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Effects of 5-Lipoxygenase Inhibitors on Interleukin Production by Human Synovial Tissues in Organ Culture: Comparison with Interleukin-1-synthesis Inhibitors*

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Abstract

Prostaglandins and leukotrienes differentially regulate the production of interleukin-1 (IL-1) in monocytes. It was, therefore, decided to investigate the effects of some 5-lipoxygenase inhibitors compared with standard IL-1-synthesis inhibitors on the production of IL-1 by human synovial tissue explants in organ culture. Human synovial (from hip/knee arthroplasty) or porcine tibio-tarsal joint synovial explants were incubated in organ culture in Dulbecco's Modified Eagle's Medium + 5% foetal calf serum in the presence of the test compounds or solvents (controls), or media alone for 1-5 days. Total bioactive IL-1 was assayed in the medium (following serial dilution or with polyethylene glycol 8000 added in some assays to remove inhibitors) using the D-10 T-cell bioassay. Some assays of interleukins 1α, 1β, 6 or 8 were performed by ELISA. Of the 5-lipoxygenase inhibitors investigated, MK-886 (3-(1-(4-chlorobenzyl)-3-*tert*-butyl-thio-5-isopropylindol-2-yl)-2,2-dimethyl propanoic acid), L-656,224 ((7-chloro-2-[4-methoxypenyl]methyl)-3-methyl-5-propyl-4-benzofuranol), PF-5901 and tepoxalin were the most potent inhibitors of IL-1 production. While the PF-5901 was effective at 5-30 μM and tepoxalin was effective at 1-10 μM, the others were the most potent having minimal inhibitory activity in the range of 0.01–0.1 μM. The presumed IL-1-synthesis inhibitors, tenidap and IX-207,887, were inactive at concentrations of 30-50 μM. Leukotriene B_4 (1-100 ng mL^{-1}) added to MK-886 (5 μM)-treated cultures reversed the inhibitory effects of the latter on IL-1, confirming the role of 5-lipoxygenase products in the regulation of IL-1 production. Addition of polyethylene glycol 8000 to MK-886-treated cultures eliminated the inhibitory effects of this drug, suggesting that this drug exerts its effects by promoting production of IL-1 inhibitors. MK-886 also inhibited synovial production of two other pleiotrophic cytokines which it regulates, IL-6 and IL-8. The results suggest that some 5-lipoxygenase inhibitors may be usefully employed in regulating production of those interleukins involved in joint cartilage destruction.

Some 5-lipoxygenase inhibitors have been reported to inhibit acute or chronic inflammation (Marshall & Chang 1989; Pfister & Ernest 1989; Salmon et al 1989) but the mechanism of their action is not fully established. By reducing production of leukotriene B_4 (LTB4) these 5-lipoxygenase inhibitors reduce leucocyte infiltration into inflamed sites and lymphocyte activation (Bray 1987; Rola-Pleszczynski 1988; Marshall & Chang 1989), and development of arthritis (Griffiths et al 1995). Inhibition of 5-lipoxygenase may reduce interleukin-1 (IL-1) production (Brandwein 1986; Otterness et al 1989). Feedback control on IL-1 production has been reported with products of eicosanoid metabolism, or from the inhibition of prostaglandin cyclo-oxygenase (Brandwein 1986). Cyclo-oxygenase inhibition, in general, enhances monocyte IL-1 production, while the reverse occurs upon addition of E-prostaglandins (Brandwein 1986). A recent report by Hoffman et al (1991) claimed that blockade of 5-lipoxygenase activity with a specific inhibitor of this enzyme, MK-886, was, however, without effect on IL-1 production by human monocytes. Thus, we decided to examine the effects of this and other 5-lipoxygenase inhibitors compared with standard IL-1 synthesis inhibitors on the production of IL-1 as well as IL-6 and IL-8 in explants of human synovial tissue from patients with inflammatory arthropathies in organ culture. In organ culture this tissue produces substantial cytokines from activated macrophage-like cells, so that interleukin production in this pathologic tissue could be considered a model of synovial reactions as in the inflamed joint (Hopkins et al 1988; Feldman et al 1990; Rooney et al 1990).

