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Anti-inflammatory Effect of FR140423, a Novel Selective Cyclo-oxygenase-2 Inhibitor, in Rat Adjuvant Arthritis Without Gastrointestinal Side Effects

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Abstract

We investigated the effect of FR140423 (3-(difluoromethyl)-1-(4-methoxyphenyl)-5-[4-(methylsulphinyl)phenyl]pyrazole), a novel and selective cyclo-oxygenase (COX)-2 inhibitor, in rat adjuvant arthritis. The results were compared with that of indomethacin.

We tested the inhibitory effects of FR140423 on paw oedema and the formation of the arachidonic acid metabolites prostaglandin (PG) E_2 and leukotriene (LT) B_4 in inflamed paws immunized with heat-killed and dried *Mycobacterium tuberculosis*. Oral administration of FR140423 showed a dose-dependent anti-inflammatory effect. This effect was two- to threefold more potent than that of indomethacin. The increase of PGE_2 and LTB_4 in inflamed paws was associated with the development of paw swelling. FR140423 and indomethacin dose-dependently suppressed the level of PGE_2 but not LTB_4 in arthritic paws. Unlike indomethacin, FR140423 did not induce gastric lesions even at doses up to $10\,mg\,kg^{-1}$ in arthritic rats.

FR140423 has a potent anti-inflammatory effect mediated by inhibition of PGE_2 produced by COX-2 in inflamed tissues. The safety profile of FR140423 appears to be an improvement on the safety profile of indomethacin.

Non-steroidal anti-inflammatory drugs (NSAIDs) are used widely for the treatment of the symptoms of arthritis. However, side effects such as gastrointestinal irritation and renal function abnormalities have arisen after long-term treatment with NSAIDs (Schleyerbach & Wedde 1984). The common mechanism of action of NSAIDs is believed to be the inhibition of cyclo-oxygenase, which is the rate limiting enzyme for the conversion of arachidonic acid into prostaglandins (PGs) (Vane 1971). Two isoforms of cyclo-oxygenase, constitutive COX-1 and inducible COX-2, have been identified (Hla & Neilson 1992; Meade et al 1993). In general, COX-1 is detected in most normal cells and tissues, including human stomach, kidney and platelets. It is needed for normal physiological function such as cytoprotection and homeostasis. COX-2 is not detected in normal cells and tissues; however, it is rapidly induced in response to inflammatory stimuli by endotoxin, mitogens and cytokines (Hla & Neilson 1992; Xie et al 1992; Cao et al 1996). In addition, recent studies have indicated that both interleukin-1 and tumour necrosis factor-α enhance production of PGE_2 and COX-2 by synoviocytes from rheumatoid arthritis and osteoarthritis patients (Crofford et al 1994; Szczepanski et al 1994). Most of the classically available NSAIDs are non-selective cyclo-oxygenase inhibitors, demonstrating an inhibitory action on both COX-1 and COX-2. This can explain their anti-inflammatory effect, via an action on COX-2, and also their ulcerogenic effect, via an action on COX-1. Thus, selective inhibitors of COX-2 are ideal therapeutic agents expected to have anti-inflammatory effects without ulcerogenic side effects.

We have recently shown that FR140423 (3-(difluoromethyl)-1-(4-methoxyphenyl)-5-[4-(methylsulphinyl)phenyl]pyrazole), which selectively inhibits COX-2 over COX-1, is a novel non-steroidal anti-inflammatory agent that does not result in gastrointestinal lesions (Tsuji et al 1997; Ochi et al 1999b). Adjuvant-induced arthritis in rats is one of the most common pharmacological animal models for human rheumatoid arthritis. It has been used

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widely as a chronic inflammation model and for the evaluation of anti-rheumatic drugs (Bartlett & Schleyerbach 1985). In this paper, we have described the pharmacological profile of FR140423, focusing on adjuvant-induced arthritis and ulcerogenic effects after chronic treatment with FR140423. The findings were compared with indomethacin.

Materials and Methods

Animals

Ethical guidelines for the experimental use of animals were followed (Zimmermann 1983). In addition, the Fujisawa Pharmaceutical Animal Experiment Committee for Animal Experimentation reviewed the experimental work.

