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Comparative Effects of Azapropazone on Cellular Events at Inflamed Sites. Influence on Joint Pathology in Arthritic Rats, Leucocyte Superoxide and Eicosanoid Production, Platelet Aggregation, Synthesis of Cartilage Proteoglycans, Synovial Production and Actions of Interleukin-1 in Cartilage Resorption Correlated with Drug Uptake into Cartilage In-vitro

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Abstract—Azapropazone (APZ) has been compared with standard NSAIDs in title systems to establish aspects of its mode of action on cellular events at inflamed sites. APZ (150 mg kg^{-1} day^{-1}) given for 10–13 days exhibited a reduction in joint pathology in established adjuvant arthritis in rats comparable with that of indomethacin (2 mg kg^{-1} day^{-1}) and clobuzarit (20 mg kg^{-1} day^{-1}). APZ was shown to be a potent inhibitor of the production of leucocyte superoxide and synovial interleukin-1 (IL-1)-like activity and stimulated articular cartilage proteoglycan synthesis, but was ineffective as an inhibitor of platelet aggregation or IL-1 induced cartilage degradation in-vitro. These in-vitro effects may have relevance to the mode of action of this weak inhibitor of prostaglandin synthesis.

Azapropazone (Prolixan, Siegfried A. G. Zofingen, Switzerland; Rheumox, A. H. Robins Co. Ltd., Crawley, UK) is a non-steroid anti-inflammatory drug (NSAID) differing from the majority of these drugs. Chemically, it is a substituted benzotriazine with physicochemical properties distinguishing it from other NSAID keto-enolates or pyrazoles (Walker 1987). The anti-inflammatory-analgesic properties of azapropazone in animals include (a) weak prostaglandin (PG) cyclo-oxygenase inhibitory activity comparable with that of aspirin in mouse macrophages in-vitro (Brune et al 1981), (b) modest acute anti-inflammatory (rat paw oedema) anti-erythema (guinea-pig UV) and analgesic activity reflecting weak effects on PG synthesis where this drug is equipotent with phenylbutazone (Jahn & Adrian 1969; Jahn & Wagner-Jauregg 1974; Brune et al 1981).

The mode of action of azapropazone relevant to its anti-inflammatory effects in arthritic conditions is, however, unclear. The available literature (see Walker 1985) indicates that there is little detailed information on the effects of this drug on cellular events in joint injury. Thus, for example, no detailed studies have been reported comparing the effects of orally-administered azapropazone with other NSAIDs in the pathological responses in adjuvant-arthritis in rats, a standard laboratory model of chronic inflammation (Billingham 1983). Previously it was claimed that azapropazone, at high concentrations (>0·1 mM) inhibits rat peritoneal PMN superoxide production (Mackin et al 1986) but a full dose response effect was not investigated. Finally, there is no published data on the effects of azapropazone on production and actions of interleukin-1 or effects of proteoglycan synthesis, all of which are important in the cellular pathology of joint inflammation (Brandt & Palmoski 1984; Billingham 1985; Cooke 1985).

Thus, in the present studies the effects were investigated of azapropazone compared with standard NSAIDs on: (a) the hind limb joint inflammation and histopathology of adjuvant-induced arthritis in rats, (b) superoxide anion production by human PMNs and its relationship to PG production, (c) the platelet aggregation elicited by stimuli responsible for PG cyclo-oxygenase activation, a standard measure of drug effects on the PG's, (d) biosynthesis of cartilage proteoglycans (PrGns) and glycosaminoglycans (GAGs), inhibition of which may promote the acceleration of joint destruction in arthritis (Brandt & Palmoski 1984; Cooke 1985), (e) interleukin-1 (IL-1) production by synovial tissue, and cartilage-resorbing actions in-vitro.

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Materials and Methods

Studies in arthritic rats

Arthritis was induced in female Sprague-Dawley rats (Tucks, Ryleigh, Essex, UK) by the s.c. injection into the tailbase of 0·05 mL of 0·5 mg mL^{-1} heat-killed and delipidated *Mycobacterium tuberculosis* (Porton strain) suspended in mineral oil (Sigma) as previously described (Whitehouse et al 1974). The animals were used at 14–18 days following induction of the disease at which stage the full joint destruction in this disease is manifest.

To determine the therapeutic regime of azapropazone and

comparator NSAIDs required for reduction of established adjuvant disease approximating to the ED50, initial studies were performed to establish the dose-response effects of once and twice daily oral administration of azapropazone compared with that of indomethacin and aspirin. These results established that at 14 days of drug treatment the ED50 values were for azapropazone 150 mg kg^{-1} day^{-1}, indomethacin 2 mg kg^{-1} day^{-1}, and aspirin 200 mg kg^{-1} day^{-1}. With the exception of aspirin, these doses were subsequently employed in the main study and as well an additional group of animals dosed with clobuzarit (Clozic, ICI) 20 mg kg^{-1} day^{-1} (Billingham & Rushton 1985), all of which were given as single daily doses for 13 days. In this study the comparative effects of these drugs were examined on joint inflammation, histopathology and gross joint morphology of the arthritic hind limbs. Paw and lateral tibio-tarsal (ankle) swellings were measured upon initiation of drug treatment and at 10 and 13 days by micrometer screw gauge. Body weights and scores of disease activity were also recorded before and at the end of drug administration.

At termination, both hind legs were severed about 1 cm above the tibio-tarsal joint and the left hand limb was fixed in formol-saline, decalcified in EDTA, embedded in paraffin and sectioned for light microscopy. Transverse sections (5 μm) through the full length of the limb were stained by haematoxylin and eosin, or toluidine blue. These sections were photographed for subsequent assessment by three observers, two of whom had no knowledge of the treatments. The scores of cartilage, bone destruction at the tibio-tarsal joint and cellular infiltration were graded on a scale of 0 (no damage or infiltration) to 4+ (for increasing severity) and subsequently averaged.

