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Effect of a potent cyclooxygenase inhibitor, 5-ethyl-4-methoxy-2-phenylquinoline (KTC-5), on human platelets

Chang-Hui Liao, Cho-Hwa Liao, Ya-Ling Chang, Jih-Hwa Guh, Shen-Chu Kuo, Li-Jiau Huang and Che-Ming Teng

Abstract

Because the metabolites of arachidonic acid participate in many physiopathological responses, including inflammation and platelet aggregation, cyclooxygenase inhibitors are important in the treatment of associated diseases. A biologically active compound, 5-ethyl-4-methoxy-2-phenylquinoline (KTC-5), selectively and concentration dependently inhibited aggregation of platelets from man and ATP release caused by arachidonic acid (200 μM) and collagen (10 μg mL^{-1}) without affecting the aggregation caused by thrombin (0.1 U mL^{-1}) and U46619 (2 μM). The IC50 value (drug concentration inhibiting maximum response by 50%) of KTC-5 for aggregation induced by arachidonic acid and collagen was 0.11$\pm$0.04 μM and 0.20$\pm$0.03 μM, respectively. This inhibitory effect of KTC-5 was reversible and time dependent. KTC-5 specifically inhibited intracellular calcium mobilization initiated by arachidonic acid or collagen without affecting that caused by thrombin or U46619 in human platelets. Furthermore, KTC-5 inhibited thromboxane B_2 and prostaglandin D_2 formation provoked by arachidonic acid. The IC50 value of KTC-5 for arachidonic-acid-induced thromboxane B_2 formation was 0.07$\pm$0.02 μM. Based on these observations, the data indicated that KTC-5 potently inhibited human platelet aggregation and ATP release mainly via the inhibition of the cyclooxygenase-1 activity. Moreover, KTC-5 inhibited lipopolysaccharide-induced prostaglandin E_2 formation in RAW264.7 cells in the presence of external arachidonic acid with an IC50 value of 0.17$\pm$0.06 μM. Immunoblot analysis showed that KTC-5 did not affect the cyclooxygenase-2 expression in the presence of lipopolysaccharide on RAW264.7 cells. This result indicated that KTC-5 affects the activity of cyclooxygenase-2. According to these data, we concluded that KTC-5 is a cyclooxygenase inhibitor for both subtypes.

Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan

Chang-Hui Liao

Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan

Cho-Hwa Liao, Ya-Ling Chang, Jih-Hwa Guh, Che-Ming Teng

Graduate Institute of Pharmaceutical Chemistry, China Medical College, Taichung, Taiwan

Shen-Chu Kuo, Li-Jiau Huang

Correspondence: C.-M. Teng, Pharmacological Institute, College of Medicine, National Taiwan University, No 1, Jen-Ai Rd., Sect. 1, Taipei, Taiwan. E-mail: cmteng@ha.mc.ntu.edu.tw

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Introduction

In acute arterial thrombosis, endothelial injury, blood clotting and platelet activation are the primary pathological factors (Wilson & Ferguson 1999). The first step in the response of platelets to vascular injury is irreversible attachment to the altered surfaces followed by platelet secretion and aggregation. Platelet activation mainly takes place by the action of endogenous agonists such as arachidonic acid, platelet-activating factor, ADP, collagen and thrombin, as well as adhesion of platelets to the site of injury (Siess 1989).

Activation of phospholipase A_2 can liberate arachidonic acid from phospholipids. Arachidonic acid is metabolized by cyclooxygenase into prostaglandin and thromboxane A_2, by lipoxygenase in leucocytes into hydroxyeicosatetraenoic acid (HETEs) and by cytochrome P450 into epoxyeicosatrienoic acid (12-HETE) (Muthalif et al 1998). Some metabolites of the arachidonic acid cascade are important secondary messengers in cells (Khan et al 1995) and in platelets (Nozawa et al 1991; Khan et al 1992). These metabolites contribute to many physiological and pathophysiological responses, such as chemotaxis (Siegel et al 1982), inflammation (Irvine 1982) and platelet aggregation. Two isozymes of cyclooxygenase have been described (Fletcher et al 1992; Meade et al 1993), and both catalyse the cyclooxygenase-dependent