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Materials and Methods

Methods

Human synovial tissue was obtained from subjects undergoing hip or knee arthroplasty. The subjects had not previously received disease-modifying anti-rheumatic drugs, and in many cases had only taken paracetamol (Tylenol) while a few had taken non-steroidal anti-inflammatory drugs. Histological examination revealed these samples were extensively inflamed. The tissues were placed in organ culture overnight to achieve stabilization in

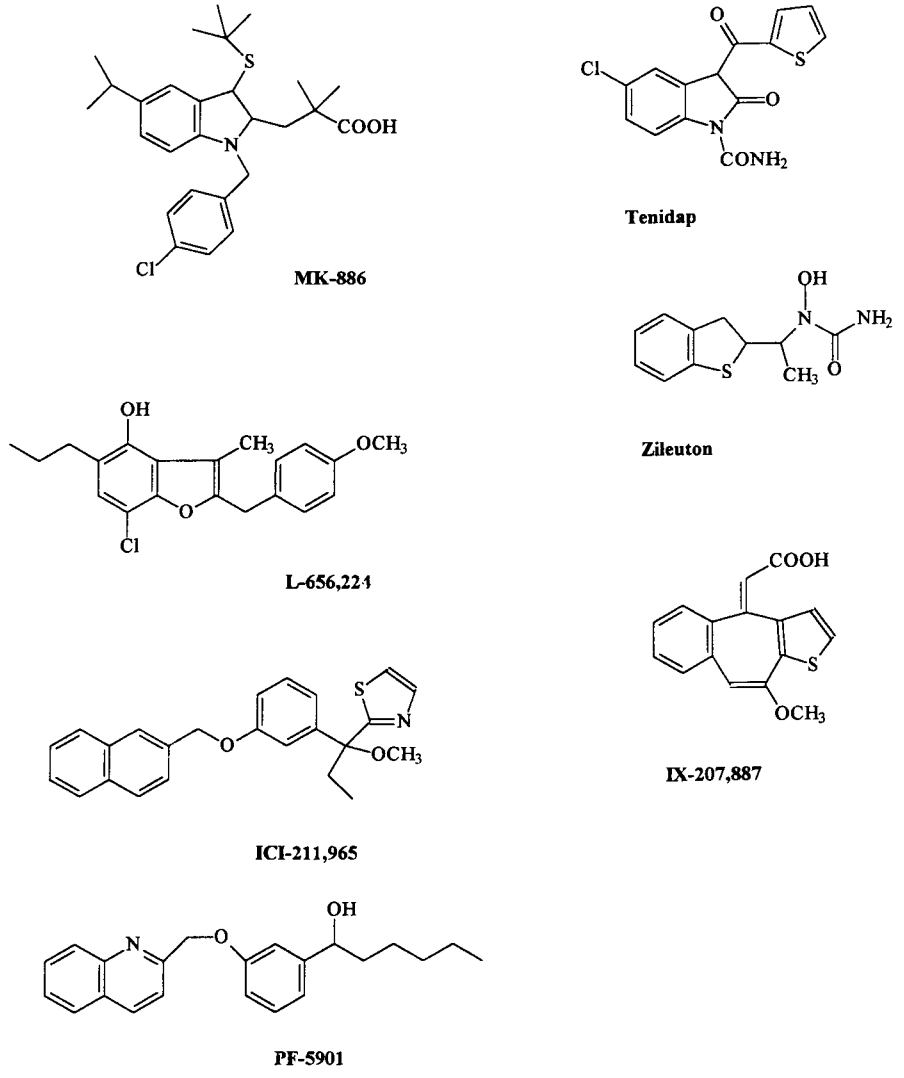

FIG. 1. Structures of 5-lipoxygenase inhibitors used in this study.