The right hind limb was fixed in ethanol and digested in 1% KOH until the bones were clearly visible, at which these were dissected free of adherent tissue (Dawson 1926). The cartilage, bone and other hard connective tissue components (including pannus) were stained with 1% alizarin red 100 mg L^{-1} in ethanol (Dawson 1926). The skeletal tissue was then cleared with 20% glycerol in 0·1% KOH before being assessed for the extent of pannus and joint destruction by scoring on a 0 to 4+ scale.

Polymorph (PMN) superoxide production

The methods employed were essentially those as described by Ginsburg et al (1987). Thus neutrophils were obtained from freshly drawn human blood in acid-citrate-dextran, following sedimentation on a Ficoll-Hypaque gradient, a procedure which yielded greater than 95% viable PMNs. Erythrocytes were removed by treatment with hypotonic saline followed by washing with normal saline buffered with 0·01M sodium phosphate, pH 7·3. The washed leucocytes were resuspended in Hanks' balanced salt solution (HBSS) buffered with 3 mM HEPES, pH 7·3, or in HBSS plus HEPES to which 10 mM sodium azide was added. Foetal calf serum (5%) was added to all leucocyte suspensions, which were kept on ice. Viability of the PMNs was evaluated either by the trypan blue exclusion technique or by measuring the amount of lactic dehydrogenase (LDH) released from the ligand-treated PMNs.

Superoxide was determined in stimulated PMNs by the reduction of cytochrome c (80 μM, type III Sigma Chemical Company, St. Louis, Missouri) according to Babior (1984). The reaction mixtures contained $1–5 \times 10^6$ PMNs mL^{-1} preincubated for 10 min with either poly-L-histidine HCl (PHSTD; mol wt 26 000, Sigma), tetrahydrophorbol 13-acetate (TPA, Sigma), or with group A streptococci (type 3 strain C203S) which had been preopsonized with PHSTD (Ginsburg et al 1987), together with the anti-inflammatory drugs, azapropazone (10–200 μM) or phenylbutazone (10–200 μM) dissolved in DMSO (10 mg^{-1} mL) and further diluted. Control mixtures contained DMSO alone. Following the preincubation cytochrome c, with or without superoxide dismutase (SOD) 20 $\mu g\,mL^{-1}$ (Gruenthal GmbH, West Germany) was added and the final volume made up to 1·0 mL.

The reaction mixtures were incubated in a water bath at 37°C for various time intervals and then centrifuged at 1000 *g* for 5 min. The absorbance of supernatant fluids were read in a type SP 1700 double-beam spectrophotometer at 550 nm (Babior 1984). The amount of superoxide was calculated from the extinction values using the formula $E_{550} = 2{\cdot}1 \times 10^{-4}$ M^{-1} and was expressed as nanomoles per given number of cells per 10 min (Babior 1984).

The chemiluminescence was measured in an LKB Wallac 1250 Luminometer at 37°C as described (Ginsburg et al 1987). Transparent polyethylene tubes (1·0 × 7·0 cm), containing 0·9 mL HBSS (without azide), 50 μL PMNs ($1–3 \times 10^5$), 50 μL of an appropriate ligand and 10 μL of luminol (Sigma), 2 mg mL^{-1}, together with the drug dissolved in DMSO, were then added. Results were expressed as peak CL after a given time of incubation.

Platelet aggregation

This was performed essentially using methods of Mustard et al (1975) on the platelet-rich fraction which was prepared as described by those authors. Platelet aggregation was measured turbidimetrically in a Born-type aggregometer using standard procedures (Buchanan et al 1982). Azapropazone was added to the reaction tubes in EtOH or DMSO either 1–5 min before or at the same time as addition of the stimuli (in stated final concentrations): ADP (10^{-5}M; Sigma), thrombin (10 u mL^{-1} Sigma), adrenaline (10^{-5}M; Sigma); while aspirin, dissolved in ethanol, was included as a positive standard (Buchanan et al 1982) for comparison. DMSO or ethanol (5 or 10 μL) were used as controls.

Eicosanoid production by monocytes and PMNs

Human peripheral blood monocytes and PMNs from healthy donors were obtained by the metrizamide (Nyegaard, Oslo) gradient-separation method of Vadas et al (1979). After being washed in Dulbecco's modified Eagle's Medium (DMEM) the monocytes were suspended in DMEM and 900 μL of $1{\cdot}2 \times 10^8$ cells mL^{-1} incubated in quadruplicate for 5 min at 37°C in a mixture comprising 50 μL of azapropazone in 10% DMSO (final concentrations 10 and 100 μM). Control mixtures received 10% DMSO alone. Following this 2·5 μL of calcium ionophore A23187, (final concentration 10 μM in 10% v/v DMSO) and 25 μL of 0·1 μCi [1-^{14}C]arachidonic acid (Amersham International, Little Chalfont, UK) in 10% DMSO were added and the mixture incubated at 37°C for a further 10 min before being terminated by the addition of 100 μL 0·2 M citric acid and

stored at −80°C. Subsequently the samples were diluted with 4 mL H_2O and the eicosanoids were extracted twice with 4 volumes of ethyl acetate. The combined extracts were evaporated to dryness under reduced pressure and redissolved in 100 μL ethyl acetate for quantitative transfer on to silica gel 60 thin layer chromatography plates (GF_{254}, Merck). These were developed in the toluene–dioxane–acetic acid (66:34:1·5) system of Harvey & Osborne (1983), which enables separation of PGE_2, $PGF_{2\alpha}$, TxB_x, HHT, 5, 12-di-HETE, 5-HETE from arachidonic acid. These components were visualized after thin layer chromatography by iodine vapour and identified by comparison with analytical standards of the prostanoids (obtained from Upjohn and ONO) or with [^{3}H]5-HETE and [^{3}H]LTB_4 (Amersham, UK). The zones corresponding to the eicosanoid standards were scraped off and suspended in Triton-PPO-dimethyl POPOP scintillant for scintillation counting.

