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Recent pharmacodynamic and pharmacokinetic findings on oxaprozin

K. D. RAINSFORD*, H. OMAR, A. ASHRAF, A. T. HEWSON, R. A. D. BUNNING, R. RISHIRAJ, P. SHEPHERD and R. W. SEABROOK

Biomedical Research Centre, Sheffield Hallam University, Sheffield S1 1WB, UK

Abstract—Oxaprozin has proven a safe and effective treatment for arthritic conditions. It has a low incidence of gastrointestinal side effects making it a particularly attractive therapy. Being an unselective COX-1 and COX-2 inhibitor the drug may have advantages in more effective pain relief related to both COX-1 as well as COX-2 effects. We have investigated the pharmacology of oxaprozin on (a) the biochemical components of joint destruction *in vitro*, and (b) the pharmacokinetic interactions of this drug with albumin *in vitro* in which drug interactions are modelled by competitive binding of the drug with endogenous ligands or drugs that are commonly prescribed with NSAIDs. The latter *in vitro* pharmacokinetic study can be considered as a basis for understanding both safety and therapeutic activity of the drug. Among the major effects of oxaprozin (10–100 μM) observed on components of joint destruction was (a) the inhibition of the production of interleukin-1β (IL-1β) from pig synovial tissues in organ culture and IL-1β, IL-6 and IL-8, as well as tumour necrosis factor-α (TNFα) from THP-1 mononuclear cells at 50–100 μM. Oxaprozin (1.0–100 μM) did not affect NO production from porcine synovial tissue, whereas indomethacin and nimesulide reduced production of NO. Oxaprozin did not exacerbate the IL-1β and/or TNFα-induced proteoglycan destruction in pig or bovine cartilage in organ culture as observed with indomethacin and aspirin. Radiolabelled oxaprozin accumulated in cartilage to a much greater extent than observed with other NSAIDs. This may be a particular advantage in enabling expression of cartilage protective effects of the drug. Albumin binding of [^{14}C]oxaprozin (10 μM) *in vitro* was unaffected by other NSAIDs (e.g., aspirin, diclofenac, ibuprofen, paracetamol, salicylate), a range of commonly co-prescribed drugs (e.g., atenolol, clonidine, cromolyn, diphenhydramine, furosemide, ketotifen, salbutamol, prednisolone, theophylline), endogenous steroids (e.g., oestradiol, progesterone) or other agents (e.g., caffeine) at concentrations of 40 μM. The free concentrations of oxaprozin were, however, increased slightly by the same concentrations of warfarin, prednisolone, diazepam and captopril. In contrast, the binding of oxaprozin to free-fatty acid depleted albumin was only appreciably affected by captopril and caffeine. The free oxaprozin was slightly increased by zinc and copper ions (which are increased in rheumatic conditions) in normal as well as fatty acid-free albumin. Tryptophan release from albumin, which has a role in central analgesic actions of anti-rheumatic drugs by enhancing CNS turnover of 5-hydroxytryptamine (serotonin), was increased by 50–100 μM oxaprozin, as well as

*To whom correspondence should be addressed. E-mail: k.d.rainsford@shu.ac.uk

other NSAIDs. This indicates that serotonergic activation could also contributed to the analgesic properties of oxaprozin like that of other NSAIDs. These studies indicate that the potentially-significant pharmacodynamic actions of oxaprozin in arthritic joints may involve inhibition of the release of pro-inflammatory cytokines from inflamed synovium and relatively high rate of drug uptake into inflamed joints. Analgesia, in addition to being a consequence of prostaglandin inhibition, may be initiated by release of albumin-bound tryptophan and uptake into the brains leading to activate serotonergic pathways of pain suppression. Oxaprozin is only slightly affected pharmacokinetically by some co-prescribed drugs or patho-physiologic metal ions but these effects may be of limited clinical consequence principally as oxaprozin has a relatively long plasma elimination half-life (50–60 h) and any small increase in free concentrations of the drug in circulating plasma increases elimination of the drug.

Key words: NSAIDs; propionic acids; arthritis; serotonin; albumin; cytokines.

1. INTRODUCTION

Oxaprozin (Wy-21,743; 4,5-diphenyl-2-oxazolepropionic acid; CAS-21256-18-8) is a non-steroidal anti-inflammatory drug (NSAID) which has some unique chemical and pharmacokinetic properties and low gastrointestinal (GI) irritancy/ulcerogenicity compared with other drugs of its class (Lussier *et al.*, 1982; Lewis *et al.*, 1985; Todd and Brogden, 1986; Bonney *et al.*, 1987; Suwa *et al.*, 1987; Rainsford, 1988; Miller, 1992; Lanza, 1993; Weaver *et al.*, 1995; Karim, 1996; Karim *et al.*, 1997; Davies, 1998; Simon *et al.*, 1998).

The biological, chemical and pharmacokinetic properties of oxaprozin have been reviewed by Lewis *et al.* (1985), Bonney *et al.* (1987) and Davies (1998). In this paper aspects of chemistry, pharmacological and pharmacokinetic properties of oxaprozin are briefly reviewed and new data are presented on the *in vitro* pharmacological effects and pharmacokinetic interactions.

1.1. Chemical aspects

Oxaprozin differs chemically from other propionic acid compounds in that the propionic acid group attached to the oxazole does not posses a chiral centre. The latter property means that there is no complex metabolic fate of enantiomers as seen with many other propionic acids (except naproxen and dexibuprofen) (Janssen *et al.*, 1980; Davies, 1998).

The tricyclic diphenyl-oxazole ring structure has some general similarities to benzodiazepines. Indeed, oxaprozin interferes with urinary and immunoassays for benzodiazepines (Pulini, 1995; Fraser and Howell, 1998) and it has been shown to bind to the site II or benzodiazepine binding site on albumin. While not having the antidepressant pharmacological properties of benzodiazepines the chemical features of oxaprozin may have some pharmacological consequences even though it is generally like that of other NSAIDs in having analgesic, anti-inflammatory and antipyretic activities.

Oxaprozin has a relatively high pK_a of 6.1 and moderate lipophilicity ($\log P_{pH7.4/octanol} = 1.33$) compared with that for aspirin (-0.47) and indomethacin

(1.36) (Rainsford, unpublished observations). The high pK_a may be important in explaining the favourable gastric mucosal tolerability of oxaprozin compared with other NSAIDs. However, the distribution coefficient log D may also be important, this being a parameter that incorporates the factor relating to ionizability of compounds like that of weak acids with variation in pH, thus

$$\log D = \log P - \log(1 + 10^{\mathrm{pH}-\mathrm{p}K_a})$$

(Barbato *et al.*, 1986). Using this relationship it is possible to show that oxaprozin has a markedly different pattern of distribution over the range of gastric pH values than that observed by other NSAIDs (e.g., diclofenac, indomethacin and naproxen) (see Figs 1 and 2). It can be seen that the variation in the distribution by oxaprozin in the pH range of 2–4 in the lipophilic medium (i.e. *n*-octanol) is appreciably lower than that of the other NSAIDs, all of which are well known to be ulcerogenic. As a consequence of this, oxaprozin may have a slower or more uniform uptake into gastric mucosal cells over the pH range in the stomach. The slow rate of absorption

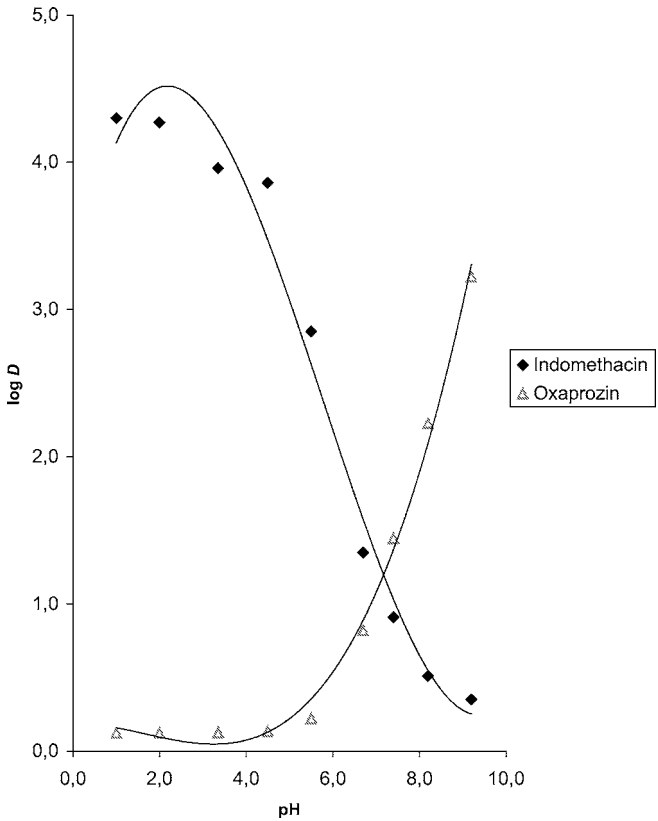


Figure 1. The distribution coefficient, log D, versus pH of oxaprozin, a drug with low irritancy, contrasted with standard NSAIDs indomethacin, and naxproxen and diclofenac (Fig. 2) that are relatively more gastro-ulcerogenic.

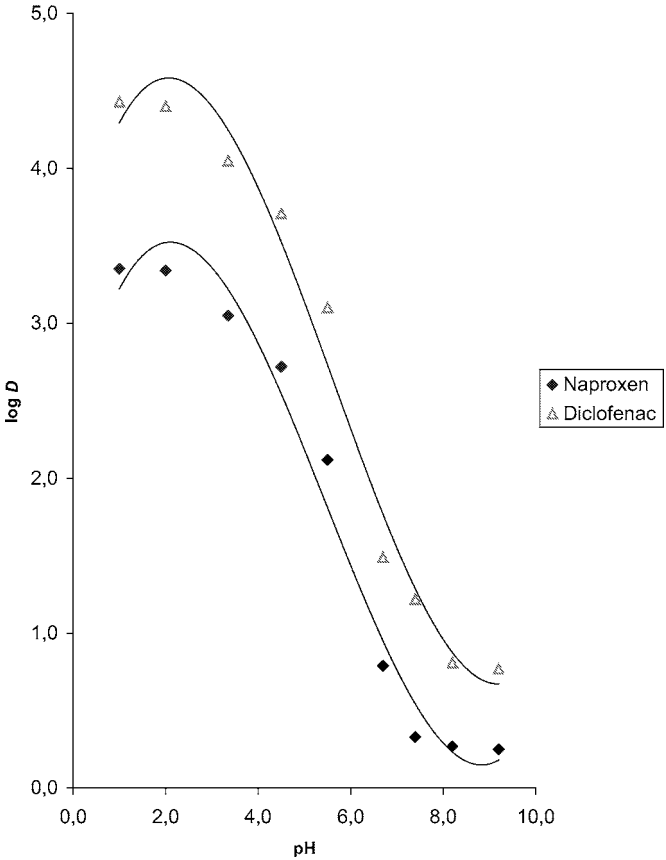


Figure 2. The distribution coefficient, log *D*, versus pH of naxproxen and diclofenac.

of oxaprozin into gastric mucosal tissues of rats supports this suggestion (Rainsford, 1988). This physicochemical feature may underly the low gastro-irritancy of this drug (Shriver *et al.*, 1977; Lanza *et al.*, 1981; Suwa *et al.*, 1987; Lucker *et al.*, 1987; Rainsford, 1988) even though it causes lowering of gastric mucosal prostaglandins (Rainsford, 1988).

1.2. Pharmacokinetics

The physiochemical properties of oxaprozin outlined above might also underly, in part, the pronounced transynovial distribution and synovial accumulation of this highly protein-bound drug (Kurowski and Thabe, 1989). The high plasma protein binding and long plasma elimination half-life ($t_{½}$ 50–60 h) (Karim, 1996; Davies, 1998) favours once-daily dosage with this drug for treatment of osteoarthritis, juvenile and adult rheumatoid arthritis and ankylosing spondylitis (Miller, 1992; see also Kean *et al.* (2002) in this issue). Oxaprozin shows relatively little systemic drug accumulation in elderly patients or in those at risk subjects with compromised hepatic or cardiovascular functions or rheumatoid arthritis (Greenblatt *et al.*, 1985;

Ochs *et al*., 1986; Davies, 1998). The occurrence of renal abnormalities is relatively low, being of the same order as ibuprofen (Mitnick *et al*., 1980; Bonney *et al*., 1987; Audet *et al*., 1988). In subjects with impaired renal function there is increased free (non-protein bound) plasma concentrations and increased clearance but this does not lead to a lower $t_{½}$ (Chiang *et al*., 1982).

A tyrosine residue on albumin at the principal binding site (Tyr^{411}, Site II) appears to act as a nucleophile in the covalent interaction of oxaprozin (acyl) glucuronide. The hydrolysis of this appears such that the consequences of this reaction may not be of major pharmacokinetic importance, except if liver proteins are so bound. This may contribute to the few cases of liver reactions reported infrequently with this drug (Freeland *et al*., 1988; Purdum *et al*., 1994).

The main variable in the pharmacokinetics of oxaprozin is in relation to doses greater than 1200 mg/day (the upper dose limit for the drug) where there is increased unbound fraction of the drug leading to an increase in the clearance and volume of distribution of the drug (Karim, 1996; Davies, 1998). Drug accumulation after repeated dosing is about half that predicted from single doses of the drug due to increased clearance of total drug and reduction in the unbound fraction (Karim, 1996; Davies, 1998). The clinical consequences of the inverse non-linear pharmacokinetics of oxaprozin have yet to be determined although it has been suggested that the importance of the plasma half-life for therapy may be overstated (Davies, 1998).

1.2.1. Drug interactions. *In vivo* pharmacokinetic studies have shown that in menopausal women who received conjugated oestrogens for at least 3 months the β-elimination $t_{½}$, volume of distribution, clearance and peak plasma concentrations of oxaprozin did not differ significantly from those women who did not take oestrogens. The time of maximal drug concentration (t_{max}) was, however, prolonged (8.9 cf., 4.0 h).

Aspirin has been shown to interact with the protein binding of oxaprozin (Kahn and Hubsher, 1983). No pharmacokinetic effects of concurrently administration of H_2 receptor antagonists, antacids or paracetamol have been observed (Scavone *et al*., 1986, 1988; Dixon and Page, 1991).

1.2.2. Pharmacokinetic consequences of displacing protein-bound tryptophan and its role in analgesia. Tryptophan is the natural amino-acid precursor of the neurotransmitter, 5-hydroxytryptamine (5-HT or serotonin) (Fig. 3). The rate of synthesis of 5-HT in the brain is principally regulated by the availability of tryptophan (Smith and Lakatos, 1971; Iwata *et al*., 1975). The concentration of tryptophan in the brain under physiological conditions is in the range of 5–25 μmol/l. Heyliger *et al*. (1998) showed that tryptophan and its metabolites were analgesic in rats at oral doses of 300 or 600 mg/kg.

5-HT has various physiopathological effects in the control of cardiovascular regulation, respiration and thermoregulation (Lucki, 1998). Pharmacological functions

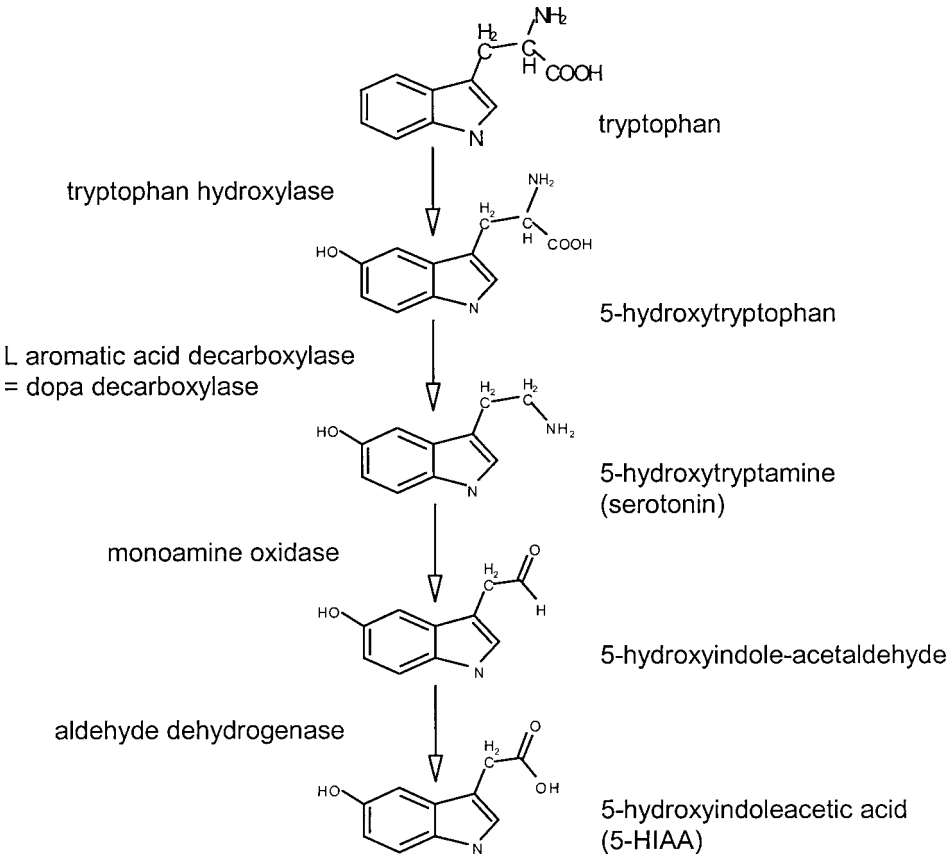


Figure 3. Pathways for the synthesis of 5-hydroxytryptamine (5-HT, serotonin) from tryptophan.

of 5-HT include, increased gastrointestinal motility, contraction of other smooth muscle and vascular constriction and dilatation, platelet aggregation, stimulation of peripheral nociceptive nerve endings and excitation/inhibition of CNS neurons.

Fernstrom and Wurtman (1971) and Knott and Curzon (1972) found that the regulation of central 5-HT levels may be partially controlled by the concentration of plasma tryptophan and, as well these may control tryptophan hydroxylase, the rate-limiting enzyme for 5-HT synthesis. In the CNS, 5-HT levels have been implicated in the pharmacological regulation of a variety of behavioural functions including sleep, depression, anxiety, temperature, sexual behaviour and, importantly, pain sensitivity (Birdsall, 1998).

The first step in the synthesis of 5-HT (Fig. 3) involves the activity of tryptophan hydroxylase and this is the rate-limiting step in the synthesis of 5-HT (Friedman *et al.*, 1972; Curzon *et al.*, 1973). If the availability of tryptophan to the brain is altered, predictable changes in the rate of 5-HT synthesis and turnover then occur. Tryptophan hydroxylase functions normally below saturation so that any potential increase in the concentration of tryptophan available for hydroxylation would be

likely to influence the rate of reaction of this enzyme (Gál, 1975). Friedman *et al.* (1972) highlighted that tryptophan hydroxylase was inhibited by an excess of tryptophan.

5-HT synthesis in the CNS has been shown to depend on carrier-mediated transport of tryptophan across the blood–brain barrier (Bender and McCreanor, 1982). The rate of uptake of tryptophan, like that of other amino acids, from the bloodstream into the brain, is extremely high (Oldendorf, 1971). Bender and McCreanor (1982) reviewed the uptake of tryptophan to the CNS and considered that the two major factors seemed to affect the rate of tryptophan accumulation in the CNS are (a) concentration of other amino acids that compete with tryptophan in blood and (b) binding of tryptophan to plasma albumin, which reduces the availability of tryptophan for transport as essentially it is bound. There could also be active transport systems for the net efflux of tryptophan like other amino acids from the brain into the circulation.