25-well dishes with 1.5 mL Dulbecco's Modified Eagle's Medium (DMEM), 5% foetal calf serum (FCS), and 200 units mL^{-1} of penicillin and streptomycin added (Rainsford 1987); the incubations were performed at 37°C in an atmosphere of 5% CO_2-95% O_2. The medium was then changed, the inhibitors added and the cultures incubated for 1, 3, or 5 days (with the medium changed each time). Additionally experiments were performed in pig non-inflamed synovial tissues from the tibio-tarsal joint of a normal untreated pig cultured as described above (Rainsford 1987). Following incubation the harvested medium was dialysed at 4°C against sterile phosphate-buffered-saline for 24 h to remove prostanoids, which affect the bioassay. The total IL-1 concentration was assayed by [^{3}H]thymidine incorporation into the D-10.(N4).M cells, a D10.G4.1 T-helper subline (Kaye et al 1983; Hopkins & Humphreys 1989; Hopkins et al 1990) (kindly provided by Dr Stephen Hopkins, University of Manchester, Manchester, UK) cultured in RPMI-1640 medium as described by those authors (Hopkins & Humphreys 1989). Following serial dilution into the assay buffer this assay is highly specific for IL-1, with no interference being evident from IL-2 or IL-6 (Hopkins & Humphreys 1989). In some experiments polyethylene glycol (PEG 8000) was added to remove endogenous IL-1 inhibitors using methods described by Hopkins et al (1990). A standard of human recombinant IL-1α, 0·4–32·5 units mL^{-1} (generously donated by the National Institute for Biological Standards and Control, Potter's Bar, Herts, UK) was used in each assay. In some experiments MK-886 with or without LTB4 was added to cultures of normal (i.e. non-inflamed) pig synovial tissues obtained from untreated animals and cultured as previously described (Rainsford 1987). Assays of interleukins 1α, 1β, 6, 8 and LTB_4 were performed by ELISA using methods described in the manufacturer's instructions (Amersham Canada Ltd, Oakville, Ontario, Canada). Protein content was determined by the method of Lowry et al (1951).

Drugs and their preparation

MK-886 (3-(1-(4-chlorobenzyl)-3-*tert*-butyl-thio-5-isopropylindol-2-yl)-2,2-dimethyl propanoic acid; Merck-Frosst

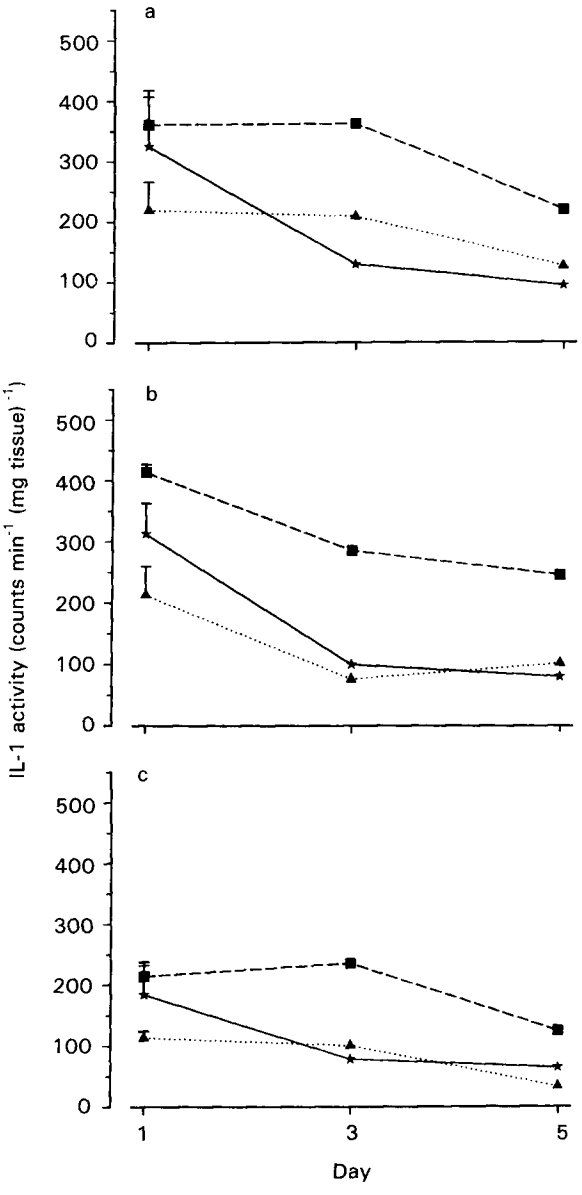

FIG. 2. Effects of the 5-lipoxygenase inhibitors, MK-886 (▲) and PF-5901 (☆) (5 μM each) on the production of IL-1 activity on days 1, 3 and 5 from human synovial explants in organ culture. Statistically significant reduction ($P < 0.05$) compared with control (■) was evident at all medium dilutions with MK-886. Assays were performed on culture medium diluted 1:10 (a), 1:20 (b) and 1:50 (c) in RPMI-1640 medium. Values are means ± s.e.m. of five replicates.