transformation of prostaglandin G_2 from arachidonic acid (Ohki et al 1979). Cyclooxygenase-1 exists constitutively in most tissues, while cyclooxygenase-2 is usually induced in the early gene by either mitogenic or inflammatory stimuli, as well as by ligands. Cyclooxygenase-1 is the major enzyme in the synthesis of thromboxanes from arachidonic acid in platelets (Smith & Marnett 1991). The inhibition of thromboxane synthesis by the blocking of cyclooxygenase-1 with acetylsalicylic acid (aspirin) leads to a decreased aggregability of platelets. Aspirin is a very effective inhibitor of platelet-mediated thrombosis at sites of vascular injury (Goodnight 1996). Therefore, aspirin is used in the prevention of secondary myocardial infarction and unstable angina (Goldstein et al 1996). Moreover, the anti-inflammatory effect of aspirin is mediated via inhibition of the cyclooxygenase-2 pathway in cells.

We have studied and developed anti-platelet drugs for many years. The major sources of compounds were obtained from components of plants (Teng et al 1994) and synthetic compounds (Liao et al 1998). 5-Ethyl-4-methoxy-2-phenylquinoline (KTC-5) (Ko et al 2001), a synthetic compound, has a potent anti-platelet effect. The purpose of this study is to address the mechanism of its inhibitory activity on platelet aggregation.

Materials and Methods

Materials

KTC-5 (Figure 1) was prepared as described previously (Ko et al 2001) and was dissolved in dimethyl sulfoxide (DMSO). Collagen (type I, bovine Achilles tendon) was homogenized in 25 mM acetic acid and stored at $-20^{\circ}C$ at a concentration of 1 mg mL^{-1}. Arachidonic acid, ethylenediaminetetra-acetic acid (EDTA; disodium salt), luciferin-luciferase, DMSO, lipopolysaccharide (LPS), bovine serum albumin (BSA), U46619 and fluo-3-acetoxymethyl ester (fluo-3-AM) were purchased from Sigma (St Louis, MO). Thrombin (bovine) was obtained from Parke Davis (Detroit, MI) and dissolved in 50% glycerol to furnish a stock solution of 100 NIH units mL^{-1}. Thromboxane B_2, prostaglandin D_2 and prostaglandin E_2 kits were obtained from Amersham, UK. Dulbecco's modified Eagle medium (DMEM) was from GIBCO/RBI Life Technologies, Grand Island, NY. Fetal bovine serum (FBS) was purchased from Life Technologies, Inc., Madison, MI. Antibody for cyclooxygenase-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish-peroxidase-coupled antibody and the enhanced chemiluminescence detection agent were purchased from Amersham Pharmacia Biotech (Piscatway, NJ).

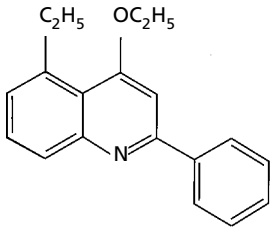

Figure 1 Chemical structure of KTC-5.

Preparation of washed platelets

The platelet suspension was prepared according to the washing procedure (Wu et al 1994). Blood was drawn from healthy, drug-free subjects. Approximately 60 mL blood was mixed directly with anticoagulant solution (ACD; 25 g trisodium citrate dihydrate, 14 g citric acid and 20 g glucose per litre) in a ratio of 9:1. Blood was centrifuged at 190 *g* for 15 min to obtain platelet-rich plasma, from which platelets were obtained by centrifugation at 1200 *g* for 15 min. The platelet pellet was gently resuspended in 10 mL of Tyrode's buffer supplemented with 2 U mL^{-1} apyrase and 50 U mL^{-1} heparin. After incubating at $37^{\circ}C$ for 15 min, the cell suspension was centrifuged at 1200 *g* for 10 min. The platelets were resuspended gently in 10 mL of Tyrode's buffer. Platelets were then pelleted at 1200 *g* for 10 min and resuspended at room temperature in Tyrode's buffer with 0.3 U mL^{-1} apyrase only. The platelet suspension was counted in an automated cell counter (Hemalaser 2; Sebia, Moulineaux, France) and suspended at a concentration of 3×10^8 mL^{-1}. All glassware was siliconized.

