Inhibitory Effects of the New Anti-platelet Agent KBT-3022 and Its Metabolite on Rabbit Neutrophil Function In Vitro

Koichi Yokota[1], Noriko Yamamoto[1], Yuji Obata[1] and Minoru Oda[2]

[1]*New Drug Research Laboratories, Kanebo, Ltd., 5-90, Tomobuchi-cho 1-chome, Miyakojima-ku, Osaka 534, Japan*
[2]*Research Laboratories, Torii Pharmaceutical Co., Ltd., 2-1, Ohnodai 1-chome, Midori-ku, Chiba 267, Japan*

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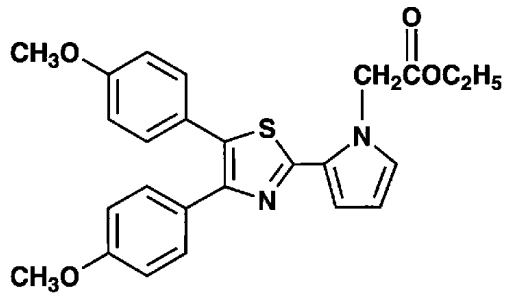
ABSTRACT—The effects of the new anti-platelet agent KBT-3022, ethyl 2-[4,5-bis(4-methoxyphenyl)-thiazol-2-yl]pyrrol-1-ylacetate, and its metabolite desethyl KBT-3022 on rabbit neutrophil function were investigated in comparison with the effects of acetylsalicylic acid (ASA), ticlopidine hydrochloride (TP), cilostazol (CIL) and indomethacin (IM). The adhesion and migration of neutrophils induced by formyl-methionyl-leucyl-phenylalanine (fMLP) were inhibited by all the compounds tested, their rank order of potency being KBT-3022 = desethyl KBT-3022 > TP = CIL = IM > ASA. KBT-3022, desethyl KBT-3022, CIL and IM all suppressed fMLP-induced increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in neutrophils, their potencies correlating with their inhibitory effects on fMLP-induced adhesion and migration. KBT-3022 (1 μM), desethyl KBT-3022 (1-10 μM) and CIL (10 μM) but not IM significantly inhibited both neutrophil migration and the increase in $[Ca^{2+}]_i$ induced by leukotriene B_4 (LTB_4). KBT-3022 (1 μM) and desethyl KBT-3022 (1 μM) suppressed the increase in $[Ca^{2+}]_i$ induced by complement C5a. Although KBT-3022 and desethyl KBT-3022 did not influence [^{3}H]LTB_4 and [^{125}I]C5a specific binding, [^{3}H]fMLP specific binding was inhibited by desethyl KBT-3022 (IC_{50}: 1.9 μM). Neutrophil adhesion and superoxide anion production stimulated by phorbol 12-myristate 13-acetate were partially inhibited by KBT-3022 (1 μM) and desethyl KBT-3022 (1-10 μM). These results suggest that KBT-3022 and desethyl KBT-3022 have a wider spectrum of action and are more potent inhibitors of neutrophil activation than ASA, TP, CIL and IM.

Keywords: KBT-3022, Anti-platelet drug, Rabbit neutrophil, Formyl-methionyl-leucyl-phenylalanine (fMLP), Leukotriene B_4

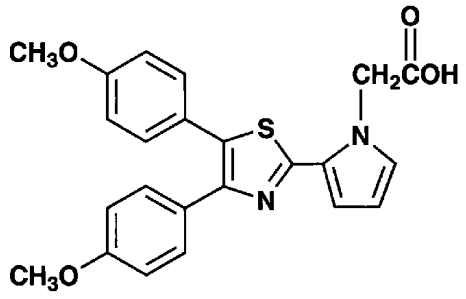
The neutrophil plays an important role not only in host defense but also in aggravation of tissue injury during ischemia and after reperfusion (1, 2). Experimental studies have demonstrated that i) neutrophils accumulate in areas of ischemia (3), with neutrophil plugging of the capillary lumen resulting in a progressive decrease in blood flow (the "no reflow" phenomenon) after reperfusion (4); and ii) activated neutrophils injure the parenchymal cells and micro-vasculature by the production of proteolytic enzymes (5), reactive oxygen species (6) and arachidonic acid metabolites (7). Therefore, in these pathological conditions, suppression of neutrophil activation may be of therapeutic benefit.

Neutrophils can be activated by receptor-mediated and non-receptor-mediated pathways. The chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), a synthetic oligopeptide resembling chemotactic factors produced by bacteria (8), leukotriene B_4 (LTB_4) (9, 10), C5a (11), platelet-activating factor (PAF) (12, 13) and interleukin-8 (IL-8) (14) are known to cause receptor-mediated activation, while phorbol esters directly activate protein kinase C (PKC) and do not require specific receptors for neutrophil activation (15). Thus, it is possible that compounds capable of inhibiting neutrophil activation by more than one mechanism may have greater therapeutic potential, since neutrophils can be activated by a variety of physiological stimuli.

KBT-3022, ethyl 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate (Fig. 1), a new anti-platelet agent, has been shown to inhibit cyclooxygenase and 5-lipoxygenase (16), platelet function (17), and thrombus formation (18). Furthermore, oral administration of KBT-3022 increases blood filterability and erythrocyte deformability ex vivo (19), and it attenuates brain edema when ischemia is followed by reperfusion (20). KBT-3022 is readily hydrolyzed to desethyl KBT-3022 (Fig. 1) and is



KBT-3022



Desethyl KBT-3022

Fig. 1. Chemical structures of KBT-3022 and its metabolite desethyl KBT-3022.

not detectable in plasma after oral administration to animals (21).