Institute for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada; Fig. 1) was provided as a sodium salt and was soluble in DMEM whereas the other 5-lipoxygenase inhibitors, L-656,224 (7-chloro-2-[4-methoxyphenyl]methyl)-3-methyl-5-propyl-4-benzofuranol) (Belanger et al 1987), ICI 211,959 (Zeneca, Macclesfield, UK) (McMillan et al 1991), tepoxalin (Robert Wood Johnson Institute, Raritan, NJ, USA) (Murray et al 1990), PF-5901 (REV-5901; Perdue-Frederick, Norwalk, CT, USA) (Gordon et al 1984; Coutts et al 1985), and zileuton (ABT-077, A64077, Abbott Laboratories, Abbott Park, IL, USA) (Sirios et al 1991), the presumptive IL-1 synthesis inhibitors, IX-207,887 (Schnyder et al 1990) (Sandoz, Basel, Switzerland), and tenidap (Pfizer, Groton, CT, USA) (McDonald et al 1988), and the cyclo-oxygenase and IL-1 inhibitor, prinomide (Ciba Geigy Corporation Orientation Brochure 1987), were all dissolved in ethanol to a final concentration of 0.1%; this concentration of ethanol being without effect on IL-1 production (studies shown in Fig. 1). Hydrocortisone (Sigma) in ethanol (0·1%) was employed as a positive control (Kragballe 1989).

Results

The initial time-course experiments showed that IL-1 production from human synovial explants assayed following serial dilution in RPMI-1640 medium at concentrations of 1:10, 1:20 and 1:50 peaked at day 1 of incubation, remained at this level on day 3 then declined by day 5 (Fig. 2). IL-1 production was significantly inhibited by 5 μM MK-866 on day 1 of culture and remained at this level of inhibition following 3 and 5 days incubation, irrespective of the effects of dilution of the medium for bioassay. The inhibitory effects of MK-886 were also apparent when the IL-1 production was assayed by specific ELISA for the α- and β-isotypes (Table 1). LTB4 production was reduced by 53% with 5 μM MK-886 (data not shown). PF-5901, 5 μM, only inhibited IL-1 production at 1:20 dilution in RPMI-1640 medium on day 1, while 30 μM significantly inhibited IL-1 production at 1:10 dilution but not 1:20 or 1:50 dilutions at this time (Fig. 2). PF-5901, 30 μM, reduced production of IL-1 at all dilutions on day 3 but not on day 5 (data not shown). In the subsequent experiments with these and other drugs the 1 or 3 day incubation periods were employed together with a 1 : 20 and 1 : 50 dilutions since these were found the minimal dilution for inhibitory effects to be manifest, even accounting for some variability between tissues from different subjects. The data in Fig. 3 show that MK-886 and L-656,224 produced a statistically significant (Student's t-test, $P < 0.05$) inhibition of IL-1 in the range of 0.1-5.0 μM. The same inhibitory effects were found in tissues from three separate experiments of tissues from four different subjects. The 5-lipoxygenase inhibitor, ICI-211,965, had no significant effects on the production of IL-1 at 1 and 10 μM, but did so at 50 μM (Table 2), whereas zileuton 10 and 50 μM stimulated production of IL-1, activating after 1 and 3 days incubation (Table 1). Prinomide also stimulated IL-1 production at 100 μM (Table 2). The combined lipoxygenase/cyclo-oxygenase inhibitor, tepoxalin, significantly reduced IL-1 production in the range 1–50 μM, although there tended to be greater inhibition at the lower concentrations (Table 2). The presumed IL-1 synthesis inhibitors, IX-207,887 and tenidap, were without any significant effects on IL-1 production at concentrations up to 50 and 30 μM, respectively (Table 2). Tenidap, was even inactive at 30 μM even following 5 day's incubation (Table 2). In comparison, the positive control, hydrocortisone showed marked inhibition of IL-1 production at 1.0 μM and this was unchanged at higher concentrations up to 10 μM (Table 2). The inhibitory effects of tepoxalin, and the lack of significant effects of IX-207,887, tenidap and prinomide were confirmed by ELISA assays for IL-1α and 1β in the culture media from these assays assayed in triplicate or duplicate (data not shown).

Table 1. Effects of the 5-lipoxygenase inhibitor, MK-886, in the presence and absence of LTB_4 on the production by human synovial tissues of IL-1α, IL-1β, IL-6 and IL-8 assayed by ELISA.

