hasten or slow the progression of the disease.

Of the corticosteroids tested, betamethasone had no inhibitory activity on MMP-2 and 9, whilst dexamethasone and MPA had

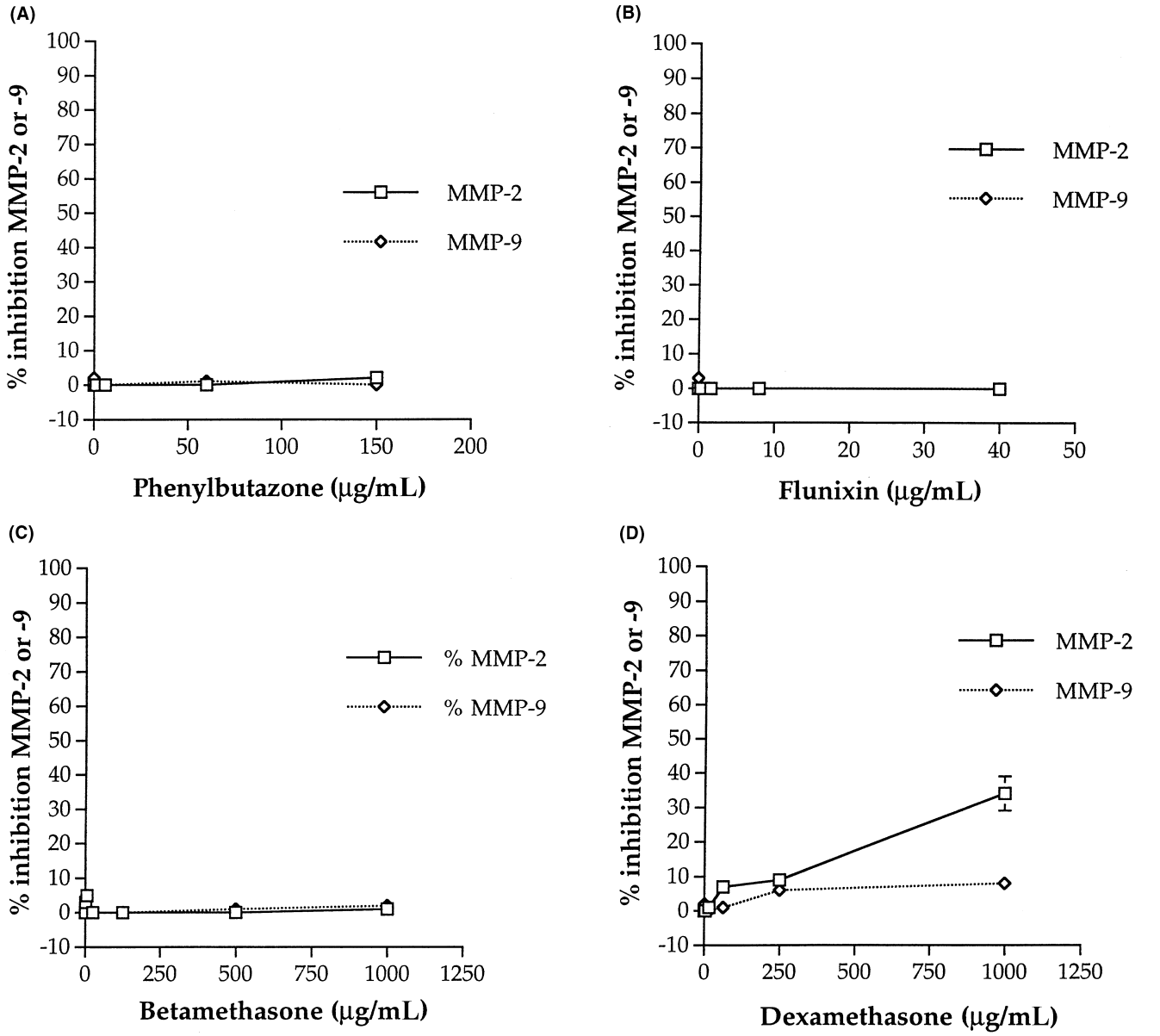

Fig. 1. Equine MMP-2 and MMP-9 inhibition by increasing concentrations of drugs measured by gelatin degradation ELISA ($n = 4$, $\pm$ 1SD). Drugs: (A) Phenylbutazone; (B) Flunixin; (C) Betamethasone; (D) Dexamethasone; (E) Methylprednisolone acetate; (F) Hyaluronan; (G) Pentosan polysulphate; (H) Polysulphonated glycosoaminoglycans (PSGAG).