In the present paper, using peripheral blood neutrophils from rabbits, the effects of KBT-3022 and desethyl KBT-3022 on adhesion, migration and the production of superoxide anion (O_2^-) induced by stimulation with fMLP, LTB_4, C5a or phorbol 12-myristate 13-acetate (PMA) have been investigated in order to evaluate their inhibitory profiles on neutrophil function in vitro in comparison with those of the other antiplatelet agents, acetylsalicylic acid (ASA), ticlopidine hydrochloride (TP), cilostazol (CIL) and indomethacin (IM). Furthermore, the effects of KBT-3022 and desethyl KBT-3022 on changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) in neutrophils stimulated by fMLP, LTB_4, C5a and PAF and their specific binding have also been investigated.

MATERIALS AND METHODS

Animals

Male Japanese albino rabbits weighing 2.5–3.5 kg, from Keari(Osaka), were used.

Chemicals

KBT-3022 (ethyl 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate) (22) and desethyl KBT-3022 were synthesized, and TP and CIL were extracted and purified from Panaldine® tablets (Daiichi Pharmaceutical, Tokyo) and Pletaal® tablets (Otsuka Pharmaceutical, Tokyo), respectively, at Kanebo. ASA was purchased from Wako Pure Chemical Industries (Osaka) and IM, from Sigma Chemical Co. (St. Louis, MO, USA). These drugs were dissolved in dimethyl sulfoxide (DMSO) before addition to the cell suspensions. The final concentration of DMSO was 0.1% or less in all the experiments. FMLP, PMA, C5a (human recombinant), tosyl-phenylalanine chloromethyl ketone (TPCK), cytochrome *c* and bovine serum albumin (BSA) were purchased from Sigma; PAF (C18), from Bachem (Bubendorf, Switzerland); dextran (M.W., 208,000), from Nacalai Tesque (Tokyo); Ficoll-Paque®, from Pharmacia LKB Biotechnology (Oslo, Sweden); LTB_4, from Cayman Chemical (Ann Arbor, MI, USA); RPMI 1640, from Nissui Pharmaceutical (Tokyo); fetal calf serum (FCS), from HyClone (Logan, UT, USA); fura 2-AM and fura 2, from Dojindo Laboratories (Kumamoto); sodium azide, from Wako Pure Chemical; and [^{3}H]fMLP, formyl-Met-Leu-Phe, *N*-[phenylalanine-ring-3,4,5-^{3}H(*N*)]-, from Du Pont Co. (Wilmington, DE, USA).

Neutrophil preparation

Twenty-seven milliliters of blood was collected from the rabbit ear artery in a syringe containing 3 ml of 3.8% sodium citrate solution. The citrated blood was mixed with 3% dextran-saline and allowed to stand at 4℃ for 40 min in order to sediment the erythrocytes. The cells in the supernatant were pelleted by centrifugation at $180 \times g$ for 10 min and then resuspended in 1 ml of phosphate-buffered saline (pH 7.4, PBS). This suspension was layered on 5 ml of Ficoll-Paque® in a plastic tube (14×100 mm) and centrifuged at $360 \times g$ for 20 min at 4℃. The supernatant containing mononuclear cells was discarded, and the pellet was resuspended in 1 ml of PBS. In order to lyse the residual erythrocytes, 9 ml of cold water was added to the cell suspension, and then this mixture was vortexed for 30 sec followed by the addition of 1 ml of 1.5 M NaCl. These cells were washed twice with PBS before finally resuspending them in each experimental buffer. The suspension contained more than 95% neutrophils, as assessed by May-Grünwald-Giemsa-stained smears, and the cell viability was more than 99% as assessed by Trypan blue dye exclusion.

Adhesion

Neutrophils (10^6 cells), suspended in 10 mM HEPES-buffered RPMI 1640 (pH 7.4) containing 5% FCS, were

incubated with either each drug or the vehicle in tissue-culture wells (3047; Falcon, Lincoln Park, NJ, USA) for 5 min at 37℃. Either fMLP (3 nM) or PMA (1 nM) was then added to the suspension and incubated for 30 min at 37℃. After the incubation, the wells were washed twice with 10 mM HEPES-buffered RPMI 1640, and the number of adherent cells was counted. Adhesion (%) was calculated as follows: (number of adherent cells / 10^6) × 100.

Migration

Chemotaxis was measured in a modified Boyden chamber using a Chemotaxicell (3-μm diameter pores; Kurabo, Osaka) and a tissue culture well (3047, Falcon). Briefly, the upper compartment containing neutrophils (10^6 cells, final incubation volume of 0.2 ml), suspended in RPMI 1640 containing 0.2% BSA together with either the drug or vehicle, was preincubated for 5 min at 37℃. The lower compartment contained 0.6 ml of 3 nM fMLP or 1 nM LTB_4 in the same culture medium containing drugs. The chamber was placed in a CO_2 incubator at 37℃ with 5% CO_2, 95% air and 100% humidity for 45 min. The number of cells migrating into the lower compartment was counted, the migration rate (%) being calculated as follows: (number of cells collected from the lower compartment / 10^6) × 100.

Production of O_2^-

The generation of O_2^- was measured by the reduction of cytochrome c as described by Goldstein et al. (23). Neutrophils (10^6 cells) suspended in Hank's balanced salt solution (HBSS) containing 0.1 mM cytochrome *c* were preincubated with either the drug or vehicle for 5 min at 37℃. PMA (10 nM) or C5a (100 nM) was added to the suspension and incubated for 10 min at 37℃. The incubation was terminated by cooling the tube in an ice-water bath, followed by centrifugation at 7,000 × *g* for 3 min at 4℃. The absorbance of the supernatant was measured at 550 nm using a spectrophotometer.