Experiment	Treatment		IL-1α (pg (mg protein)$^{-1}$)	IL-1β	IL-6 (ng (mg protein)$^{-1}$)		IL-8
1	Control		21·3	ND	343·4		255·6
	MK-886	0·1 μM	19·2	ND	486·8		202·6
		1·0 μM	5·3	ND	286·7		260·5
		5·0 μM	4·6	ND	260·0		117·4
2	Control		397·8	1130	335·7	15·57 ± 1·47	123·5
	MK-886	5·0 μM	15·6	396	123·1	6·84 ± 1·55	97·0
	MK-886	5·0 μM					
	with LTB_4	1 ng mL^{-1}	26·2	461	159·8	9·46 ± 0·46	103·4
		10 ng mL^{-1}	140·8	985	183·4	6·97 ± 0·85	142·0
		100 ng mL^{-1}	480·3	806	143·5		224·0

Incubations of synovial tissues were performed for one day with the drugs, and interleukin assays were performed on the harvested media. The assays were performed at least twice and the means compiled from pooled data, except for data form the right hand side column for IL-6 which was performed in 4–5 replicate samples (means ± s.d.). ND denotes not determined.

Addition of 1–100 ng mL^{-1} LTB4 reversed the inhibitory effects of 5 μM MK-886 in a concentration-dependent manner on IL-1 production, assayed by both D10 cell bio-assay (Table 2) and ELISA for the α- and β-isoforms of IL-1 (Table 1). The highest concentration of LTB4 (100 ng mL^{-1}) stimulated IL-1 production above that of the control (Tables 1, 2). Addition of PEG 8000 to media from MK-886 (5μM)-treated tissues to remove inhibitors of IL-1 resulted in increased IL-1 production when determined by bioassay (Table 2) or ELISA for the α- and β-isotypes. Addition of PEG 8000 to cultures from tissues incubated with 1-50 μM IX-207,887 did not reveal any inhibitory effects of this presumptive IL-1 synthesis inhibitor (Table 2). Addition of 1.0–10 ng mL-1 LTB4 also reversed the effect of this inhibitor and the higher concentrations of 100 ng mL^{-1} LTB4 further stimulated IL-1 production (Tables 1, 2). Addition of the same concentrations of LTC4 did not reverse the inhibitory effects of 5 μM MK-886, whereas 1-100 ng mL-1 5-hydroxyeicosatetraneoic acid did reverse this inhibitory effect (data not shown). In addition to inhibiting IL-1 production, MK-886 5 μM, inhibited the production of IL-6 and IL-8, and this was also partially reversed by 1-100 ng mL^{-1} LTB4 (Table 1). Interleukin production was increased by 10–50 μM zileuton and IL-8 was also reduced by this drug (data not shown). The inhibitory effects of the 5-lipoxygenase inhibitors (e.g. MK-886, PF-5908, L-656,224, tepoxalin) were evident at both high and low IL-1 activities, there being no alteration by media dilution or variation in capacity of the tissue to produce this cytokine (Table 2, Figs 2, 3). It was found that MK-886 (5 μM alone or in the presence of 1.0-100 mg mL^{-1} LTB4, with or without PEG 8000 treatment of culture media) failed to affect IL-1 production from normal (i.e. non-inflamed) pig synovial tissues incubated under the same conditions as for the human tissues (data not shown).

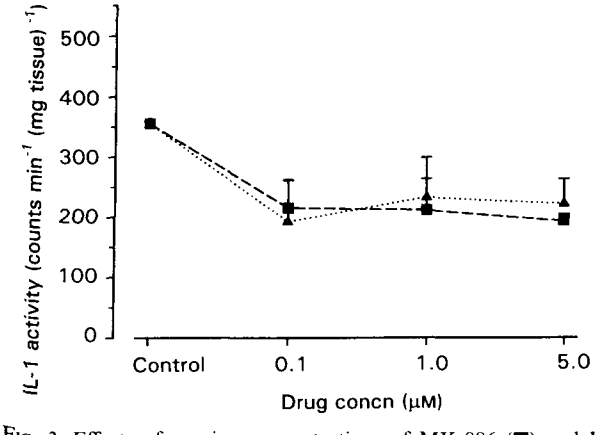

FIG. 3. Effects of varying concentrations of MK-886 (■) and L-656,224 (▲) on human synovial IL-1 production (determined by the D-10 cell assay, on culture medium diluted 1:20) Statistically significant reduction in IL-1 production was observed in the range 0.1-5.0 (μM of these drugs. Values are means ± s.e.m. (n = 5).