Platelet aggregation and ATP release

Platelet aggregation was measured by the turbidimetric method (O'Brien 1962). The absorbance of the platelet suspension was taken as 0% aggregation and that of Tyrode's solution as 100% aggregation. ATP released from platelets was measured by the bioluminescence method (DeLuca & McElory 1978). Both aggregation and ATP release were measured by a Lumi-aggregometer (Model 1020; Payton, Scarborough, Canada) connected to two dual-channel recorders. For evaluation of the reversible effect of KTC-5, KTC-5 (0.5 μM) was incubated with human platelet suspension at $37^{\circ}C$ for 3 min, then the cell suspension was centrifuged at 1200 *g* for 10 min and suspended in Tyrode's buffer.

Intracellular calcium measurement

Washed platelets in modified Tyrode Hepes buffer containing 20 ng mL^{-1} prostaglandin E_1 (PGE_1) were incubated with 3 μmol L^{-1} fluo-3-AM at $37^{\circ}C$ for 30 min, washed twice with Tyrode Hepes buffer containing 20 ng mL^{-1} PGE_1 and resuspended in modified Tyrode Hepes buffer at a concentration of 5×10^7 cells mL^{-1}. Probenecid (2.5 mM) was added to prevent leakage of the dye from platelets throughout the whole experiment. The fluo-3-AM-loaded platelets were stimulated with arachidonic acid (200 μM), collagen (10 μg mL^{-1}), thrombin (0.1 U mL^{-1}) and U46619 (2 μM), and the cytosolic free-calcium concentration was determined on a fluorescence spectrofluorometer (Model F4000; Hitachi, Tokyo, Japan) at wavelengths of 505 nm

and 530 nm (excitation and emission, respectively). The suspension was gently stirred during the measurement.

Thromboxane B_2 and prostaglandin D_2 assay

Six minutes after the challenge of platelets with the aggregation inducers, 2 mM of EDTA and 50 μM of indometacin were added to stop thromboxane and prostaglandin D_2 formation. After centrifugation in an Eppendorf microcentrifuge (model 5414, Hamburg, Germany) for 2 min, the supernatant was collected. The thromboxane B_2 and prostaglandin D_2 were assayed by enzyme immunoassay (EIA) kits.

Cell line and cell culture

RAW264.7 cells (mouse peritoneal macrophage cell line) were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM supplement with 5% FBS, 2 mM glutamine and 1000 U mL^{-1} penicillin–streptomycin.

Prostaglandin E_2 determination

The production of prostaglandin E_2, one of the mediators released after activation of cyclooxygenase, was used as a marker for cyclooxygenase-2 activity. The induction assay was slightly modified according to the method of Salvemini et al (1993). Briefly, RAW264.7 cells were pre-treated with lipopolysaccharide (1 μg mL^{-1}) for 18 h and then equilibrated in fresh Tyrode buffer (composition (mM): 137 NaCl, 2.7 KCl, 2.1 $MgCl_2$, 0.4 NaH_2PO_4, 12 $NaHCO_3$, 2 EDTA). The cells were thereafter incubated in the absence or presence of KTC-5 for another 30 min, and 30 μM arachidonic acid was added for 15 min. All incubations were terminated by adding 4 mM EDTA and 30 μM indometacin. The supernatants were assayed for prostaglandin E_2 and determined by EIA system. All determinations were performed in duplicate.

Immunoblot analysis of cyclooxygenase-2

RAW264.7 cells were co-incubated with KTC-5 and lipopolysaccharide for 18 h. After lipopolysaccharide treatment, RAW264.7 cells were washed twice in ice-cold phosphate-buffered saline (PBS), solubilized in buffer A (composition (mM): 20 Tris-HCl, 0.5 EGTA, 2 EDTA, 2 DDT, 0.5 *p*-methylsulphonyl fluoride and 10 mg mL^{-1} leupeptin, pH 7.5) and then sonicated. Samples of equal amount of protein (50–100 μg) were subjected to 9% SDS-PAGE under reducing conditions, and the separated proteins were transferred onto a nitrocellulose membrane, which was then incubated in NaCl 150 mM, Tris 20 mM and Tween 0.02%, pH 7.4, containing 5% milk, before being probed with antibody specific for cyclooxygenase-2. After washing, the blots were probed with horseradish-peroxidase-conjugated immunoglobulin G (IgG) and immunoreactivity was detected by enhanced chemiluminescence (ECL), following the manufacturer's instructions. Densitometrical analyses were performed on a Molecular Dynamic densitometer.