Measurement of $[Ca^{2+}]_i$

Neutrophils suspended in Ca-HEPES buffer (10 mM HEPES, 1 mM $CaCl_2$, 1% BSA, pH 7.4) supplemented with 2 μM fura 2-AM were incubated at 37℃ for 45 min. The cells were cooled and then washed twice with Ca-HEPES buffer before continuing the incubation for another 30 min at 37℃. After further cooling and washing with Ca-HEPES buffer, the neutrophils were then resuspended in HEPES buffer. The neutrophils (10^6 cells) were incubated with either 1 mM $CaCl_2$ or 0.2 mM EGTA in the presence of either the drug or vehicle for 5 min at 37℃ while constantly stirring. The stimulants fMLP (3 nM), LTB_4 (1 nM), C5a (1 nM), PAF (1 nM) or PMA (10 nM) were added to the suspension and fluorescence measured at both 340 and 380 nm for excitation and at 500 nm for emission using a calcium analyzer (CAF-100; Nihon Bunko, Tokyo). $[Ca^{2+}]_i$ was calculated as described previously (24).

Binding assay of fMLP

The binding of [^{3}H]fMLP was measured as described by Williams et al. (25) with minor modifications. Briefly, neutrophils (10^6 cells) suspended in CF-PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 1 mM $MgCl_2$ and 137 mM NaCl, pH 7.4) were incubated with either each drug or vehicle for 5 min at 37℃ in the presence of 10 mM sodium azide and 100 μM TPCK. Subsequently, [^{3}H]fMLP (0.25 – 10 nM) was added and incubated for 30 min at 37℃. The incubations were terminated by addition of ice-cold CF-PBS (4 ml) followed by rapid filtration of the mixture through a glass fiber filter (GF/C; Whatman, Maidstone, England). The filters were then washed four times with ice-cold CF-PBS (4 ml) before they were dried, and any retained radioactivity was counted by a liquid scintillation counter. Nonspecific binding was determined in the presence of unlabeled fMLP (10 μM). Specific binding was defined as the total amount of [^{3}H]fMLP bound minus the nonspecific binding.

Binding assays of LTB_4, C5a or PAF

The binding assays of [^{3}H]LTB_4, [^{125}I]C5a and [^{3}H]-PAF were performed as a study using Nova Screen® (Hanover, MA, USA). The conditions used to perform the binding assays were as follows: i) LTB_4, [^{3}H]LTB_4 (0.4 nM) in a phosphate buffer containing NaCl, $MgCl_2$, EDTA and bacitracin was incubated at 0℃ for 2 hr with guinea pig splenic membrane (26); ii) C5a, [^{125}I]C5a (1.0 pM) in HEPES buffer containing $CaCl_2$, $MgCl_2$, BSA, PMSF and bacitracin was incubated at 4℃ for 2 hr with U937 cells (27); and iii) PAF, [^{3}H]PAF (1 nM) in HEPES buffer containing BSA were incubated at 0℃ for 2 hr with rabbit platelets (28).

Statistical analyses

The results are each expressed as a mean ± S.E. Statistical analyses were performed by one-way ANOVA followed by Dunnett's test. Differences in P values of less than 0.05 were considered to be statistically significant. The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]fMLP were estimated by Scatchard-Rosenthal analysis of the saturation data using the LUNDON program (29), purchased from Lundon Software (Chagrin Falls, OH, USA). The IC_{50} values of the drugs for specific binding of [^{3}H]fMLP were calculated by probit analysis.

RESULTS

Effects on fMLP- and PMA-stimulated adhesion

The effects of KBT-3022, desethyl KBT-3022, ASA, TP, CIL and IM on fMLP- and PMA-stimulated adhesion of neutrophils are shown in Figs. 2 and 3, respectively. In the absence of stimulants, adhesion was 2–4%, but this was significantly increased by fMLP (3 nM) to 26–32%. KBT-3022 and desethyl KBT-3022 (both at 0.3 and 1 μM) significantly inhibited fMLP-stimulated neutrophil adhesion in a concentration-dependent manner to a similar degree. CIL (10 μM) and IM (10 μM) also significantly inhibited fMLP-stimulated adhesion. In contrast, ASA (100 μM) and TP (1 μM) only showed a tendency to inhibit adhesion. PMA (1 nM) also significantly increased neutrophil adhesion to 48%. KBT-3022 (1 μM) and desethyl KBT-3022 (1–10 μM) significantly inhibited PMA-stimulated adhesion of neutrophils while ASA, TP, CIL or IM had no effect.

Effects on fMLP- and LTB_4-induced migration

The effects of KBT-3022, desethyl KBT-3022, ASA, TP, CIL and IM on fMLP- and LTB_4-stimulated migration of neutrophils are shown in Figs. 4 and 5, respectively. In the absence of stimulants, there was no neutrophil migration, while fMLP (3 nM) induced 41–45% of the neutrophils to migrate from the upper to the lower compartment of the Boyden chamber. KBT-3022 and desethyl KBT-3022 (both at 0.1–1 μM) significantly inhibited fMLP-induced neutrophil migration in a concentration-dependent manner to a similar degree. ASA (100 μM), TP (1 μM), CIL (1 and 10 μM) and IM (1 and 10 μM) also significantly inhibited fMLP-induced migration. LTB_4 (1 nM) also induced 45% of the neutrophils to migrate. KBT-3022 (1 μM) and desethyl KBT-3022 (1–10 μM) significantly inhibited LTB_4-induced migration. CIL (10 μM) also inhibited LTB_4-induced migration, while ASA, TP and IM had no effect.