Tagliamonte and co-workers (1973a, b) investigated whether the level of tryptophan in the brain might be controlled by the concentration of free tryptophan in serum. In order to test their hypothesis they investigated the relationship between free serum and brain tryptophan in two conditions associated with increased levels of brain tryptophan in rats following (a) administration of exogenous tryptophan, and (b) in animals fasted for 24 h. They found that administration of 50 mg/kg of L-tryptophan, the total and free tryptophan in plasma increased over 30 to 60 min then returned to normal values after 120 min. Their work is in agreement with studies by Ashcroft *et al.* (1973) who found in patients with various conditions that increase in tryptophan caused an increase in brain 5-HT and 5-HIAA levels.

Using [^{3}H]tryptophan Tagliamonte *et al.* (1973a, b) added further support to their hypothesis and they found that this amino acid passed through the blood–brain barrier into the brain of rats. Subsequently, a number of studies have revealed that administration of loading doses of L-tryptophan leads to an increase in the rate of turnover of 5-HT and a significant increase in the concentration of 5-HIAA in the brain. (Weil-Fugazza *et al.*, 1980; Kelly and Franklin, 1985; Sandyk, 1992; Kema *et al.*, 2000).

L-Tryptophan is the only amino acid that is non-covalently bound to serum albumin (McMenamy and Oncley, 1958; Francesconi and Mager, 1975). In one study the albumin binding was been claimed to be specific for the L-isomer of tryptophan while D-tryptophan was not bound (Bender, 1982). However, Fitos and Simonyi (1986) found that the L-isomer exhibited a high degree of secondary binding, whereas D-tryptophan showed increased binding when the concentration of human serum albumin was decreased. It was concluded that the steroselectivity of binding of both forms, is much lower than generally believed. The binding of tryptophan to circulating albumin is relatively specific since only a limited number of analogues and metabolites will compete for binding. Pardridge (1977) demonstrated that the dissociation constant of the tryptophan–albumin complex was of the same order of magnitude as the K_m for tryptophan transport.

Non-esterified fatty acids in plasma proteins displace tryptophan from albumin binding. Curzon and Knott (1972) found that plasma fatty acid changes have a role in determining the availability of tryptophan to the brain and hence 5-HT.

Weil-Fugazza and co-workers (1979) investigated the total and free-serum tryptophan levels in arthritic rats. They found that there is a decrease in total serum tryptophan levels with increase in the free levels of tryptophan in the circulation 15 and 21 days after induction of arthritis by Freund's adjuvant.

McArthur *et al.* (1971a, b) proposed that anti-rheumatic drugs act by displacing tryptophan and some di-peptides from their binding sites to circulating proteins and that the free-fractions of these peptides protect susceptible tissues against the effect of chronic inflammatory reactions. In patients with rheumatoid arthritis, they found that the peptides are bound to an abnormal extent to the serum proteins. They suggested the role of the drugs is to restore this binding to normal (McArthur *et al.*, 1971a, b; McArthur, 1979) and this was confirmed by Aylwood and Maddock (1973) in rheumatic patients.

Davis *et al.* (1968) presented evidence to suggest that part of the anti-inflammatory action of salicylate is mediated via tryptophan. They found that administration of tryptophan inhibited the infiltration of leucocytes into an area of local inflammation of rats. Subsequently, Igari *et al.* (1979) and Igari and Shimamura (1979) found that tryptophan was metabolised mainly to anthranilic acid through kynurenine in the kynurenine pathway in rheumatoid arthritis but in osteoarthritis the pathway of kynurenic acid and nicotinic acid was prominent. 5-HT and 5-HIAA were not significantly changed in the synovial fluid in patients with rheumatoid- or osteoarthritis.

McArthur *et al.* (1971a, b) found that (a) phenylbutazone, indomethacin, prednisolone, chloroquine and gold salts all displaced L-tryptophan from its binding to human serum at therapeutic drug concentrations and (b) certain dipeptides bind to albumin and are displaced by a therapeutic concentrations of salicylate. Of the dipeptides used, they found that all the anti-rheumatic drugs affect the binding of L-phenylalanyl-L-phenylalanine. They concluded that when the drugs bind to circulating albumin they displace other biologically active small molecules from their binding sites on serum proteins. In rheumatoid disease, it was proposed that the unbound forms of some peptides may exert a protective effect against the actions of mediators of chronic inflammatory insults and the drugs act by increasing the proportion of free peptides in the blood (McArthur *et al.*, 1971a).

Iwata *et al.* (1975) demonstrated the effects of various drugs on serum free and total tryptophan levels in rats. Their results showed that both in vitro and in vivo salicylate, benzoate and indomethacin displaced tryptophan from its binding site on serum albumin and increased the concentration of tryptophan and 5-HIAA in rat brain. However, neither phenylbutazone nor sulphathiazole increased the concentrations of tryptophan and 5-HIAA in the brain hence no displacement effect of bound tryptophan. Guerinot *et al.* (1974) reported that acetylsalicylic acid increased free plasma tryptophan and increased brain tryptophan and 5-HIAA in

rats, which in turn indicates an increase in the brain serotonin turnover. Their results are in agreement with Spano *et al*. (1971); Tagliamonte *et al*. (1971a, b).

Another mechanism proposed by Badawy (1982) was that the breakdown of tryptophan in brain by tryptophan pyrrolase could be inhibited by salicylate. Although he showed this could occur in rats with low doses of salicylate, the displacement of tryptophan from albumin by this drug would be expected to have greater consequences because of the rate control of tryptophan hydroxylase on the pathway to 5-HT.

Overall, these studies show that an important part of analgesia by NSAIDs may be related to the displacement by these drugs of tryptophan from circulating albumin which then is taken up into the brain and increases brain turnover of 5-HT.

1.3. Joint reactions in arthritis

As mentioned previously, oxaprozin has a long plasma half-life of elimination ($t_{½}$ approx. 55 h) and relatively high rate of accumulation in inflamed synovial tissue and synovial fluids (compared with that in plasma and synovial fluids) (Kurowski and Thabe, 1989; Davies, 1998). Following oral administration of 1200 mg oxaprozin for 2.5 days to patients with rheumatoid- and osteo-arthritis the synovial tissue concentrations 12 h after the last dose (at what is probably plateau blood levels) average 25 μg/ml (85.2 μmol/l) (Kurowski and Thabe, 1989). These values considerably exceed the levels in synovial fluid (4.9–7.6 μg/ml; 16.7–25.9 μmol/l) and in plasma (10–17 μg/ml; 34–58 μmol/l). This suggests that there is appreciable accumulation of oxaprozin in the inflamed synovium.

Thus, for any meaningful pharmacological effects to be determined *in vitro* concentrations of oxaprozin that should be employed should range around about 80–100 μmol/l to approximate the synovial tissue concentrations. For a full concentration–response to be derived that would be of pharmacological significance it would be necessary to investigate drug effects in the range of 1.0 to 100 μM.

The control of inflammatory destructive events by oxaprozin may be related to inhibition of the release of metalloproteinases (Barracchini *et al*., 1998; 2001). This and other effects in joints may be more effectively controlled by oxaprozin than with some other NSAIDs as a consequence of appreciable accumulation of this drug in synovial tissue and synovial fluids. There is only a limited amount of information available on the spectrum of pharmacological effects of oxaprozin in joint destructive events and the relationship of these events to uptake of the drug into inflamed and normal cartilage.

Oxaprozin has been shown to inhibit human platelet cyclooxygenase-I (COX-I) in the prostaglandin synthase isoform-I with an IC_{50} of 2.2 μmol/l (Kawai *et al*., 1998). Human synovial prostaglandin production stimulated by interleukin-1β largely has been found to be inhibited by oxaprozin with an IC_{50} of 36 μmol/l (Kawai *et al*., 1998). The latter has been considered to be a reflection of COX-2 inhibition but it is now established that appreciable COX-1 activity is present in the epithelial cells of the synovium (Crofford *et al*., 1994; Crofford, 1997;

Siegle *et al.*, 1998). Thus, estimates of COX-1/COX-2 ratios based on comparisons of this activity in synovial tissues requires revision in the light of these new findings.

Couchman and Sheppeard (1986) reported that oxaprozin (100 μmol/l) along with some other NSAIDs and DMARDs inhibited the release from porcine synovial tissue of factors that degrade cartilage *in vitro*. Previously, Rainsford (1985) had established that NSAIDs were only weak inhibitors of the release from synovia of what was then described as "catabolin-like activity" that was responsible for cartilage degradation *in vitro*, one of the principle cytokine components of which was later shown to be porcine IL-1α (Saklatvala *et al.*, 1984). Other pro-inflammatory cytokines and enzymes that induce cartilage destruction have been observed in the protein macromolecular fractions that constitute catabolin (Saklatvala, unpublished studies). The release of cartilage-degrading, catabolin-like activity (CDA) from synovial tissue in organ culture probably represents the major part of the combined inflammatory and destructive components that are produced from inflamed synovia *in vitro* to cause destruction of both cartilage and underlying bone in the diathrodal joints of patients with osteo- and rheumatoid arthritis. Thus, determination of the effects of NSAIDs, DMARDs and other agents for their effects on the release of CDA from synovial tissues is an important way of establishing the potential for these drugs to control the arthritic degradative changes in the joints *in vivo*. Another pharmacological component of the actions of drugs in controlling cartilage destruction is to determine if they affect the pro-inflammatory cytokines (IL-1α, TNFα) affect the release of reactive oxygen species and nitric oxide, and the metalloproteinases that are released by inflamed synovium. These approaches are, therefore, the two central pharmacological components of cartilage destruction that form the rationale for the studies reported herewith.

The earlier studies by Couchman and Sheppeard (1986) claiming that oxaprozin reduced the release of CDA were performed using assay methods which lacked adequate controls. Using more precise and rigorous assay methods involving use of serial dilution techniques we have developed a modified assay for the effects of drugs on the release of CDA (Rainsford *et al.*, 1989, 1995). Such bioassays enable any potential effects of the drugs/metabolites interfering with the bioassay to be eliminated. Furthermore, controls to check for the possibility of drug interference with the bioassay can be identified and where necessary correction factors applied to the data that is obtained in these assays. Such controls were not applied by Couchman and Sheppeard (1986) either in their studies of the effects of oxaprozin and other drugs they studied which could account for the reason why the results they obtained with some other NSAIDs have not been confirmed in other studies (Rainsford, 1985; Dingle, 1985).

1.4. Aims and objectives

The aims of these studies are to (a) determine the potential for drug interactions to occur at the level of albumin binding such that they may contribute to the

development of adverse reactions by drug displacement, (b) to explore the potential for oxaprozin to displace tryptophan from its binding sites on albumin and thus contribute to its analgesic activity via serotonergic activation and (c) determine the key biochemical effects of oxaprozin on various component systems involved in joint destruction in arthritic diseases involving the synovial production of key mediators of inflammation and joint destruction and relate these effects to synovial uptake of oxaprozin *in vitro*.

The effects of oxaprozin on the joint manifestation of arthritic diseases include studies on the (a) pro-inflammatory cytokines (interleukins (ILs)-1, -6 and -8), and tumour necrosis factor-α (TNFα), from synovial tissues and the mononuclear cell line, THP-1; (b) prostaglandin E_2 by pig synovial tissues and THP-1 cells; (c) lipid peroxidation products (as an index of oxyradical damage); (d) synovial and chondrocyte nitric oxide (measured as nitrite); (e) synovial metalloproteinases and (f) synovial cartilage-resorptive activity (CDA) that probably reflects the combination of cartilage-destructive events in (a) to (e) above. These effects were compared with the uptake of radioactivity of ^{14}C-labelled oxaprozin and nimesulide into pig articular cartilage in organ culture *in vitro* in the presence or absence of pro-inflammatory cytokines. Also, in this study the effects of oxaprozin were compared with reference NSAIDs on the basal cartilage destruction and that stimulated by IL-1α with and without TNFα, as well as the total cartilage resorptive activity released following exposure of pig synovial tissue to prolonged organ culture (known as "catabolin"-like activity (CDA)).

2. METHODS

2.1. Effects of drugs and metal ions on binding of oxaprozin to albumin

Ultrafiltration was carried out by using disposable Centrifree^{TM} Micropartition System (Millipore 4104, Hertfordshire, UK), with MYT membrane (molecular weight cut-off of 30 000). The drugs were diluted in the appropriate medium then further diluted in PBS containing human serum albumin (HSA, 40 g/l) to their therapeutic plasma concentrations, and the mixtures then maintained on ice.

[^{14}C]Oxaprozin was synthesized according to the scheme shown in Fig. 4 (Lewis *et al.*, 1985). The product was purified by thin-layer chromatography (TLC) using a solvent system comprising of dichloromethane : methanol : acetic acid (28 : 2 : 1) with the radioactive content being determined by scintillation counting of the TLC zones; the purity of the final product being 98%. The structural formula of the compound with the position of the ^{14}C-label is shown in Fig. 4. [^{14}C]Oxaprozin, final concentration 10 μM, was placed in the reservoir of the ultrafiltration cartilage and the following drugs were then added: racemic ibuprofen (100 μM), atenolol (44.5 μM), diazepam (7 μM), aspirin (55 μM), diclofenac (12.5 μM), paracetamol (20 μM), salbutamol (40 μM), ketotifen (40 μM), cromolyn (40 μM) or theophylline (40 μM); all the other drugs were added at a con-

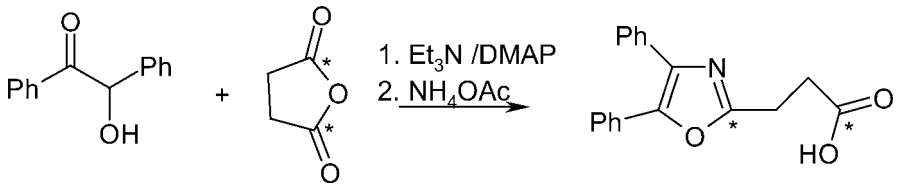


Figure 4. Preparation of [^{14}C]oxaprozin. The procedure involved reacting [1,4-^{14}C]succinic anhydride (Sigma; 6.7 mg, 1 mCi) and unlabelled succinic anhydride (43.3 mg) which were dissolved in dichloromethane (5 cm^3). Benzoin (106 mg), triethylamine (0.1 cm^3) and 4-dimethylaminopyridine (10 mg) were added and the solution was stirred at room temperature for 16 h. The solution was extracted with 2 M hydrochloric acid (2 × 5 cm^3), dried over magnesium sulphate and evaporated. The residue was dissolved in acetic acid (5 cm^3) and ammonium acetate (150 mg) was added. The mixture was refluxed for 2 h, cooled and poured into water (50 cm^3) and the resulting off-white solid product was filtered off, washed with water (10 cm^3) and dried to give the ^{14}C-labelled oxaprozin (99 mg, 68%); m.p. 161–163 °C (lit. 163–165 °C).

centration of 90 μM. The same drugs were also incubated at two different concentrations (40 μM and 90 μM) with the oxaprozin solution.

In the experiments with the metal ions Cu^{2+}, Zn^{2+} and Se^{2+}, their salts were added at concentrations 1 μM and 5 μM. In another experiment the metal ions (5 μM) were added in combination with salicylate at 5 μM and 40 μM to establish if the chelation of the metal ions by salicylate affects their binding. In all experiments drugs and metal ions were incubated with oxaprozin for 2 h at 37 °C in the reservoir of the ultrafiltration device then centrifuged for 30 min at 2000 *g* in a fixed angle rotor (35°) at 37 °C. The free fraction of the drug (the ultrafiltrate) and the bound drugs left in the reservoir of the device were collected in scintillation vials. Next scintillation fluid was added, each device opened, and the membrane was placed in a scintillation vial and then the β-radioactivity was counted on scintillation counter (Packard, UK). Vials containing the same media only served as blanks providing background counts. A total of 5 replicates were performed for each experiment and the free fraction was calculated as a percentage of the total drug.

2.2. Effects of oxaprozin and NSAIDs on binding of tryptophan to albumin

In the tryptophan displacement experiments, ^{3}H-labelled tryptophan was diluted accordingly until a final concentration of 10 μM was achieved. The NSAIDs indomethacin, oxaprozin, diclofenac, nimesulide and salicylate were added to the reservoir of the ultrafiltrate devices at concentrations of 1.0, 10, 50 and 100 μM. Subsequently, the labelled tryptophan was dispensed appropriately into the devices ready for incubation that lasted 30 min at 37 °C. After incubation, all devices were centrifuged for 30 min at 800 *g* in a fixed rotor angle of 35°. Controls consisted of radio-labelled tryptophan without the presence of the selected NSAIDs. For every concentration of drug, including the controls, a series of three replicates were performed. Once centrifuging had been completed the free fraction of the drug (the ultrafiltrate) and the bound fraction (reservoir) were carefully dispensed

in scintillation vials containing scintillation fluid and water. The membrane was also placed into a vial and then the radioactivity was determined by liquid scintillation β-assay and counted for 5 min. The reservoir section measured is the bound fraction whereas the filtrate at the bottom of the devices is the free fraction.

2.3. Uptake of radiolabelled NSAIDs

[^{14}C]Oxaprozin and [^{14}C]nimesulide (100 μM, approx.10 μCi radiolabelled drug) were added to porcine articular cartilage in organ culture. The tissues were cultured for 1–4 days. The radioactivity present in the tissues, after washing with cold drug and medium, and that remaining in the medium was determined (following solubilization) using scintillation counting. Experiments were also performed in which [^{3}H]inulin was added in the presence of 100 μM oxaprozin or nimesulide to establish the relative uptake of the radiolabelled drug in respect of that of [^{3}H]inulin which is an indicator of uptake into tissue water space. The [^{14}C]nimesulide was supplied by Helsinn Healthcare SA (Pambio-Noranco, Lugano, Switzerland) from drug that was originally synthesized by Amersham International (UK).

2.4. Effects of oxaprozin and other NSAIDs on cartilage destruction induced by cytokines and CDA

Bovine nasal cartilage was cultured in the presence of oxaprozin and other comparator drugs with IL-1α for 3 days, or IL-1α with TNFα for 5 days. Oxaprozin solution were prepared by adding 2.93 mg of the drug, a few drops of 0.1 M NaOH, and the mixture made up to 10 ml 4% bovine serum albumin in phosphate-buffered saline. This was then diluted in DMEM to final concentrations of 10–100 μM. All other drugs were prepared in either DMSO, ethanol or prepared as sodium salts. Controls contained the respective solvents. The glycosaminoglycans (GAGs) released into the medium and those remaining in the papain-digested cartilage discs were assayed by the dimethylmethylene blue (Taylor's blue) dye technique (Farndale *et al.*, 1986).

2.5. Effects on release from porcine synovium of catabolin-like CDA and cytokines

Oxaprozin and comparator NSAIDs (prepared as described in Section 2.4 above) were added to synovial explants in culture as described (Rainsford *et al.*, 1989, 1995). The medium was collected and bioassayed after serial dilution in the bovine nasal cartilage resorption assay as above.

2.6. Cytokine and eicosanoid production by THP-1 cells

The THP-1 cells were cultured in Dubecco's Modified Eagle's Medium (DMEM) to which was added 5% (v/v) heat-inactivated foetal calf serum (FCS). Prior to incubation with drugs the cells were centrifuged and resuspended in DMEM without FCS and 0.5 ml of 10^6 cells plated out in 24 well dishes. The cells were incubated

with 10, 50 and 100 μM oxaprozin (prepared as described in Section 2.4 above) or solvent controls for 24 h, then centrifuged and the supernatants assayed for IL-1β, TNFα, IL-6 and IL-8 by ELISA using the manufacturer's (R&D Systems) instructions.

2.7. Production of nitric oxide

The pig synovium was cultured in the presence of the test drugs for 4 days. The medium was collected and assayed for nitrite (as an index of nitric oxide produced) using the Griess reagent (Monaghan *et al.*, 1997). Standard curves were constructed in the presence of the test drugs in order to establish if there was any interference of these drugs in the assay and appropriate correction factors applied to account for any interference.

2.8. Synovial PGE_2 production

The pig synovium was cultured as above in the presence of the test drugs for 4 days. The medium was collected and assayed for PGE_2 using ELISA (R & D systems).

