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Analgesic and anti-inflammatory activities in rats of α-(3,5-di-t-butyl-4-hydroxybenzylidene)-γ-butyrolactone (KME-4), and its intestinal damage

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α-(3,5-Di-t-butyl-4-hydroxybenzylidene)-γ-butyrolactone (KME-4), an anti-inflammatory drug, possesses analgesic activity in rat models. In the acetic acid-induced writhing test, the oral ED50 values for KME-4, indomethacin, naproxen and ibuprofen were 5·2, 3·8, 7·0 and 18·6 mg kg^{-1}, respectively, and the relative order of potency of these drugs correlated with their inhibitory effect on acetic acid-induced vascular permeability in rats. KME-4 also had analgesic activity in the tests of Randall–Selitto and adjuvant arthritic flexion, but the dose required was greater than that needed in the writhing test. KME-4 (10 mg kg^{-1} day^{-1} orally) has a preventive effect against adjuvant-induced arthritis in rats, and its efficacy was more potent than indomethacin (2 mg kg^{-1} day^{-1}) as judged from various parameters determined. When administered orally to rats once daily for 12 days, KME-4 caused perforating ulceration of the small intestine but this action was less potent than the effect of indomethacin, naproxen and ibuprofen.

The recently synthesized compound, α-(3,5-di-t-butyl-4-hydroxybenzylidene)-γ-butyrolactone (KME-4) possesses potent anti-inflammatory and antipyretic activities (Hidaka et al 1984) and it may belong to a new class of non-steroidal anti-inflammatory drugs (NSAIDs) since it inhibits both arachidonate cyclooxygenase and 5-lipoxygenase (Hidaka et al 1984, 1985) unlike most known NSAIDs, which are selective cyclooxygenase inhibitors. More recently, this drug has been also reported to be effective in reducing migration of leucocytes (neutrophils and monocytes) in rat carrageenan-induced pleurisy (Hidaka et al 1986). We have extended tests on the drug to include measurement of analgesic and additional anti-inflammatory activities in rat models with the aim of clarifying the spectrum of pharmacological activity. Intestinal damage by repeated treatment with the drug was also examined.

MATERIALS AND METHODS

Animals

Male Wistar rats from Kitayama Labs and Shizuoka Laboratory Animal Center were used.

Drugs

KME-4 was prepared in Biochemical Research Laboratories of Kanegafuchi Chemical Industry Co., Ltd. Indomethacin (Hachidai Pharmaceutical Co.), naproxen (Nissan Chemical Industry Co.) and ibuprofen (Yonezawahamari Pharmaceutical Co.) were used as the standard anti-inflammatory drugs. All the test drugs were suspended in a 5% tragacanth gum for the pharmacological tests but were suspended in a 2·5% arabic gum for the toxicity test. Control groups received vehicle alone in each experiment.

Acetic acid-induced writhing

Groups of 8 rats, 110–155 g, were injected intraperitoneally with a 1% acetic acid solution in a volume of 0·5 ml/100 g weight (Niemegeers et al 1975). KME-4 was administered orally 60 min and the other drugs 30 min before the acid. The number of writhes were counted for 20 min starting 10 min after injection of the acid. The ED50 values were calculated by Litchfield & Wilcoxon's method (1949).

Acetic acid-induced vascular permeability

The method of Whittle (1964) was used. The test drugs were administered orally to groups of 8 rats of 130–150 g. Thirty min later, i.v. injection of a 0·1% pontamine sky-blue solution and i.p. injection of a 1% acetic acid solution were made in a volume of 0·5 ml/100 g weight. The animals were killed 30 min later and the peritoneal cavity was washed with 8 ml of distilled water. This was filtered and then made up to

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10 ml by adding water and 0·1 ml of 1 M NaOH. The amount of dye was measured at 590 nm.