The experiments with PMNs were performed using the same incubation procedures with the exception that the radioactively-labelled arachidonic acid was omitted. Following termination of incubation the media were subjected to purification on SepPak (Millipore) C-18 reverse-phase mini columns (Harvey & Osborne 1983) and the methyl formate or methanol fractions thus obtained (Harvey & Osborne 1983) were dried and the residues assayed for content of PGE_2, 5-HETE and LTB_4 by radioimmunoassay (RIA) after being resuspended in recommended RIA buffer.

Synovial production of cartilage-resorbing IL-1-like activity

Porcine synovial tissue was dissected under sterile conditions from the metacarpophalangeal joints of freshly slaughtered (< 2 h) pigs. This tissue was finely minced and incubated in proportions of approximately 5–10% by wet weight in 1 mL Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (600 μg mL^{-1}), 5% foetal calf serum, and penicillin/streptomycin (200 units mL^{-1} (referred to as DMEM/FCS medium), together with the drugs or their appropriate solvent ethanol (0·2%) mixtures (as controls) added. The incubations were performed in groups of 5 replicates as described (Rainsford 1987), with the media being harvested at 4 and 8 days (the latter with medium change at 4 days) and assayed by the bovine nasal cartilage bioassay described below at serial dilutions (DMEM/FCS) of 1:0 and 1:4, respectively. The protein content of the synovial tissue was determined by the method of Lowry et al (1951) after digestion in 1 mL 1M NaOH.

Bovine nasal cartilage bioassay for IL-1-like CRA and effects of drugs on IL-1-induced cartilage resorption

Bovine nasal septal cartilage from freshly slaughtered (< 2 h) adult cattle was dissected under sterile conditions and transverse slices (ca 1 mm thick) cut, from which discs (2 mm in diameter) were obtained with the aid of a sterile leather punch. The cartilage discs were pre-incubated in 200 μL DMEM/FCS medium (in Microtiter dishes) in an atmosphere of 5% CO_2/95% O_2 for 2 days, and then subsequently incubated for 4 days (Rainsford 1985a, 1986) (n = 8/treatment) in either:

(a) the serially-diluted media from the synovial cultures as above to enable determination of IL-1-like cartilage-resorbing activity (CRA) (Rainsford 1987), or

(b) the anti-inflammatory drugs in 0·2% ethanol added together with 5 units mL^{-1} human recombinant p15 αIL-1 (obtained from Genzyme Corp., Boston, MA, USA, or as a gift from the National Institute for Biological Standards and Control, Potters Bar, Herts., UK Code No. 86/632). Controls were incorporated in which the drugs were added alone without IL-1 and blanks without both the drugs or IL-1.

The extent of cartilage degradation was determined by assaying with dimethyl-methylene blue reagent the glycosaminoglycan (GAG) content of the culture medium and dividing by that remaining in papain digests of the cartilage discs using procedures described by Farndale et al (1982).

Histology and correlated proteoglycan degradation by IL-1 of pig articular cartilage incubated with azapropazone or indomethacin

Articular cartilage dissected under sterile conditions from porcine metacarpo-phalangeal joints was sectioned into pieces of approximately 3 mm long by 1 mm wide by 0·5 mm deep, and incubated in 1·5 mL DMEM/FCS medium placed in 25 well Sterilin 103 incubation dishes to which IL-1 (20 u mL^{-1}) with or without either 100 μM azapropazone or 30 μM indomethacin (made up as the sodium salts from equimolar Na_2CO_3) was added. The tissues were incubated for 4 days (Rainsford 1985a, 1986) and the medium harvested for assay of released GAGs (i.e from degradation of cartilage proteoglycans) using the dimethylmethylene blue method of Farndale et al (1982). The cartilage pieces were fixed in phosphate-buffered formol-saline, and the paraffin-embedded histological sections were stained with either toluidine blue for proteoglycans or Van Giesen's stain for collagen-like material, or haematoxylin & eosin.

Proteoglycan and GAG synthesis by pig articular cartilage

Cartilage pieces prepared as described above were incubated with 10 μCi/well of $Na^{35}SO_4$ (Amersham, UK) in DMEM alone and either 25–250 μM azapropazone (as Na salt prepared as above) 5–50 μM indomethacin (as Na salt prepared as above) or 50–375 μM sodium salicylate. Controls were incubated with medium alone. A separate series of experiments were performed in which the tissues were incubated with 10 μCi/well $Na_2{}^{35}SO_4$ in Iscove's medium with 10–100 μM azapropazone, 10 or 50 μM indomethacin and 10 or 100 μM sodium salicylate. In both of the experiments the incubations were terminated 6 and 24 h later. The GAGs in the media were isolated by w/v 2% CPC precipitation and the content of radiosulphate in the precipitated fraction determined by scintillation counting and of GAG's by the dimethyl methylene blue assay Farndale et al (1982). The PrGns in the cartilage pieces were extracted from thinly sliced sections (ca 0·01–0·05 mm) as described (Pottenger et al 1985) and precipitated with 2% w/v CPC. The radioactivity in the precipitate and supernatant fractions was determined by liquid scintillation counting and the concentration of GAGs by dimethyl-methylene blue assay (Farndale et al 1982) with shark chondroitin sulphate A (Sigma) as a standard.

