Pharmacological Properties of a New Anti-Inflammatory Compound, α-(3,5-Di-Tert-Butyl-4-Hydroxybenzylidene)-γ-Butyrolactone (KME-4), and Its Inhibitory Effects on Prostaglandin Synthetase and 5-Lipoxygenase

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Accepted June 8, 1984

Abstract—The pharmacological effects of a new anti-inflammatory compound, α-(3,5-di-tert-butyl-4-hydroxybenzylidene)-γ-butyrolactone (KME-4), and its inhibitory effects on arachidonate prostaglandin synthetase and 5-lipoxygenase activities were examined. KME-4 showed anti-inflammatory activity. It was less active than indomethacin, but more active than naproxen and ibuprofen in carrageenin-induced paw edema in rats; and it was less active than indomethacin, equipotent as naproxen, but more active than ibuprofen in granuloma formation in rats. The ulcerogenic activity of KME-4 was weaker than indomethacin and naproxen, but stronger than ibuprofen in starved rats. The ratio of UD50 stomach to ED30 carrageenin edema or to ED25 granuloma for KME-4 showed higher values than those of the reference drugs. KME-4 showed antipyretic activity in yeast-induced fever in rats. It also inhibited platelet aggregation induced by arachidonic acid and protected rabbits from arachidonic acid-induced death. Furthermore, KME-4 was found to be equipotent in inhibiting both prostaglandin synthetase and 5-lipoxygenase activities of rat basophilic leukemia cells, unlike indomethacin, naproxen and ibuprofen. It also inhibited the prostaglandin synthetase activity of bovine seminal vesicle. The present findings indicate that KME-4 may be a new type of anti-inflammatory drug with dual prostaglandin synthetase and 5-lipoxygenase inhibition.

During a search for anti-inflammatory compounds, a series of 3,5-di-tert-butyl-styrene derivatives were found to have anti-inflammatory activity and inhibition of platelet aggregation in our laboratories (manuscript in preparation). Of them, α-(3,5-di-tert-butyl-4-hydroxybenzylidene)-γ-butyrolactone (KME-4), with a chemically new structure (Fig. 1), was the most active. We further found that it inhibited not only prostaglandin synthetase but also 5-lipoxygenase, unlike known nonsteroidal anti-inflammatory drugs such as indomethacin, naproxen and ibuprofen.

Fig. 1. Chemical structure of KME-4

In this paper, we describe pharmacological properties of KME-4 and its inhibitory effects on arachidonate prostaglandin synthetase and 5-lipoxygenase.
Materials and Methods

Animals: Male Wistar rats from the Shizuoka Agricultural Cooperative for Laboratory Animals and male Japanese White rabbits from Funabashi Farm were used.

Drugs and reagents: KME-4 and BW755C (3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline) were prepared in our laboratories. Indomethacin was obtained from Sigma; naproxen from Tanabe Seiyaku Co.; ibuprofen from Kaken Pharmaceutical Co.; [1-{14C}] arachidonic acid (55.4 mCi/mmol) from New England Nuclear; [5, 6, 8, 9, 11, 12, 14, 15(n)-3H] leukotriene B4 (LTB4) (144 Ci/mmol) from Radiochemical Centre (Amersham); arachidonic acid from Nu-Chek-Prep; sodium arachidonate from Sigma; Prostaglandin (PG) E₂, F₂α and D₂ from Funakoshi Yakuhin Co.; 5-hydroxy-eicosatetraenoic acid (5-HETE) methyl ester from Paesel GMBH & Co.; prostaglandin synthetase of lyophilized bovine seminal vesicle microsome (BSVM) from Miles Laboratories; and rat basophilic leukemia (RBL-1) cells from American Type Culture Collection. All other reagents were of the highest quality commercially available.

All the test compounds were suspended in 2.5% arabic gum solution for oral administration, and for in vitro assays, they were used after dissolving them in ethanol.