Yeast-induced paw oedema in rats (Randall & Selitto test)

Rats (about 100 g) were injected s.c. into the right hindpaw with 0·1 ml of 10% brewer's yeast suspension. Four hours later, the pressure threshold of inflamed and normal paws was measured (Randall & Selitto 1957), then the test drugs were administered orally to groups of 12 animals. The pressure threshold of both paws was measured again, hourly for 3 h after the drug. Analgesic potency was expressed as analgesic index which was calculated as follows.

$$\text{Analgesic index} = \frac{\text{(pressure threshold (mmHg) at 1, 2, or 3 h after vehicle or drug treatment)}}{\text{(pressure threshold (mmHg) before vehicle or drug treatment)}}$$

Adjuvant arthritic pain

Rats (about 200 g) were injected intradermally into the right hindpaw with 0·6 mg of *Mycobacterium butyricum* (Difco) suspended in 0·1 ml of liquid paraffin (Walz et al 1971). On day 15 after the injection, animals with established arthritis were selected, and then animals responding five successive times after five flexions of the tarso-tibial joint of uninjected paw at about 5 s intervals, were further selected for the test (Kuzuna & Kawai 1975). The test drugs were administered orally to groups of 10 animals, and these responses were recorded hourly for 5 h after the drug as described above.

Adjuvant arthritis

Groups of 10 rats, 250–290 g, had adjuvant arthritis induced by the same method as above. The test drugs were administered orally once daily for 28 days starting on the first day (day 1) after the adjuvant. Both hindpaw volume and body weight were measured at the indicated times. The weight of several organs and erythrocyte sedimentation rate (ESR) were also determined on day 29.

Toxicity by repeated treatment

The test drugs were administered orally to groups of 6–10 rats (160–190 g) once daily for 12 days at indicated doses. At death, animals were checked and autopsy was carried out to determine the cause. As mortality from intestinal perforation was observed, the dose killing 50% of animals (PD50) was determined according to Schiantarelli & Cadel (1981). In addition, autopsies were subsequently carried out on surviving animals on the day after the final treatment to examine macroscopically whether intestinal ulcers were induced.

Statistics

Significance was assessed with Student's *t*-test.

RESULTS

Analgesic activity

KME-4 produced marked inhibition of acetic acid-induced writhing as did the standard drugs. The ED50 values for KME-4, indomethacin, naproxen and ibuprofen were 5·2, 3·8, 7·0 and 18·6 mg kg^{-1}, respectively (Table 1).

Effects on acetic acid-induced vascular permeability

KME-4 at 3 to 30 mg kg^{-1} showed significant inhibition of vascular permeability induced by acetic acid as did indomethacin, naproxen and ibuprofen (Table 2). The activity of these drugs correlated with their anti-writhing activity.

Table 1. Analgesic activity of KME-4 and standard drugs in acetic acid-induced writhing in rats. The detailed method is described in Materials & Methods.

Drug	ED50* (mg kg^{-1}) (95% confidence limits)
KME-4	5·2 (3·4– 8·0)
Indomethacin	3·8 (2·0– 7·3)
Naproxen	7·0 (2·9–17·1)
Ibuprofen	18·6 (9·3–37·2)

* Litchfield & Wilcoxon (1949)

Table 2. The inhibitory effect of KME-4 and standard drugs on acetic acid-induced vascular permeability in rats (Whittle's method).

Drug	Dose (mg kg^{-1})	Leakage of dye (μg/10 ml)	Inhibition (%)
Control	Vehicle	331·2 ± 19·7	0
KME-4	3	120·1 ± 29·6**	63·7
	10	147·8 ± 20·5**	55·4
	30	69·6 ± 15·3**	79·0
Indomethacin	1	217·0 ± 25·4**	34·5
	3	152·6 ± 21·0**	53·9
	10	99·5 ± 19·0**	70·0
Naproxen	3	130·9 ± 15·1**	60·5
	10	104·1 ± 11·2**	68·6
	30	89·2 ± 12·4**	73·1
Ibuprofen	10	135·4 ± 7·6**	59·1
	30	100·0 ± 12·9**	69·8
	100	83·3 ± 9·2**	74·8

Each value indicates the mean ± s.e.m. of 8 animals.
** $P < 0·01$: statistically significant difference from the control.

Analgesic activity in the Randall–Selitto test

KME-4 showed dose-related elevation of the pressure threshold in the inflamed foot that was significant at 30 mg kg^{-1}, while indomethacin was effective at 2·5 and 5 mg kg^{-1}. Naproxen and ibuprofen also had significant effect at 50 and 100 mg kg^{-1} (Table 3). Except for indomethacin (2·5 mg kg^{-1}) at 1 h, the other drugs tested had no effect on the normal foot.