no inhibitory effect on MMP-9. Matrix metalloproteinase-2 was inhibited significantly by the high doses tested of dexamethasone (1 mg/mL) and MPA (1.2 and 5 mg/mL). These high doses of steroids are unlikely to be achieved *in vivo* for any length of time. There is little evidence that corticosteroids have an effect directly on MMP activity, although one recent report has demonstrated MMP inhibition by dexamethasone (Hirata *et al.*, 1996). Corticosteroids are able to affect MMP levels in disease by preventing synthesis at a transcriptional level (Vincenti *et al.*, 1994) and this has been demonstrated in the horse (Richardson & Dodge, 1996).

Neither hyaluronan nor pentosan polysulphate had any direct inhibitory action on MMPs-2 and 9. The finding for hyaluronan is in agreement with that of May *et al.* (1988). Despite the lack of direct inhibitory activity, hyaluronan may have a DMOAD role as it has profound effects on leucocyte migration and function (Howard & McIlwraith, 1996). We have previously shown that leucocytes are a rich source of equine MMP-9 (Clegg *et al.*, 1997a) and thus hyaluronan, by virtue of its anti-inflammatory action, may decrease the levels of MMPs in the joint. Although we demonstrated that pentosan polysulphate had no effect on MMPs-2 and 9 activity, this drug has previously been shown to inhibit MMP-3, at concentrations much lower than those used in this experiment, though paradoxically it had the opposite effect in increasing the MMP collagenase activity (Nethery *et al.*, 1992).