Statistical analysis

Results are expressed as the means$\pm$s.e.m. for the indicated number of separate experiments. Statistical significance between drug-treated and untreated groups were evaluated by Student's *t*-test and *P* values less than 0.05 were considered significant.

Results

Effect of KTC-5 on platelet aggregation and ATP release

Thrombin (0.1 U mL^{-1}), U46619 (2 μM), collagen (10 μg mL^{-1}) and arachidonic acid (200 μM) all caused about 90% aggregation in washed platelets from man. KTC-5 specifically inhibited arachidonic-acid- and collagen-induced aggregation of the washed platelets in a concentration-dependent manner, with IC50 values of 0.11 ± 0.04 and 0.20 ± 0.03 μM, respectively (Figure 2). In addition to the inhibition of platelet aggregation, KTC-5 also concentration-dependently inhibited arachidonic-acid- (Figure 3) and collagen-induced ATP release (data not shown). However, when the concentration of KTC-5 was increased to 100 μM, KTC-5 did not affect platelet aggregation and ATP release caused by thrombin (Figure 2) and had only a minor effect on those caused by U46619 (Figure 2). KTC-5 (0.1 μM) inhibited arachidonic-acid-induced platelet aggregation with a time-dependent pattern. The inhibitory values of KTC-5 (0.1 μM) on arachidonic-acid-induced aggregation were 2.9 ± 1.0% (3 min), 26.1 ± 1.7% (10 min), 55.1 ± 1.2% (20 min) and 100% (30 min), respectively (n = 6). KTC-5 (0.5 μM)

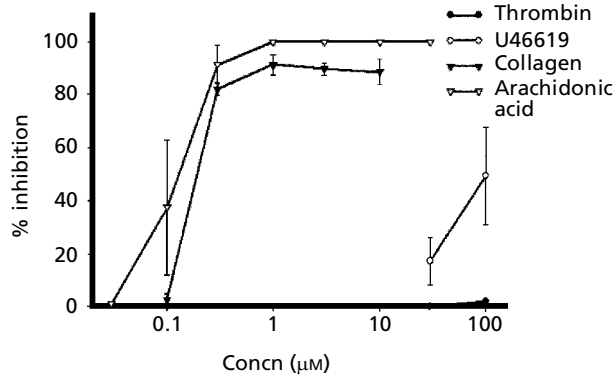

Figure 2 Concentration-dependent inhibition by KTC-5 on washed platelets (from man) induced by thrombin (0.1 U mL^{-1}), U46619 (2 μM), collagen (10 μg mL^{-1}) and arachidonic acid (200 μM). Washed platelets from man were incubated with DMSO (0.5%) or various concentrations of KTC-5 at 37°C for 3 min, and then the inducer was added to trigger the aggregation. Values are presented as means$\pm$s.e.m. (n = 6).