Eight-week-old female Lewis rats (140–180 g, Charles River Japan, Yokohama, Japan) were housed for at least five days in a controlled environment. Food and water were freely available.

Drugs

Indomethacin was obtained from Sigma (St Louis, MO). FR140423 was chemically synthesized at Fujisawa Pharmaceutical (Osaka, Japan).

Induction of adjuvant arthritis

A suspension of heat-killed and dried *Mycobacterium tuberculosis* H37 rheumatoid arthritis (0·5 mg; DIFCO, Detroit, MI) in 0·05 mL liquid paraffin was administered by intradermal injection into the plantar surface of the rat right hind paw (day 0) to induce adjuvant arthritis (Newbould 1963; Walz et al 1971). The drugs, suspended and diluted in 0·5% methylcellulose, were given orally once a day therapeutically from day 15 to day 24 after adjuvant injection. For the time course of oedema, paw volume was measured before and 1, 4, 10, 15, 18, 21 and 24 days after adjuvant injection using a Volume Meter TK-105 (Neuroscience, Tokyo, Japan). For pharmacological studies, paw volume was measured before and 15, 18, 21 and 24 days after adjuvant injection. The anti-inflammatory effect was expressed as the ED50 value on day 24.

Biochemical measurements

The technique of Opas et al (1987) was used. At selected times after adjuvant injection, rats were killed by CO_2 inhalation and both hind paws were amputated. The paws were then placed immediately into n-hexane, cooled by dry ice acetone, for 30 s. Frozen paws were then stored at −70°C until needed for biochemical analysis of arachidonic acid metabolites.

Frozen paw tissue was homogenized under cooling in 5 mL extraction buffer (75% methanol, 25% 0·1 M sodium acetate, adjusted to pH 3 with HCl). The extracted tissue was centrifuged at 3000 rev min^{-1} for 10 min at 4°C. The resulting supernatant fluid was filtered through gauze and diluted with distilled water to a final concentration of 15% methanol. This solution was applied to a C18 Sep-Pak cartridge (Waters, Milford, MA), preconditioned with 10 mL methanol, distilled water and 15% methanol. After loading Sep-Pak, the columns were sequentially washed with 5 mL 15% methanol, distilled water and petroleum ether. The samples were eluted with 2 mL methyl formate (Powell 1980, 1982), evaporated under nitrogen gas, taken up in 1 mL phosphate-buffered saline and assayed for arachidonic metabolites, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), by radioimmunoassay (Amersham, Buckinghamshire, UK).

The efficiencies of recovery as determined by injection of radiolabelled arachidonic acid metabolites into amputated paws were as follows (mean percent ± s.e.m., n = 3): PGE_2 42·0 ± 2·1% and LTB_4 30·1 ± 2·3%.

Gastric ulcerogenic activity

The ulcerogenic activity was expressed on day 24. After the rats were killed, the stomachs were removed and placed in 2% formalin (Kanto Chemical, Tokyo, Japan). Each stomach was opened by cutting along the greater curvature, and the lesion index was assessed by scoring zero to four gastric lesions. Petechiae were assigned a score of 1, and erosion was assigned a score of 2. The gastric mucosal lesions were scored according to their number (a score of 3 for one to four lesions, and a score of 4 for five or more lesions).

Statistical analysis

Results were expressed as means ± s.e.m. Statistical significance was analysed using the one-way analysis of variance followed by Dunnett's multiple comparison test. ED50 values and 95% confidence limits (95% CL) were calculated from the dose–percent inhibition relations by computer log-linear regression analysis (Litchfield & Wilcoxon 1949).