2.9. Proteoglycan synthesis

^{35}S-labelled sulphate in combination with oxaprozin, nimesulide and indomethacin were added to porcine articular cartilage in organ culture (Rainsford, 1985; Rainsford *et al.*, 1989). The tissues were cultured for 24 h. The cartilage was then soaked in 1 M sodium sulphate for about 10 min, washed again 1 M sodium sulphate to remove all unincorporated ^{35}S. The radioactivity remaining in the tissues (after solubilization) and that remaining in the medium was determined using scintillation counting.

2.10. Production of synovial oxygen radicals

Pig synovium was cultured as above in the presence of the test drugs for 4 days. The amount of thiobarbituric acid reactive substances (TBARS) produced in culture was determined (Dickens *et al.*, 1992).

3. RESULTS

3.1. Effects of drugs and metal ions on the binding of oxaprozin to albumin

Using normal HSA a statistically significant ($P < 0.05$, Student's t-test) increase in free oxaprozin was observed when incubated with ibuprofen, diazepam, atenolol and captopril; the increase in free oxaprozin being 6.8, 3.9, 2.15 and 1.52% respectively, compared with the control samples (0.2%) (Fig. 5).

Results from higher and lower concentrations of the same drugs are summarized in Figs 6 and 7. At the low concentration (40 μM), the drugs had small effect on oxaprozin binding to HSA, except for captopril, prednisolone, warfarin,

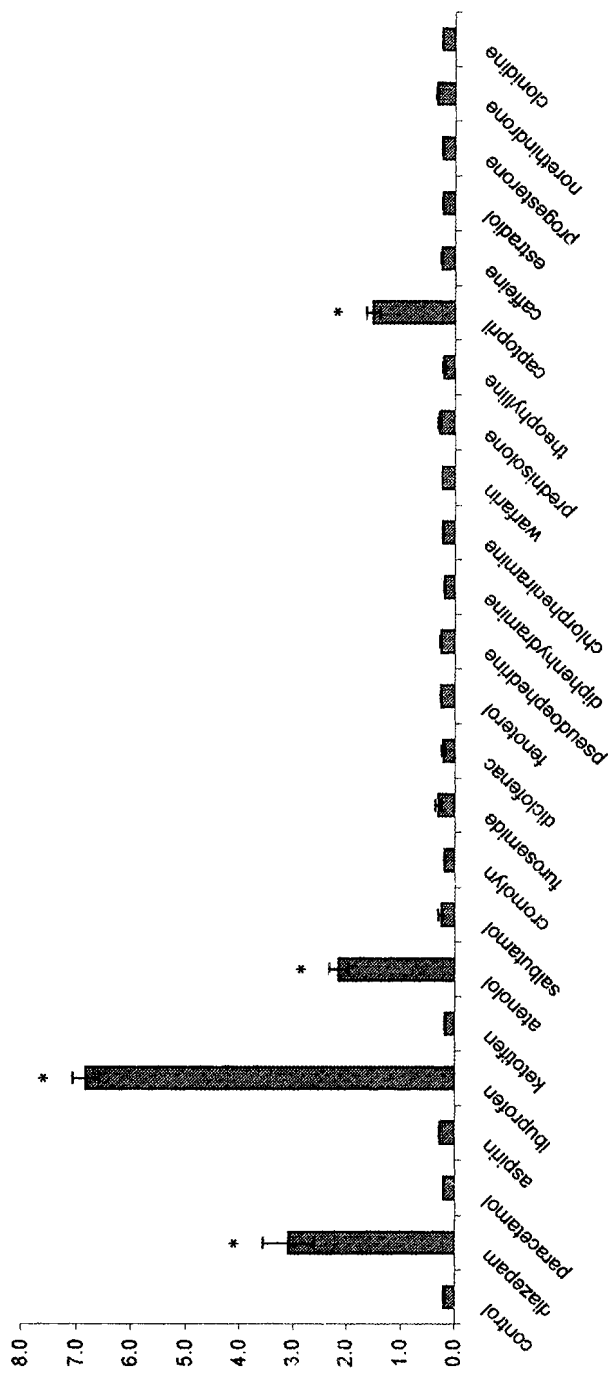


Figure 5. Oxaprozin (10 μM) plus drugs: diazepam (7 μM), paracetamol (20 μM), aspirin (55 μM), ibuprofen (100 μM), ketotifen (40 μM), atenolol (44.5 μM), salbutamol (40 μM), cromolyn (40 μM), furosemide (90 μM), diclofenac (12.5 μM), fenoterol (90 μM), pseudoephidrine (90 μM), diphenhydramine (90 μM), chlorpheniraminie (90 μM), warfarin (90 μM), prednisonlone (90 μM), theophylline (40 μM), captopril (90 μM), caffeine (90 μM), estradiol (90 μM), progesterone (90 μM), norethindrone (90 μM) and clonidine (90 μM). Data are mean±S.E.M. $^*P < 0.05$, compared with control.

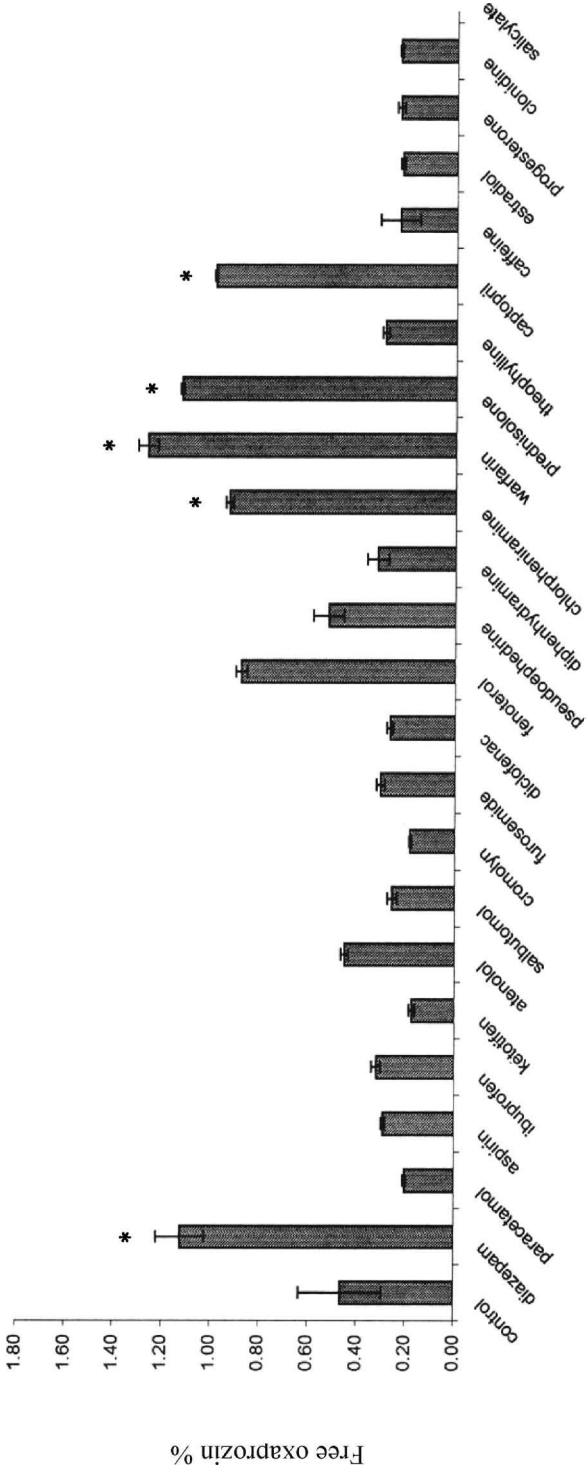


Figure 6. Oxaprozin (10 μM) + drug (40 μM). Data are mean ($n = 5$) $\pm$ S.E.M. $^{*}P < 0.05$, compared with control.

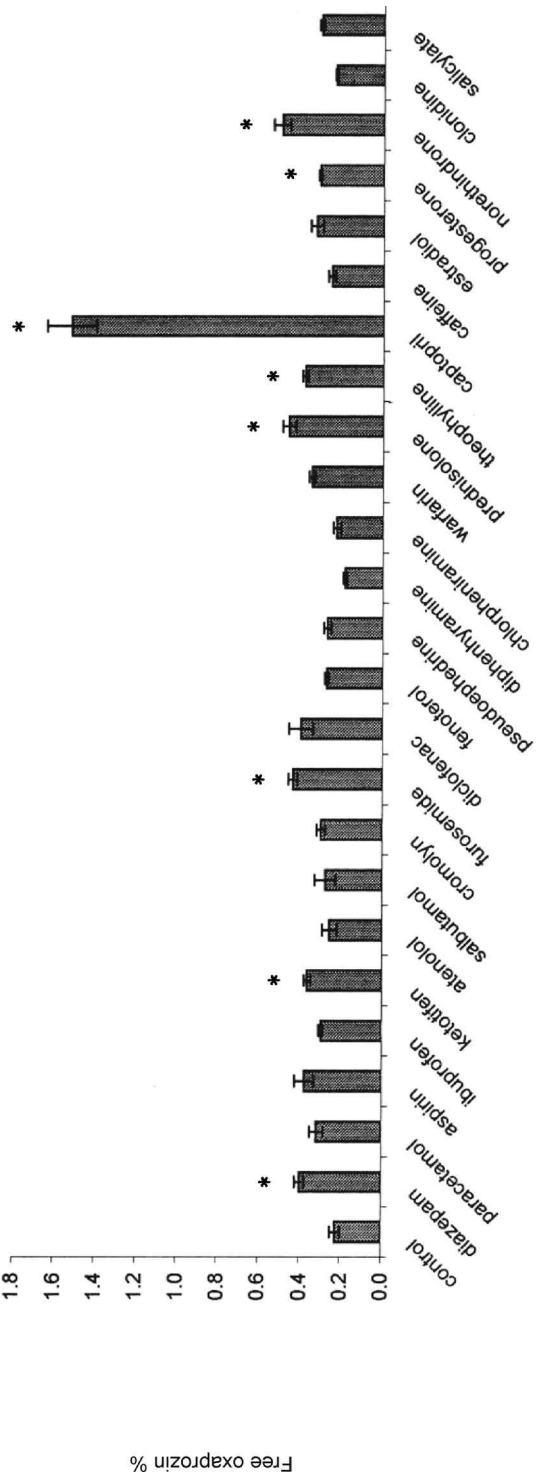


Figure 7. Oxaprozin (10 μM) + drug (90 μM). Data are mean ($n = 5$) $\pm$ S.E.M. * $P < 0.05$, compared with control.

chlorpheniramine and diazepam, where a small but statistically significant increase in free oxaprozin was observed. The increase was 0.99, 1.1, 1.2, 0.03 and 1.1%, respectively, compared with the control samples (Fig. 6).

At a concentration of 90 μM, most drugs increased the free fraction of oxaprozin. However, this increase was statistically significant, when oxaprozin was incubated with northindrone, progesterone, captopril, theophylline, prednisolone, furosemide, ketotifen and diazepam, the values being 0.49, 0.2, 1.5, 0.4, 0.5, 0.4, 0.4 and 0.4%, respectively, compared with the control. In contrast a small decrease in the free oxaprozin was observed when oxaprozin was incubated with diphenhydramine (Fig. 7).

When the drugs were incubated with fatty acid-free human serum albumin (FAF-HSA), a statistically significant increase in the free oxaprozin was observed only with captopril and caffeine, the increase being 1.9 and 3.5%, respectively, compared with the control samples (0.4%). The other drugs had either a small decrease in the free oxaprozin or no significant effect (Fig. 8).

The metal ions Cu^{2+}, Zn^{2+} and Se^{2+} had effects on the binding characteristics of oxaprozin. Free oxaprozin levels were increased significantly when the metal ions were incubated with HSA at concentrations of 5 μM and 1 μM (Fig. 9). When the metal ions were incubated with FAF-HSA there was also an increase in free oxaprozin. However, this increase was statistically significant only at a metal ion concentration of 5 μM (Fig. 9). At a metal ion concentration of 1 μM, the increase in free oxaprozin was not statistically significant (Fig. 10).

When copper and zinc were combined with salicylate (40 μM) and incubated with HSA no alterations were observed in the free portion of oxaprozin. However, when salicylate was incubated with selenium there was small increase in free oxaproxin compared with the control samples (Fig. 11). Incubating salicylate at a concentration of 5 μM with the three metal ions showed no effect on the free portion of oxaprozin (Fig. 11).

3.2. Effects of oxaprozin and other NSAIDs on binding of tryptophan to albumin

The results (Fig. 12, Table 1) show that indomethacin, diclofenac, nimesulide and salicylate significantly displaced L-tryptophan from its binding sites on serum albumin in a concentration-dependent manner. Statistically significant ($P < 0.05$) effects of the NSAIDs on the binding of L-tryptophan to serum albumin were observed at concentrations of 50 and 100 μM respectively with diclofenac, indomethacin, nimesulide and salicylate, although there seemed to be little displacement at 10 and 1 μM. Approximately 68% of free serum tryptophan levels was observed with 100 μM salicylate. Indomethacin, nimesulide and diclofenac showed similar effects by displacing up to 53, 56 and 47% free serum tryptophan levels, respectively, at 100 μM. Oxaprozin (100 μM) exerted statistically significant displacement of 41% free tryptophan.

Figure 8. Oxaprozin (10 μM) + drug. Drug concentration was 90 μM, except for progesterone (50 μM), estradiol (50 μM) and norethindrone (50 μM). Data are mean ($n = 5$) $\pm$ S.E.M. * $P < 0.05$, compared with control.

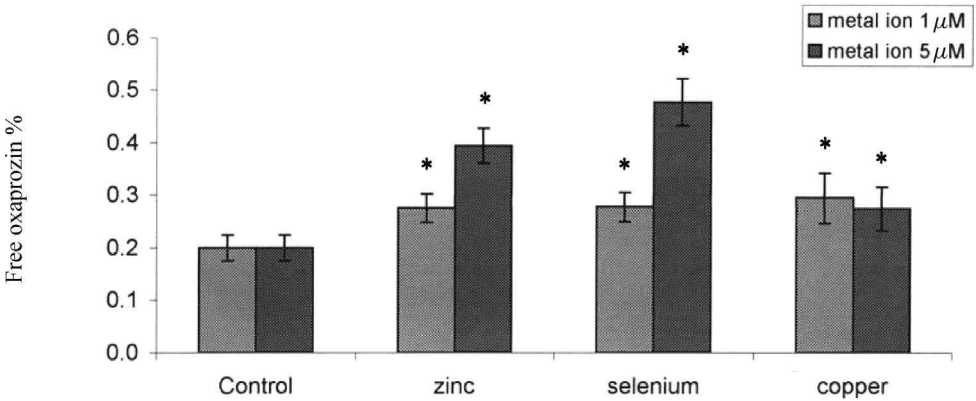


Figure 9. Oxaprozin (10 μM) + metal ion. Data are mean ($n = 5$) ± S.E.M. $^*P < 0.05$, compared with control.

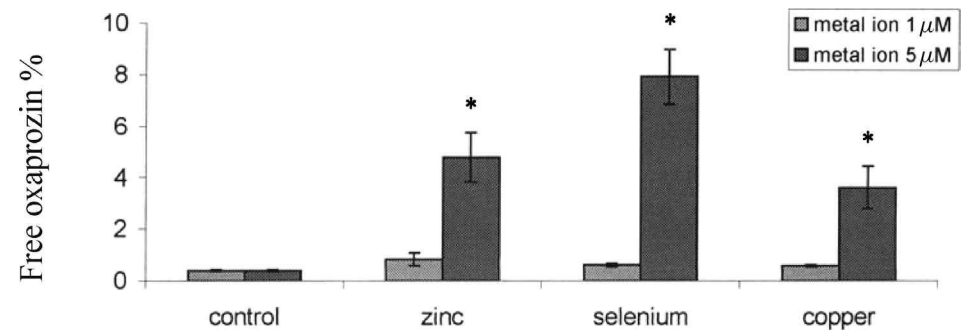


Figure 10. Oxaprozin (10 μM) + metal ion with FAF-HSA. Data are mean ($n = 5$) ± S.E.M. $^*P < 0.05$, compared with control.

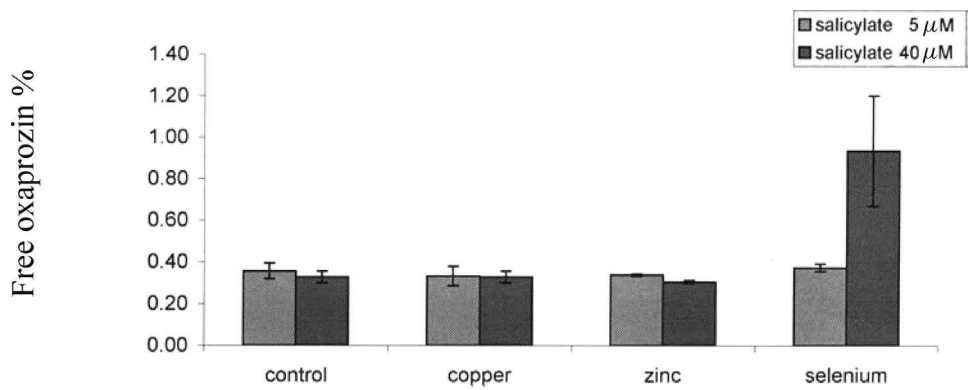


Figure 11. Oxaprozin (10 μM) + salicylate + metal ion (5 μM).

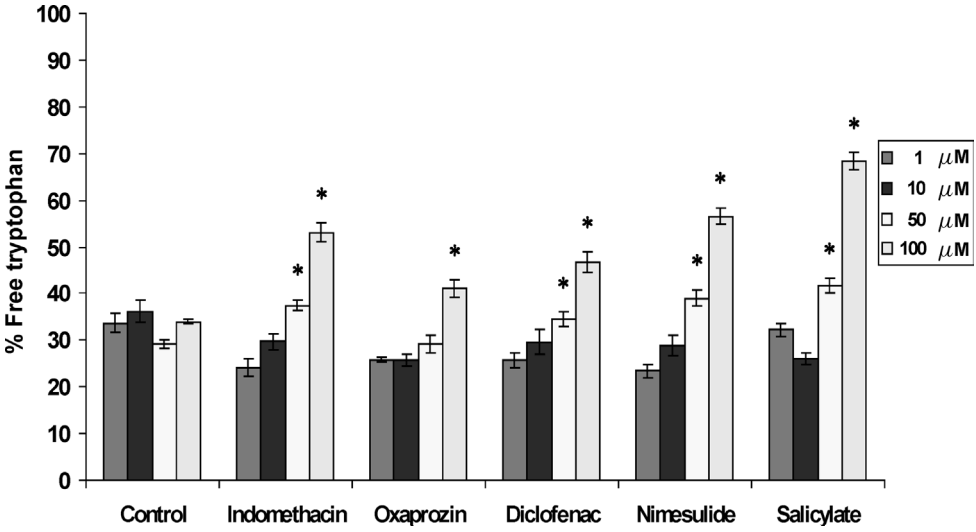


Figure 12. Effects of various NSAIDs (1–100 μM) on the binding of tryptophan (10 μM) to HSA. Values are mean ± S.E.M. ($n = 3$). Statistical analysis was performed by using the two-tailed Student's t-test. *Significantly different from control ($P < 0.05$).

3.3. Uptake of radiolabelled oxaprozin and nimesulide into cartilage

Figures 13A and 14A show the uptake of [^{14}C]oxaprozin and [^{14}C]nimesulide into pig articular cartilage using the same system as employed in our previous studies with [^{14}C]indomethacin, [^{3}H]diclofenac and [^{14}C]azapropazone (Rainsford *et al.*, 1989 and unpublished studies). The difference in these experiments was that IL-1β and TNFα alone or in combination were added to stimulate cartilage matrix breakdown which might be considered a factor in enabling drug penetration. It would also be expected to replicate conditions that occur in arthritic joints where the cartilage is undergoing degradation.

It appears that there is appreciable uptake of [^{14}C]oxaprozin within the first day of culture and this would appear to be consistent over the next 4 days of incubation (Fig. 13A). These results suggest that the uptake of oxaprozin may be initially relatively quick and then remains constant. The effects of 100 μM oxaprozin on the uptake of [^{3}H]inulin with time are shown in Fig. 13B.