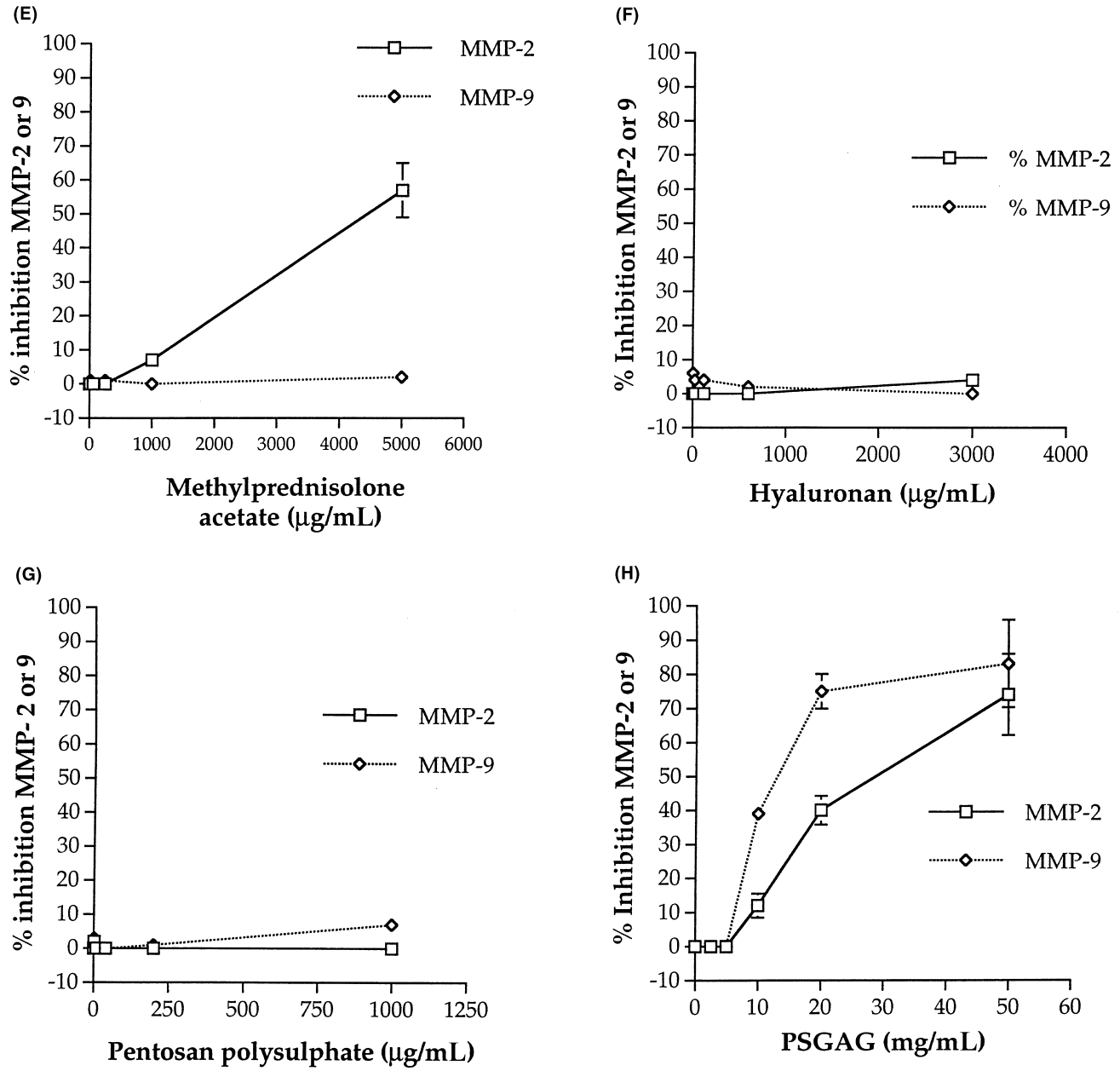

Fig. 1. Continued

Polysulphated glycosaminoglycan had strong inhibitory activity, when at high concentrations (> 10 mg/mL), against both MMP-2 and 9. It should be noted that this drug concentration is much higher than that used to test pentosan polysulphate. It has previously been shown that PSGAG had a similar effect on MMP caseinase activity produced by equine synovial cells in monolayer culture which had been 'nutritionally deprived' (May *et al.*, 1988). The authors attributed the MMP activity in this experiment to MMP-3 (stromelysin), though no attempt was made to specifically identify the MMP activity. We demonstrate here that equine MMP-9 is able to degrade casein and is also inhibited by PSGAG. We have previously shown that unstimulated equine synovial fibroblasts produce MMP-2 (Clegg *et al.*, 1997a), though it has been shown that human synovial fibroblasts when stimulated by interleukin-1 a or by phorbol myristate acetate, produce MMP-9 (Tetlow *et al.*, 1993). Synovial membrane explants from equine joints with clinical osteoarthritis, when grown in culture, also produce MMP-9 (results not shown). Therefore, it is possible that some of the casein degrading activity in the May, Hooke and Lees experiment (1988) is attributable to MMP-9. Whilst significant inhibitory activity to both MMPs-2 and 9 is seen with PSGAG, this only occurs at high concentrations. It is to be noted that in the rabbit, following systemic administration of PSGAG at a dose of 7.5 mg/kg, articular cartilage contained only 1.7 μg/g of PSGAG 48 h later. Similarly in humans, levels of only