Analgesic activity in adjuvant arthritis

Indomethacin (2·5 and 5 mg kg^{-1}), naproxen (50 and 100 mg kg^{-1}) and ibuprofen (50 and 100 mg kg^{-1}) showed relatively strong analgesic activity as compared with KME-4 (30 mg kg^{-1}) (Fig. 1).

Effect on adjuvant arthritis

The results are shown in Fig. 2 and Table 4. KME-4 (10 mg kg^{-1} day^{-1}) and indomethacin (2 mg kg^{-1} day^{-1}) were nearly equiactive in reducing the swelling of adjuvant-injected paws, but KME-4 was more effective in causing a significant increase of body weight. Both drugs also produced a significant recovery of the weights of thymus, spleen, adrenals, lymph nodes, and moved erythrocyte sedimentation rate (ESR) towards normal ranges. The degrees of improvement of growth rate and ESR were greater with KME-4 (10 mg kg^{-1} day^{-1}) than by indomethacin (2 mg kg^{-1} day^{-1}). KME-4 also inhibited the paw swelling (1 mg kg^{-1} day^{-1}) which was almost equiactive with ibuprofen (40 mg kg^{-1} day^{-1}), but no drug caused a significant increase in body weight or the significant recovery of the other parameters measured. The swelling of uninjected paws was similarly inhibited by the test drugs (data not shown).

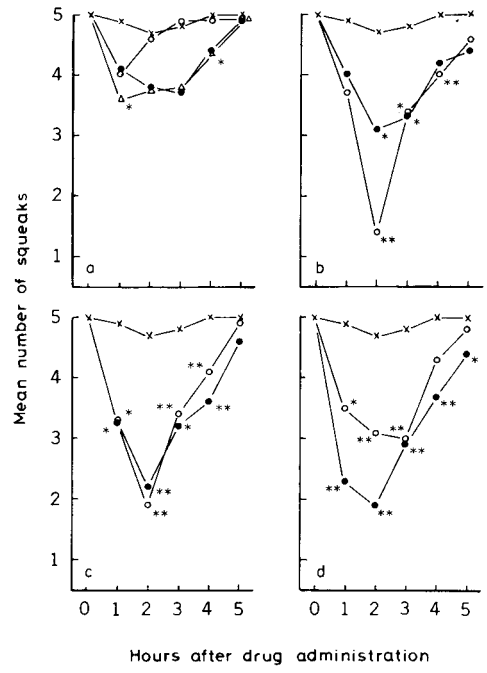

FIG. 1. Analgesic effect of KME-4 and standard drugs on adjuvant arthritic pain in rats. Each point indicates the mean value of 10 animals. —×—; control, * $P < 0.05$ and ** $P < 0.01$: Statistically significant difference from the control. Key: (a) KME-4; ○ 3, ● 10, △ 30 mg kg^{-1}. (b) Indomethacin; ○ 2·5, ● 5 mg kg^{-1}. (c) Naproxen; ○ 50, ● 100 mg kg^{-1}. (d) Ibuprofen; ○ 50, ● 100 mg kg^{-1}.

Toxicity by repeated treatment

As shown in Table 5, when administered orally once daily for 12 days, all the drugs tested induced death at the indicated doses. Intestinal perforation and/or peritonitis was observed in all the dead animals. The PD50 values for KME-4, indomethacin, naproxen

Table 3. Analgesic activity of KME-4 and standard drugs on yeast-induced paw oedema in rats (Randall–Selitto method).