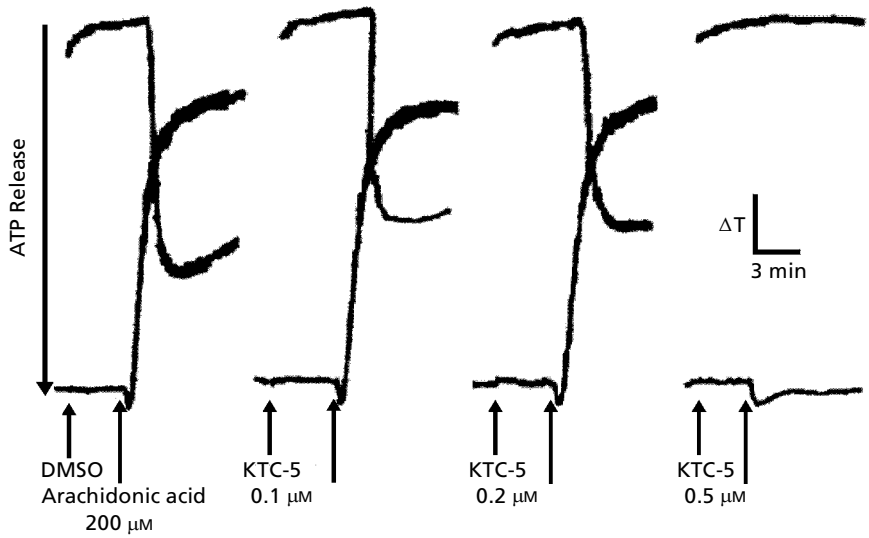

Figure 3 Typical pattern of KTC-5 inhibition on aggregation of washed platelets from man and ATP release induced by arachidonic acid (200 μM). Washed platelets were pre-incubated with various concentrations of KTC-5 or DMSO (0.5%) at 37°C for 3 min, and then aradchidonic acid (200 μM) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

completely inhibited arachidonic-acid-induced platelet aggregation and ATP release (Figures 2 and 3); after washing out KTC-5, arachidonic acid induced full platelet aggregation and ATP release (data not shown).

Effect of KTC-5 on thromboxane B_2 and prostaglandin D_2

In the unstimulated condition, thromboxane B_2 formation in human platelet suspension was very low (0.6 ± 0.1 ng mL^{-1}). Table 1 shows the thromboxane B_2 formation in platelets 6 min after challenge with arachidonic acid (200 μM), collagen (10 μg mL^{-1}) and thrombin (0.1 U mL^{-1}). Thromboxane B_2 was increased to 1297.3 ± 172.4, 44.1 ± 7.9 and 10.0 ± 2.5 ng mL^{-1}, respectively. However, U46619 failed to raise the thromboxane B_2 level significantly (data not shown). KTC-5 inhibited thromboxane B_2 formation caused by arachidonic acid and collagen in a concentration-dependent manner. The IC50 value of KTC-5 for arachidonic-acid-induced thromboxane B_2 formation was 0.07 ± 0.02 μM. The formation of thromboxane B_2 from thrombin stimulation was abrogated

Table 1 Effect of KTC-5 on arachidonic-acid-, collagen- and thrombin-induced thromboxane B_2 formation in washed platelets from man.

		Thromboxane B_2 formation (ng mL^{-1})		
		Arachidonic acid	Collagen	Thrombin
Control		1297.3 ± 172.4	44.1 ± 7.9	10.0 ± 2.5
Indometacin	1 μM	10.6 ± 3.4***	–	–
Imidazole	1 mM	17.9 ± 0.3***	–	–
KTC-5	0.02 μM	945.8 ± 67.3	–	–
	0.05 μM	620.0 ± 67.5*	–	–
	0.1 μM	319.0 ± 31.9***	24.0 ± 1.1	–
	0.2 μM	128.8 ± 23.2***	–	–
	0.5 μM	22.6 ± 0.6***	–	–
	1 μM	–	1.0 ± 0.2***	–
	100 μM			1.90 ± 0.05*

The thromboxane B_2 level of resting platelets was 0.6 ± 0.1 ng mL^{-1}. Values are presented as mean$\pm$s.e.m. (n = 3 or 4). *$P<0.05$, ***$P<0.001$ compared with the respective control. Final concentration: arachidonic acid, 200 μM; collagen, 10 μg mL^{-1}; thrombin, 0.1 U mL^{-1}. KTC-5 was incubated with platelets 3 min before various inducers were added.

by a high concentration of KTC-5 (100 μM). Both imidazole (1 mM), a thromboxane synthase inhibitor, and indometacin (1 μM) inhibited arachidonic-acid-induced thromboxane B_2 formation (Table 1). The prostaglandin D_2 level in resting platelets was low (0.28 ± 0.05 ng mL^{-1}), but was increased to 114.2 ± 40.8 ng mL^{-1} in the presence of arachidonic acid. This action was inhibited by indometacin (1 μM; 10.4 ± 4.6 ng mL^{-1}) but was enhanced dramatically by imidazole (1 mM; 10207.2 ± 7102.2 ng mL^{-1}). KTC-5 (0.5 μM) inhibited the prostaglandin D_2 induced by arachidonic acid.