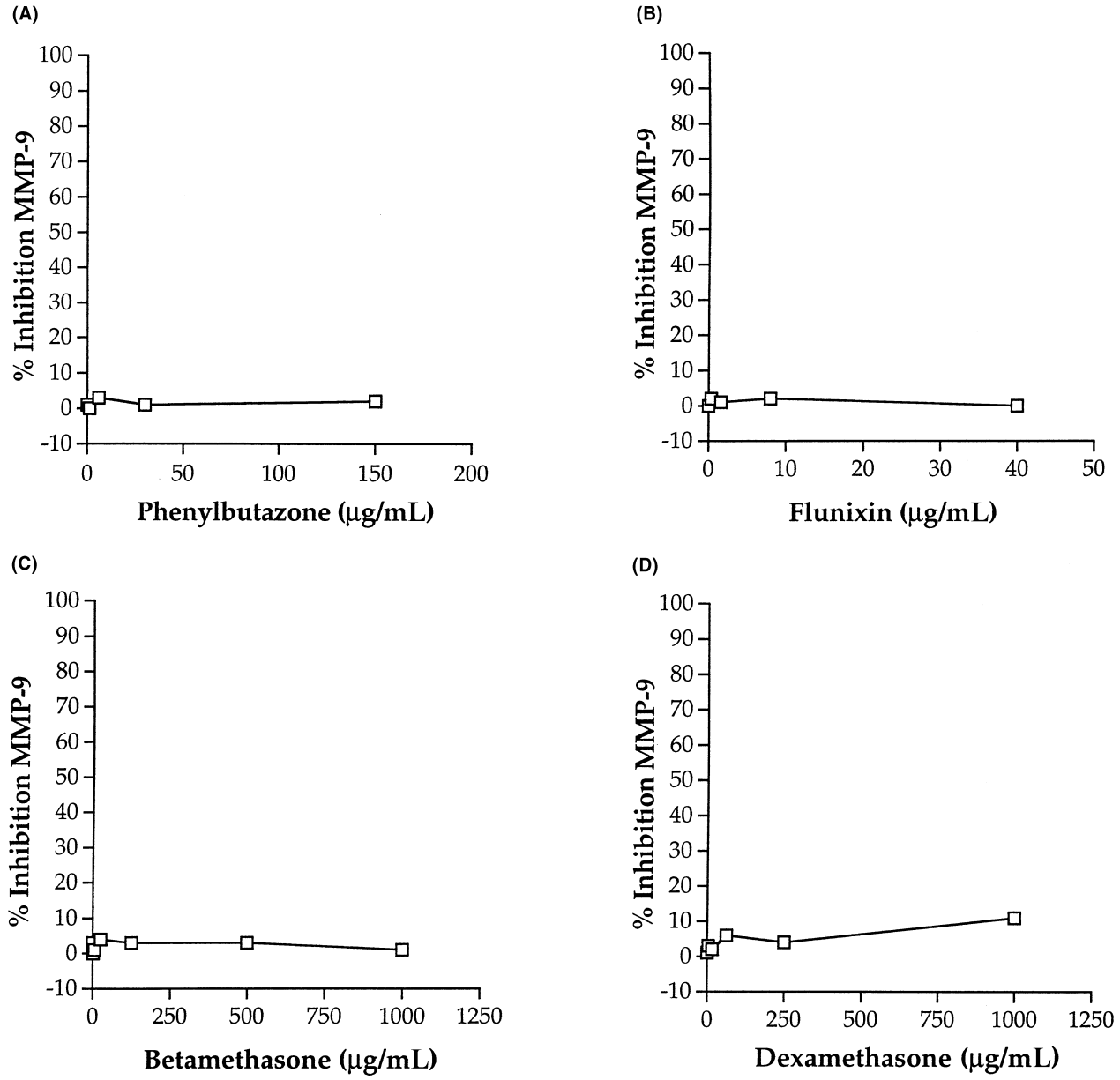

Fig. 2. Equine MMP-9 inhibition by increasing concentrations of drugs measured by casein degradation assay ($n = 4$, $\pm$ 1SD). Drugs: (A) Phenylbutazone; (B) Flunixin; (C) Betamethasone; (D) Dexamethasone; (E) Methylprednisolone acetate; (F) Hyaluronan; (G) Pentosan polysulphate; (H) Polysulphonated glycosoaminoglycans (PSGAG).

1.45 μg/g of PSGAG were found in OA cartilage, 24 h after 50 mg of PSGAG were administered intramuscularly (Trotter, 1996). Thus whilst PSGAG has an *in vitro* inhibitory action against MMPs, it is questionable whether the inhibitory activity demonstrated would be significant *in vivo* for any useful length of time.

It is unlikely that any of the drugs tested here, presently used in the management of equine joint disease, are able to therapeutically inhibit MMPs-2 and 9 *in vivo* for any length of time. In recent years there have been great advances made in the design of synthetic inhibitors to the MMPs (Beckett *et al.*, 1996). These specific synthetic, zinc binding MMP inhibitors may have a role in the future as a treatment for various joint diseases (Gordon *et al.*, 1993). Some of these compounds are at present in clinical trials in human medicine as treatment for various MMP mediated diseases such as neoplasia, multiple sclerosis and arthritis. We have shown that some of these synthetic inhibitors are able to inhibit equine MMPs at low concentrations (data not shown). Whilst these synthetic agents show considerable promise for the future, it will be necessary to overcome problems of bioavailability, effects during circulation through the body and successful delivery to the articular cartilage at effective concentrations (Vincenti *et al.*, 1994). Further work is now required to assess the effect of pharmacological intervention on the secretion of MMPs from cells, and to assess whether this would also be a potential route to modify degradative pathways within articular cartilage.