Drug	Dose (mg kg^{-1})	Analgesic index inflamed foot			Analgesic index normal foot		
		1 h	2 h	3 h†	1 h	2 h	3 h†
Control	Vehicle	0·76 ± 0·06	0·67 ± 0·07	0·56 ± 0·09	0·85 ± 0·07	0·90 ± 0·06	0·74 ± 0·08
KME-4	3	0·95 ± 0·16	0·82 ± 0·14	0·76 ± 0·12	1·06 ± 0·12	1·04 ± 0·08	0·87 ± 0·08
	10	1·25 ± 0·35	0·84 ± 0·11	0·89 ± 0·15	1·03 ± 0·11	1·06 ± 0·12	0·89 ± 0·09
	30	1·70 ± 0·34*	1·32 ± 0·27*	1·10 ± 0·14**	0·99 ± 0·10	0·98 ± 0·08	0·76 ± 0·09
Indomethacin	2·5	1·02 ± 0·16	1·00 ± 0·14*	0·92 ± 0·14*	1·26 ± 0·16*	1·18 ± 0·17	0·96 ± 0·11
	5	1·30 ± 0·11**	1·20 ± 0·16**	1·16 ± 0·18**	0·95 ± 0·06	0·85 ± 0·07	0·69 ± 0·10
Naproxen	50	1·08 ± 0·09*	1·07 ± 0·11**	0·99 ± 0·14*	0·91 ± 0·09	0·84 ± 0·07	0·78 ± 0·06
	100	1·19 ± 0·13**	1·32 ± 0·12**	1·13 ± 0·14**	1·02 ± 0·07	0·96 ± 0·08	0·83 ± 0·10
Ibuprofen	50	1·12 ± 0·16*	1·09 ± 0·23	0·99 ± 0·21	0·99 ± 0·14	1·17 ± 0·14	0·82 ± 0·08
	100	1·18 ± 0·16*	1·05 ± 0·09**	1·13 ± 0·20*	0·94 ± 0·07	0·82 ± 0·08	0·76 ± 0·08

Each value indicates the mean ± s.e.m. of 12 animals.
* $P < 0.05$ and ** $P < 0.01$: statistically significant difference from the control.
† Hours after drug administration.

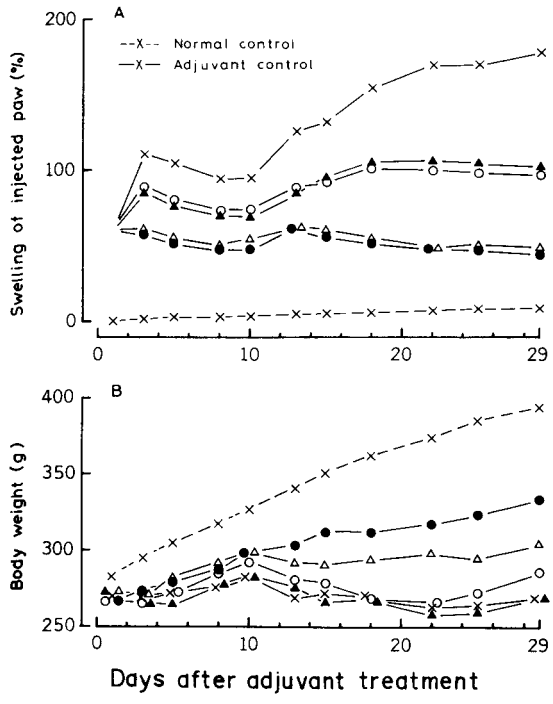

FIG. 2. Effect of KME-4 and standard drugs on swelling of injected hindpaw (A) and body weight (B) in adjuvant arthritic rats. Drugs were administered orally daily on day 1 to day 28 after the adjuvant (day 0). Each point indicates the mean value of 10 animals. (○), KME-4 1·0 mg kg^{-1}; (●), KME-4 10 mg kg^{-1}; (△), Indomethacin 2 mg kg^{-1}; (▲), Ibuprofen 40 mg kg^{-1}.

and ibuprofen were greater than 400, 5·9, 83 and 222 mg kg^{-1}, respectively, indicating KME-4 to have the lowest intestinal toxicity of the drugs tested. A similar result with indomethacin has been reported by Schiantarelli & Cadel (1981). Those rats that underwent autopsy on day 13, all showed intestinal ulcers. The severity of damage was almost dose-dependent, and reflected the degree of body weight loss.