Uptake of radiolabelled azapropazone and indomethacin

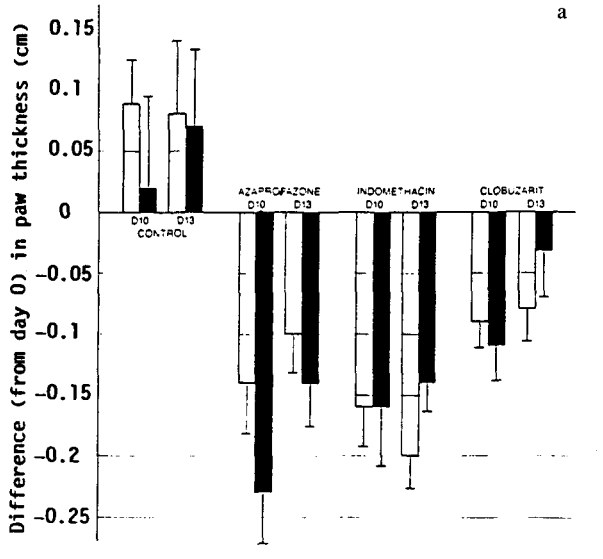
Bovine nasal cartilage discs were prepared (as described earlier) and incubated in 200 μL DMEM + 5% foetal calf serum with porcine α-IL-1 in sufficient quantity to give > 80% release of proteoglycans to which was added 100 μM [2-^{14}C] azapropazone, 210 d min^{-1} nmol^{-1} or [2-^{14}C]indomethacin, 484 d min^{-1} nmol^{-1}. The radiolabelled azapropazone was a gift from Mr F. S. Walker, A. H. Robins Co. Ltd., Horsham and was purified by thin layer chromatography (TLC) in silica gel F_{254} developed in chloroform–methanol (80 > 20 v/v). The plates were placed in tanks surrounded by aluminium foil and pre-flushed with N_2 gas. The radiolabelled indomethacin (Amersham International) was re-purified by TLC on silica gel F_{254} developed in benzene–dioxane–(gl) acetic acid (2:1:1 v/v/v). Both drugs were diluted in chloroform from scraped zones corresponding to pure standards chromatographed at the same time. The chloroform–methanol (50:50) fractions were placed in glass vials and the appropriate unlabelled drug added and mixed with the solution of radioactive drug. The chloroform–methanol mixture was then evaporated over N_2 gas with gentle warming and the residual labelled drug solubilized with 20 μL 1M $NaHCO_3$ with DMEM/5% FCS mixture added thereafter. Groups of 3–6 discs were incubated for 1 h, and 1, 2 and 3 days in 5% CO_2/air at 37°C. Upon removal from the culture media the discs were dried, weighed and digested in 500 μL soluene (Packard, Chicago, USA), followed by neutralizing with 20 μL acetic acid, for subsequent radioactive counting. Aliquots (20 μL) of the media were taken for radioactive counting. The radio of cartilage/medium uptake of ^{14}C was determined as:

$$\text{Uptake} = \frac{\text{Total d min}^{-1}\text{ in disc}}{\text{Total d min}^{-1}\text{ in medium}}$$

Results

Adjuvant arthritis

The results show that oral administration of 150 mg kg^{-1} day^{-1} azapropazone for 10–13 days produced a significant reduction of the swelling in the rear-paws (Fig. 1a) and ankle joints (Fig. 1b) comparable with that obtained from the two standard NSAIDs, indomethacin (2 mg kg^{-1} day^{-1}) and clobuzarit (20 mg kg^{-1} day^{-1}). As mentioned in the methods section the preliminary dose–response studies had shown that these doses of the drug were comparable following 14 days. Furthermore, no differences were evident between once daily compared with divided daily doses of azapropazone (100 or 200 mg kg^{-1} day^{-1}) and indomethacin (2 mg kg^{-1} day^{-1}) (data not shown). No appreciable improvements were observed in body weights of azapropazone or clobu-



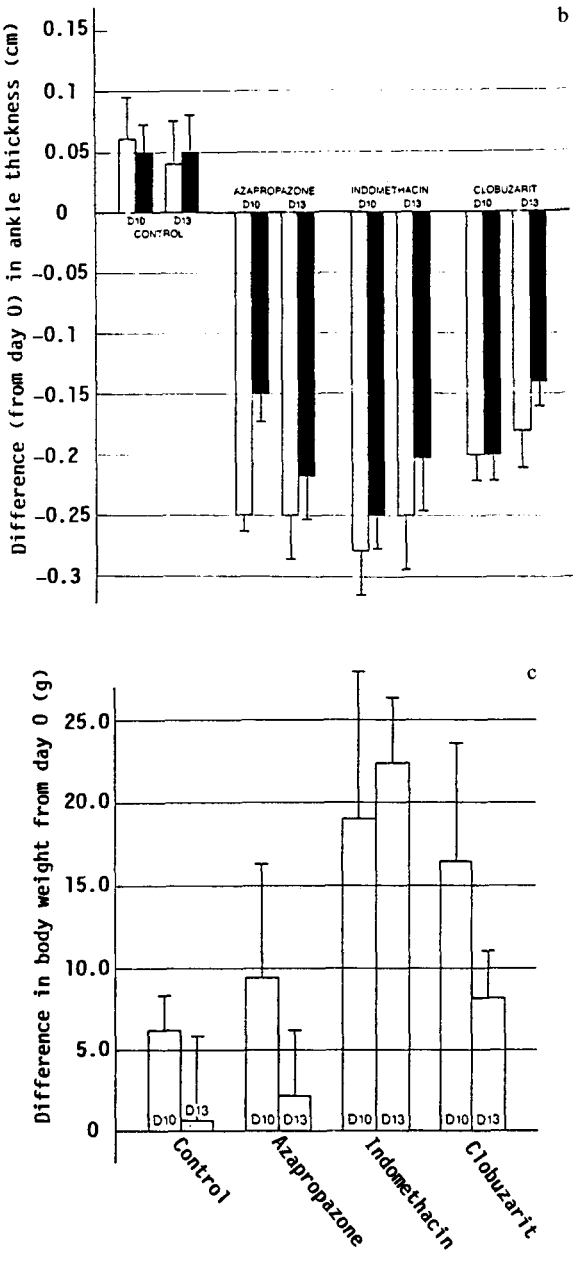


FIG. 1. Effects of azapropazone 150 mg kg^{-1} day^{-1}, compared with indomethacin 2 mg kg^{-1} day^{-1} and clobuzarit 20 mg kg^{-1} day^{-1} on rear paw swelling (a), tibio-tarsal (ankle) diameter (b) and body weight changes (c) in developed adjuvant arthritis in Sprague-Dawley rats. Values are means ± s.d. of N = 5–7 per group. D10 and D13 in the columns refers to the measurements on days 10 and 13 following initiation of daily drug dosage, respectively.

Table 1. Histological grading of joint pathology in rats with established adjuvant arthritis and treated with azapropazone and standard NSAIDs.