The uptake of [^{14}C]nimesulide was lower than compared with that of the same concentration (100 μM) of oxaprozin. The concentrations of radiolabelled nimesulide also appear to be quite constant with time of incubation (Fig. 14A). The drug did not vary the uptake of [^{3}H]inulin uptake with time (Fig. 14B). Overall, the uptake of oxaprozin appears to be about 50% greater than that of the same concentration of nimesulide. These results were confirmed in a second experiment under similar conditions. When compared with the uptake of [^{3}H]inulin the uptake of oxaprozin was slightly greater than that of nimesulide (Fig. 13C, cf. Fig. 14C). Thus, these differences in the uptake of oxaprozin compared with nimesulide are

Table 1.
Effects of various NSAIDs on the binding of tryptophan to HSA

Concentration (μM)	NSAID added	Mean (% free)	S.E.M.
1	Control	33.66	1.97
	Indomethacin	24.25	1.92
	Oxaprozin	25.81	0.39
	Diclofenac	25.67	1.53
	Nimesulide	23.40	1.47
	Salicylate	32.18	1.45
10	Control	36.15	2.32
	Indomethacin	29.63	1.85
	Oxaprozin	25.79	1.19
	Diclofenac	29.61	2.68
	Nimesulide	28.84	2.26
	Salicylate	25.94	1.24
50	Control	29.22	0.90
	Indomethacin	37.46*	1.03
	Oxaprozin	29.20	1.97
	Diclofenac	34.57*	1.56
	Nimesulide	38.99*	1.75
	Salicylate	41.67*	1.60
100	Control	34.00	0.51
	Indomethacin	53.12*	2.02
	Oxaprozin	41.11*	1.94
	Diclofenac	46.78*	2.13
	Nimesulide	56.53*	1.65
	Salicylate	68.37*	1.91

* Significantly different from control ($P < 0.05$, Student's t-test).

probably related to the greater uptake by oxaprozin into more lipophilic (non-tissue water space) compartments.

3.4. Effects of oxaprozin and nimesulide on cytokine-induced cartilage resorption

The data in Figs 15–21 are from all the experiments that have been performed to examine the effects of various concentrations of oxaprozin and nimesulide on the resorption of bovine nasal cartilage induced by varying concentrations of IL-1α (Figs 15–18) or IL-1α with TNFα (Figs 19–21).

These results, overall, show that there did not appear to be consistent statistically significant effects of either oxaprozin or nimesulide on the resorption of cartilage induced by the various cytokine concentrations or combinations. While in one experiment nimesulide (10 and 100 μM) produced a concentration related reduction in the cartilage resorption induced by a high concentration of 500 U/ml IL-1α this effect was not shown when the drug was incubated with other concentrations of

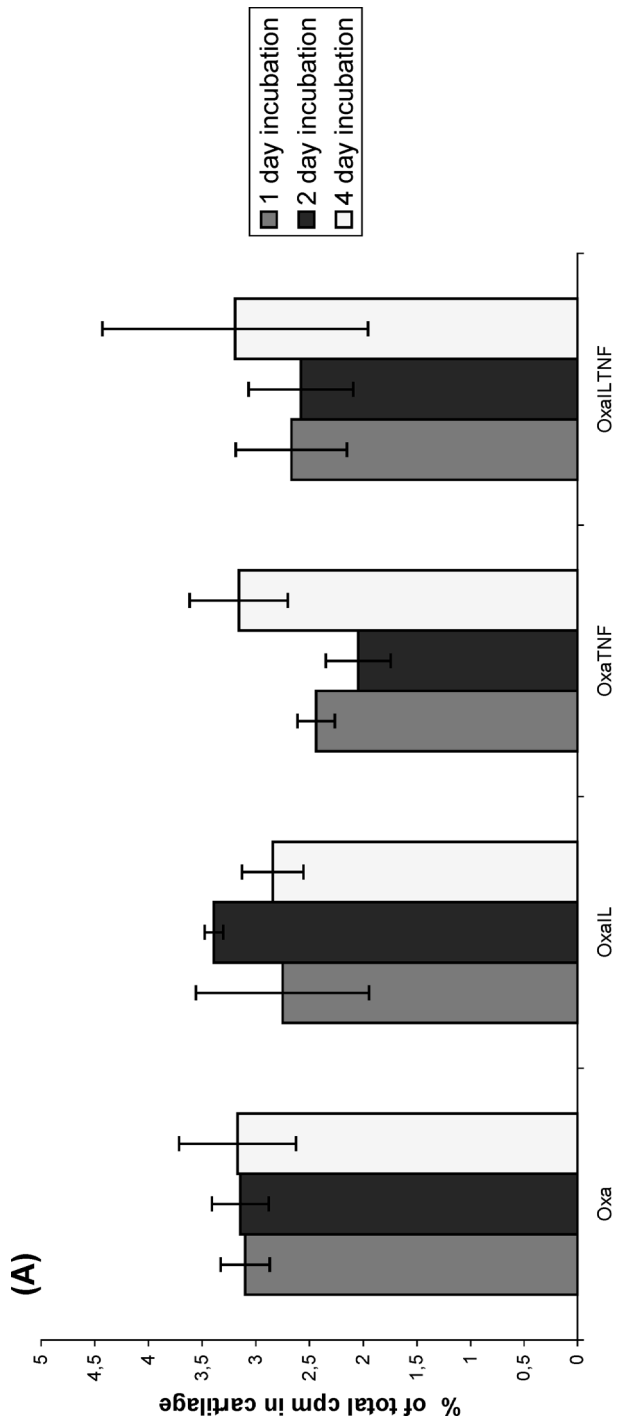


Figure 13. (A) Percentage uptake of [^{14}C]oxaprozin (Oxa, 100 μM) into porcine articular cartilage in the prescence of IL-1α (IL, 100 U/ml) and TNFα (200 U/ml). Values are mean ($n = 4$) $\pm$ S.E.M. (B) Percentage uptake of [^{3}H]inulin per mg of porcine articular cartilage after incubation with oxaprozin (100 μM), IL-1α (100 U/ml) and TNFα (200 U/ml). Values are mean ($n = 4$) $\pm$ S.E.M. (C) Ratios of oxaprozin/inulin uptake in porcine articular cartilage when treated with IL-1α (100 U/ml) and TNFα (200 U/ml). Values are mean ($n = 4$) $\pm$ S.E.M.

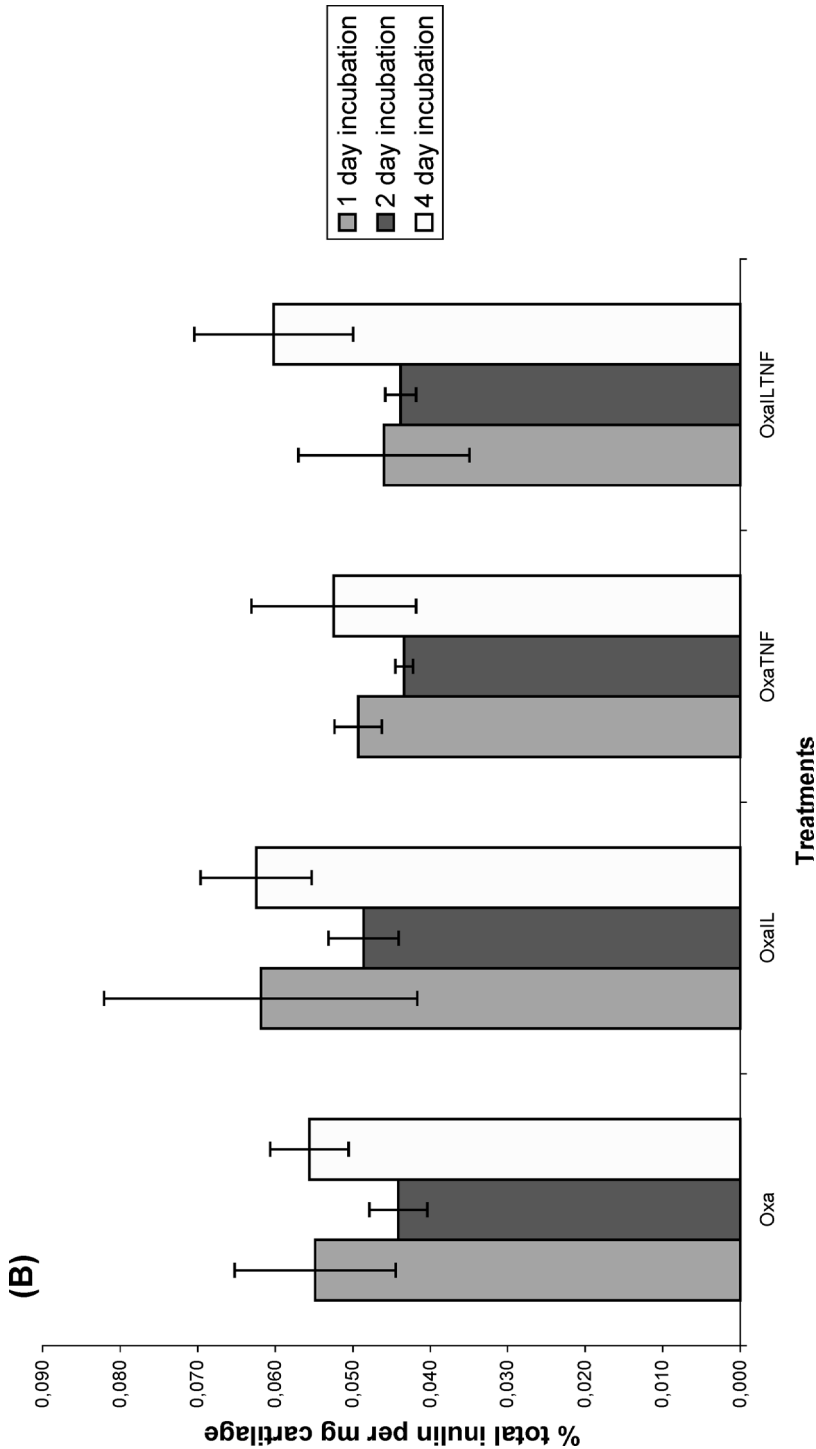
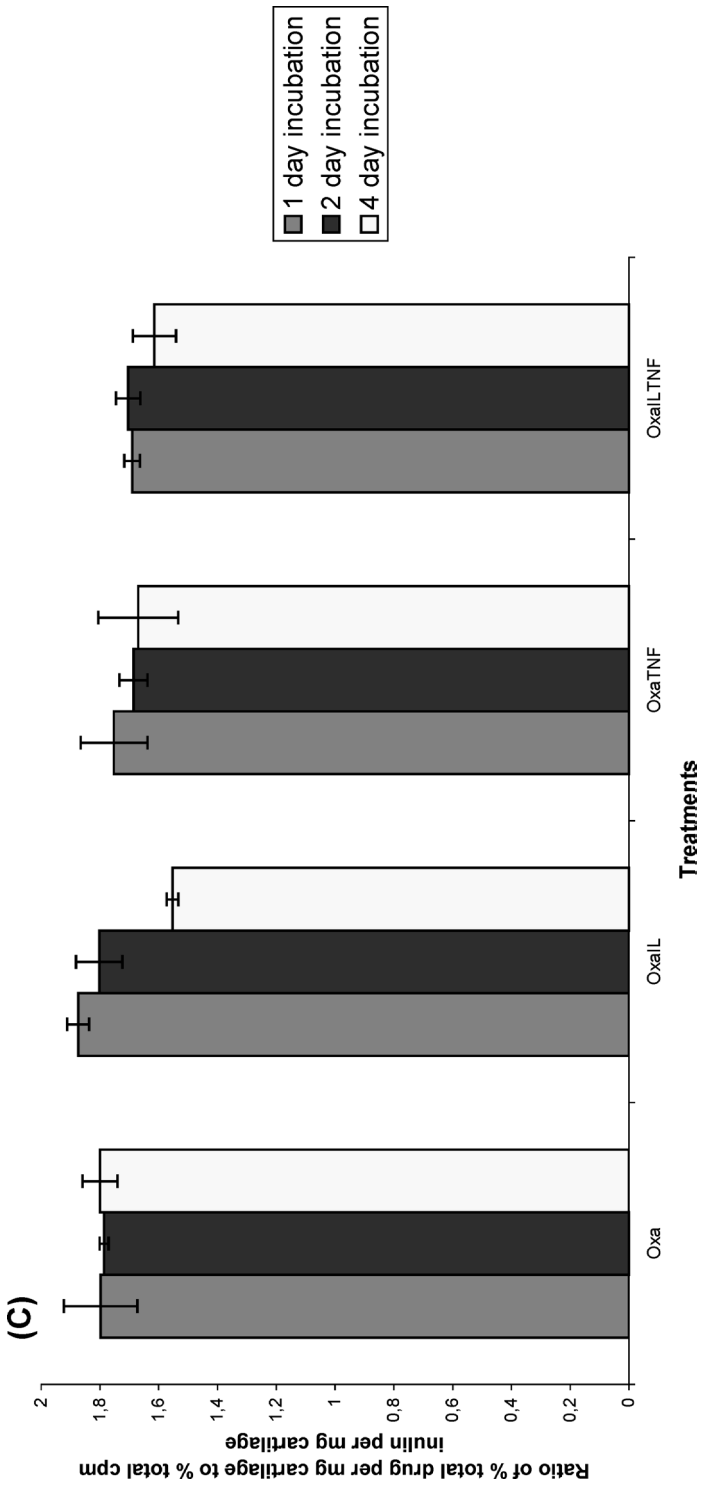


Figure 13. (Continued).



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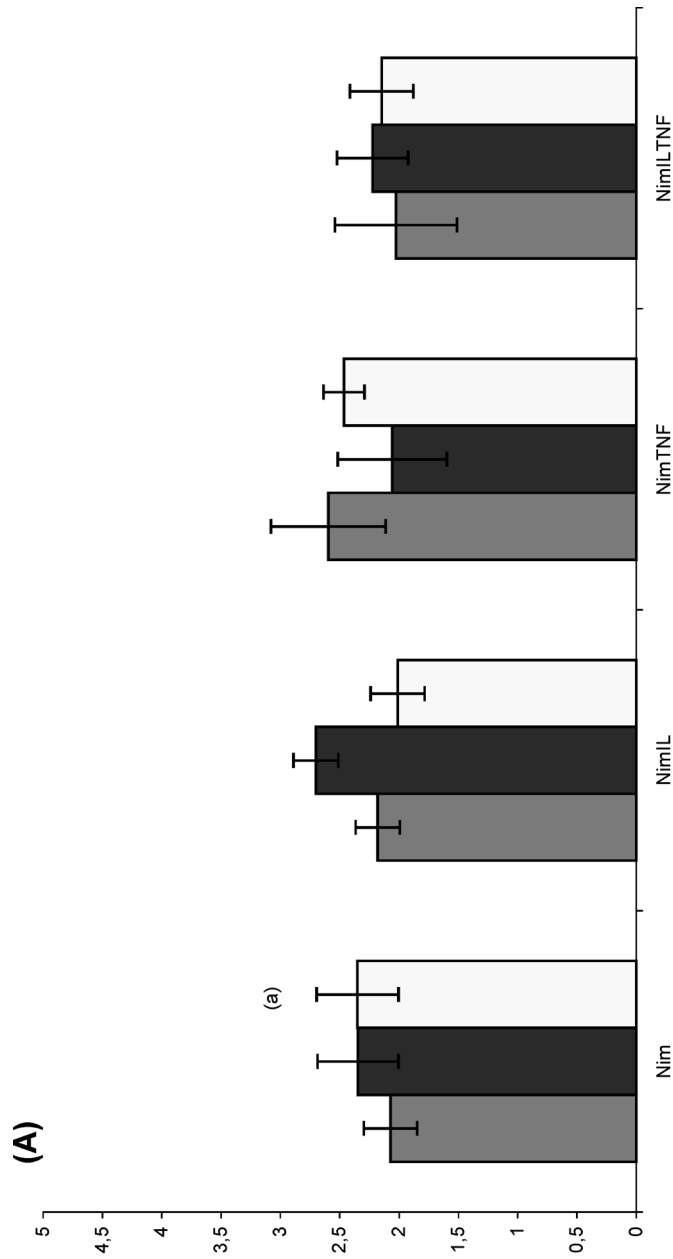
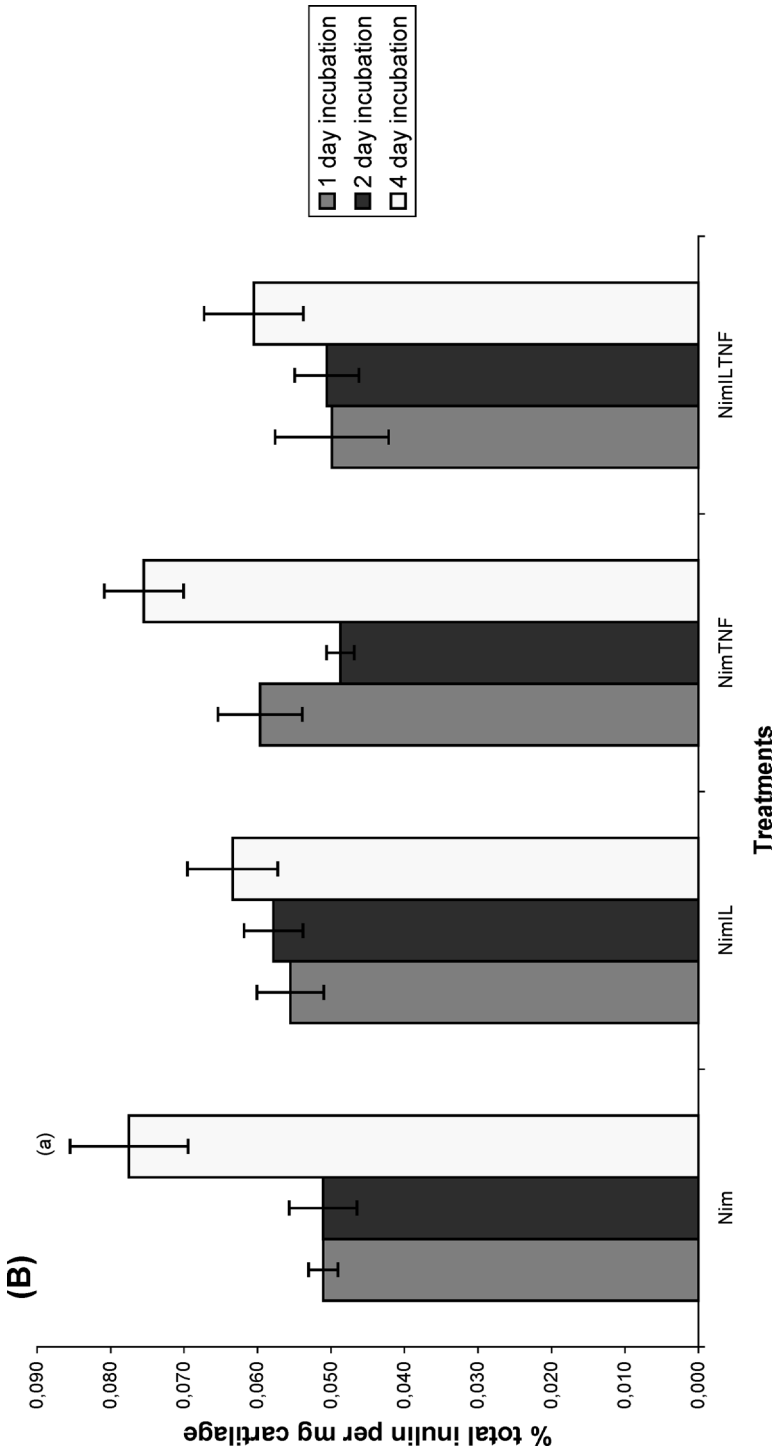


Figure 14. (A) Percentage uptake of [^{14}C]nimesulide (Nim, 100 μM) into porcine articular cartilage in the prescence of IL-1α (IL, 100 U/ml) and TNFα (200 U/ml). Values are mean ($n = 4$) $\pm$ S.E.M. (B) Percentage uptake of [^{3}H]inulin per mg of porcine articular cartilage after incubation with nimesulide (100 μM), IL-1 (100 U/ml) and TNFα (200 U/ml). Values are mean ($n = 4$) $\pm$ S.E.M. (a) $n = 3$. (C) Ratios of drug nimesilide/inulin uptake in porcine articular cartilage when treated with IL-1α (100 U/ml) and TNFα (200 U/ml). Values are mean ($n = 4$) $\pm$ S.E.M. (a) $n = 3$.