Carrageenin-induced paw edema: The assay was done by the method of Winter et al. (1). Ten rats weighing 150-200 g were used in each group. Test compounds were administered p.o. 1 hr before s.c. injection of 0.1 ml of 1% carrageenin (picrin A®, Zushi Chemical Laboratory) in 0.9% NaCl solution into right hind paw. The foot volume was measured with a plethysmometer 5 hr after carrageenin.

Paper disk-induced granuloma formation: The assay was done by the method of Hara and Tomizawa (2). Six rats weighing 150-200 g were used in each group. A sterilized paper disk (Toyo Roshi, 29±1 mg) was implanted s.c. into bilateral area of axilla under anesthesia with amobarbital sodium. Test compounds were administered p.o. once daily for 7 days, starting on the day of surgery. The animals were killed 7 days after the implantation, and the paper disks with granulomatous tissues were removed, dried at 60°C overnight and weighed.

Gastric damage: The assay was carried out essentially based on the methods of Okabe et al. (3) and Hitchens et al. (4). Six rats weighing 150-200 g were used in each group. The animals were starved for 18 hr, and then test compounds were administered p.o. Four hr later, the animals were killed by decapitation 10 min after the injection of 5% pontamine sky blue solution (0.5 ml/rat) via the tail vein, and their stomachs were removed, fixed with 2% formalin for 10 min and opened along the greater curvature. The mucosal membranes were examined for the lesions under a dissecting microscope. The animals showing at least two lesions (erosions and/or ulcers) that were 0.5 mm and over in length were regarded as positive.

Yeast-induced fever: The assay was done by the method of Yanagi et al. (5). Rats weighing 180-200 g were used. A 20% baker’s yeast (Wako Pure Chemical Co.) suspension in 0.9% NaCl was injected s.c. into the dorsal region of animals in a volume of 1.0 ml/100 g body weight. Eighteen hr later, the animals that had a rectal temperature of 39.2°C and over were selected, divided into groups of six each, and then test compounds were administered p.o. The rectal temperature was measured with a thermometer every hour for 5 hr after the administration of test compounds.

Platelet aggregation: The assay was carried out based on the method of Silver et al. (6). Platelet-rich plasma (PRP) was prepared from citrated rabbit blood (blood: 3.8% sodium citrate=9:1, by volume). PRP was preincubated with or without test compounds (ethanol concentration: 1%) at 37 °C for 1 min, and then sodium arachidonate (final concentration: 100 μg/ml) or ADP (final concentration: 2 μM) was added to the mixture to induce platelet aggregation. Platelet aggregation was monitored as the change in light transmission using an aggregometer.

Arachidonic acid-induced sudden death: Five rabbits weighing 2.5–3.5 kg were used in each group. Test compounds were administered p.o. 0.2 hr before i.v. injection of 1.4 mg/kg of arachidonic acid dissolved in 0.9% saline
containing 7% HCO-60. The animals were observed for death within 5 min after arachidonic acid.

**Assay of prostaglandin synthetase activity of BSVM:** The assay was carried out based on the methods of Flower et al. (7) and Taylor and Salata (8). The reaction mixture (0.5 ml) consisted of 100 mM tris-HCl (pH 8.2), 0.2 mM [1-14C] arachidonic acid (0.2 μCi), 3 mM epinephrine bitartrate, 3 mM reduced glutathione, 2 mg lyophilized BSVM and test compound. The reaction was started by the addition of enzyme, and the mixture was incubated with or without test compounds (ethanol concentration: 4%) at 37°C for 4 min with shaking. The reaction was terminated by the addition of 3N HCl (20 μl) and extracted twice with ethyl acetate (2 ml). After the extracts were evaporated under a N2 gas stream, the residues were redissolved in ethanol, spotted quantitatively on silica gel coated plastic sheets (Eastman Kodak) and developed in solvent system A (ethyl acetate/water/2,2,4-trimethyl-pentane/acetic acid=11/10/5/2 by volume, upper phase). Authentic arachidonic acid and PGE2 were run on the same sheet and were located by iodine vapor. The radioactive bands corresponding to PGE2 were cut out and counted in a liquid scintillation counter. The radioactive PGE2 formed from [1-14C] arachidonic acid was designated as prostaglandin synthetase activity.