Effects on PMA- and C5a-induced O_2^- production

The effects of KBT-3022, desethyl KBT-3022, ASA, TP, CIL and IM on PMA- and C5a-stimulated production of O_2^- are shown in Figs. 6 and 7, respectively. PMA (10 nM) induced O_2^- production by neutrophils

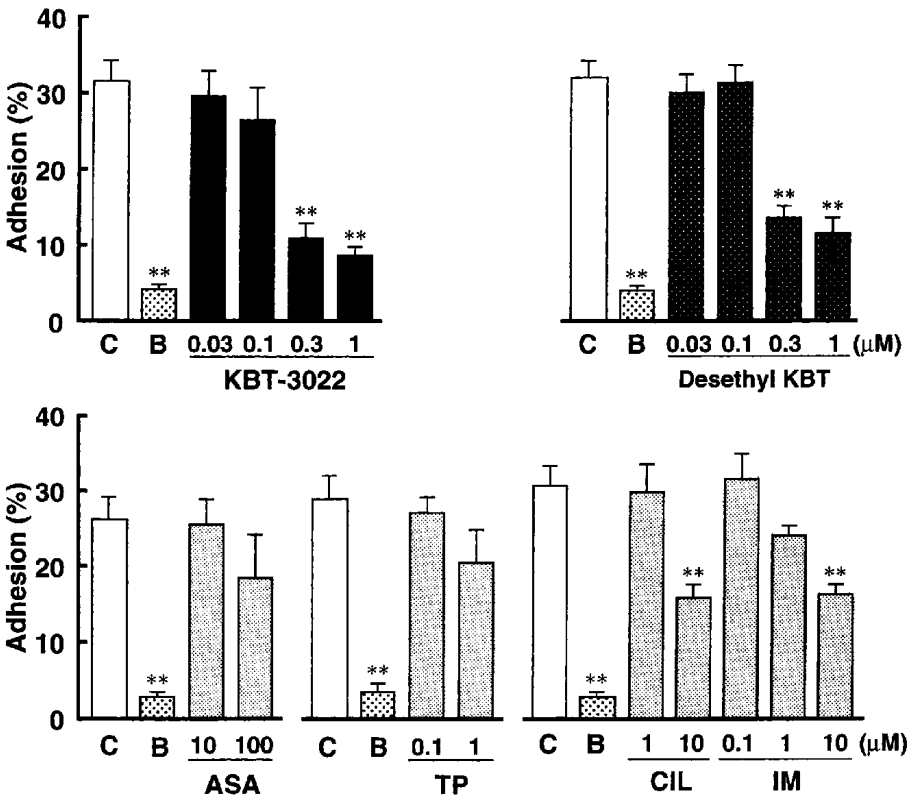


Fig. 2. Effects of KBT-3022, desethyl KBT-3022, ASA, TP, CIL and IM on fMLP-induced neutrophil adhesion. The cells and each drug were placed in wells and preincubated for 5 min at 37℃. fMLP (3 nM) was added to the cell suspension and incubated for 30 min at 37℃. After incubation, nonadherent cells were discarded, the wells were washed twice and the number of adherent cells counted. Each column and bar represents the mean and S.E. of 4 experiments (performed in duplicate), respectively. **$P<0.01$, significantly different from the control (Dunnett's test). C: control, B: blank, Desethyl KBT: desethyl KBT-3022.

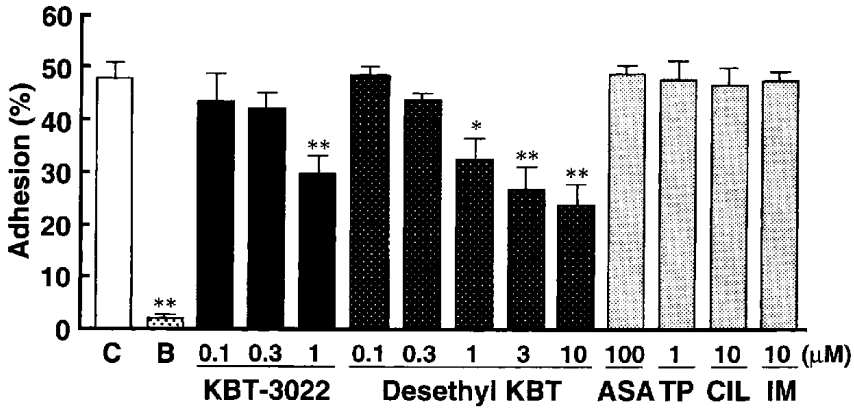


Fig. 3. Effects of KBT-3022, desethyl KBT-3022, ASA, TP, CIL and IM on PMA-induced neutrophil adhesion. The cells and each drug were placed in wells and preincubated for 5 min at 37℃. PMA (1 nM) was added to the cell suspension and incubated for 30 min at 37℃. After incubation, nonadherent cells were discarded, the wells were washed twice and the number of adherent cells counted. Each column and bar represents the mean and S.E. of 4 experiments (performed in duplicate), respectively. *P<0.05, **P<0.01, significantly different from the control (Dunnett's test). C: control, B: blank, Desethyl KBT: desethyl KBT-3022.

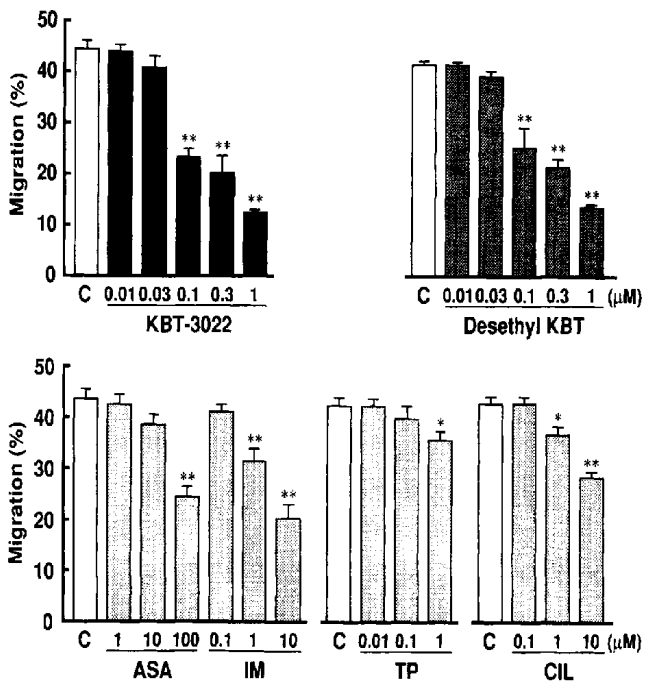


Fig. 4. Effects of KBT-3022, desethyl KBT-3022, ASA, IM, TP and CIL on fMLP-induced neutrophil migration. fMLP (3 nM) was present in the lower compartment of the Boyden chamber. After preincubation with each drug for 5 min at 37℃, the cells were allowed to migrate for 45 min at 37℃. Each column and bar represent the mean and S.E. of 4 experiments (performed in duplicate), respectively. *P<0.05, **P<0.01, significantly different from the control (Dunnett's test). C: control, Desethyl KBT: desethyl KBT-3022.