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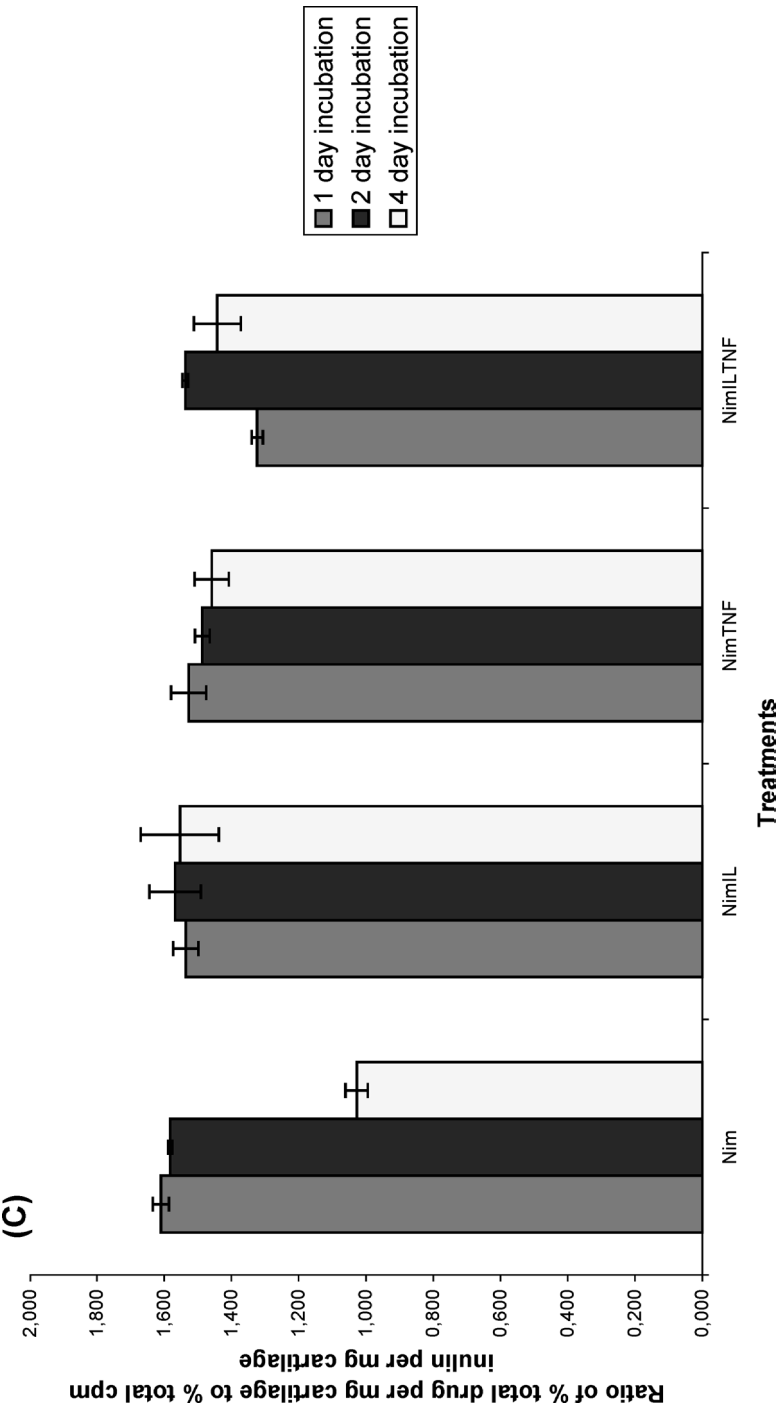

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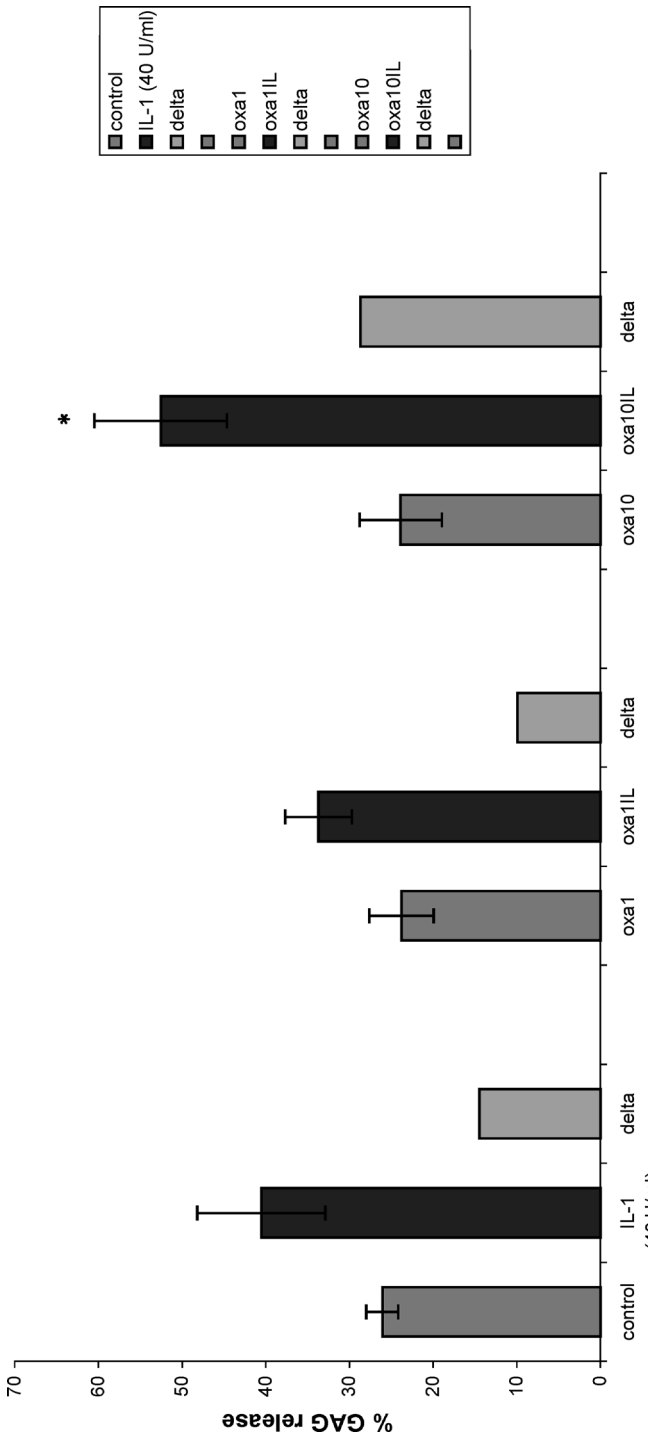

Figure 15. Effects of oxaprozin (Oxa, 0–10 μM) on IL-1α (IL, 40 U/ml) stimulated cartilage resorption. Values are mean ($n = 6$) $\pm$ S.E.M. $^*P < 0.05$, compared with control. delta = difference between IL-1 ($\pm$ drug) treated and control (or drug alone).

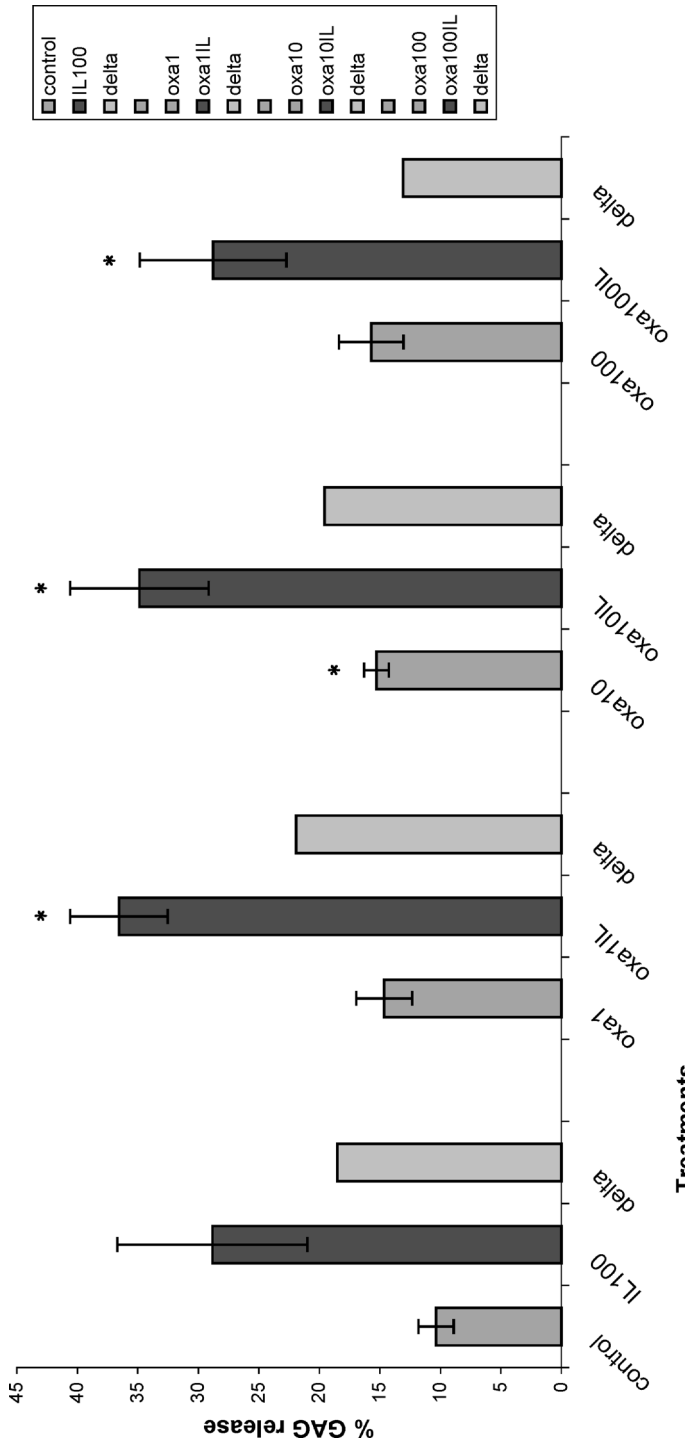

Figure 16. Effects of oxaprozin (0–100 μM) on IL-1α (100 U/ml) stimulated cartilage resorption. Values are mean ($n = 6$) $\pm$ S.E.M. * $P < 0.05$, compared with control. delta = difference between IL-1 ($\pm$ drug) treated and control (or drug alone).

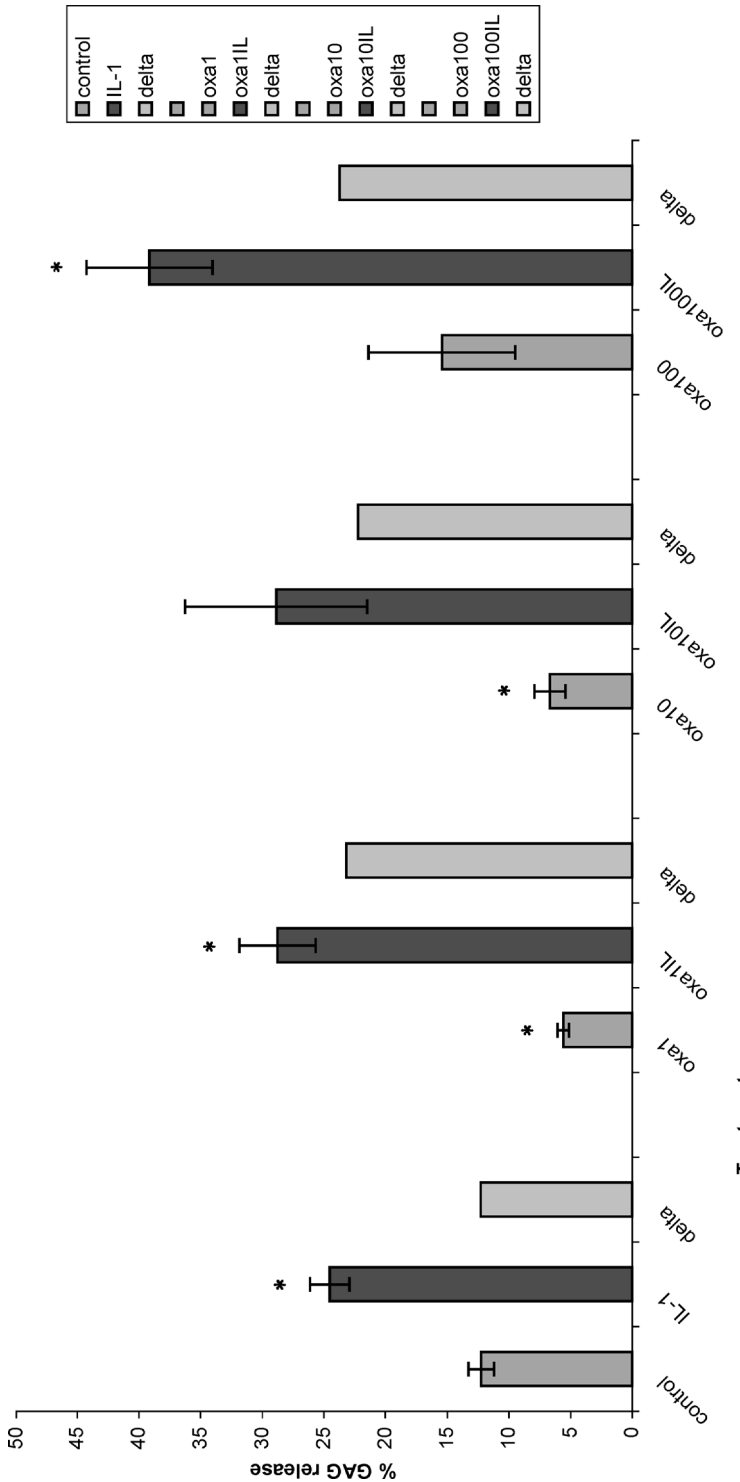

Figure 17. Effects of oxaprozin (0–100 μM) on IL-1α (500 U/ml) stimulated cartilage resorption. Values are mean ($n = 5$) $\pm$ S.E.M. * $P < 0.05$, compared with control. delta = difference between IL-1 ($\pm$ drug) treated and control (or drug alone).

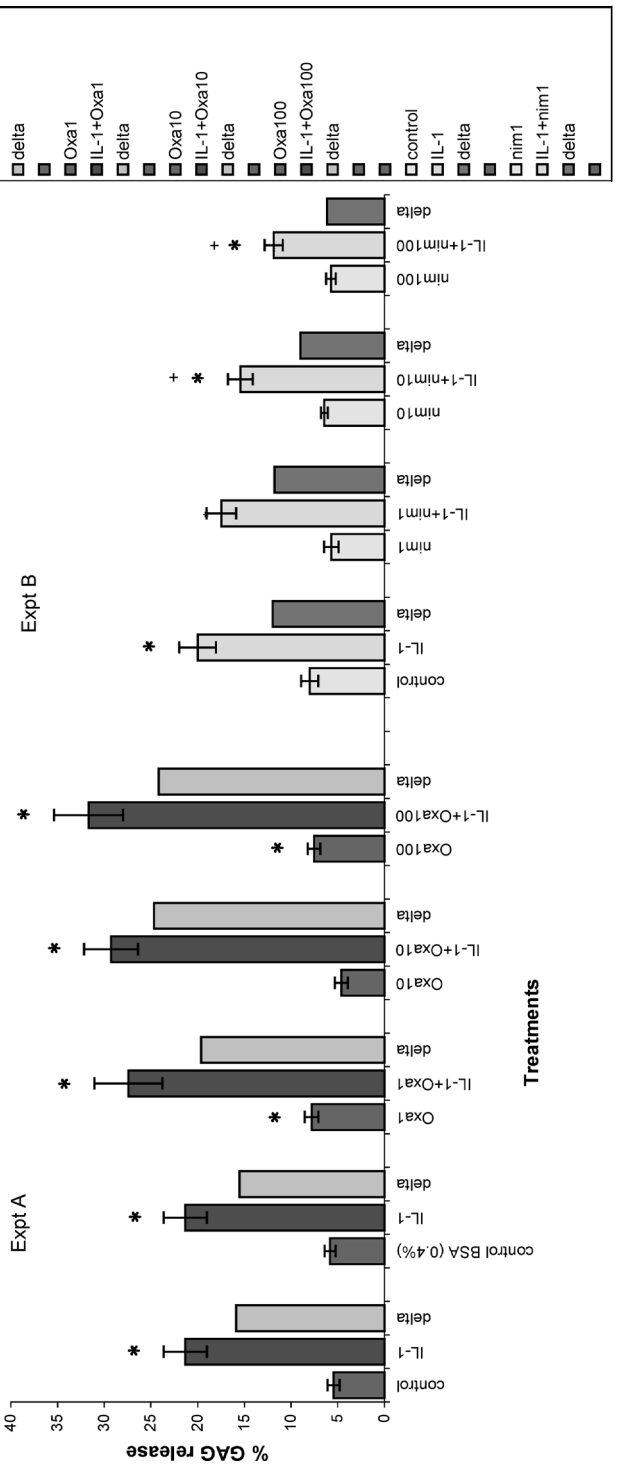

Figure 18. Effects of IL-1α (500 U/ml) stimulated cartilage resorption in the presence of 0–100 μM oxaprozin ($n = 12$) and 0–100 μM nimesulide ($n = 11$). Data shown are means $\pm$ S.E.M from two separate experiments combined. $^{*}P < 0.05$, compared with control; $^{+}P < 0.05$, compared with IL-1 ocntrol. Oxa = oxaprozin in 0.4% BSA, Nim = nimesulide, delta = difference between GAGs release with IL-1 with or without drug.