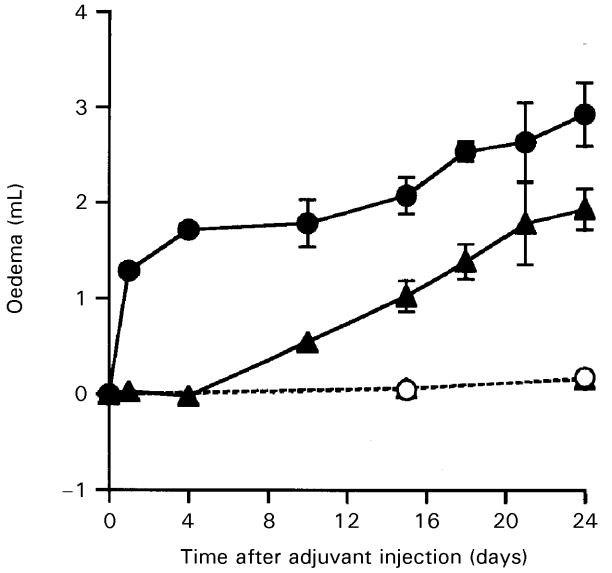

Figure 1. Time course of adjuvant arthritic rat paw oedema. Heat-killed and dried *Mycobacterium tuberculosis* H37 rheumatoid arthritis (0·5 mg/paw) was injected into the plantar surface of the right hind paw. Paw volume was measured before and various times after injection of vehicle or adjuvant and pre-injection volume was subtracted from these values. Adjuvant-injected right hind paws (●), adjuvant-uninjected left hind paws (▲) and vehicle-treated hind paws (○). Values are means ± s.e.m., n = 5.

Results

Induction of paw swelling in adjuvant arthritic rats

In the adjuvant control group, primary swelling of adjuvant-injected hind paws (right hind paws) was observed between day 1 and day 4 after mycobacterial adjuvant injection, and secondary swelling occurred with a delay of approximately 10 days after adjuvant injection (Figure 1). The secondary swelling was characterized by inflammation of both the paws and legs, especially adjuvant-uninjected hind paws (left hind paws). In the vehicle-treated group, swelling was not observed in either paw.

Anti-inflammatory effect of FR140423 in adjuvant arthritic rats

Oral administration of FR140423 (0·01–3·2 mg kg^{-1}) to arthritic rats rapidly reversed paw oedema (Figure 2). After 10 days of therapeutic treatment, paw swelling was dose-dependently reduced in the FR140423-treated rats with ED50 values (95% CL) of 0·11 (0·048–0·47) and 0·064 (0·0042–0·32) mg kg^{-1} for adjuvant-injected paws and adjuvant-uninjected paws, respectively. The anti-inflammatory effect of FR140423 was two- to threefold that of indomethacin with ED50 values (95% CL) of 0·24 (0·047–1·8) and 0·18 (0·035–0·89) mg kg^{-1} for adjuvant-injected paws and adjuvant-uninjected paws, respectively (Figure 3).