(12.1±0.5 nmol/10 min, n=10) as monitored by the reduction of cytochrome *c*. This was completely abolished by 100 U/ml superoxide dismutase. Both KBT-3022 and desethyl KBT-3022 at 1 μM inhibited the production of O_2^- by 22.1% and 39.6%, respectively. ASA (100 μM) showed a tendency to inhibit O_2^- produc-

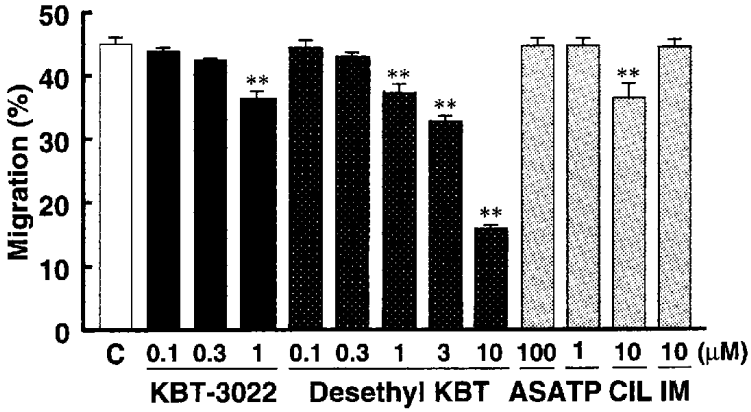


Fig. 5. Effects of KBT-3022, desethyl KBT-3022, ASA, TP, CIL and IM on LTB_4-induced neutrophil migration. LTB_4 (1 nM) was present in the lower compartment of the Boyden chamber. After preincubation with each drug for 5 min at 37℃, the cells were allowed to migrate for 45 min at 37℃. Each column and bar represent the mean and S.E. of 4 experiments (performed in duplicate), respectively. $^{**}P<0.01$, significantly different from the control (Dunnett's test). C: control, Desethyl KBT: desethyl KBT-3022.

tion, but TP, CIL or IM did not have any effect. C5a (100 nM) also induced neutrophils to produce O_2^- (4.7±0.4 nmol/10 min, n=10). Desethyl KBT-3022 inhibited the O_2 production in a concentration-dependent manner, but KBT-3022 up to 1 μM did not have any effect. ASA (100 μM) and CIL (10 μM) partially inhibited the O_2^- production, while TP and IM had no effect.

Effects on fMLP-, LTB_4-, C5a- and PAF-induced increases in $[Ca^{2+}]_i$ in neutrophils

The $[Ca^{2+}]_i$ in fura 2-loaded neutrophils was calculated to be between 240 and 330 nM; this was not influenced by the addition of each drug in the presence of 1 mM Ca^{2+}. FMLP (3 nM) and LTB_4 (1 nM) both elicited an increase in $[Ca^{2+}]_i$ in two phases, an initial rapid rise and a continuous delayed rise (Fig. 8). KBT-3022 and desethyl KBT-3022 (both at 0.3 and 1 μM) inhibited both phases of the rise in $[Ca^{2+}]_i$ in fMLP-stimulated neutrophils in a concentration-dependent manner in the presence of 1 mM Ca^{2+}. CIL and IM (both at 10 μM) also suppressed the

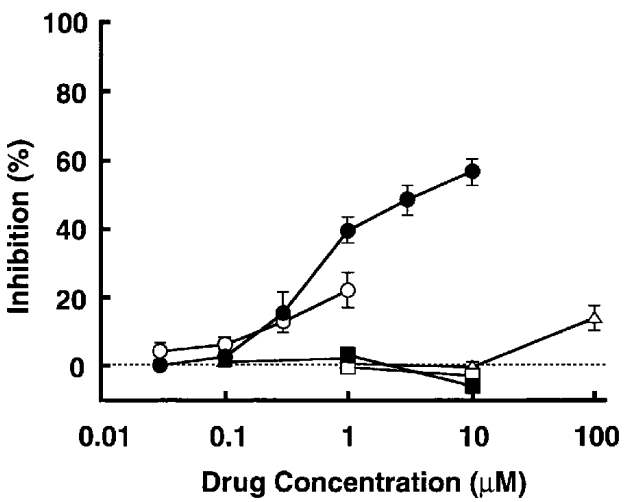


Fig. 6. Effects of KBT-3022 (○), desethyl KBT-3022 (●), ASA (△), TP (▲), CIL (□) and IM (■) on O_2^- production from neutrophils stimulated by PMA. After preincubation with each drug for 5 min at 37℃, the cells were stimulated by PMA (10 nM). The reduction of cytochrome *c* for 10 min was measured and the percentage inhibition determined using the response of PMA-stimulated control cells. Each point and vertical bar represents the mean and S.E. of 4 experiments, respectively.