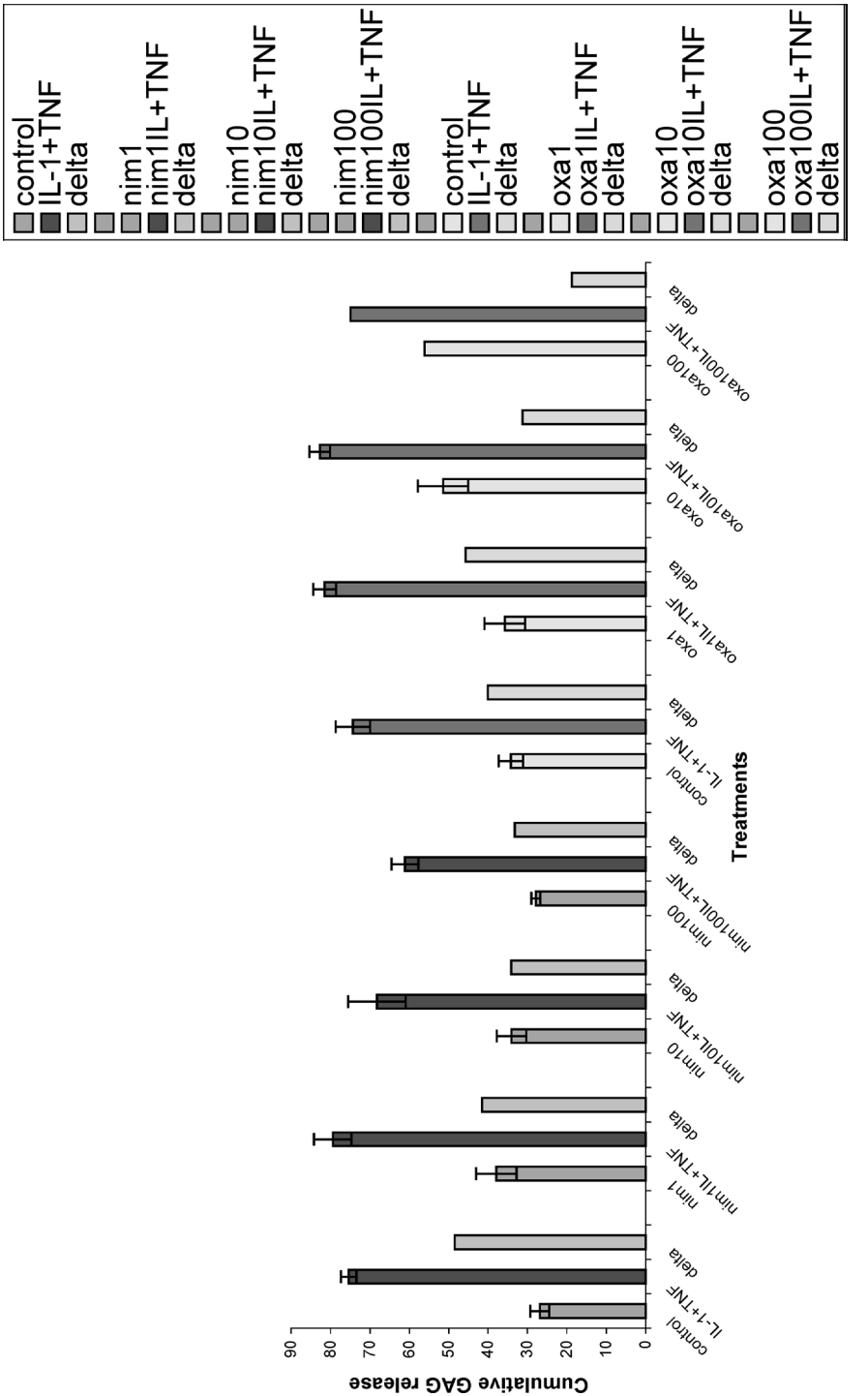

Figure 19. Effects of nimesulide (1.0–100 μM) and oxaprozin (1.0–100 μM) on IL-1α (40 U/ml) and TNFα (2000 U/ml) stimulated cartilage resorption. Values are mean ($n = 6$) $\pm$ S.E.M. delta = difference between cytokine ($\pm$ drug) treated and control (or drug alone).

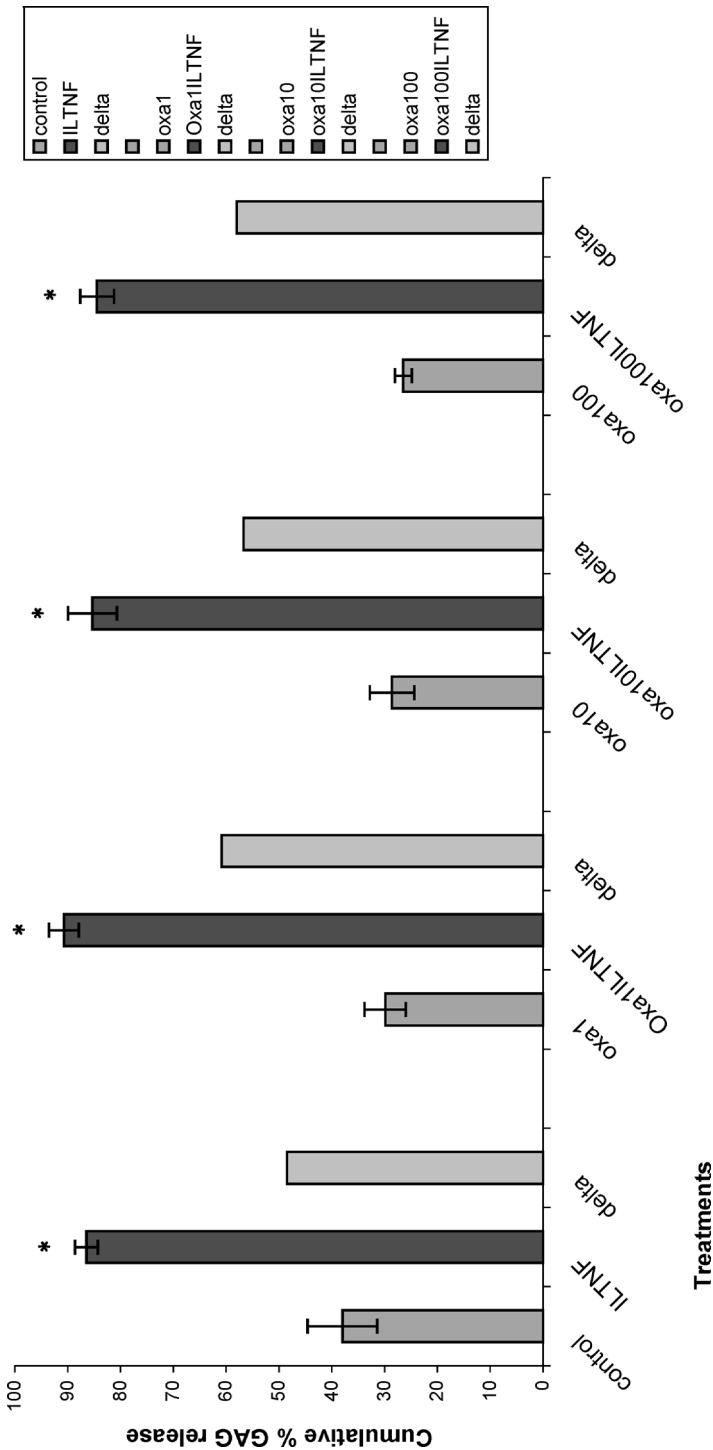

Figure 20. Effects of oxaprozin (0–100 μM) on IL-1α (100 U/ml) and TNFα (2000 U/ml) stimulated cartilage resorption. Values are mean ($n = 6$) $\pm$ S.E.M. * $P < 0.05$, compared with control. delta = difference between IL-1 ($\pm$ drug) treated and control (or drug alone).

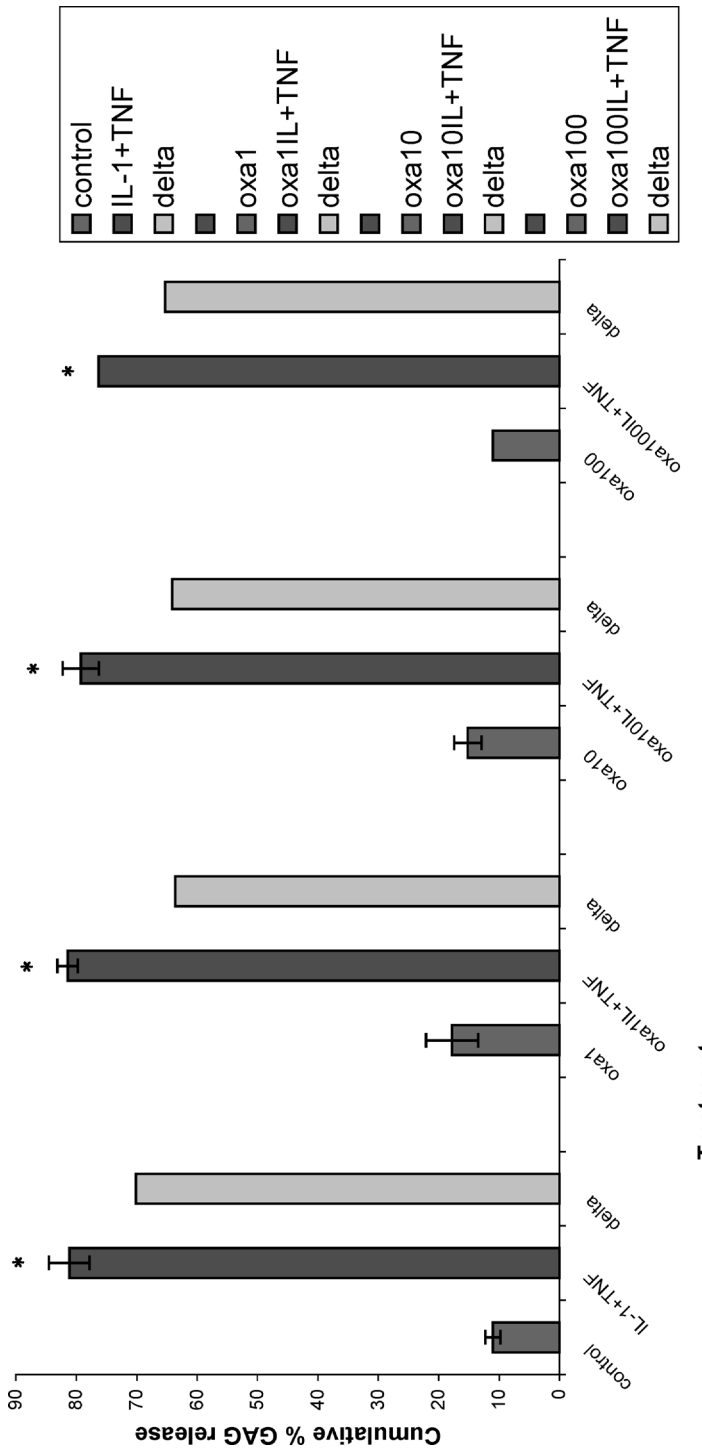

Figure 21. Effects of oxaprozin (0–100 μM) on IL-1α (500 U/ml) and TNFα (2000 U/ml) stimulated cartilage resorption. Values are mean ($n = 6$) $\pm$ S.E.M. * $P < 0.05$, compared with control. delta = difference between IL-1 ($\pm$ drug) treated and control (or drug alone).

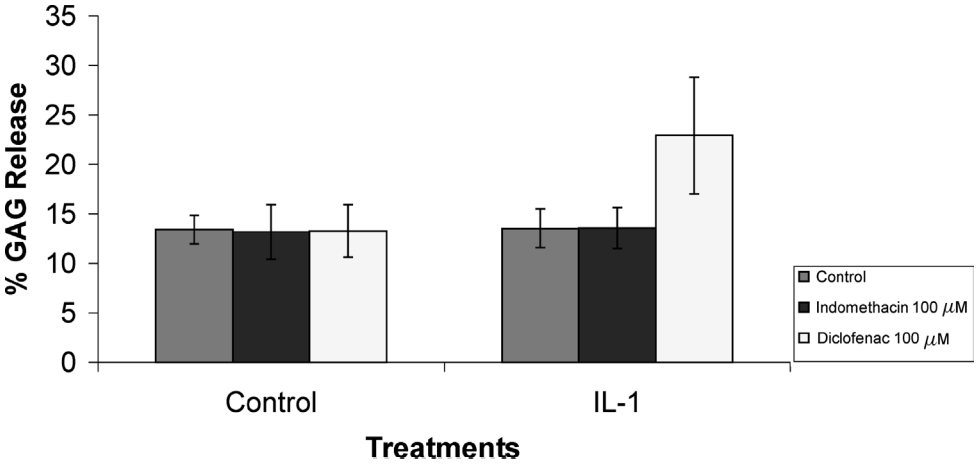

Figure 22. Effects of indomethacin and sodium diclofenac on IL-1 (40 U/ml) stimulated cartilage resorption. Values are mean ($n = 8$).

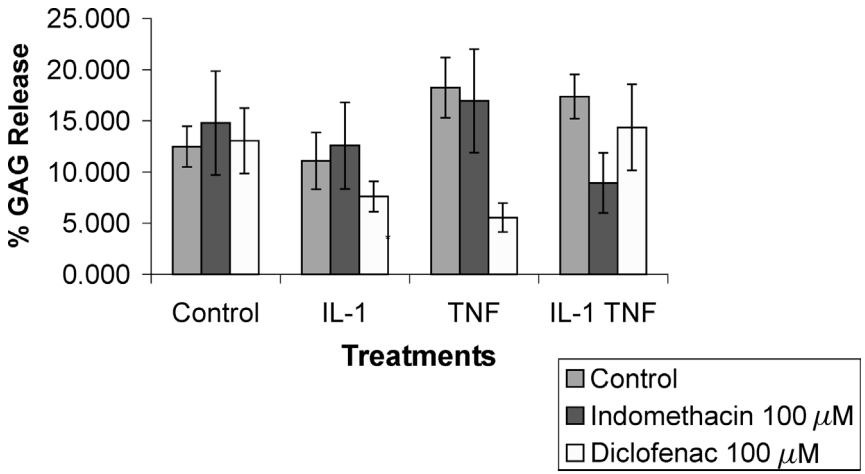

Figure 23. The effects of indomethacin and sodium diclofenac on IL-1α (40 U/ml) and TNFα (200 U/ml) stimulated cartilage resorption. Values are mean ($n = 6$) $\pm$ S.E.M. $^{*}P < 0.05$, compared with TNF control.

the cytokines (Fig. 18). Some minor inter-experiment variation can be seen but this is normal with this type of organ culture model. The use of relatively large numbers of replicates per experiment allows for clear statistical analysis of the data. In comparison, the data in Figs 22 and 23 with the comparitor drugs, diclofenac sodium and indomethacin (with one exception in the case of diclofenac with TNFα), show no significant effects on cytokine-mediated cartilage resorption.

These experiments were performed over a wide range of cytokine and drug concentrations. This has enabled unequivocal establishment of the negative effects of the drug treatments.

3.5. Release of cartilage degrading activity (catabolin) from porcine synovium in vitro

The results in Fig. 24A show that oxaprozin (1.0–100 μM) did not inhibit the release from porcine synovial tissue of cartilage-degrading activity (CDA) following 4 days incubation. Neither did oxaprozin affect the IL-1α-induced cartilage degradation when added directly to the nasal cartilage assays (Fig. 24B). These negative results were evident irrespective of the dilution of the synovial culture media, thus showing that there is no effect of putative inhibitors released in the presence of the drugs. Similar data were obtained after 6 days incubation. The results in Fig. 24C show that nimesulide significantly decreased the IL-1α stimulated cartilage resorption at 100 μM but not at the lower concentrations of 1 μM and 10 μM. The effect was also seen with prednisolone (10 μM) which was included as a positive control (Fig. 24C).

3.6. Production of cytokines from porcine synovium and THP-1 cells

The production of the pro-inflammatory cytokines, IL-1β, TNFα, IL-6 and IL-8 was uniformly inhibited at concentrations of 50–100 μM (data not shown).

3.7. Production of nitric oxide by isolated bovine articular chondrocytes and porcine synovium in vitro

The results in Fig. 25 show that nitric oxide production did not appear to be affected by oxaprozin. This was irrespective of whether the experimental data was grouped into low or high NO responding cells (data not shown). In contrast, both indomethacin and nimesulide reduced the NO production stimulated by IL-1α (Figs 26 and 27). The effects of nimesulide on IL-1α-induced NO production were greater with low NO rather than high NO producing cells.

With the exception of the lowest concentration of oxaprozin (1.0 μM) the production of NO from porcine synovial tissue did not appear to be affected by oxaprozin (10–100 μM) (data not shown).

3.8. Production of PGE_2

The results in Fig. 28 show that IL-1α-stimulated PGE_2 production in porcine synovial explants was reduced by 100 μM oxaprozin in the absence of IL-1β. At lower concentrations oxaprozin (1 and 10 μM) inhibited the IL-1α-stimulated PGE_2 production.

3.9. Proteoglycan synthesis

The results in Fig. 29 show that oxaprozin (1.0–100 μM) stimulates while addition of IL-1α negated this effect on the incorporation of [^{35}S]sulphate into the porcine articular cartilage. A stimulatory effect was also seen with 10–100 μM nimesulide (both basal and IL-1α stimulated) (Fig. 30). However, indomethacin alone inhibited

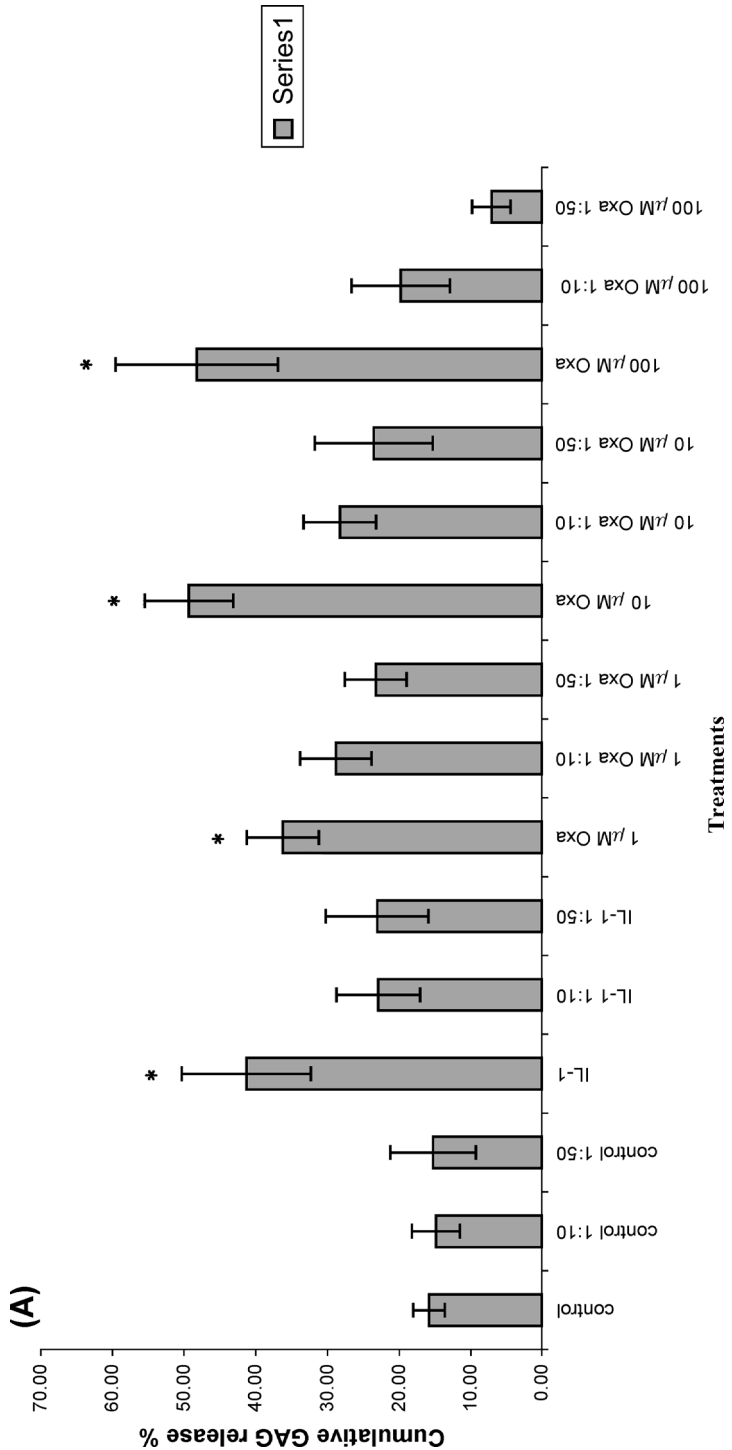

Figure 24. (A) Effects of oxaprozin (0–100 μM) at various dilutions on cartilage resorption. Values are mean ($n = 6$). Incubation time 4 days. $^{*}P < 0.05$, compared with control. (B) Effects of oxaprozin (0–100 μM) with or without IL-1α (100 U/ml) at various dilutions on cartilage resorption. Values are mean ($n = 8$). Incubation time 4 days. $^{*}P < 0.05$, compared with the 1 : 10 control. (C) Effect of nimesulide on IL-1α (100 U/ml) stimulated cartilage resorption. Values are means ($n = 12$) $\pm$ S.E.M. $^{*}P < 0.05$, compared with IL-1 control.