Treatment	Histopathological assessment		
	Cartilage destruction	Periostitis new bone growth	Pannus
Control	3·2	4·0	4·0
Azapropazone 150 mg kg^{-1} day^{-1}	1·2	2·8	2·8
Clobuzarit 20 mg kg^{-1} day^{-1}	2·1	2·8	3·0
Indomethacin 2 mg kg^{-1} day^{-1}	2·0	3·3	2·7

Grading of histopathology assessed on scale of 0 (no destruction or other pathology) to 4+ (maximal pathology).

zarit-treated animals but there was a significant improvement in those animals given indomethacin (Fig. 1c).

The scores of overall clinical appearance of the arthritic rats when graded on a scale of 0 (no improvement) to 3+ (most improvement) were $2·3 \pm 0·5$ (mean $\pm$ s.d.) for azapropazone, $2·3 \pm 0·7$ for clobuzarit, $2·7 \pm 0·3$ for indomethacin compared with 0·0 for controls. Thus the overall affects of all these drugs was essentially comparable at the doses given.

The histopathological assessments of the effects of drug treatment on the destruction of cartilage, periostitis, new perichondral bone formation, pannus and cellular infiltration are shown in Table 1. All three drugs appeared to reduce equally the extent of cartilage destruction but were notably less effective in reducing the extent of periostitis, bone renewal and pannus formation (Fig. 2). The reduction in bone symptoms by these NSAIDs was comparable to that in the Alizarin-stained hind limbs although it was more difficult to discern the extent of joint pathology in this material (data not shown).

Human PMN superoxide generation

Azapropazone inhibited the generation of superoxide anion by human polymorphonuclear leucocytes (PMNs) stimulated with optimal concentrations (Ginsburg et al 1987) of polyhistidine (PHST) and phorbol myristyl acetate (PMA = TPA) but not by histone-coated streptococci with cytochalasin B (HSG) (Fig. 3). The inhibitory effects were concentration-dependent with the half-maximal concentrations for azapropazone-induced inhibition of generations by both PHST and PMA being approximately 15 μM. Phenylbutazone by comparison elicited similar effects to azapropazone on PHST-induced superoxide generation but was weakly inhibitory against that induced by HSG (Fig. 3).

Eicosanoid production by monocytes

As shown in Table 2, azapropazone 100 μM, but not 10 μM, caused a statistically significant reduction in the production of prostaglandins (PG) E_2 and $F_{2\alpha}$ but not of the 5-lipoxygenase products, 5, 12-dihydroxyeicosatetraenoic acid (5, 12-diHETE) or 5-hydroxy-eicosatetraenoic acid (5-HETE), or hydroxyheptaenoic acid (HHT) by human monocytes in-vitro. The percent conversion of arachidonic acid to the respective oxygenated cyclo-oxygenase and lipoxygenase products was the same with azapropazone (10 and 100 μM) as controls, i.e. ca 10% (Table 2). Similar results were obtained

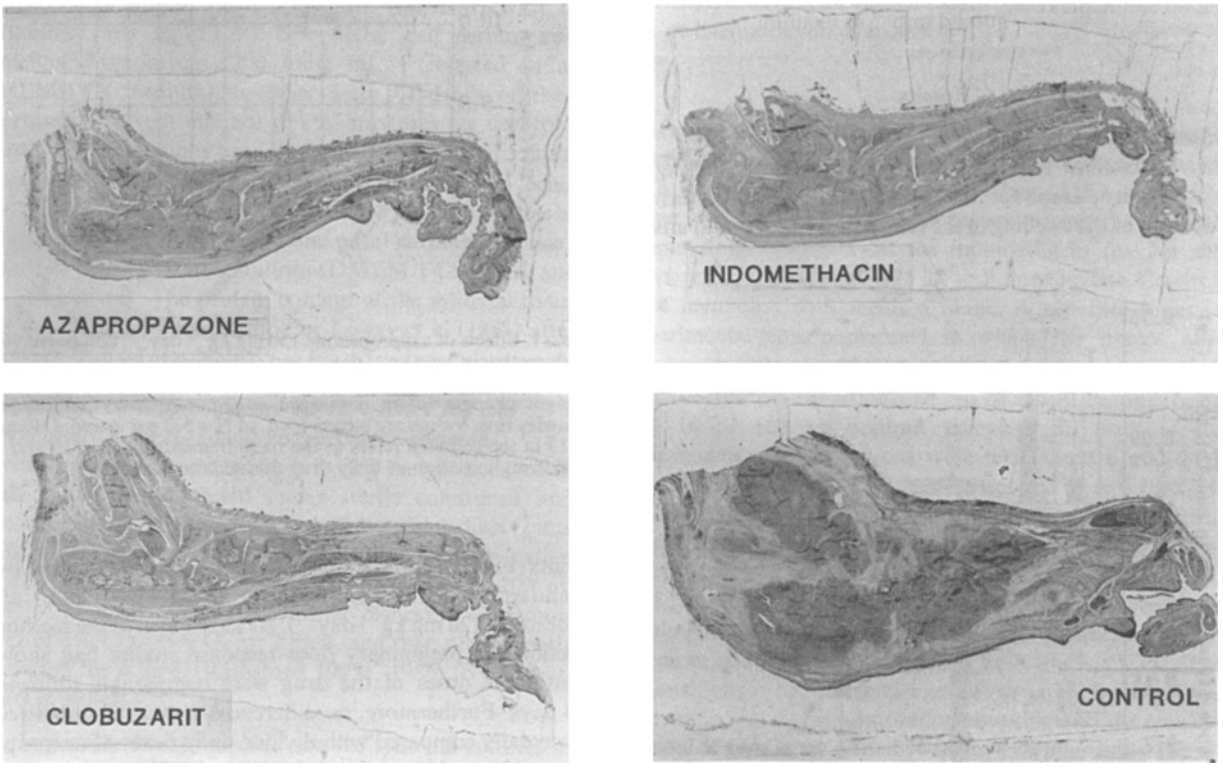


FIG. 2. Comparison of the effects of azapropazone, 150 mg kg^{-1} day^{-1} with indomethacin, 2 mg kg^{-1} day^{-1}, and clobuzarit 20 mg kg^{-1} day^{-1} on the histological appearance of transverse sections of representative hind limb skeletons stained with haematoxylin and eosin.