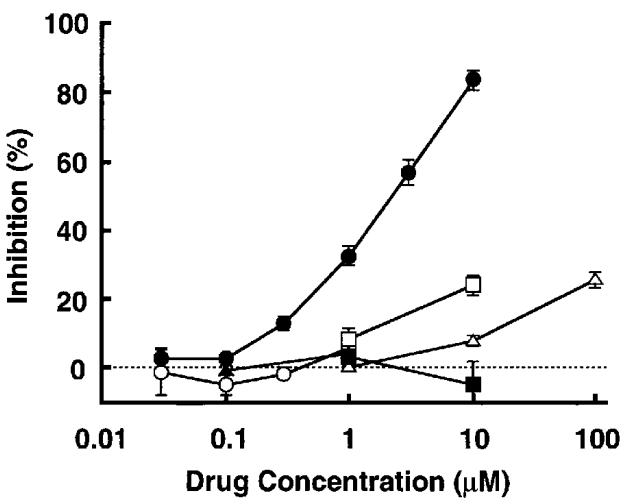


Fig. 7. Effects of KBT-3022 (○), desethyl KBT-3022 (●), ASA (△), TP (▲), CIL (□) and IM (■) on O_2^- production from neutrophils stimulated by C5a. After preincubation with each drug for 5 min at 37℃, the cells were stimulated by C5a (100 nM). The reduction of cytochrome *c* for 10 min was measured and the percentage inhibition determined using the response of the C5a-stimulated control cells. Each point and vertical bar represents the mean and S.E. of 4–5 experiments, respectively.

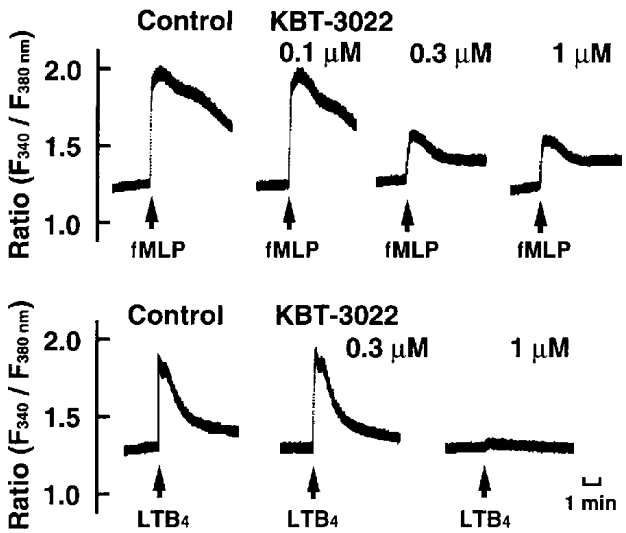


Fig. 8. Effects of KBT-3022 on the elevation of $[Ca^{2+}]_i$ in neutrophils stimulated by fMLP and LTB_4. fMLP (3 nM) or LTB_4 (1 nM) was added to fura 2-AM-loaded cells preincubated with either KBT-3022 or vehicle for 5 min at 37℃, and the ratio of $F_{340\,nm}$ and $F_{380\,nm}$ was recorded.

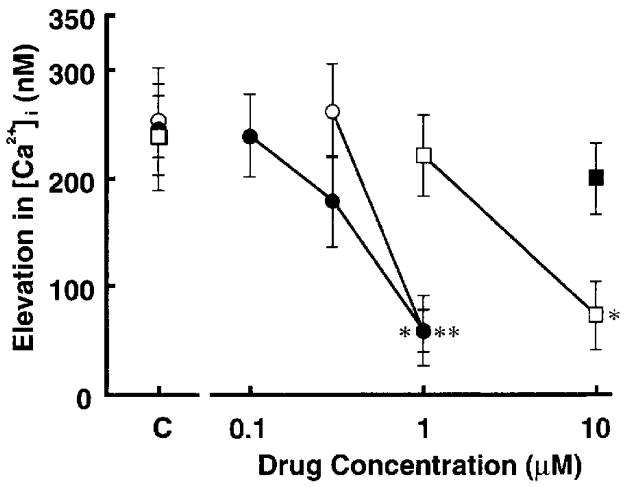


Fig. 10. Effects of KBT-3022 (○), desethyl KBT-3022 (●), CIL (□) and IM (■) on the elevation of $[Ca^{2+}]_i$ in neutrophils stimulated by LTB_4. Fura 2-loaded cells were preincubated with either each drug or vehicle for 5 min at 37℃, and then LTB_4 (1 nM) was added. $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. Each point and vertical bar represents the mean and S.E. of 4 determinations, respectively. *P<0.05, **P<0.01, significantly different from the control (Dunnett's test). C: control.

increase in $[Ca^{2+}]_i$ in fMLP-stimulated neutrophils (Fig. 9). KBT-3022 and desethyl KBT-3022 (both at 1 μM) significantly suppressed the increase in $[Ca^{2+}]_i$ in LTB_4-stimulated neutrophils in a concentration-dependent manner in the presence of 1 mM Ca^{2+}, while IM had no effect (Fig. 10). CIL (10 μM) also significantly suppressed the increase in $[Ca^{2+}]_i$ in LTB_4-stimulated neutrophils. PMA (10 nM) did not elevate $[Ca^{2+}]_i$ (data not shown). In a calcium-free medium (in the presence of 0.2 mM EGTA), fMLP (3 nM) and LTB_4 (1 nM) elicited only the first rapid rise in $[Ca^{2+}]_i$; this was suppressed by KBT-3022 and desethyl KBT-3022 at concentrations similar to those observed in the presence of 1 mM Ca^{2+} (data not shown). C5a (1 nM) and PAF (1 nM) also elicited an increase in $[Ca^{2+}]_i$, the pattern being similar to that seen with fMLP and LTB_4. Both KBT-3022 and desethyl KBT-3022 at 1 μM significantly inhibited the increase in $[Ca^{2+}]_i$ in neutrophils stimulated by C5a but not PAF (Table 1).