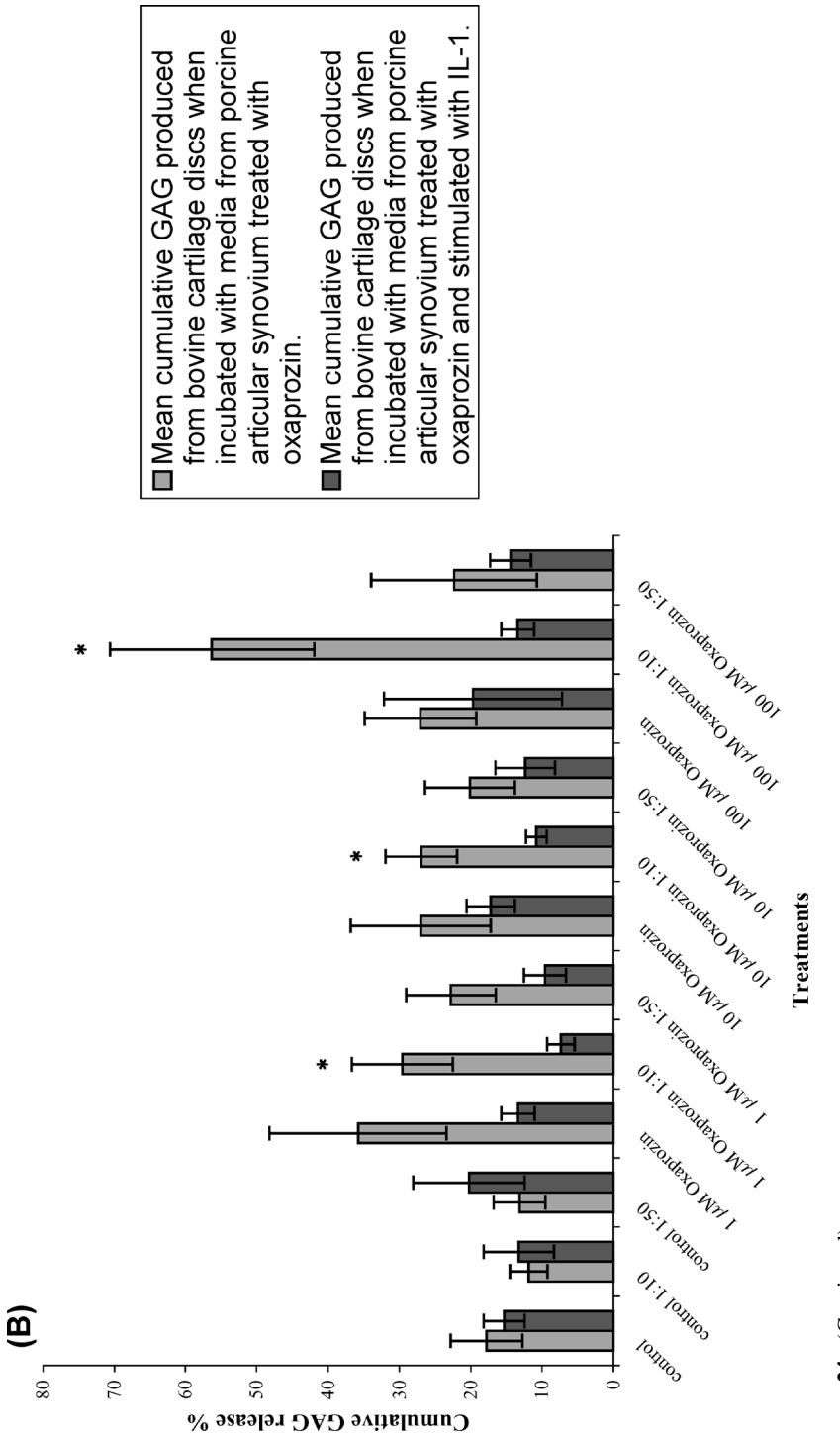

Figure 24. (Continued).

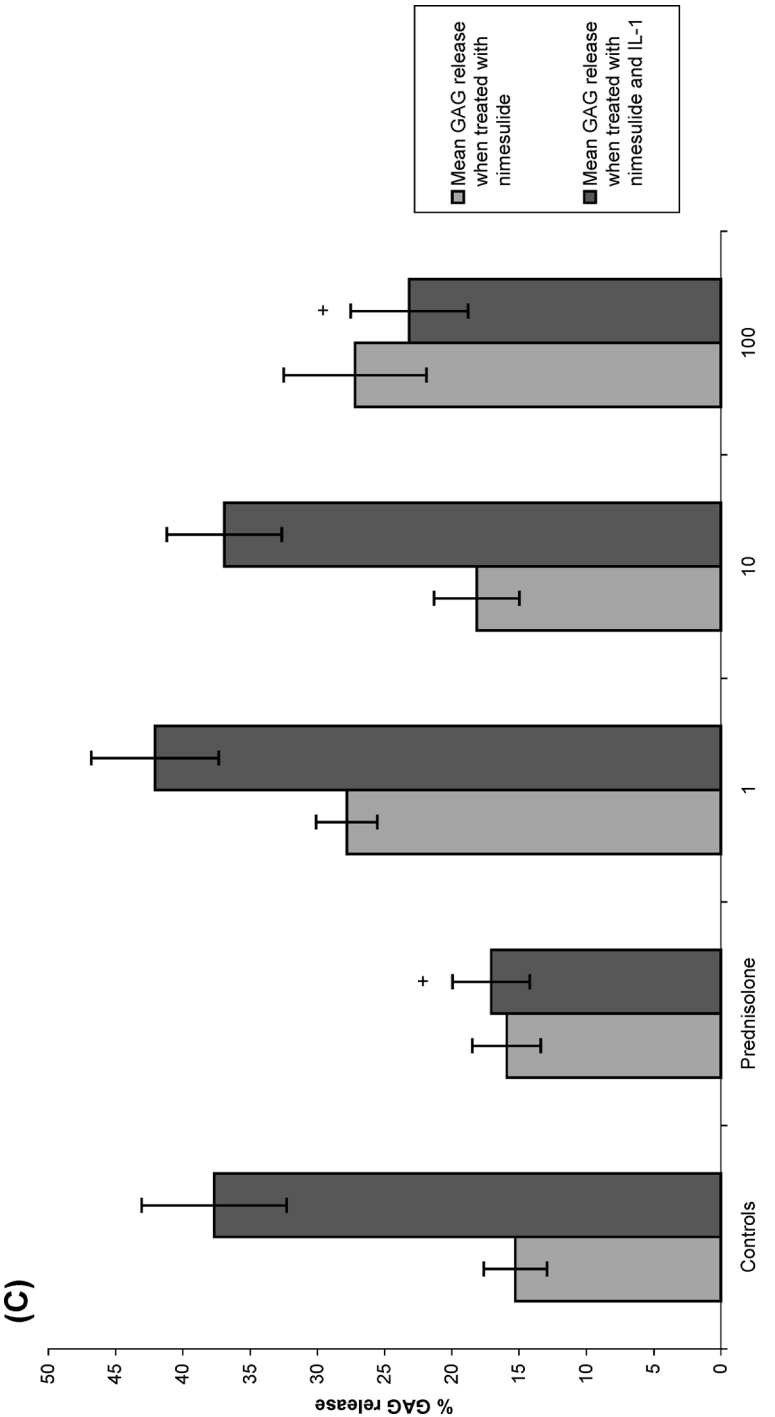

Figure 24. (Continued).

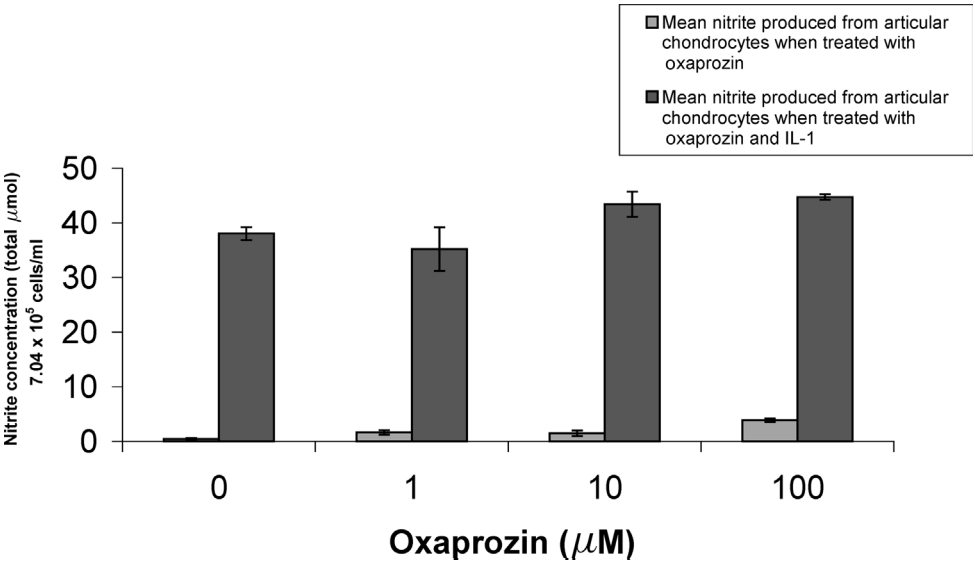

Figure 25. The effect of oxaprozin on nitric oxide production by IL-1α (100 U/ml) stimulated articular chondrocytes. Values are means ($n = 8$) $\pm$ S.E.M.

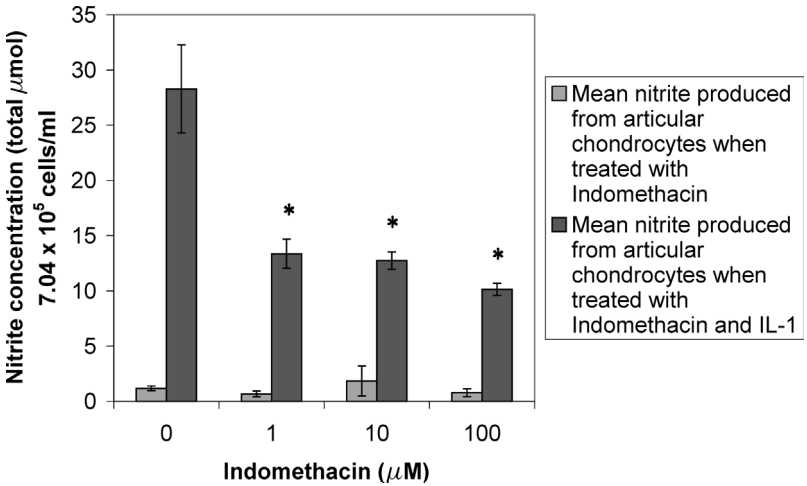

Figure 26. The effect of indomethacin on nitric oxide production by IL-1α (100 U/ml) stimulated articular chondrocytes. Values are mean ($n = 4$) $\pm$ S.E.M. $^*P < 0.05$, compared with IL-1 control.

proteoglycan synthesis at 24 h and exerted a biphasic response with IL-1α added (Fig. 31).

3.10. Production of oxygen radicals

The results in Fig. 32 show that increasing concentrations of oxaprozin alone or with IL-1α caused variable but nonsignificant decrease in the production of TBARS.

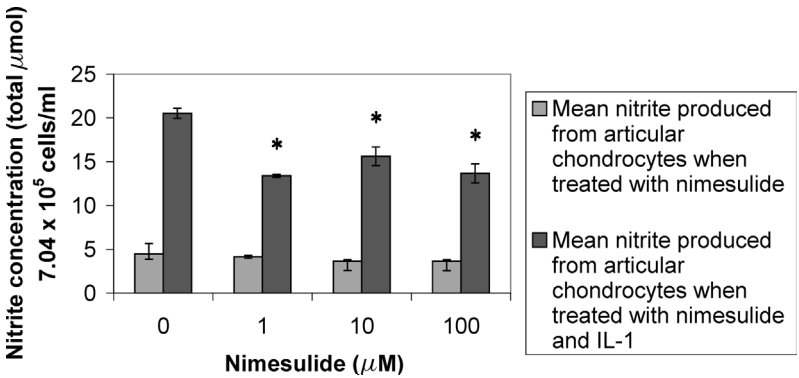

Figure 27. The effect of nimesulide on nitric oxide production by IL-1α (100 U/ml) stimulated articular chondrocytes. Values are means ($n = 4$) $\pm$ S.E.M. $^*P < 0.05$, compared with IL-1 control.

The results seen in Fig. 33 show the effects that nimesulide (1–100 μM) has on the same system, although the results are somewhat variable.

4. DISCUSSION

Nonsteroidal anti-inflammatory drugs (NSAIDs) bind to circulating plasma proteins with high affinity. The results show that oxaprozin also has this effect and this is in agreement with earlier studies (Aubry *et al*., 1995; Karim, 1996: Davies, 1998). Albumin is a major carrier for NSAIDs and a competition between co-administered drugs on the albumin binding sites has been hypothesised (Aubry *et al*., 1995; Davies, 1980). In previous studies Aubry *et al*., (1995) studied the effects of some commonly prescribed drugs on the binding of oxaprozin to albumin. These authors did not use radiolabelled oxaprozin but instead high performance liquid chromatography to assay the drug. Neither did they examine the binding to fatty acid-free albumin, which would enable the effects of fatty acids on binding to be established. Results from the current study indicate that there is an effect of some commonly used drugs on the binding of oxaprozin to HSA. These results are of particular importance in determining the optimal therapeutic dose, as well as the toxic effect of oxaprozin due to an excess of the free drug available to the target cells, when oxaprozin is administered in combination with other commonly used drugs.

In the present study there was a slight but statistically significant increase in free oxaprozin with therapeutic plasma concentrations of ibuprofen, diazepam, atenolol and captopril (Fig. 5).

To investigate the effect of varying the drug concentrations on oxaprozin binding to HSA, the drugs were added to oxaprozin at a lower (40 μM) and a higher concentration (90 μM). Results from the experiments with lower concentrations of drugs showed that captopril, prednisolone, warfarin, chlorpheniramine and diazepam increased the free oxaprozin and this increase was statistically significant

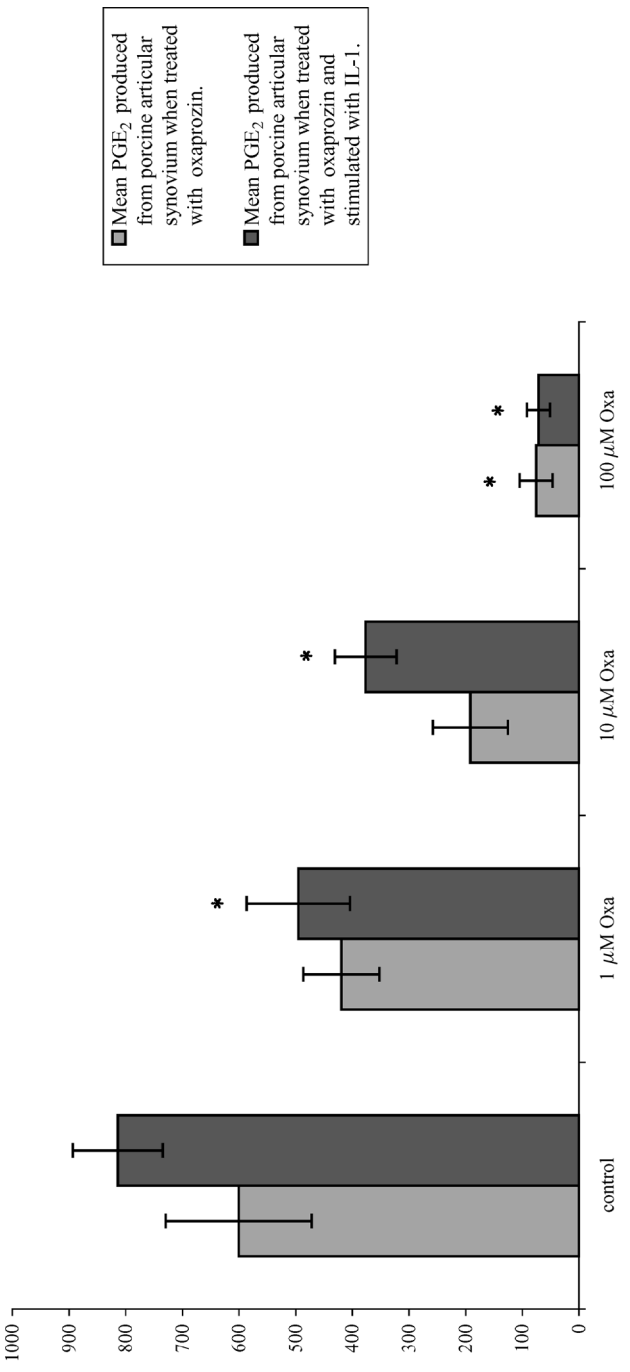

Figure 28. Effects of oxaprozin (0– 100 μM) with or without IL-1α on PGE_2 production from porcine articular synovium. * $P < 0.05$, compared with the control and the IL-1 control. Data are mean ± S.E.M. ($n = 3$).

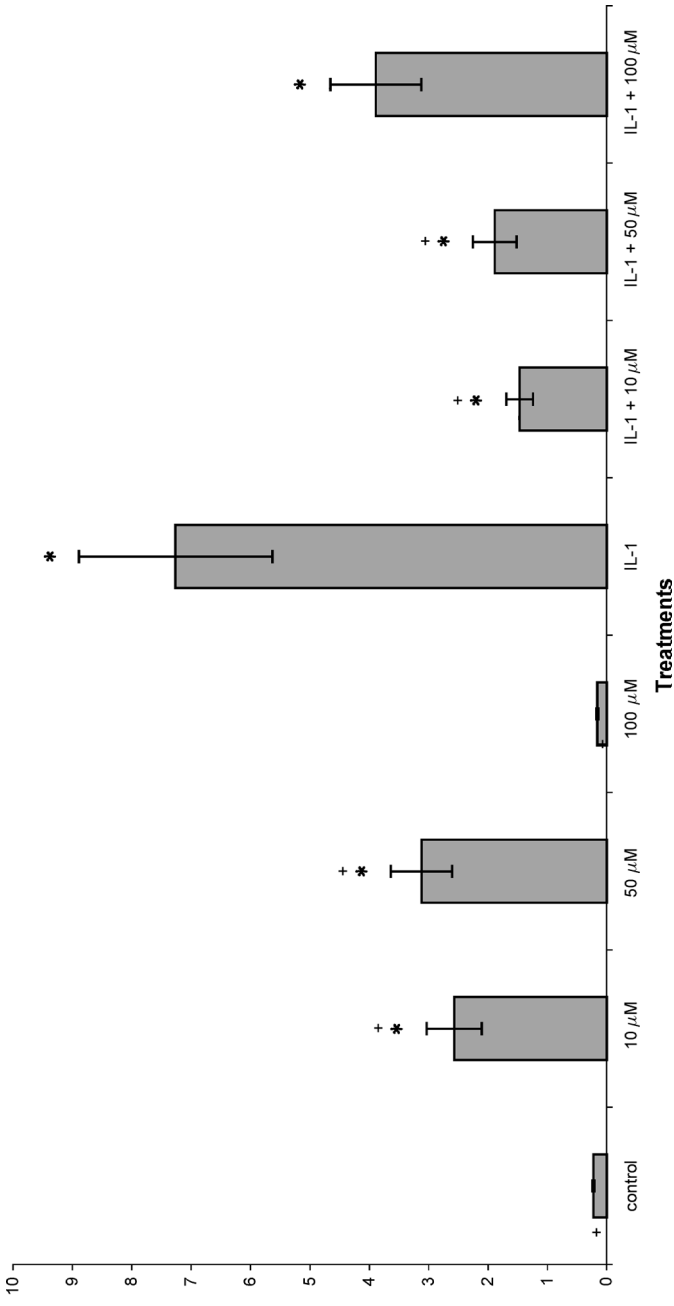

Figure 29. Effects of oxaprozin (0–100 μM) with or without IL-1α (100 U/ml) on proteoglycan synthesis in bovine nasal cartilage after 48 h incubation. Values are mean ($n = 6$) $\pm$ S.E.M. * $P < 0.05$, compared with control, $^{+}$ $P < 0.05$, compared with IL-1 control.