Effect of KTC-5 on intracellular calcium

Collagen (10 μg mL^{-1}), arachidonic acid (200 μM), thrombin (0.1 U mL^{-1}) and U46619 (2 μM) all caused a significant increase in intracellular calcium concentration from a resting level of 15.5 ± 4.5 nM to 93.8 ± 10.4, 281.7 ± 34.3, 377.0 ± 46.7 and 302.2 ± 38.8 nM, respectively (Table 2). KTC-5 inhibited collagen- and arachidonic-acid-induced calcium mobil- ization without affecting that caused by thrombin and U46619 (Table 2). The increment in fluorescence signal was only short-lived and decreased towards the resting level within a few minutes due to interference with the fluorescence signal by aggregation (data not shown), which is a limitation of the technique.

Effect of KTC-5 on prostaglandin E_2 formation in RAW264.7 cells

The basal prostaglandin E_2 level in RAW264.7 cells was 4.05 ± 1.14 ng mL^{-1} (Table 3). Lipopolysaccharide (1 μg mL^{-1}) dramatically increased the prostaglandin E_2 level to 21.74 ± 2.78 ng mL^{-1} and this action was inhibited by indometacin (1 μM) to the basal level and by KTC-5 (2 μM) in a concentration-dependent manner. The IC50 value of KTC-5 for lipopolysaccharide-induced prostaglandin E_2 formation was 0.17 ± 0.06 μM.

Table 3 Effect of KTC-5 on lipopolysaccharide-induced prostaglandin E_2 formation in RAW264.7 cells.

		Prostaglandin E_2 formation (ng mL^{-1})
Basal		4.05 ± 1.14
Control		21.74 ± 2.78
Indometacin	1 μM	3.90 ± 0.82***
	10 μM	3.36 ± 1.15***
KTC-5	0.01 μM	15.84 ± 4.41
	0.02 μM	15.43 ± 3.56
	0.05 μM	14.33 ± 3.02
	0.1 μM	8.98 ± 0.92***
	0.2 μM	7.06 ± 1.18***
	0.5 μM	5.83 ± 0.96***
	1 μM	5.91 ± 0.63***
	2 μM	4.06 ± 0.8***

The prostaglandin E_2 level of resting RAW264.7 cells was 0.72 ± 0.17 ng mL^{-1}. Values are presented as mean$\pm$s.e.m. (n = 5). ***$P < 0.001$ compared with the control (1 μg mL^{-1}). KTC-5 was incubated with platelets 30 min before lipopolysaccharide was added.

Effect of KTC-5 on cyclooxygense-2 expression in RAW264.7 cells

RAW264.7 cells expressed slight cyclooxygenase-2 protein in the absence of stimulants (Figure 4). Cyclooxygenase-2 expression significantly increased after treatment with lipopolysaccharide (1 μg mL^{-1}) for 18 h (3.9 ± 0.8 fold). KTC-5 (5 μM) caused only a minor increase in cyclooxygenase-2 expression. It did not, however, affect lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 cells (3.4 ± 0.2 fold compared with resting level).

Discussion

The purpose of this study was to elucidate details of the mechanisms involved in the anti-platelet action of KTC-5. KTC-5 specifically and significantly inhibited platelet ag-

Table 2 Effect of KTC-5 on collagen-, arachidonic-acid-, thrombin- and U46619-induced intracellular calcium increase in washed platelets from man.

		Increased intracellular calcium concn (nM)			
		Collagen	Arachidonic acid	Thrombin	U46619
Control		93.8 ± 10.4	281.7 ± 34.3	377.0 ± 46.7	302.2 ± 38.8
KTC-5	0.5 μM	–	243.8 ± 56.8	–	–
	5 μM	34.8 ± 2.3***	102.1 ± 16.6***	–	–
	50μM	–	–	307.7 ± 50.2	332.6 ± 46.9

Values are expressed as mean$\pm$s.e.m. (n = 3–8). ***$P < 0.001$ compared with control. Final concentration: collagen, 10 μg mL^{-1}; arachidonic acid, 200 μM; thrombin 0.1 U mL^{-1}; U46619, 2 μM. KTC-5 was incubated with platelets 3 min before various inducers were added. The resting level of calcium was 15.5 ± 4.5 nM. KTC-5 did not alter the basal level of calcium.