**Assays of prostaglandin synthetase and 5-lipoxygenase activities of RBL-1 cells:** The assays were carried out based on the methods of Jakschik and Lee (9) and Steinhoff et al. (10). RBL-1 cells were grown in Eagle's minimum essential medium (Nissui) supplemented with 15% fetal bovine serum (Flow Laboratories) in a CO2 incubator at 37°C for 3 days. The harvested cells were washed twice with buffer A (50 mM phosphate buffer, pH 7.4, 1.0 mM EDTA and 0.1% gelatin). The washed cells were resuspended in buffer A at 5×10^7 cells/ml, sonicated and centrifuged at 10,000×g for 20 min. The supernatant fraction was used as the enzyme source for both assays.

The assay conditions (0.5 ml) for prostaglandin synthetase activity were as follows: The supernatant (0.4 ml) was preincubated with or without test compounds (ethanol concentration: 2%) at 37°C for 1 min. After the addition of [1-14C] arachidonic acid (0.1 μCi, 10 μM), the mixture was incubated at 37°C for 20 min with shaking. For the assay of 5-lipoxygenase, to the above incubation mixture, 2 mM CaCl2 and 20 μM indomethacin were added, and then it was incubated as described in the assay of prostaglandin synthetase activity. Both reactions were terminated by the addition of acetone (1.0 ml) and 0.9% NaCl (0.5 ml). The mixture was acidified with 5N formic acid and extracted twice with chloroform (2 ml). After each extract was evaporated under a N2 gas stream, the residue was redissolved in ethanol, spotted quantitatively on silica gel glass plate (Kieselgel F254, Merck) and developed in solvent A. Radioactive bands were located by radioautography, scraped off and counted in a liquid scintillation counter. The sum of radioactivity of PGD2 and PGF2α formed from [1-14C] arachidonic acid was designated as prostaglandin synthetase activity, and the sum of radioactivity of 5-HETE and 5,12-diHETE formed was designated as 5-lipoxygenase activity. The products were identified by cochromatography with authentic standards on silica gel plates.

**Statistical analysis:** Statistical significance was evaluated by Student’s t-test.

**Results**

**Effect on carrageenin-induced paw edema of rats:** As shown in Fig. 2, KME-4 was found to inhibit carrageenin-induced paw edema at 5 hr in a dose-dependent manner, as well as the other reference compounds. The ED30 values calculated for indomethacin, KME-4, naproxen and ibuprofen were 4, 8, 13 and 42 mg/kg, respectively.

**Effect on paper disk-induced granuloma formation:** As shown in Table 1, KME-4 showed significantly a dose-dependent inhibition of granuloma formation at doses of 2–10 mg/kg/day. The ED25 for KME-4 was 2.2 mg/kg/day, and it was 0.3 times as active as indomethacin, equipotent as naproxen and 6.4 times as active as ibuprofen. KME-4 at the indicated doses did not reduce body weight gain or increase adrenal weights (data
Ulcerogenic activity in rats: KME-4 caused gastric lesions in starved rats. The UD50 value for KME-4 was 43 mg/kg, and it was about 0.2 times as irritating as indomethacin and naproxen and 1.7 times as irritating as ibuprofen (Table 2).

Antipyretic activity in rats: Figure 3 shows the comparison of antipyretic activity of KME-4 and indomethacin. KME-4 showed a significant reduction of the increase in body temperature induced by yeast in rats at doses of 0.5–10 mg/kg. Indomethacin did not significantly reduce it at 0.5 mg/kg, but it was effective at 2–10 mg/kg. In addition, KME-4 did not affect the body temperature of normal rats at doses up to 50 mg/kg (data not shown).