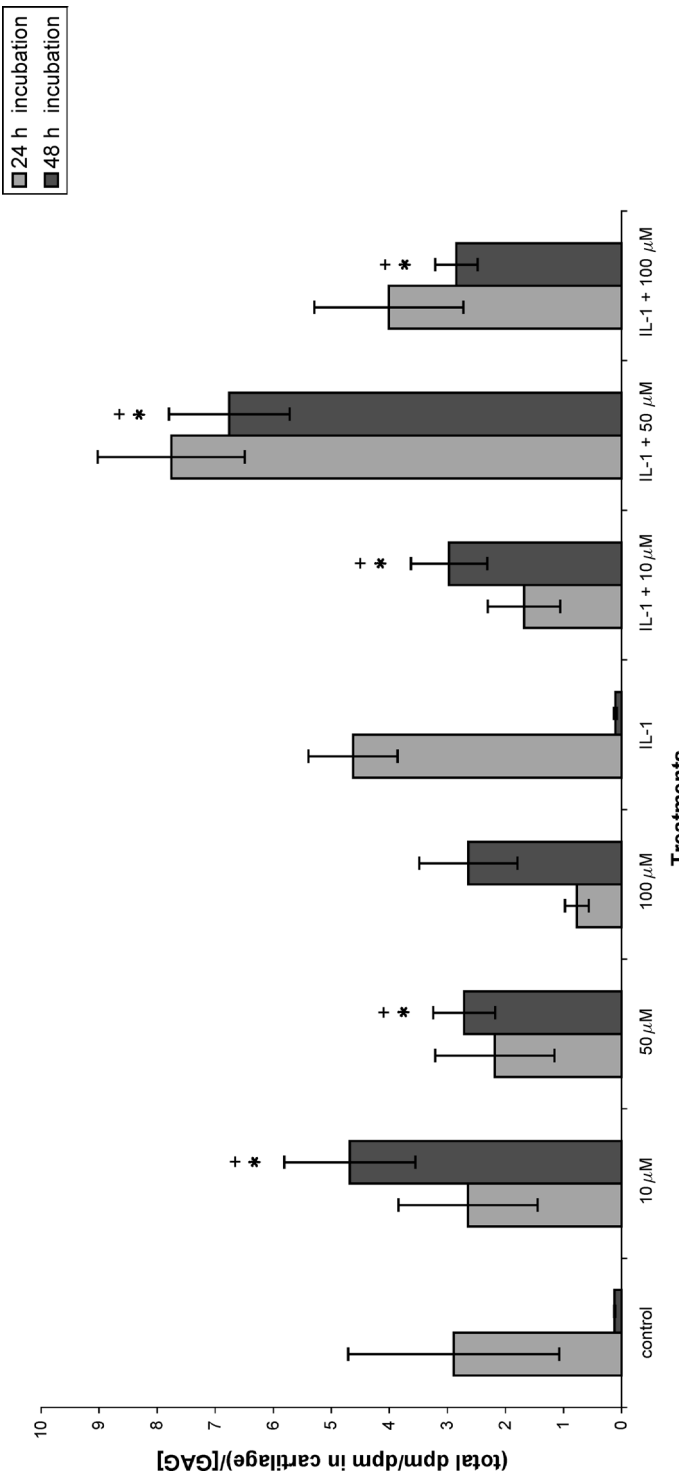

Figure 30. Effects of nimesulide (0–100 μM) with or without IL-1α (100 U/ml) on proteoglycan synthesis in bovine nasal cartilage after 24 h and 48 h incubations. Values are mean ($n = 6$) $\pm$ S.E.M. $^{*}P < 0.05$, compared with control, $^{+}P < 0.05$, compared with IL-1 control.

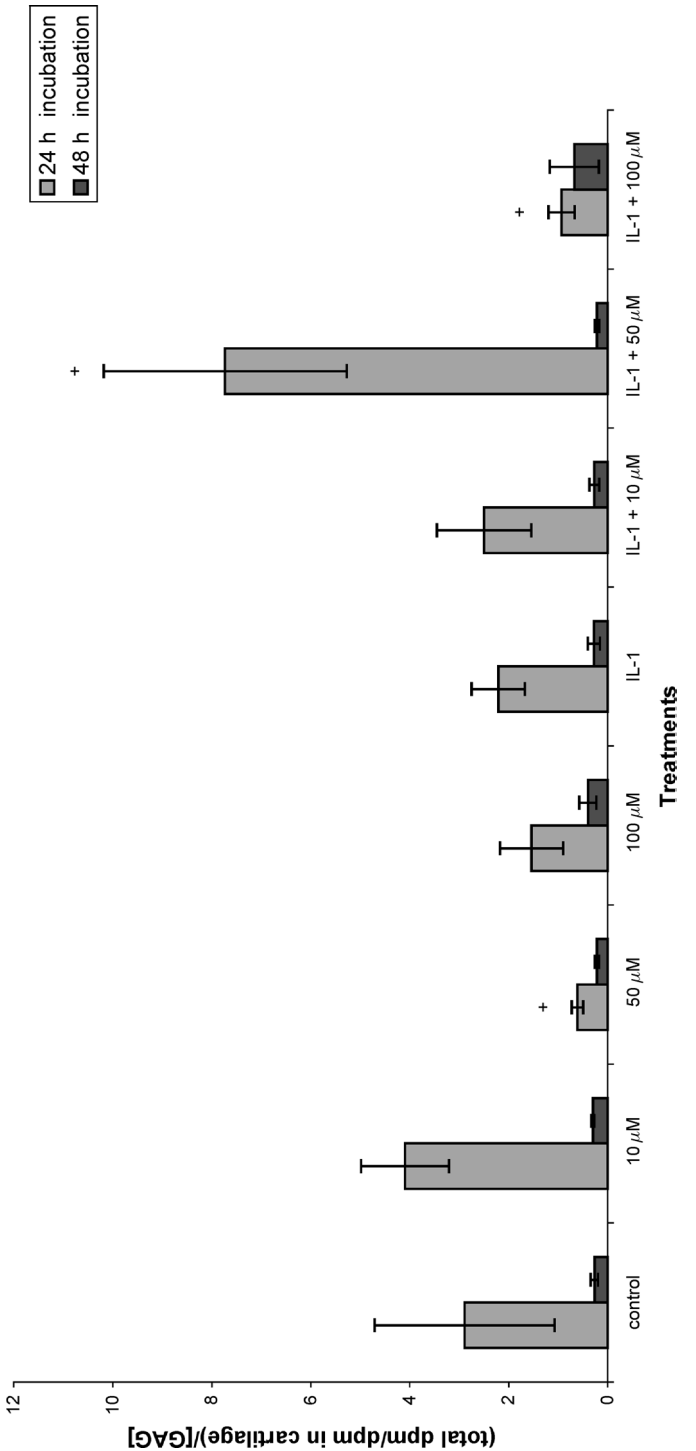

Figure 31. Effects of indomethacin (0–100 μM) with or without IL-1α (100 U/ml) on proteoglycan synthesis in bovine nasal cartilage after 24 h and 48 h incubations. Values are mean ($n = 6$) $\pm$ S.E.M. $^{+}P < 0.05$, compared with IL-1 control.

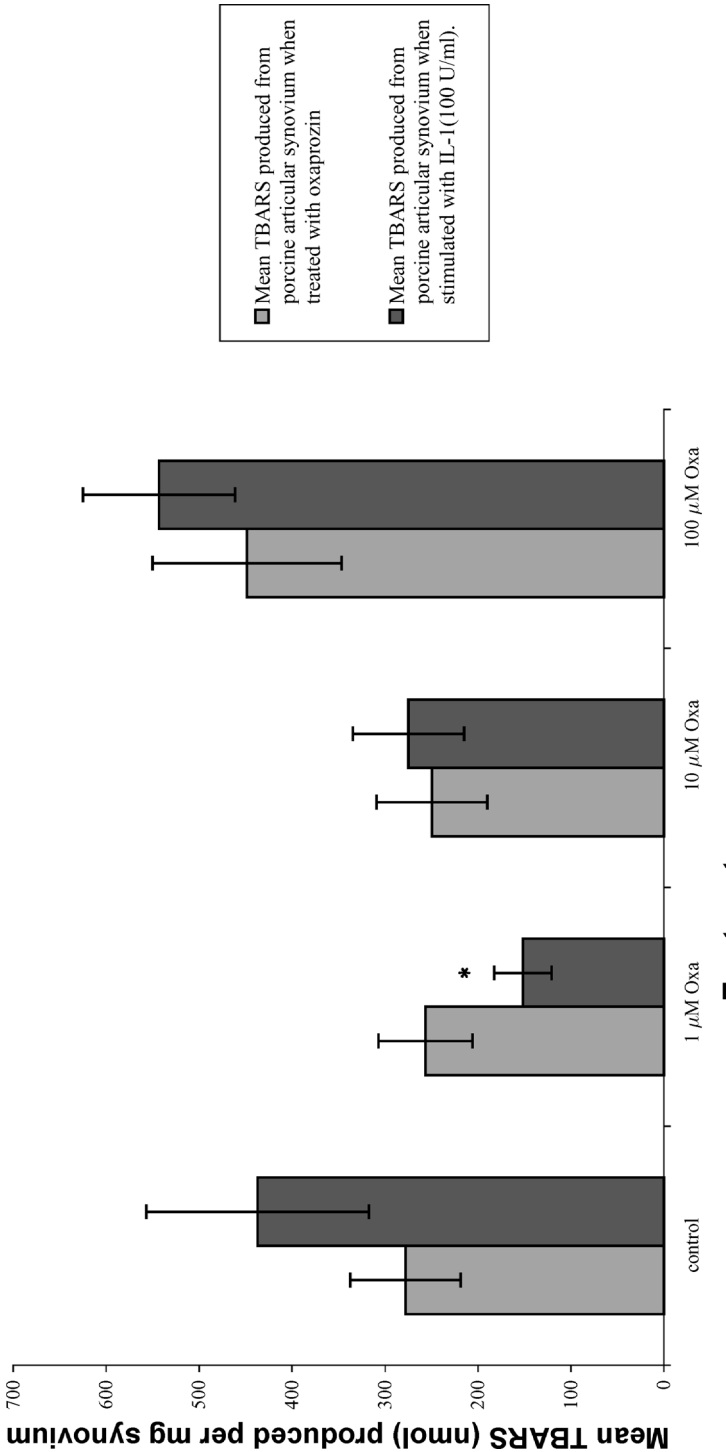

Figure 32. Effects of oxaprozin on TBARS production in incubation media from porcine articular synovium. Incubation time 4 days. Values are mean ($n = 6$) $\pm$ S.E.M. * $P < 0.05$, compared with the IL-1 control.

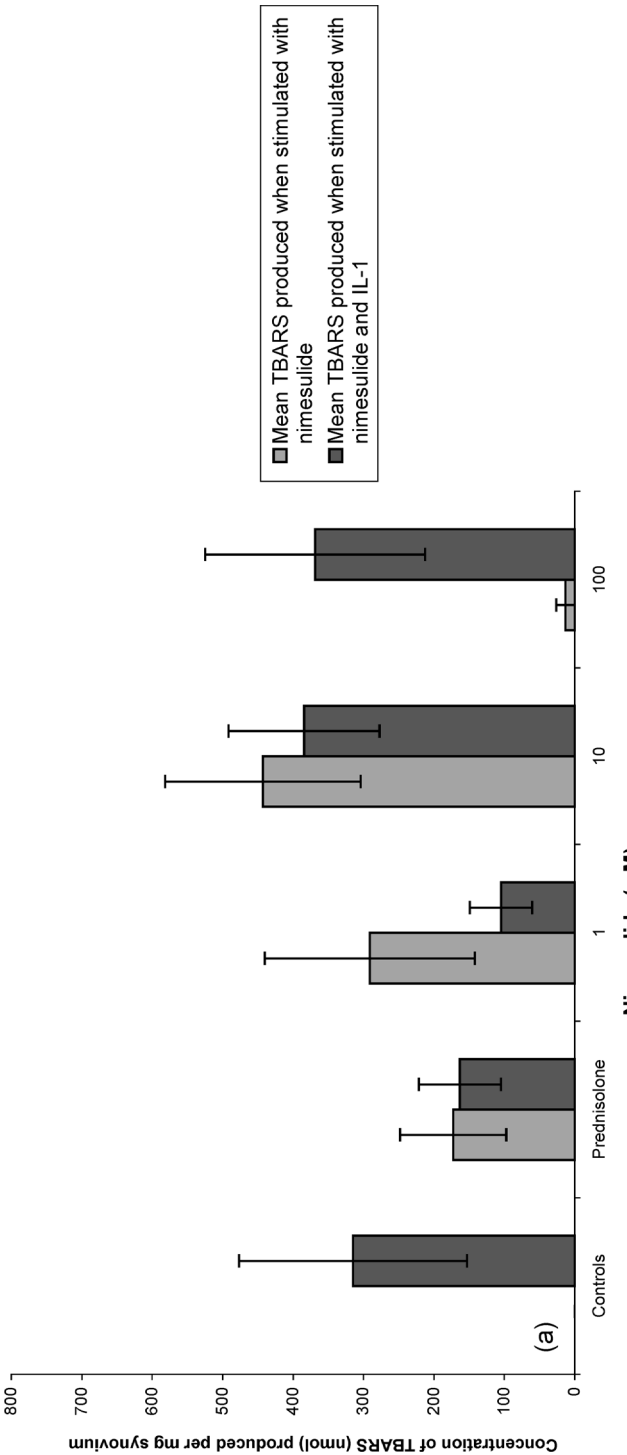

Figure 33. Effects of nimesulide on TBARS production in incubation media from porcine articular synovium. Incubation time 4 days. Values are mean ($n = 6$) $\pm$ S.E.M. (a) No value obtained due to values not falling within the standard curve.

(Fig. 5). At the higher concentration of oxaprozin it was found that diazepam, norethindrone, progesterone, captopril, theophylline, prednisolone, furosemide and ketotifen also significantly increased the free proportion of oxaprozin when compared with the control samples (Fig. 7).

The binding of oxaprozin to FAF-HSA was affected by captopril and caffeine since there was a statistically significant increase in the oxaprozin compared with other drugs (Fig. 8). These results suggest that fatty acids may influence the binding of those drugs that were found to increase free oxaprozin (Fig. 7).

Some metal ions whose levels are altered during inflammation and are known to be important in the inflammatory process (Rainsford *et al.*, 1981, 1998) were found to affect the binding of oxaprozin to HSA. Copper, zinc and selenium increased the free concentration of oxaprozin when incubated with both FAF-HSA and normal (i.e., fatty-acid replete) HSA at a metal ion concentration of 5 μM (Figs 9 and 10). The possibility that salicylate-metal ion complexes might form was examined by incubating a known metal chelating drug with the metal ions at concentrations of 5 and 40 μM, to determine if salicylate may prevent the metal ions from competing with the oxaprozin on the binding sites on HSA (Rainsford *et al.*, 1981, 1998). The results indicated that the metal ions combined with salicylate at 5 μM did affect binding of oxaprozin to albumin since the free oxaprozin in the treated samples was similar to that in the controls (Fig. 11). At a concentration of 40 μM, salicylate appears to also form a complex with the copper and the zinc but not with the selenium, as the free oxaprozin was slightly increased with the selenium (Fig. 11).

The fact that some of the site-I binding drugs still increased free oxaprozin in the current study may be because these drugs partially bind to site II and therefore displace oxaprozin. For example, Aubry and co-workers (1995) demonstrated that racemic warfarin reduced oxaprozin binding to HSA from 98.5 to 96%, therefore increasing the free proportion of oxaprozin.

The distribution of a drug is considered to be largely dependent on its binding to the tissues as well as other blood components. In addition, the enantio-selectivity of some drugs could also alter the binding characteristics of oxaprozin. Thus, competition between drugs for blood and tissue binding sites and enantio-selective competition for the binding sites of HAS has been suggested (Lagrange *et al.*, 2000; Bickel and Gerny, 1980).

It is well known that many NSAIDs interact with one another in binding to HSA (Grifin and D'Arcy, 1997). The clinical significance of these interactions with some drugs may be minor but can be at high doses of the drugs leading to adverse reactions (e.g., to increased GI and renal). Clearly, this is might be of some importance for oxaprozin.

The possibility that influences of NSAIDs on the binding of the amino acid, tryptophan may have pharmacological consequences is of particular interest, especially for their analgesic effects. L-Tryptophan is the only amino acid bound to human serum albumin (McArthur *et al.*, 1972). It is displaced *in vivo* (Smith and Lakatos, 1971) and *in vitro* (McArthur and Dawkins, 1969) by salicylates. The present

work has shown that tryptophan is displaced from its binding site on albumin by oxaprozin and a range of other NSAIDs. It is presumed that as the levels of free plasma tryptophan increase in the circulation after treatment with these NSAIDs that this leads to increased levels of tryptophan in the brain. As 5-HT (serotonin) synthesis in the brain is dependent upon the availability of the precursor amino acid, L-tryptophan, increased synthesis of 5-HT and production of the excretory metabolite, 5-HIAA, will follow.

The present results from the tryptophan binding studies are in agreement with the work of Guerinot *et al.* (1974) who showed that increases in free plasma tryptophan resulted in increased brain tryptophan levels and 5-HIAA, which indicates an increase in brain serotonin turnover. The results also demonstrate that the most of the NSAIDs displaced tryptophan from its binding site at concentrations of 50 and 100 μM, respectively (Fig. 10).

The results showed that the effect of oxaprozin was like that of indomethacin, diclofenac and nimesulide in causing an increase in the free concentrations of tryptophan. Oxaprozin is somewhat less potent than the other drugs but this concentration difference has the same order of potency of these drugs in pain relief. The hypothesis of a relationship between changes in plasma-free tryptophan levels and the severity of pain is an aspect that requires further investigation.

The uptake of radiolabelled oxaprozin into cartilage was about 50% greater than that of nimesulide (Fig. 13A, cf. 14A) but this would appear to reflect greater uptake of the drug into lipoidal non-aqueous compartments as indicated from comparisons with the [^{3}H]inulin uptake (Fig. 13C, cf. 14C). Oxaprozin is more lipophilic than nimesulide (see Introduction; Figs 1 and 2) so the uptake of oxaprozin compared with nimesulide would appear to be related to its greater liposolubility.

Overall, these studies show that while oxaprozin does not directly influence cytokine-induced cartilage degradation (Figs 15–21) and the release of synovial-derived cartilage-degrading activity (CDA) (Fig. 24A, B), the drug does inhibit production of IL-1β and TNFα from pig synovium and IL-1β, TNFα, IL-6 and IL-8 by THP-1 cells coincident within the range of inhibitory effects on PGE_2 production. Oxaprozin did not affect the production of IL-1α-stimulated nitric oxide from articular chondrocytes (Fig. 25) or synovium (data not shown). These lack of effects on NO production contrast with the effects of oxaprozin on IL-1α-stimulated PGE_2 production in synovial cultures which is reduced by 100 μM oxaprozin but not lower concentrations of the drug (Fig. 28). While oxaprozin did not appear to affect IL-1α-stimulated or basal nitric oxide production, the production of NO induced by IL-1α was inhibited by nimesulide and indomethacin (Figs 26 and 27).

The lack of effects of oxaprozin on cartilage degradation as akin to that with nimesulide and diclofenac sodium (Figs 22 and 23) and previous studies with indomethacin and other NSAIDs (Rainsford *et al.*, 1989, 1997, 1999). Oxaprozin alone did, however, increase synthesis of proteoglycans (Fig. 22A) and this may confer some joint protection although this requires further study.

In conclusion, oxaprozin has at least two pharmacologically significant effects on parameters of joint destruction involving effects on the production of proinflammatory cytokines and cartilage proteoglycan production which may be relevant to the actions of this drug in osteoarthritis. The pronounced accumulation of the drug in cartilage might be important in enabling control of cartilage destruction.

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