DISCUSSION

As previously reported, KME-4 possesses potent anti-inflammatory and antipyretic activities with a dual inhibition of cyclooxygenase and 5-lipoxygenase (Hidaka et al 1984, 1985). The present report demonstrates the analgesic effect of KME-4 in rat models. In the writhing test, KME-4 had relatively potent analgesic activity as did the standard drugs (indomethacin, naproxen and ibuprofen) used. The relative order of potency among these drugs fairly correlated with their anti-inflammatory activity obtained in the carrageenan paw oedema test (Hidaka et al 1984). This method had been originally reported by Niemegeers et al (1975) who suggested that NSAIDs might exhibit anti-writhing activity by inhibiting prostaglandin biosynthesis. It has been postulated also that the analgesic action of NSAIDs is due to prevention of synthesis of prostaglandins, which sensitize pain receptors at the inflammatory site (Ferreira et al 1972; Moncada et al 1975).

The intraperitoneal injection of acetic acid has been shown to cause not only writhing but also an increase in vascular permeability in mice (Whittle 1964). This was also observed in rats in the present study. KME-4 and standard drugs produced a significant inhibition of vascular permeability (leakage of dye) (Table 2), and the potency of the drugs almost paralleled their anti-writhing activity. Whittle (1964) has reported that non-narcotic analgesics inhibit both leakage of dye and writhing induced by acetic acid in mice, whereas narcotic

Table 4. Effect of KME-4 and standard drugs on organ weight and erythrocyte sedimentation rate (ESR) in adjuvant arthritic rats.

		Organ weight (mg% of body weight)					
					Iliac lymph nodes		
Drug	Dose (mg kg^{-1})	Thymus	Spleen	Adrenals	Treated side	Other side	ESR (mm h^{-1})
Intact control	Vehicle	133·8 ± 5·9	250 ± 20	13·7 ± 0·4	6·1 ± 0·6	4·3 ± 0·3	0·46 ± 0·05
Adjuvant control	Vehicle	68·4 ± 5·5	510 ± 60	33·4 ± 2·7	39·3 ± 3·0	28·1 ± 5·1	9·54 ± 1·55
KME-4	1	87·5 ± 8·5	370 ± 40	24·0 ± 1·3**	33·0 ± 2·9	17·7 ± 3·6	4·14 ± 0·49**
	10	123·8 ± 7·3**	320 ± 30**	17·4 ± 0·6**	23·5 ± 3·4**	13·6 ± 2·1*	1·94 ± 0·32**
Indomethacin	2	112·5 ± 10·4**	300 ± 10**	20·5 ± 0·6**	28·1 ± 3·2*	14·8 ± 2·6*	3·10 ± 0·85**
Ibuprofen	40	83·3 ± 5·4	430 ± 40	28·6 ± 2·0	33·3 ± 7·6	20·8 ± 2·4	5·74 ± 1·15

Drugs were administered orally daily on day 1 to day 28 and the results were obtained on day 29.
Each value indicates the mean ± s.e.m. of 10 animals.
* $P < 0·05$ and ** $P < 0·01$: Statistically significant difference from the adjuvant control.

Table 5. Lethal toxicity and intestinal ulceration in rats by repeated oral treatment with KME-4 and standard drugs.

Drug	Dose (mg kg^{-1})	Mean weight gain(g) (Days 13–1)	Mortality	Incidence[b] of rat with 1–5 ulcers	Incidence[b] of rat with over 6 ulcers	PD50[c] (mg kg^{-1} day^{-1})
Control	Vehicle	46·1	0/30	0/30	0/30	
KME-4	50	35·3	0/10	7/10	3/10	
	200	16·5	1/10	3/9	6/9	>400
	400	12·9	3/10	0/7	7/7	
Indomethacin	4·63	42·0	0/6	6/6	0/6	
	5·56	12·0	1/6	3/5	2/5	5·9
	5·92	6·8	2/6	1/4	3/4	(5·6–6·1)
	6·12	—	6/6	—	—	
Naproxen	55·9	−17·3	0/6	1/6	5/6	
	64·3	−43·1	1/6	0/5	5/5	83
	73·9	−41·8	4/6	0/2	2/2	(62–143)
	85·0	−59·6	2/6	0/4	4/4	
Ibuprofen	172	1·5	0/6	1/6	5/6	
	197	2·2	1/5[a]	3/4	1/4	222
	227	−47·6	5/6	0/1	1/1	(201–216)
	261	−59·5	4/6	0/2	2/2	

Drugs were administered orally once daily for 12 days. Five separate experiments were conducted using 6 to 10 animals and control data were pooled.