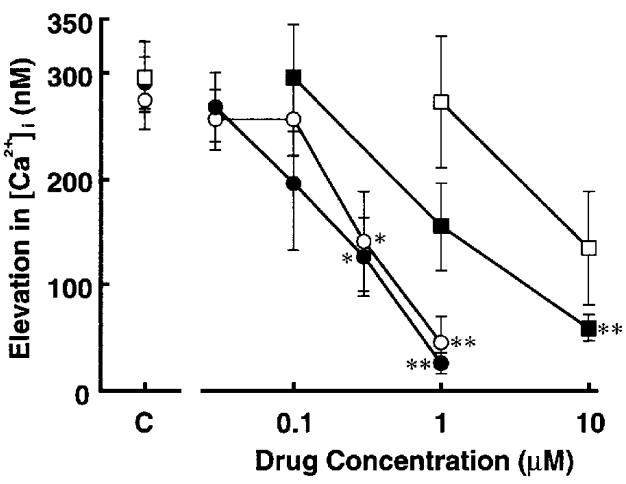


Fig. 9. Effects of KBT-3022 (○), desethyl KBT-3022 (●), CIL (□) and IM (■) on the elevation in $[Ca^{2+}]_i$ in neutrophils stimulated by fMLP. Fura 2-loaded cells were preincubated with either each drug or vehicle for 5 min at 37℃, and then fMLP (3 nM) was added. $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. Each point and vertical bar represents the mean and S.E. of 4 determinations, respectively. *P<0.05, **P<0.01, significantly different from the control (Dunnett's test). C: control.

Effects on specific binding of fMLP, LTB_4, C5a and PAF

The effects of KBT-3022, desethyl KBT-3022, ASA,

Table 1. Effects of KBT-3022, desethyl KBT-3022 and IM on the increase in $[Ca^{2+}]_i$ of neutrophils stimulated by C5a and PAF

Drug	Concentration (μM)	Increase in $[Ca^{2+}]_i$ (nM)	
		C5a	PAF
Control		279±24	264±36
KBT-3022	1	115±26**	201±36
Desethyl KBT-3022	1	93±32**	176±40
IM	10	285±21	301±48

Each value represents the mean±S.E. of 4 experiments. **P<0.01, significantly different from the control (Dunnett's test).

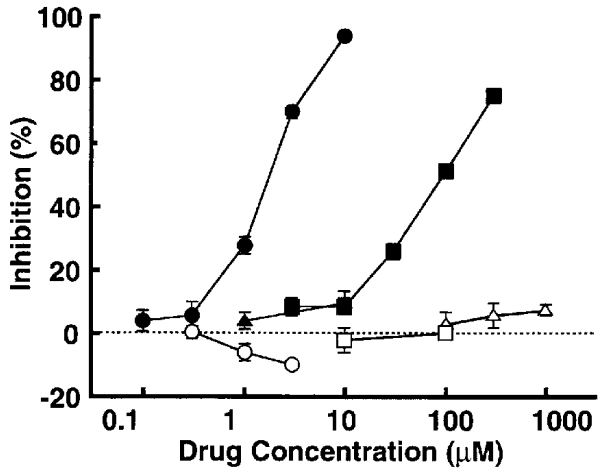


Fig. 11. Effects of KBT-3022 (○), desethyl KBT-3022 (●), ASA (△), TP (▲), CIL (□) and IM (■) on the specific binding of [^{3}H]-fMLP to neutrophils. [^{3}H]fMLP (0.5 nM) was incubated with neutrophils and each drug for 30 min at 37℃ followed by rapid filtration. Each point and vertical bar represents the mean and S.E. of 4 determinations, respectively.

TP, CIL and IM on the specific binding of [^{3}H]fMLP to neutrophils are shown in Fig. 11. Desethyl KBT-3022 but not KBT-3022 inhibited the specific binding of [^{3}H]fMLP (0.5 nM) to the neutrophils in a concentration-dependent manner, its IC_{50} value being 1.9 μM. Kinetic analysis of the specific binding of [^{3}H]fMLP showed the K_d value to be 0.82 nM, with B_{max} being 8,700 sites/cell. The addition of desethyl KBT-3022 (1.2 μM) increased the K_d and B_{max} values of the specific binding of [^{3}H]fMLP to 1.8 nM and 9,700 sites/cell, respectively, which is indicative of competitive inhibition (Fig. 12). IM also inhibited the specific binding of [^{3}H]fMLP to neutrophils yielding an

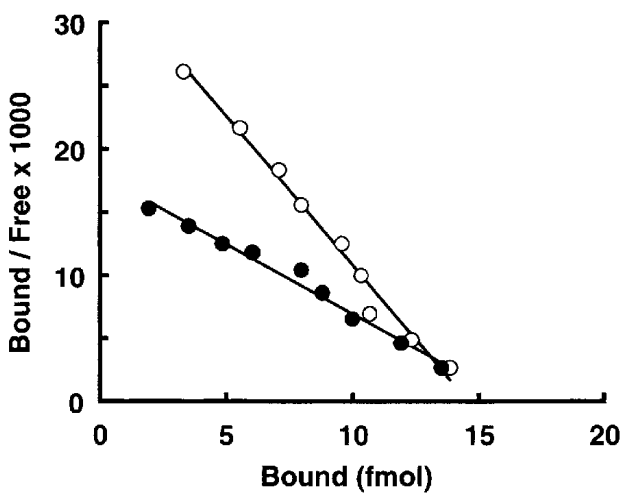


Fig. 12. Scatchard analysis from the saturation data with (●) and without (○) 1.2 μM desethyl KBT-3022.

Table 2. Effects of KBT-3022 and desethyl KBT-3022 on the specific bindings of LTB_4, C5a and PAF

Drug	Concentration (μM)	Inhibition (%)		
		LTB_4	C5a	PAF
KBT-3022	0.1	18.9	3.7	−7.5
	1	13.1	17.8	4.8
Desethyl KBT-3022	0.1	7.5	7.2	1.8
	1	1.1	6.7	15.9
	10	14.4	12.2	5.9

Each value represents the average of duplicate determinations.