Effect on platelet aggregation: As shown in Fig. 4, KME-4 at the low concentration of 0.2 μM inhibited completely platelet aggregation induced by arachidonic acid. Indomethacin was also effective, but it required the concentration of 6 μM to cause complete inhibition. ADP-induced platelet aggregation was not affected by either KME-4 or indomethacin at concentrations up to 400 μM.

Effect on arachidonic acid-induced death: It was confirmed that the i.v. injection of 1.4 mg/kg of arachidonic acid caused acute death in rabbits as reported by Silver et al. (11). Under this condition, KME-4 was effective in preventing rabbits from arachidonic acid-induced death at doses of 1–10 mg/kg, while indomethacin was more effective at doses of 0.1–0.4 mg/kg (Table 3).

Effect on prostaglandin synthetase and 5-lipoxygenase: KME-4 inhibited prostaglandin synthetase activity of BSVM in a concentration—dependent manner, as well as the other anti-inflammatory compounds (Fig. 5). The IC50 values estimated from dose-

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### Table 1. Effect of KME-4, indomethacin, naproxen and ibuprofen on paper disk-induced granuloma formation in rats

| Exp. No. | Compound     | Dose (mg/kg/day) | No. of rats | Granuloma weight (mg) | Inhibition (%) | ED25 (mg/kg/day) |
|----------|--------------|------------------|-------------|-----------------------|---------------|------------------|
| 1        | Control      | —                | 6           | 56.4±2.8              | 0             | —                |
|          | KME-4        | 2                | 6           | 42.5±4.9*             | 25            | 2.2              |
|          |              | 5                | 6           | 38.9±3.3**            | 31            | —                |
|          |              | 10               | 6           | 33.7±3.0***           | 40            | —                |
|          | Indomethacin | 0.5              | 6           | 45.8±2.5*             | 19            | 42               |
|          |              | 1                | 6           | 36.9±4.2**            | 35            | 0.67             |
|          |              | 2                | 6           | 32.6±1.9***           | 42            | —                |
| 2        | Control      | —                | 6           | 47.0±3.2              | 0             | —                |
|          | KME-4        | 10               | 6           | 27.2±1.4***           | 42            | —                |
|          | Naproxen     | 2                | 6           | 36.3±2.6*             | 23            | 2.5              |
|          |              | 10               | 6           | 28.4±1.9***           | 40            | —                |
|          | Ibuprofen    | 5                | 6           | 42.6±3.1*             | 9             | 16               |
|          |              | 20               | 6           | 33.8±3.4*             | 28            | —                |

*P<0.05, **P<0.01, ***P<0.001: Significant difference from the control.
Table 2. Ulcerogenic activity of KME-4, indomethacin, naproxen and ibuprofen in rat stomachs

| Compound    | Dose (mg/kg) | No. of rats* with ulcer | UDG50 (mg/kg) (95% confidence limit) |
|-------------|--------------|-------------------------|-------------------------------------|
| KME-4       | 5            | 0/6                     |                                     |
|             | 10           | 0/6                     |                                     |
|             | 20           | 3/6                     | 43                                  |
|             | 50           | 2/6                     | (16~97)                             |
|             | 100          | 4/6                     |                                     |
|             | 200          | 6/6                     |                                     |
| Indomethacin| 2            | 0/6                     | 7.9                                 |
|             | 5            | 3/6                     |                                     |
|             | 10           | 5/6                     | (2.1~13.0)                          |
|             | 20           | 5/6                     |                                     |
|             | 30           | 6/6                     |                                     |
| Naproxen    | 2            | 0/6                     | 9.3                                 |
|             | 5            | 2/6                     |                                     |
|             | 10           | 3/6                     | (6.3~12.3)                          |
|             | 15           | 5/6                     |                                     |
|             | 20           | 6/6                     |                                     |
| Ibuprofen   | 10           | 0/6                     | 74                                  |
|             | 20           | 2/6                     |                                     |
|             | 50           | 2/6                     | (43~110)                            |
|             | 100          | 4/6                     |                                     |
|             | 200          | 6/6                     |                                     |

*Showing at least two lesions that were 0.5 mm and over in length. The UDG50 values were calculated by the probit method.