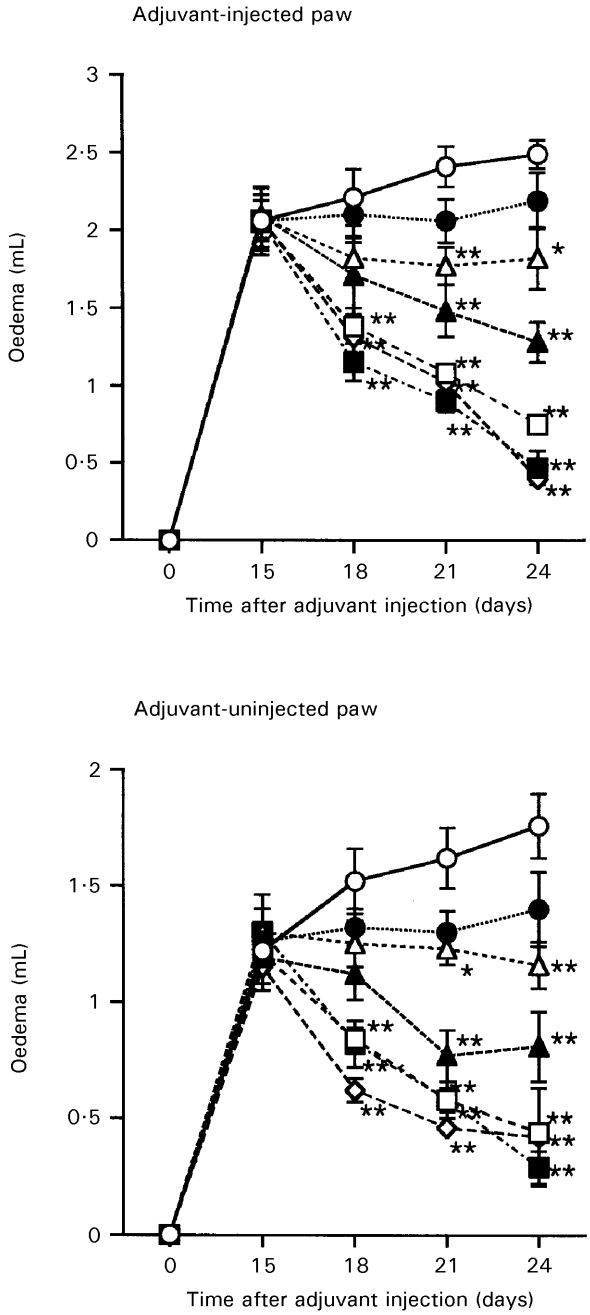

Figure 2. Therapeutic effect of FR140423 on adjuvant arthritis in rats. FR140423 at doses of 0·01 (●), 0·032 (△), 0·1 (▲), 0·32 (□), 1 (■) and 3·2 (◇) mg kg^{-1} and vehicle-treated control (○) were given orally once a day from day 15 to day 24 after adjuvant injection. Increase in paw volume in the vehicle-treated control group was 2·06 ± 0·22 and 1·22 ± 0·10 mL 15 days after immunization for adjuvant-injected paws and adjuvant-uninjected paws, respectively. *$P < 0·05$, **$P < 0·01$ compared with control. Values are means ± s.e.m., n = 5.

Formation of PGE_2 in adjuvant arthritic rat paws

The level of PGE_2 in adjuvant-injected rat paws increased dramatically after adjuvant injection

between day 0 and day 4 (Figure 4). PGE_2 in paw exudate reached a maximum of $75{\cdot}8 \pm 4{\cdot}7$ ng/paw on day 4. The level of PGE_2 gradually decreased between day 10 and day 15 and was steady at 40–50 ng/paw during the development of arthritis. In the vehicle-treated group, the level of PGE_2 in both rat paws did not increase during the experiment.

The level of PGE_2 in adjuvant-uninjected rat paws did not increase within four days after adjuvant injection (Figure 4). An increase in the level of