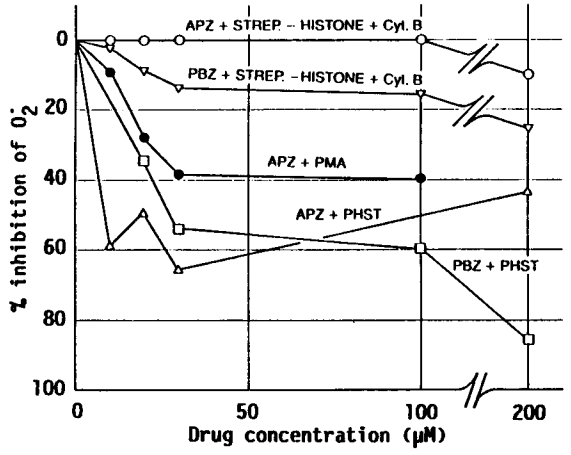


FIG. 3. Effects of azapropazone (APZ) compared with phenylbutazone (PBZ) on human polymorphonuclear leucocyte (PMN) generation of superoxide anion in the presence of various stimuli. Cyt B = cytochalasin B, PHST = polyhistidine PMA = phorobol myristyl acetate (TPA).

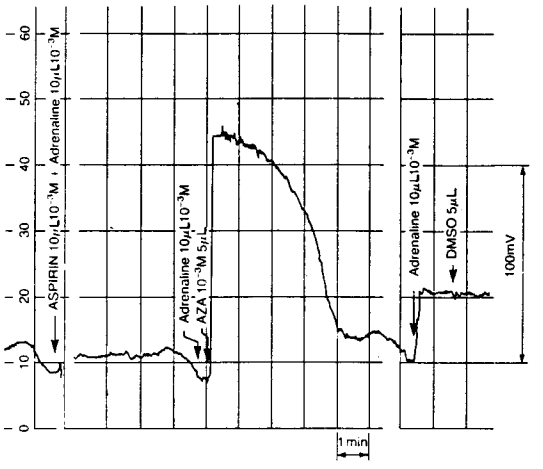


FIG. 4. Effects of azapropazone with adrenaline on platelet aggregation in human platelet-rich plasma fractions. Tracing runs from right to left.

Table 2. Effects of azapropazone on the synthesis of eicosanoids from [1-^{14}C]arachidonic acid in human peripheral monocytes stimulated with calcium ionophore (A23187).

	d min^{-1} ^{14}C/5 × 10^6 cells (mean ± s.d.)		
		Azapropazone	
Eicosanoid	Control	10 μM	100 μM
PGE_2	5523 ± 228	3978 ± 1889	3722 ± 860*
PGF_2	1700 ± 112	1109 ± 577	1410 ± 168*
5, 12-diHETE	1123 ± 207	772 ± 363	1270 ± 492
5-HETE	1032 ± 81	896 ± 225	1127 ± 33
HHT	1380 ± 153	1515 ± 876	1200 ± 50
Arachidonic acid	100517 ± 3781	74838 ± 42277	73826 ± 32889

* Denotes statistically significant reduction c.f. control in incorporation of [1-^{14}C] arachidonic acid into the eicosanoid (Student's t-test, $P < 0.95$, n = 4 group).

in human PMNs in which the eicosanoids were assayed by radio-immunoassay (data not shown).

The results show that azapropazone is a weak cyclo-oxygenase inhibitor in this system (cf. indomethacin IC50 ca 2 μM in comparable experiments), without influencing either the 5-lipoxygenase pathway or overall turnover of arachidonic acid.

Platelet aggregation

Azapropazone 5 μM failed to inhibit the platelet aggregation induced by maximally-effective concentrations of ADP or thrombin (not shown) but did inhibit the effects of adrenaline (Fig. 4). Aspirin at standard inhibitory concentrations of 10 μM completely inhibited aggregation induced by all three of these stimuli (not shown). Higher concentrations of azapropazone proved troublesome because of insolubility.

However, the lack of inhibitory effects by azapropazone on aggregation induced by ADP and thrombin is in agreement with the weak cyclo-oxygenase inhibitory effects of this drug.

Synovial IL-1-like cartilage resorption activity

Azapropazone 10-200 μM significantly (Student's t-test, $P < 0.05$) inhibited the production of IL-1-like cartilage resorbing activity (CRA) in undiluted media from pig synovial tissue cultured for 4 and 8 days (Fig. 5). There were no effects in the 1:4 diluted media indicating that the drug

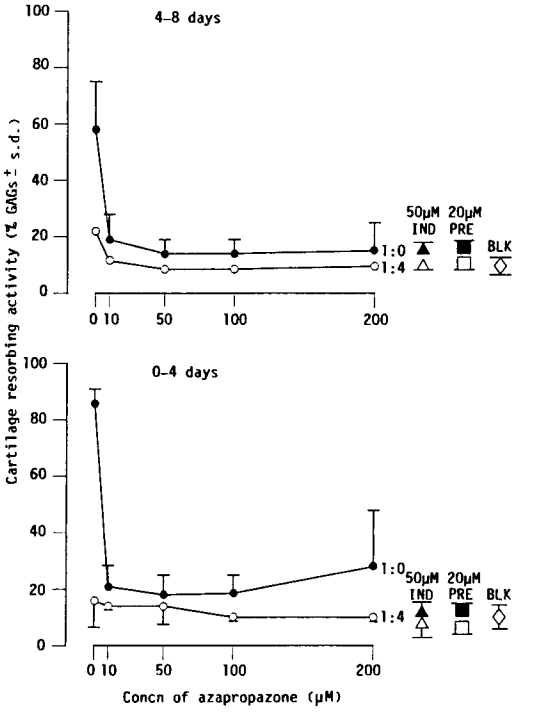


FIG. 5. Effects of azapropazone on the release from pig synovial tissue of IL-1-like cartilage-resorbing activity (CRA) following incubation for 4 and 8 days. Following incubation of the synovial tissues with the drugs, the media was harvested and the CRA determined in the bovine nasal cartilage assay at 1:0 (i.e. no dilution) or 1:4 dilution in the corresponding DMEM/5% FCS medium. Standards of indomethacin (50 μM, IND) and prednisolone (20 μM, PRE) were incorporated in these experiments. BLK represents the blank value obtained in the bovine nasal cartilage bioassay from incubation of the cartilage with DMEM/5% FCS alone.

had no effects on the production of any possible activators or inhibitors likely to affect the actions of IL-1-like activity. Prednisolone (20 μM) and indomethacin (50 μM) were maximally effective in inhibiting CRA in these experiments in agreement with previous observations (Rainsford 1987).