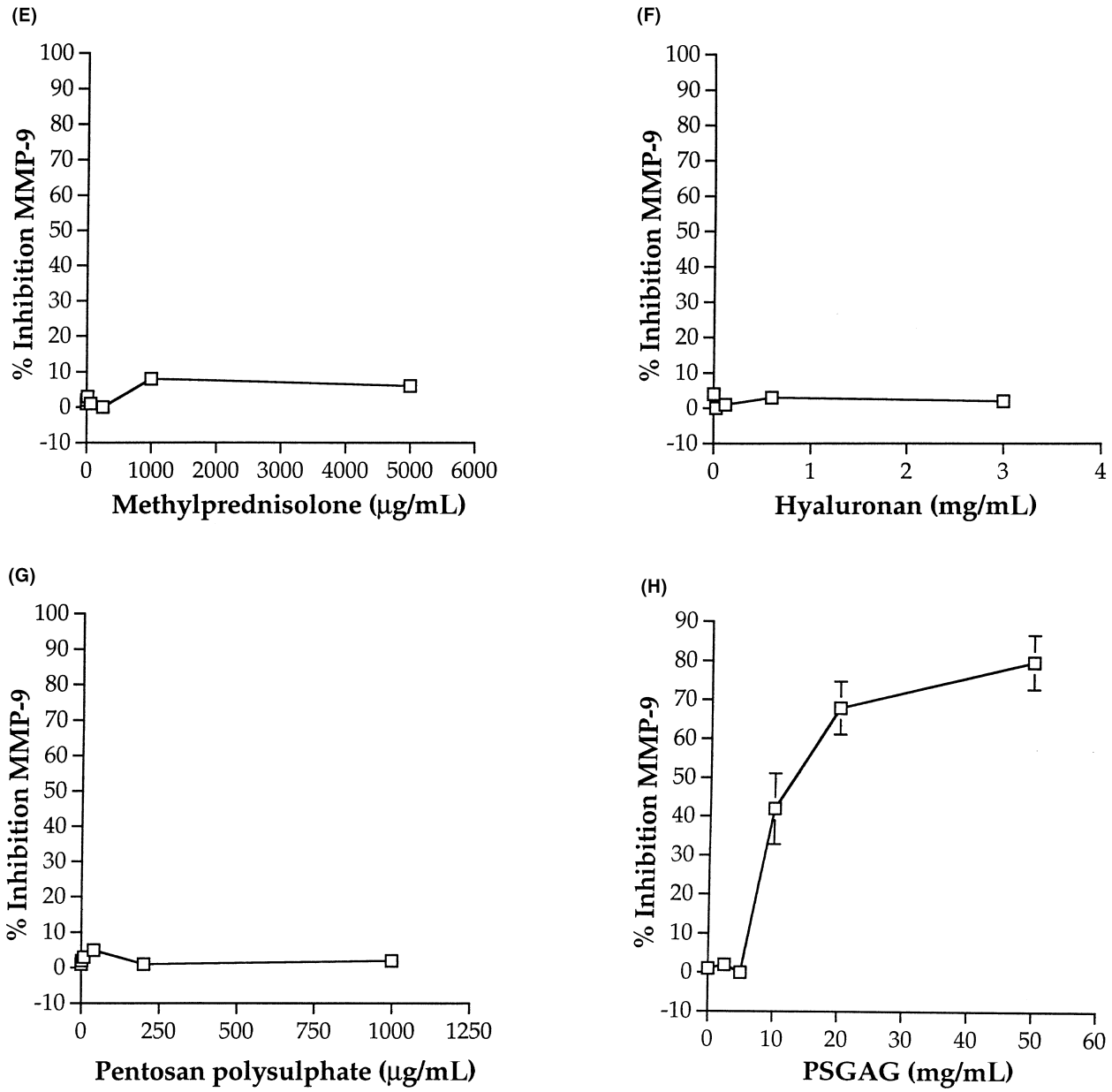

Fig. 2. Continued

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