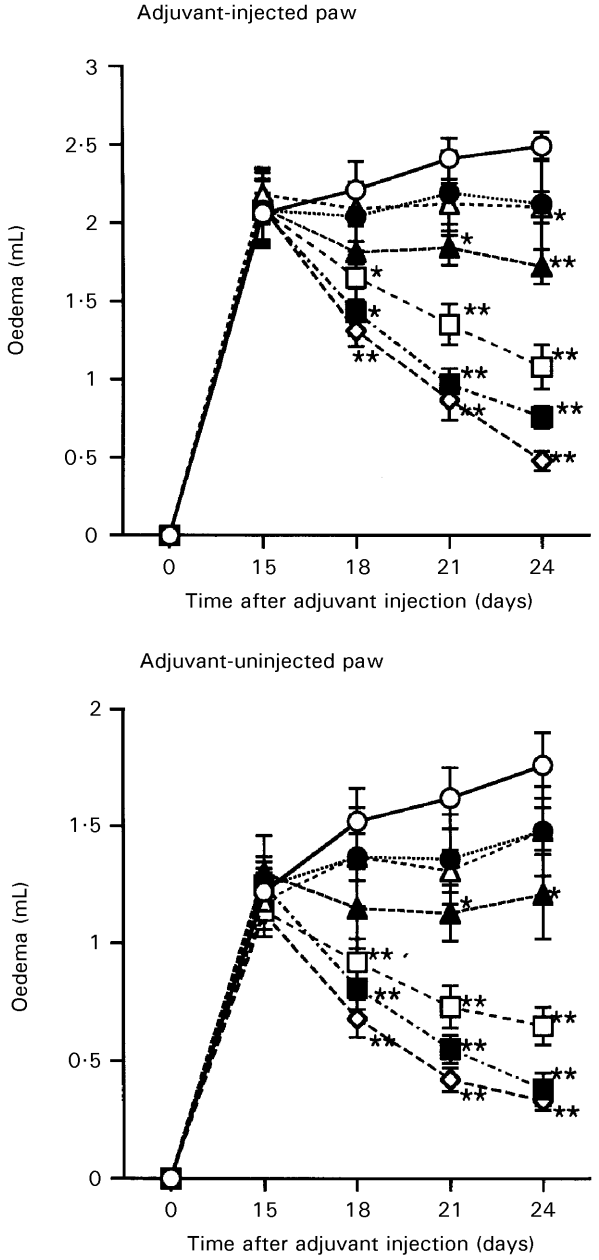

Figure 3. Therapeutic effect of indomethacin on adjuvant arthritis in rats. Indomethacin at doses of 0·01 (●), 0·032 (△), 0·1 (▲), 0·32 (□), 1 (■) and 3·2 (◇) mg kg^{-1} and vehicle-treated control (○) were given orally once a day from day 15 to day 24 after adjuvant injection. Increase in paw volume in the vehicle-treated control group was $2{\cdot}06 \pm 0{\cdot}22$ and $1{\cdot}22 \pm 0{\cdot}10$ mL 15 days after immunization for adjuvant-injected paws and adjuvant-uninjected paws, respectively. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ compared with control. Values are means ± s.e.m., n = 5.

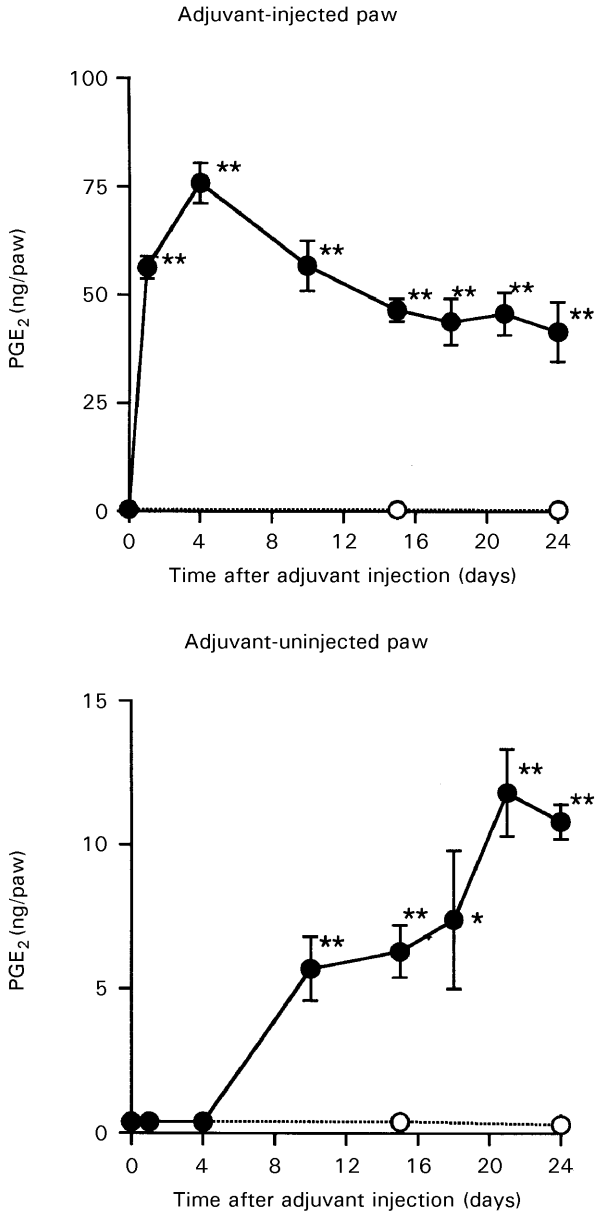

Figure 4. Time course of PGE_2 formation in adjuvant arthritic rat paws. Heat-killed and dried *Mycobacterium tuberculosis* H37 rheumatoid arthritis (0·5 mg/paw) was injected into the plantar surface of the right hind paw. Rats were killed by CO_2 inhalation at various times, and PGE_2 in adjuvant-injected right hind paws and in adjuvant-uninjected left paws were extracted and analysed by radioimmunoassay. Symbols indicate the adjuvant-treated rats (●) and vehicle-treated rats (○). $**P < 0{\cdot}01$ compared with the value on day 0. Values were corrected for recovery efficiency and expressed as ng/paw ± s.e.m., n = 5.