Bovine nasal cartilage resorption induced by α-IL-1 in-vitro

No effects were observed of azapropazone (20-100 μM) on the cartilage resorption induced by human recombinant or porcine native α-IL-1. Thus, in the presence of the former cytokine the CRA measured as the percent release of GAGs (means $\pm$ s.d., n = 8) was 86·2 $\pm$ 5·4, 76·5 $\pm$ 9·6 and 91·8 $\pm$ 3·7 in the presence of 100, 50 and 10 μM azapropazone, respectively, compared with a control of 89·2 $\pm$ 4·2. Corresponding values of CRA in the absence of α-IL-1 were 20·4 $\pm$ 7·5, 18·8 $\pm$ 5·9 and 15·9 $\pm$ 7·3, respectively, with that in the absence of the drug being 29·2 $\pm$ 13·9. Clearly, there were no unspecific or toxic effects of the drug on the cartilage chondrocytes.

Histology of pig articular cartilage in the presence and absence of IL-1

Pig articular cartilage cultured for 4 days in the presence of 20 units of human recombinant α-IL-1 showed pronounced loss of toluidine blue staining in about 1/4–1/3rd the way through the cartilage from the articular surface, reflecting the loss of proteoglycans. Van Giesens-stained collagen-like material appeared unaffected in this way by the treatment although it was slightly weaker than in control tissue without IL-1. In the presence of azapropazone (100 μM) and IL-1 the toluidine blue-stained tissue appeared the same as that without the drug; while the drug alone was without effect compared with control tissue, indomethacin (50 μM) exhibited comparable effects.

Proteoglycan synthesis in-vitro

The results in Fig. 6 show that azapropazone (25–250 μm) stimulated at 24 h, but not 6 h, of incubation the incorporation of [^{35}S] sulphate into the proteoglycans isolated from porcine articular cartilage. Sodium salicylate (250–375 μM) likewise stimulated the radiosulphate incorporation at 24 but not 6 h of culture; no effects of indomethacin (5–50 μM) were observed at either time. At 24 h a typical bell-shaped profile was evident in the concentration-response observed with azapropazone and salicylate. The reduction in the stimulatory effects of these drugs observed at their highest concentration is probably a partial reflection of some toxic effects of the drugs since histological observations of tissues incubated with the higher concentrations or greater showed chondrocyte abnormalities (data not shown).

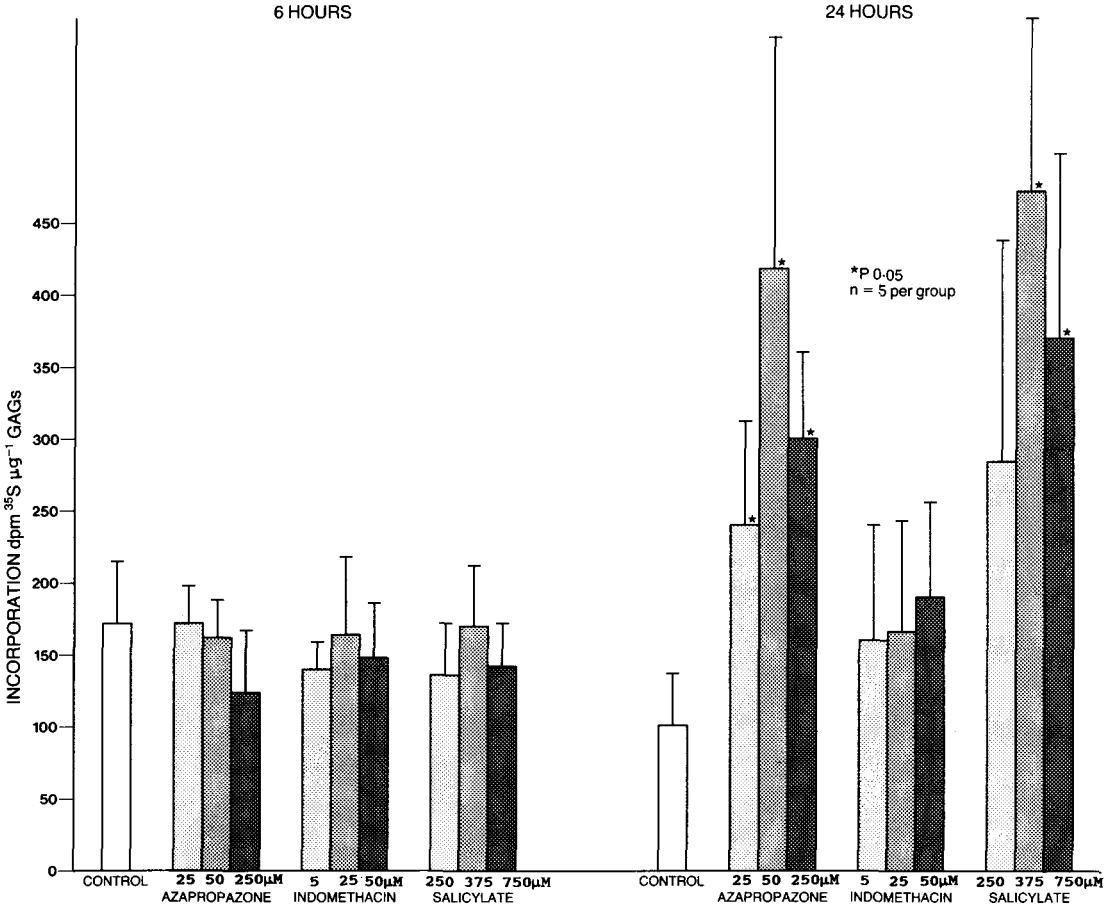


FIG. 6. Effects of azapropazone compared with indomethacin and salicylate on the synthesis of proteoglycans (PrGns) determined by the incorporation of [^{35}S-]sulphate into extracted PrGns. The 6 and 24 h experiments, respectively, were performed at different times with different batches of cartilage tissue so that the control values at these two times are not strictly comparable. All values are means $\pm$ s.d. (bar).