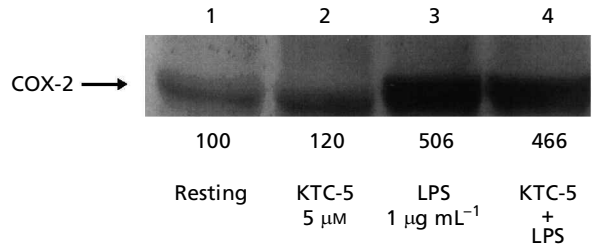

Figure 4 Typical pattern of KTC-5 effect on cyclooxygenase-2 protein expression on RAW264.7 cells. Cells were treated with various agents as indicated for 18 h, then cyclooxygenase-2 protein levels were determined. The data below each trace indicate the percentage of immunoreactivity as compared with the resting level (100). The results are representative of three experiments. Lane 1, DMSO only; lane 2, KTC-5 (5 μM); lane 3, lipopolysaccharide (LPS, 1 μg mL^{-1}); lane 4, KTC-5 (5 μM)+LPS (1 μg mL^{-1}).

gregation and ATP release stimulated by collagen and arachidonic acid without affecting those induced by thrombin and U46619. These results indicate that KTC-5 acts not at the receptor level of individual agonists but at a common step shared by collagen and arachidonic acid.

Exogenously added arachidonic acid was converted by cyclooxygenase to the prostaglandin endoperoxides, prostaglandin G_2 and H_2, which in turn were converted by thromboxane synthase to thromboxane A_2, a potent aggregating agent, which then caused platelet aggregation (Parise et al 1984). We found that KTC-5 (0.5 μM) completely inhibited arachidonic acid-induced platelet aggregation and ATP release. However, at the concentration of 100 μM, KTC-5 slightly inhibited U46619-induced platelet aggregation and ATP release without affecting that caused by thrombin. These data indicate that the anti-aggregatory effect of KTC-5 has high selectivity to arachidonic acid and reveals a similar effect to indometacin. Therefore, we evaluated the effect of KTC-5 on thromboxane B_2 and prostaglandin D_2 formation induced by arachidonic acid. KTC-5 significantly inhibited thromboxane B_2 and prostaglandin D_2 formation increased by arachidonic acid. However, KTC-5 did not increase prostaglandin D_2 formation in the presence of arachidonic acid. These results indicate that inhibition of thromboxane B_2 formation in human platelets by KTC-5 may be through a direct action on cyclooxygenase activity in platelets instead of it affecting the activity of thromboxane synthase. For this reason, the effect of KTC-5 on arachidonic acid-induced thromboxane B_2 formation was an index for its effect on cyclooxygenase. Furthermore, the concentrations of KTC-5 affecting arachidonic-acid-induced thromboxane B_2 formation were paralleled by its anti-aggregation effect. This result indicates that KTC-5 may inhibit arachidonic-acid-induced platelet aggregation by dampening the cyclooxygenase activity. In platelets from man, collagen-induced aggregation and ATP release were suppressed by a cyclooxygenase inhibitor, indicating that prostaglandin endoperoxides or thromboxane A_2 play a crucial role in collagen-induced activation of platelets (Shiraishi et al 2000). Thus, KTC-5 or a cyclooxygenase inhibitor may inhibit collagen-induced platelet aggregation, ATP release and thromboxane B_2 formation. According to our results, KTC-5 (0.1–10 μM) inhibited those responses by collagen. Obviously, KTC-5 (0.1 μM) only partly inhibited thromboxane B_2 formation caused by collagen; therefore, KTC-5 did not inhibit collagen-induced platelet aggregation. Thromboxane A_2 was also formed after thrombin stimulation in human platelets. However, the platelet aggregation induced by thrombin was not mediated by thromboxane A_2 synthesis, while platelet aggregation induced by U46619, a PGH_2/thromboxane A_2-receptor agonist, was not through the cyclooxygenase pathway (Teng et al 1987). Thus, platelet aggregation and ATP release induced by thrombin and U46619 were not affected by indometacin. Indometacin (1 μM) completely inhibited platelet aggregation caused by arachidonic acid and partly inhibited that caused by collagen, but had no influence on platelet aggregation caused by thrombin and U46619 (data not shown). KTC-5 had a similar effect to indometacin on human platelets in this study.