PGE_2 was evident 10 days after adjuvant injection and was sustained through to day 24. The production of PGE_2 in adjuvant-uninjected paws was associated with the secondary swelling.

Formation of LTB_4 in adjuvant arthritic rat paws

The level of LTB_4 in adjuvant-injected rat paws rose to a peak on day 1 and showed a maximum of $3{\cdot}4 \pm 0{\cdot}1$ ng/paw (Figure 5). During the development of arthritis the level of LTB_4 was steady at 3 ng/paw.

The level of LTB_4 in adjuvant-uninjected rat paws did not increase within four days after adjuvant injection. An increase in the level of LTB_4 was evident 10 days after adjuvant injection and was sustained through to day 24. The production of LTB_4 in adjuvant-uninjected paws was associated with the secondary swelling. In the vehicle-treated group, the level of LTB_4 in both rat paws did not increase during the experiment.

Effects of FR140423 on the formation of arachidonic acid metabolites (PGE_2 and LTB_4) in adjuvant arthritic rat paws

Oral administration of FR140423 (0·01–$3{\cdot}2\,mg\,kg^{-1}$) dose-dependently reduced the level of PGE_2 in arthritic paws (Table 1) with ED50 values (95% CL) of 0·17 (0·020–0·91) and 0·053 (0·000014–0·17) $mg\,kg^{-1}$ for adjuvant-injected paws and adjuvant-uninjected paws, respectively. However, treatment with FR140423 at doses up to $3{\cdot}2\,mg\,kg^{-1}$ (p.o.) did not affect the level of LTB_4 in either arthritic paw (data not shown). Indomethacin also showed a dose-dependent inhibition of the formation of PGE_2 (Table 1) with ED50 values (95% CL) of 0·20 (0·025–1·9) and 0·13 (0·022–0·60) $mg\,kg^{-1}$ for adjuvant injected paws and adjuvant-uninjected paws, respectively, but not LTB_4, in the arthritic rat paws.

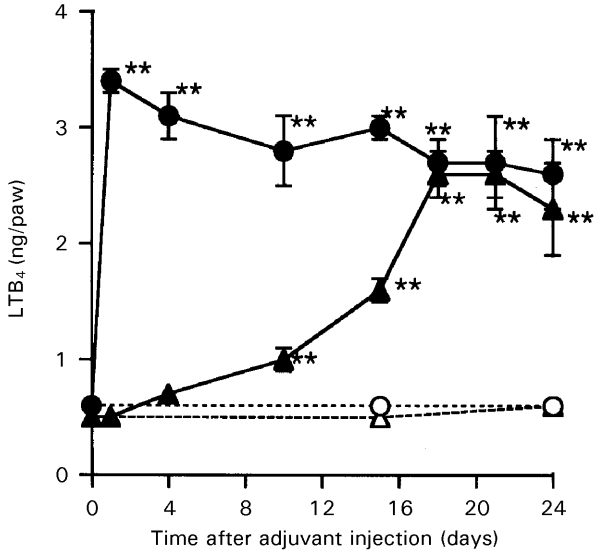

Figure 5. Time course of LTB_4 formation in adjuvant arthritic rat paws. Heat-killed and dried *Mycobacterium tuberculosis* H37 rheumatoid arthritis (0·5 mg/paw) was injected into the plantar surface of the right hind paw. Rats were killed by CO_2 inhalation at various times, and LTB_4 in both adjuvant arthritic rat paws was extracted and analysed by radioimmunoassay. Right hind paw of adjuvant-treated rats (●), left hind paw of adjuvant-treated rats (▲) and vehicle-treated hind paws (○, △). $^{**}P < 0{\cdot}01$ compared with the value on day 0. Values were corrected for recovery efficiency and expressed as ng/paw ± s.e.m., n = 5.

Gastric tolerability of drugs in adjuvant arthritic rats

Drugs were administered orally once a day from day 15 to day 24. Therapeutic treatment in adjuvant arthritis with FR140423 at doses between 1 and $10\,mg\,kg^{-1}$ did not induce any mucosal lesions. In contrast, indomethacin at a dose of $3{\cdot}2\,mg\,kg^{-1}$ produced marked gastric lesions in one of five rats. In the rats administered indomethacin $10\,mg\,kg^{-1}$, one of five rats died on day 19 and four of five rats died on day 20 (Table 2).