Discussion

The results show that IL-1 production when assayed by the D-10 cell-specific bioassay for this cytokine as well as by ELISA for its α- and β-isoforms, was reduced by some but not all of the 5-lipoxygenase inhibitors. MK-886, L-656,224, PF-5901 and tepoxalin appeared to be among the most potent inhibitors of IL-1 production, their effects being comparable with the standard hydrocortisone. The inhibitory effects of MK-886 on IL-1 production were overcome by addition of LTB4, and to a lesser extent by 5-hydroxyeicosatetraneoic acid in a concentration-related manner but not by LTC4, showing the specific role of the 5-lipoxygenase products involving leucocyte activation by non-smooth muscle contractile properties in the regulation of IL-1 production. The mechanism of the inhibitory effects of MK-886 on 5-lipoxygenase activity (via the binding to the 5-lipoxygenase activating protein) are different to those of the other direct-acting drugs (Gillard et al 1992) which were studied. The differences in effects of the other direct acting 5-lipoxygenase inhibitors on IL-1 production may be related to their relative potency and possibly to differences in their binding to serum components in the media. When PEG 8000 was added to remove natural inhibitors of IL-1 (Hopkins et al 1990) this overcame the inhibitory effects of MK-886. Thus, it seems possible that MK-886 acts by enhancing

Table 2. Effects of 5-lipoxygenase and cyclo-oxygenase inhibitors, compared with IL-1 inhibitors on the production of IL-1 by human synovial tissue explants in organ culture.

Experiment	Drug	Concn (μM)	Incubation (days)	IL-1 activity (counts min^{-1} (mg protein)$^{-1}$)	Dilution
Lipoxygenase/cyclo-oxygenase inhibitors					
1	Control		1	12803 ± 1401 (3)	1:10
	MK-886	5·0		8029 ± 1983 (3)*	
	+ LTB4 (1 ng mL^{-1})			9507 ± 824 (3)*	
	(10 ng mL^{-1})			15026 ± 420 (3)†	
	(100 ng mL^{-1})			29922 ± 2565 (4)†	
PEG separated fractions					
2	Control			20865 ± 1511 (3)	1:10
	MK-886	5·0		18483 ± 4264 (3)	
	+ LTB4 (1 ng mL^{-1})			20326 ± 4242 (3)	
	(10 ng mL^{-1})			23604 ± 4989 (3)	
	(100 ng mL^{-1})			35541 ± 2540 (4)†	
3	Control		1	1346 ± 306	1:20
	MK-886	0·1		656 ± 190*	
		1·0		692 ± 142*	
PEG separated fractions					
	Control			972 ± 259	1:20
	MK-886	0·1		709 ± 214	
		1·0		731 ± 25	
PEG separated fractions					
	Control			1014 ● 237	1:10
	MK-886	0·1		941 ± 373	
		1·0		913 ± 71	
4	Control		1	4651 ± 1482 (5)	1:6
	Zileuton	10·0		8220 ± 1072 (5)†	
	Control			3127 ± 677 (5)	1:12
	Zileuton			9103 ± 2751 (5)	
5	Control		1	23538 ± 9559 (5)	1:20
	Zileuton	10·0		23072 ± 1839 (5)	1:2
		50·0		21351 ± 1102 (5)	
	Control		3	29050 ± 8660 (5)	1:2
	Zileuton	10·0		33610 ± 5920 (5)	
		50·0		24225 ± 5630 (5)	
6	Control		1	6817 ± 2356 (4)	1:10
	Tepoxalin	1·0		1886 ± 243 (5)*	
		10·0		2563 ± 624 (4)*	
		50·0		4530 ± 1986 (4)	
	ICI 211, 965	1·0		5857 ± 1977 (5)	
		10·0		6331 ± 1552 (4)	
		50·0		2577 ± 447 (5)*	
	Control		1	10513 ± 3760 (5)	1:20
	Tepoxalin	1·0		3680 ± 1344 (5)*	
		10·0		5055 ± 2479 (4)*	
		50·0		6300 ± 2439 (4)*	
	ICI 211, 965	1·0		9770 ± 3105 (5)	
		10·0		10151 ± 3758 (5)	
		50·0		5455 ± 1063 (5)*	
7	Control		1	5832 ± 10502 (5)	1:24
	Tepoxalin	1·0		27895 ± 1927 (5)	
		10·0		44340 ± 16695 (5)	
8	Control		1	6574 ± 2073 (5)	1:12
	Prinomide	100·0		13485 ± 4622 (5)†	
	Control			8891 ± 1713 (5)†	
	Prinomide	100·0		17558 ± 3118 (4)†	
9	Control		1	807 ± 160 (4)	1:50
	IX 207, 887	1·0		879 ± 273 (5)	
		5·0		671 ± 174 (4)	
		50·0		745 ± 118 (4)	
PEG separated fractions					
	Control		1	750 ± 216 (3)	1:50
	IX 207, 887	1·0		788 ± 247 (5)	
		5·0		673 ± 195 (4)	
		50·0		630 ± 136 (4)	
10	Control		1	214 ± 24 (5)	1:50
	Tenidap	30·0		709 ± 294 (5)†	
	Control		3	414 ± 12 (5)	
	Tenidap	30·0		1033 ± 370 (5)†	
	Control		5	361 ± 46 (5)	
	Tenidap	30·0		1026 ± 362 (3)†	
Steroid standard					
11	Control		1	387 ± 10·9 (5)	1:50
	Hydrocortisone	1·0		134 ± 13·4 (5)*	
		10·0		138 ± 10 (5)*	