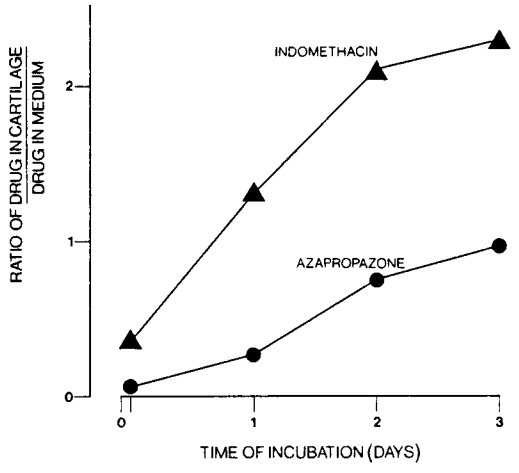


FIG. 7. Uptake into bovine nasal cartilage of [2-^{14}C]azapropazone compared with [2-^{14}C]indomethacin in the presence and absence of pig α-IL-1.

Uptake of radiolabelled drugs into bovine nasal cartilage

As shown in Fig. 7, azapropazone and indomethacin both achieved plateau concentrations in the presence of IL-1 by 2–3 days of incubation, with azapropazone having about one-half the plateau concentration compared with that of indomethacin.

Discussion

The results show that azapropazone (150 mg kg^{-1} day^{-1}) exhibited effects comparable with those of standard doses of indomethacin (2 mg kg^{-1} day^{-1}) and clobuzarit (20 mg kg^{-1} day^{-1}) in (a) reducing soft tissue inflammation (Fig 1a), (b) in clinical scores of disease severity (see Results), and (c) in the qualitative assessment of histological changes of the cartilage and bone of the affected hind limb joints (Table 1). All the drugs only exhibit modest effects in the control of periostitis, bone renewal and pannus development. The cartilage destruction in azapropazone-treated animals appeared slightly less than that in arthritic rats given the other drugs but it must be emphasized that this is a qualitative assessment.

Inhibitory effects of azapropazone (20 or 40 mg kg^{-1}) have been reported in the paw swelling of adjuvant-induced arthritis when the drug was given by i.p. injection (Lewis et al 1977), but no other parameters of joint function were determined. Szanto et al (1976) reported that azapropazone 500 μg i.p. (in 150–200 g rats) reduced the arthritis induced by the local s.c. injection of formalin alone or with PGE_2 into the hind limb, although no quantitative data were given. They also observed that the histochemical staining for succinic and malate dehydrogenases was increased but gave no reasons for this effect of the drug.

The in-vitro studies showed that pronounced inhibitory effects of azapropazone were evident on PMN superoxide productions (Fig. 3) and on the synovial production of cartilage-degrading IL-1-like activity (Fig. 5), both of which occurred in the range of 10–50 μM. The effects of superoxide production occurred at concentrations well below those for inhibition of PG production (ca 100 μM). Azapropazone failed to affect platelet aggregation stimulated by ADP and fibrin both of which result in prostaglandin production, so that the lack of effects on platelets appear to reflect the weak PG synthesis-inhibitory effects of the drug. The inhibitory effects of azapropazone on adrenaline-induced aggregation suggest drug effect at the level of adenylate cyclase activation, but this requires further investigation.

In addition to inhibiting cartilage resorption by IL-1, azapropazone, like salicylate stimulates proteoglycan synthesis in articular cartilage while indomethacin has no effects (Fig. 6). These effects of salicylate contrast with previous reports of inhibition of proteoglycan synthesis by the drug (Brandt & Palmoski 1984) which cannot be explained except through differences in the source material. These authors used canine articular cartilage in contrast to the pig cartilage which was employed here. While the upper concentration range required for these effects of azapropazone (25–250 μM) is greater than that required for inhibiting synovial IL-1 release and superoxide production, it is still within the range of plasma concentrations of the drug (upper range 0·5 mmol L^{-1}, or 150 mg L^{-1}) achieved during therapy (Jahn et al 1973; Jones 1976; Rainsford 1985b).

The lack of effects on α-IL-1-induced cartilage resorption are in agreement with previous reports of the non-effects of other NSAIDs, with the exception of the anti-malarials e.g. chloroquine (Rainsford 1985a, 1986). The lack of these effects is obviously not due to poor uptake of the drug into the cartilage (Fig. 7). While gross uptake may not represent concentration at the surface of the chondrocyte in-vitro, the overall high drug concentrations, coupled with the uptake being comparable to medium values, suggests that the chondrocytes in the cartilage may be unresponsive to the effects of IL-1.

Assuming these in-vitro observations have relevance to the actions of azapropazone in-vivo, it appears that inhibition of synovial IL-1 and superoxide production may represent the potent effects of the drug in excess of those on PG synthesis (Brune et al 1981) and lysosomal enzyme release (Lewis et al 1971). Since IL-1 stimulates phospholipase A_2 which in turn enhanced PG production (Chang et al 1986) it is possible that control at the level of IL-1 release may be considered an earlier locus in the sequence of IL-1 effects responsible for reducing PG production by azapropazone as well as by other anti-inflammatory agents which inhibit IL-1 release (Rainsford 1987). The relevance of these suggestions remains to be determined at inflamed sites in arthritic conditions in-vivo.

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