Discussion

Adjuvant arthritis of rats is one of the most important models of chronic inflammation such as rheumatoid arthritis. This model has been widely used for evaluation of traditional NSAIDs such as

Table 1. Effects of FR140423 on the formation of PGE_2 in adjuvant arthritic rat paws.

Drug ($mg\,kg^{-1}$, p.o.)		Adjuvant injected paw	Adjuvant uninjected paw
Non-treated		1·7 ± 0·1**	2·0 ± 0·2**
Control		58·6 ± 2·0	29·6 ± 0·8
FR140423	0·010	49·0 ± 4·9	25·6 ± 1·8
	0·032	38·5 ± 2·2**	19·3 ± 0·7**
	0·100	27·5 ± 2·3**	12·8 ± 0·9**
	0·320	22·0 ± 1·3**	9·3 ± 1·5**
	1·000	16·0 ± 0·7**	6·7 ± 0·6**
	3·200	7·5 ± 0·4**	4·4 ± 0·8**
Indomethacin	0·010	46·7 ± 3·9*	27·2 ± 1·6
	0·032	45·8 ± 2·1**	20·6 ± 0·5**
	0·100	39·8 ± 0·8**	17·5 ± 0·7**
	0·320	15·5 ± 1·0**	12·5 ± 0·7**
	1·000	16·0 ± 2·4**	8·3 ± 0·3**
	3·200	11·4 ± 1·2**	5·7 ± 0·2**

Drugs were given orally once a day therapeutically from day 15 to day 24 after adjuvant injection. Rats were killed by CO_2 inhalation 24 days after immunization, and PGE_2 in inflamed rat hind paws was extracted and analysed by radioimmunoassay. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with control. Values were corrected for recovery efficiency and expressed as ng/paw ± s.e.m., n = 5.

Table 2. Gastric ulcerogenicity of the drugs in adjuvant-induced arthritic rats.

Drug (mg kg^{-1}, p.o.)		Ulcer index	Incidence (%)
Non-treated		0·0 ± 0·0	0·0
Control		0·0 ± 0·0	0·0
FR140423	1	0·0 ± 0·0	0·0
	3·2	0·0 ± 0·0	0·0
	10	0·0 ± 0·0	0·0
Indomethacin	1	0·0 ± 0·0	0·0
	3·2	0·6 ± 0·6	20·0
	10	n.d.	n.d.

Drugs were administered orally once a day from day 15 to day 24 in adjuvant-treated rats. On day 24, visible gastric lesions were scored (score scales: petechiae = 1, erosion = 2, lesions between one and four = 3, lesions greater than five = 4). Values are means ± s.e.m., n = 5. n.d., No data because all the rats died.

indomethacin and aspirin (Ishizuki et al 1984). In this study, we have investigated the anti-inflammatory effect and gastric irritation of FR140423 on adjuvant-induced arthritis, and compared the actions of FR140423 with those of indomethacin. We have previously reported that FR140423 was a selective inhibitor of COX-2 (Ochi et al 1999b). In recombinant human COX-1 and COX-2 enzyme assays, FR140423 inhibited PGE_2 formation with IC50 values of 19 ± 9 and 0·13 ± 0·06 μM for COX-1 and COX-2, respectively. This result was 150-times more selective for COX-2 than COX-1, while indomethacin is a non-selective cyclo-oxygenase inhibitor. Therapeutic administration of FR140423 showed an anti-inflammatory effect in adjuvant-arthritic rat paws in a dose-dependent manner. Other well-known selective COX-2 inhibitors, NS-398 (N-[2-cyclohexyloxy-4 nitrophenyl]-methanesulphonamide), celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl) 1H-pyrazol-1-yl]benzensulphonamide) and rofecoxib (4-(4′-methylsulphonylphenyl)-3 phenyl-2-(5H)-furanone), have been reported to give similar results in this model (Futaki et al 1993, 1994; Penning et al 1997; Chan et al 1999). These results suggest that COX-2 plays an important role in the inflammation associated with adjuvant arthritis.