* Significantly less than control value, † significantly greater than control value ($P < 0·05$). PEG = polyethylene glycol 8000.

production of an IL-1 inhibitor. While MK-886, L-656,224, PF-5901 and tepoxalin might have other, as yet, unexplored actions, it seems likely from these results that these drugs act by the depletion of leukotrienes. MK-886 appears among the most potent inhibitors of IL-1 production of the drugs tested. The high potency is difficult to reconcile with claims for inhibition of this interleukin reported to be due to putative IL-1 synthesis inhibitors e.g. IX-207,887 (Schnyder et al 1990) and tenidap (McDonald et al 1988; Otterness et al 1989). A recent study has shown that IL-1 release from rat aortic smooth-muscle cells was inhibited by the selective LTD4 antagonist, MK-571 (Perreca et al 1994), giving further support to the role of leukotrienes in regulating IL-1 production. It was also found that MK-886 inhibited production of IL-6 and IL-8, although the effects on the production of the latter may be less potent than on IL-1. These potent cytokines also exhibit pleiotrophic effects akin to IL-1 being also involved in the cartilage destructive and associated effects on joints during inflammation (Helle et al 1988; Houssiau et al 1988; Elford & Cooper 1991; Thomsen et al 1991). Moreover, IL-1 induces production of IL-6 and via the recruitment of leucocytes (Zhang et al 1988), and promotes the production of IL-8 (Thomsen et al 1991; Elford & Cooper 1991; Koch et al 1991). By inhibiting both the effects of this primary stimulant cytokine, IL-1, as well as reducing the amplified production of IL-6 and IL-8, it appears MK-886 and related drugs have multiple actions in preventing synthesis of many of the cytokines involved in cartilage destruction. These involve those interleukins directly affecting cartilage resorption (IL-1 and IL-6) as well as the IL-8 involved in chemo-attraction of leucocytes into inflamed sites and activation to release cartilage destructive enzymes. Since MK-886 and related drugs inhibit production of all three of these cytokines it would seem that this drug could be a prototype or suitable regulator of the spectrum of cytokine-mediated cartilage (or joint) destruction. The lack of inflammatory state of the pig tissue would seem to be a possible reason for this lack of effect of MK-886 in this system. The previously observed lack of effects of MK-886 on monocyte IL-1 production (Hoffman et al 1991) may also be due to the lack of the inflamed state in these cells compared with that in synovial tissues. The cell populations in the inflamed synovia would be expected to be more complex and the nature of the inflammatory stimuli they produce are different from the isolated monocyte system previously studied (Hoffman et al 1991). These results emphasize the importance of defining drug effects in diseased states. The potency of effect of MK-886 as well as L-656,224 are indications for disease-modifying activity at the level of IL-1 production as well as for the well-defined role in decreasing leukotrienes in inflammatory events (Bray 1987; Rola-Pleszczynski 1988; Marshall & Chang 1989; Pfister & Ernest 1989; Salmon et al 1989).

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