Calcium plays a pivotal role in several platelet functions. Membrane-bound calcium is essential to the integrity of the fibrinogen receptor and, hence, platelet aggregation. The changes in cytosolic calcium concentrations are also important for the shape change and secretion responses of platelets. According to this study, KTC-5 specifically inhibited arachidonic-acid- and collagen-induced intracellular calcium increment without affecting that induced by thrombin and U46619 (Table 3). This specific effect supports the experimental results that KTC-5 inhibited the platelet aggregation, ATP release and cyclooxygenase activity induced by arachidonic acid and collagen in platelets from man.

Cyclooxygenase is the first rate-limiting enzyme in the synthesis of prostacyclin, prostaglandins and thromboxanes from arachidonic acid. Two isozymes of cyclooxygenase have been described. Both catalyse the cyclooxygenase-dependent transformation of prostaglandin G_2 from arachidonic acid and the subsequent peroxidation of prostaglandin G_2 to prostaglandin H_2. While cyclooxygenase-1 is expressed constitutively in most tissues, cyclooxygenase-2 is usually induced as an early gene by mitogenic or inflammatory stimuli as well as by ligands that act via G-protein- and protein-kinase-C-mediated pathways (Herschman 1994). Several studies have shown that regularly taking aspirin or other conventional non-steroidal anti-inflammatory drugs (NSAIDs) provides a 40–50% reduction in relative risk of death by colon cancer. This indicates that inhibition of cyclooxygenase in man has a chemopreventive effect (Dubois et al 1998; Masferrer et al 2000). These studies also showed that celecoxib, a selective cyclooxygenase-2 inhibitor, demonstrates potent anti-angiogenic and antitumour activity in-vivo and suggested the potential use of this anti-inflammatory drug in the treatment of cancer in man. In our study, we proved that KTC-5 inhibited cyclooxygenase-1 by the measurement of thromboxane B_2 levels in human platelets. Therefore, we also evaluated the effect of KTC-5 on the activity of cyclooxygenase-2. Cyclooxygenase-2 was

activated by long-term treatment of RAW264.7 cells with lipopolysaccharide, and the activity was evaluated by prostaglandin E_2 formation in the presence of external arachidonic acid. According to our data, KTC-5 concentration-dependently inhibited lipopolysaccharide-induced prostaglandin E_2 formation in the presence of external arachidonic acid in RAW264.7 cells. To determine whether KTC-5 inhibited the cyclooxygenase-2 protein expression, KTC-5 was co-incubated with lipopolysaccharide in RAW264.7 cells for 18 h. Our result showed that KTC-5 did not affect the cyclooxygenase-2 protein expression by lipopolysaccharide stimulation. This data indicate that KTC-5 inhibited the activity of cyclooxygenase-2 and its potency was compared with that of indometacin. More experiments are needed to prove whether or not KTC-5 can be used as an antitumour agent.

According to our studies, the effect of KTC-5 on arachidonic-acid-induced platelet aggregation was reversed by a washing procedure (data not shown). This phenomenon is different from that seen with aspirin which has an irreversible effect on cyclooxygenase. This may predict that KTC-5 has less bleeding risk.

Conclusion

Our findings suggest that KTC-5 inhibits human platelet aggregation, probably via initial inhibition of cyclooxygenase-1 activity, which leads to inhibition of thromboxane A_2 formation and calcium mobilization. Our studies also indicate that KTC-5 inhibits the activity of cyclooxygenase-2. These findings suggest that KTC-5 may be not only an effective tool in treating thromboembolic disorders, but also a drug for treating inflammatory diseases or tumours.

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