[a] One rat was omitted from data because it died accidentally.

[b] Survivors showing intestinal ulcers over 3 mm in length after autopsy on day 13.

[c] Calculated by the probit method and values in parentheses indicate 95% confidence limits.

analgesics only inhibit writhing, not leakage of dye, and corticosteroids are inactive in inhibiting either parameter. This supports KME-4 as a non-narcotic analgesic like known NSAIDs. The Randall–Selitto method is also widely used for assessing the analgesic activity of compounds. In this test, KME-4 was active only in the inflamed foot as were the standard drugs used. This effect distinguishes KME-4 and the standard drugs from narcotic analgesics such as morphine, which have been shown to raise the threshold in both inflamed and normal feet (Winter & Flataker 1965; Roszkowski et al 1971; Capetola et al 1980). However, KME-4 required a higher dose for its activity than that in the writhing or carrageenan oedema test; so, too, did naproxen. Roszkowski et al (1971) have shown that the analgesic doses of non-narcotic NSAIDs in a similar test using yeast were higher than their anti-inflammatory doses in the carrageenan oedema test.

In the adjuvant arthritic flexion test, KME-4 showed weak analgesic activity but the effective dose was higher than the anti-inflammatory dose. This was unexpected because the analgesic potency of known NSAIDs, including indomethacin, in adjuvant-induced pain has been shown to parallel their known anti-inflammatory activity (Kuzuna & Kawai 1975; Winter et al 1979). The difference between analgesic and anti-inflammatory doses for KME-4 cannot be explained at present. However, Capetola et al (1980) reported that indomethacin is inactive in the adjuvant arthritic flexion test at 3 mg kg^{-1} which is greater than its anti-inflammatory dose in adjuvant arthritic rats.

In the present experiments, KME-4 (10 mg kg^{-1} day^{-1}) has a marked effect on various indicators (inflamed paw volume, immunological organ weight, ESR) in adjuvant arthritis, indicating that it has a systemic effect in the model. This property is favourable for an anti-inflammatory drug because adjuvant arthritis, which is a typical chronic inflammatory animal model, is similar to human rheumatoid disease in many clinical and pathological events (Lewis et al 1985). As has been reviewed by Lewis et al (1985), as well as NSAIDs, anti-inflammatory steroids and immunosuppressive agents are also effective in this model which involves an immunological mechanism. However, the immunology of KME-4 has yet to be examined.

It is necessary to clarify the gastrointestinal damage caused by potential NSAIDs because many known NSAIDs cause gastrointestinal side effects (Rainsford 1982). KME-4 causes gastric damage in starved rats after a single dose, its potency being weaker than indomethacin and naproxen but slightly stronger than ibuprofen (Hidaka et al 1984). In the present study, when the drug was given orally once a day for 12 days, KME-4 induced intestinal irritation or death resulting from intestinal perforation in rats, but its toxicity was lower than that of indomethacin, naproxen or ibuprofen. Gastrointestinal damage by NSAIDs has been suggested to be associated with their ability to inhibit prostaglandin biosynthesis (Whittle et al 1980; Whittle 1981a, b) and interestingly, these authors have shown that BW755C, a dual cyclooxygenase and lipoxygenase inhibitor, does not affect or stimulate prostacyclin (PGI2) formation by gastric or intestinal mucosa and has little effect on gastric damage, unlike indomethacin and aspirin. Whittle (1981a) and Boughton-Smith & Whittle (1983) have also commented on the possibility of a relationship between lipoxygenase products, prostaglandin formation and gastrointestinal damage. In this respect, KME-4 is a BW755C-like drug in terms of having a dual inhibition of arachidonic acid metabolism (Hidaka et al 1984, 1985), and it is possible, therefore, that the weak ability of KME-4 to cause gastrointestinal damage may be, in part, related to its ability to influence prostaglandin formation in the gastrointestinal tract. Also, other factors such as pharmacokinetics need to be considered.

In summary, KME-4 possesses analgesic and anti-inflammatory effects with relatively low intestinal damage.

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