As shown in Fig. 6A and B, KME-4 showed a marked inhibition of both prostaglandin synthetase and 5-lipoxygenase activities of RBL-1 cells at almost equimolar concentration, being as inhibitory as BW755C which is known as a dual inhibitor of cyclooxygenase and lipoxygenase. The IC50 values
Fig. 4. Effect of KME-4 and indomethacin on arachidonic acid and ADP-induced platelet aggregation in rabbit platelet-rich plasma in vitro. Arrows show the addition of samples and inducers, and indicated values show the final concentration. EtOH: ethanol, AA: sodium arachidonate, ADP: adenosine diphosphate.

Fig. 5. Effect of KME-4, BW755C, indomethacin, naproxen and ibuprofen on the prostaglandin synthetase activity of bovine seminal vesicle. The results were expressed as percent inhibition of the control. Each point represents the mean of 2–4 assays or value of one assay.

Table 3. Preventive effect of KME-4 and indomethacin on arachidonic acid-induced acute death in rabbits

| Compound  | Dose (mg/kg) | Mortality |
|-----------|--------------|-----------|
| Control   | –            | 5/5       |
| KME-4     | 1            | 3/5       |
|           | 5            | 1/5       |
|           | 10           | 0/5       |
| Indomethacin | 0.1  | 3/5       |
|           | 0.2          | 1/5       |
|           | 0.4          | 1/5       |
for prostaglandin synthetase and 5-lipoxygenase were 0.74 and 1.3 \( \mu \text{M} \) for KME-4 and 4.5 and 1.1 \( \mu \text{M} \) for BW755C, respectively. On the other hand, indomethacin required a high concentration to inhibit 5-lipoxygenase activity (IC\( 50 \): 220 \( \mu \text{M} \)), although it inhibited strongly prostaglandin synthetase activity (IC\( 50 \) : 0.23 \( \mu \text{M} \)). In addition, neither naproxen nor ibuprofen inhibited 5-lipoxygenase activity by 50% at the high concentration of 300 \( \mu \text{M} \) (Fig. 6B).

**Discussion**

In this study, we have demonstrated that KME-4 possesses anti-inflammatory, antipyretic and anti-platelet aggregatory activities, and it inhibits both prostaglandin synthetase and 5-lipoxygenase. The anti-inflammatory activity of KME-4 was weaker than indomethacin, but stronger than naproxen and ibuprofen in the rat carrageenin edema test. Its activity was weaker than indomethacin, similar to naproxen, but stronger than ibuprofen in the rat granuloma test. As most non-steroidal anti-inflammatory drugs (NSAIDs) cause gastrointestinal damage, it is important to clarify the distinction between an anti-inflammatory dose and an ulcerogenic dose. KME-4 also produced gastric lesions in starved rats, and it was less irritating than indomethacin and naproxen, but more irritating than ibuprofen. On the basis of these results, when the ratio of UD50 stomach to ED30 carrageenin edema or to ED25 granuloma was compared for KME-4 and the other anti-inflammatory drugs, KME-4 showed higher values than those of the reference drugs (Table 4). This suggests that KME-4 shows anti-inflammatory activity.