We investigated the relationship between the development of adjuvant arthritis and the level of arachidonic acid metabolites, PGE_2 and LTB_4, in the inflamed rat paws. When *Mycobacterium tuberculosis* was injected into the plantar surface of the right hind paw, a dramatic increase in the levels of PGE_2 and LTB_4 occurred in the adjuvant-injected paws between day 0 and day 4, nearly paralleling the increase in right hind paw volume. A gradual increase in the levels of PGE_2 and LTB_4 occurred in the adjuvant-uninjected paws 10 days after adjuvant injection, nearly paralleling the increase in left hind paw volume. Cyclo-oxygenase expression is upregulated in inflammatory joint diseases, and anti-inflammatory glucocorticoids suppress both arthritis and cyclo-oxygenase expression in female Lewis rats (Sano et al 1992). Oral administration of FR140423, a novel selective COX-2 inhibitor, dose-dependently inhibited the level of PGE_2 but not LTB_4 in both inflamed rat paws similarly to indomethacin. The ED50 values of FR140423 for inhibition of PGE_2 formation in both inflamed paws were almost the same as the ED50 values of FR140423 for its anti-inflammatory effect in adjuvant arthritis.

Therefore, it may be safely assumed that COX-2 is the enzymatic source of pro-inflammatory prostaglandin in adjuvant arthritic rat paws. Anderson et al (1996) reported that COX-2 mRNA and protein were elevated in arthritic rat paws without significant changes in COX-1 expression. These findings suggest that the development of adjuvant arthritis is associated with the upregulation of PGE_2 produced exclusively by COX-2. However, in contrast to continuous increase of adjuvant-injected paw volume, the level of PGE_2 was a maximum on day 4 and then gradually decreased. In addition to this, there are some contradictions in the level of PGE_2 and paw volume of both inflamed paws. One explanation for this discrepancy is to assume that some inflammatory mediators such as cytokines play a role in rat adjuvant arthritis in addition to PGE_2 produced by COX-2. For example, anti-intercellular adhesion molecule 1 (ICAM-1) antibody, 1A29, and angiogenesis inhibitor, AGM-1470, cause significant suppression of the development of chronic arthritis in rats (Iigo et al 1991; Peacock et al 1995). Heat-killed *Mycobacterium tuberculosis* is found to be a strong inducer of interleukin-6 production by spleen cells in-vitro, and serum interleukin-6 levels increase in adjuvant arthritis of rats (Theisen-Popp et al 1992). Klickstein et al (1980) detected LTB_4 in synovial fluid of patients with rheumatoid arthritis. Future studies will require the use of specific 5-lipoxygenase inhibitors to discuss the role of LTB_4 in this model.

Unlike indomethacin, FR140423 has a morphine-like analgesic effect (Ochi et al 1999b). The analgesic effect of FR140423 in yeast-induced rat hyperalgesia was antagonized by pretreatment of animals with the opioid-receptor antagonist naloxone (Ochi et al 1999a). Thus, FR140423 produced a naloxone-reversible analgesia. However, the anti-inflammatory effect of FR140423 in rat adjuvant arthritis was not reversed by naloxone (Sigma, St Louis, MO) at 2 mg kg^{-1} subcutaneously (data not shown). Thus, it is considered that the inflamma-

tory response in adjuvant arthritis was not mediated through opioid receptors.

The most common adverse effect of NSAIDs is the induction of gastrointestinal lesions. It is thought that these lesions are caused by the inhibition of COX-1. In this study, indomethacin caused gastric mucosal damage at a low dose of $3{\cdot}2\,mg\,kg^{-1}$. FR140423 did not cause gastric lesions at doses up to $10\,mg\,kg^{-1}$, which are 100-fold those which showed anti-inflammatory effects in adjuvant arthritic rats with ED50 values of 0·11 and $0{\cdot}064\,mg\,kg^{-1}$ for adjuvant-injected paws and adjuvant uninjected paws, respectively. This demonstrated that FR140423 had a high apparent safety index in this model.

FR140423 may be clinically useful in the treatment of rheumatoid arthritis.

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