![Graph](image)

**Table 4. Summary of anti-inflammatory and ulcerogenic activities of KME-4, indomethacin, naproxen and ibuprofen**

| Compound    | Anti-inflammatory activity | Ulcerogenic activity | Ratio |
|-------------|---------------------------|----------------------|-------|
|             | Carrageenin edema         | Granuloma            |       |
|             | ED30 (mg/kg)              | ED25 (mg/kg)         | UD50 (mg/kg) | UD50 |
| KME-4       | 8                         | 2.2                  | 43     | 5.4  | 19.5 |
| Indomethacin| 4                         | 0.67                 | 7.9    | 2.0  | 11.8 |
| Naproxen    | 13                        | 2.5                  | 9.3    | 0.7  | 3.7  |
| Ibuprofen   | 42                        | 16                   | 74     | 1.8  | 4.6  |

ED30 indicates the dose producing 30 percent inhibition of edema. ED25 indicates the dose producing 25 percent inhibition of granuloma formation. UD50 indicates the dose producing gastric lesions in 50 percent of rats. Data were taken from Fig. 2 and Tables 1 and 2.
at a lower dose which is far from the dose producing ulcerogenic activity.

In the antipyretic activity test, KME-4 was effective in reducing fever induced by yeast at relatively low doses (0.5 to 10 mg/kg), but did not affect the body temperature of normal rats at a higher dose (50 mg/kg). This characteristic of KME-4 is considered to be desirable as a drug.

KME-4 was found to inhibit platelet aggregation induced by arachidonic acid and prostaglandin synthetase activity of bovine seminal vesicle. The NSAIDs which are the inhibitors of prostaglandin synthetase and platelet aggregation have been shown to prevent respiratory distress and death induced by i.v. injection of arachidonic acid (11, 12). As for this, we also confirmed that KME-4 had preventive effects on arachidonic acid-induced acute death in rabbits as well as indomethacin. This effect may be mainly due to the inhibition of thromboxane A2 formation from arachidonic acid via cyclooxygenase.

In the present study, the most interesting findings are that KME-4 is almost equipotent in inhibiting both prostaglandin synthetase and 5-lipoxygenase activities, unlike indomethacin, naproxen and ibuprofen which were confirmed to inhibit selectively prostaglandin synthetase. It has been generally accepted that many NSAIDs such as aspirin and indomethacin exhibit their anti-inflammatory effects by inhibiting the biosynthesis of prostaglandin since Vane (13) discovered the inhibition of prostaglandin synthetase by aspirin-like drugs. Furthermore, it has been shown recently that lipoxygenase products such as mono-HETEs and 5,12-diHETE (14, 15) have a potent chemokinetic or chemotactic activity for leukocytes in vitro, and LTB4 (16, 17) causes leukocyte accumulation in vivo. They have suggested that lipoxygenase metabolites have important roles in the inflammatory process as well as prostaglandins. Therefore, the drugs which inhibit two pathways of arachidonic acid metabolism may be expected to have a unique anti-inflammatory activity. In fact, unlike known NSAIDs, dual inhibitors of cyclooxygenase and lipoxygenase, for example, BW755C (17, 18) and timegadine (19, 20), have been shown to have an unusual profile of anti-inflammatory effects which may account for the inhibition of both cyclooxygenase and lipoxygenase. In these respects, the present results indicate that KME-4 is also a dual prostaglandin synthetase and 5-lipoxygenase inhibitor with anti-inflammatory activity, and it is suggested that KME-4 offers more different pharmacological effects than selective prostaglandin synthetase inhibitors. Especially, whether KME-4 shows the inhibitory effect on leukocyte migration should be demonstrated. This, however, is now under investigation.

In addition, slow reacting substance of anaphylaxis (SRS-A) that has recently been identified as leukotrienes C4, D4 and E4, which are also 5-lipoxygenase products, has been recognized as the important mediator in bronchial asthma and anaphylaxis (for review, 21). Therefore, 5-lipoxygenase inhibitors including KME-4 may be effective in the treatment of allergic asthma. The inhibitory effects of KME-4 on SRS-A biosynthesis and allergic reactions also remain to be examined.

Acknowledgments: We thank Dr. H. Fujimura, President of Kyoto Pharmaceutical University for valuable advice and encouragement throughout the present study.

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