IC_{50} value of 96 μM; its inhibition was also competitive (data not shown). ASA, TP and CIL did not have any effects on binding. KBT-3022 and desethyl KBT-3022 had no effect on the specific binding of LTB_4, C5a and PAF (Table 2).

DISCUSSION

Our results show that stimulation with fMLP elicited significant adhesion, migration and an increase in $[Ca^{2+}]_i$ in rabbit peripheral blood neutrophils as described previously (30, 31). KBT-3022 and desethyl KBT-3022 as well as the cyclooxygenase inhibitors, IM and ASA, suppressed the activation of neutrophils stimulated by fMLP. The inhibition of neutrophil function by cyclooxygenase inhibitors such as ASA is known to be independent of inhibition of prostaglandin synthesis (32). Moreover, fMLP-induced Mac-1 upregulation, aggregation and O_2^- production in human neutrophils were not affected by nonantioxidant 5-lipoxygenase inhibitors (33). Therefore, the inhibitory effects of KBT-3022 and desethyl KBT-3022 on cyclooxygenase and 5-lipoxygenase (16) may not contribute to the suppression of fMLP-induced responses of neutrophils.

ASA (100 μM) partly inhibited fMLP-induced neutrophil migration. ASA has been reported to inhibit fMLP-induced lysozyme release without affecting specific fMLP binding, suggesting interference with signal transduction from the fMLP receptor (34). ASA had no effect on PMA-induced neutrophil adhesion but did partially inhibit O_2^- production. Moreover, ASA also partially inhibited C5a-induced O_2^- production. As the inhibitory effect of ASA on O_2^- production is agonist-independent, it may result from direct inhibition of NADPH oxidase (35).

TP (1 μM) partially inhibited only fMLP-induced neutrophil migration, but had no effect on LTB_4-induced migration and PMA-induced adhesion of neutrophils. TP has been reported to exert less effects on rat neutrophils

(36).

The K_d and B_{max} values of the specific binding of [^{3}H]-fMLP to rabbit neutrophils were 0.82 nM and 8,700 sites/cell, respectively; these values are similar to those described previously (30, 37). Desethyl KBT-3022 and IM inhibited the specific binding of [^{3}H]fMLP to rabbit neutrophils in a competitive manner. IM (38), like other cyclooxygenase inhibitors such as diclofenac (39), piroxicam (40) and tenoxicam (41), has been previously reported to inhibit specific [^{3}H]fMLP binding and the consequent production of O_2^-. However, the relative potencies of these drugs in inhibiting the binding of [^{3}H]fMLP to neutrophils were not correlated with their potencies in inhibiting fMLP-induced cellular responses (34, 42). In this study, inhibition of the specific binding of [^{3}H]fMLP by desethyl KBT-3022 and IM was negligible at concentrations that completely inhibited adhesion, migration and the increase of $[Ca^{2+}]_i$ in neutrophils, although the ligand concentration used in the binding assay was lower than that used in the other experiments. Therefore, desethyl KBT-3022 and IM may act on sites other than the fMLP receptor to prevent subsequent cellular responses.

IM, a cyclooxygenase inhibitor, inhibited fMLP-induced neutrophil adhesion, migration and $[Ca^{2+}]_i$ elevation. IM has been reported to interfere with signal transduction from the fMLP receptor that results in PLC activation (43).

CIL, KBT-3022 and desethyl KBT-3022 inhibited the elevation of $[Ca^{2+}]_i$ within the same concentration range at which fMLP- and LTB_4-induced migration was inhibited. CIL (44) is one of the most potent cAMP phosphodiesterase (PDE) inhibitors, which suppress the increase in $[Ca^{2+}]_i$ by increasing the intracellular concentration of cAMP (45). Consequently, CIL also partly inhibited other cellular responses, i.e., fMLP-induced adhesion and C5a-induced O_2^- production, that accompany the increase in $[Ca^{2+}]_i$. Desethyl KBT-3022 inhibits cAMP PDE activity in platelets with an IC_{50} value of 47 μM, which is approximately 300 times less potent than that of CIL (K. Yokota et al., unpublished data). Therefore, inhibition of cAMP PDE by desethyl KBT-3022 is unlikely to contribute to its inhibition of ligand-mediated neutrophil responses.

It is known that LTB_4 and C5a, as well as fMLP, also bind to a specific receptor on neutrophils, activating phospholipase C (PLC) and Ca^{2+} influx which, in turn, induces neutrophil adhesion, migration and release of O_2^- and other intracellular components (25, 46–49). FMLP and LTB_4 caused a two-phase increase of $[Ca^{2+}]_i$ in fura 2-loaded neutrophils, as described by Chandler and Kazilek (31); KBT-3022 and desethyl KBT-3022 inhibited both phases of the $[Ca^{2+}]_i$ increase within the same concentration range at which migration was inhibited. The effects of KBT-3022 and desethyl KBT-3022 on LTB_4-induced migration may be characteristic because there are few cyclooxygenase inhibitors that affect LTB_4-induced responses (50, 51). KBT-3022 and desethyl KBT-3022 also inhibited C5a-induced $[Ca^{2-}]_i$ elevation in neutrophils. Neither KBT-3022 nor desethyl KBT-3022 affected the specific binding of [^{3}H]LTB_4 and [^{125}I]C5a. Furthermore, neither compound affected the specific binding of [^{3}H]inositol triphosphate (IP_3) to rat cerebellar membrane (data from the study of Nova Screen®). These observations suggest that KBT-3022 and desethyl KBT-3022 may prevent signal transduction from these receptors to PLC.

KBT-3022 and desethyl KBT-3022 inhibited fMLP-, LTB_4- and C5a-induced, but not PAF- or IL-8-induced increases of $[Ca^{2+}]_i$ in neutrophils (data on IL-8 not shown). It is known that binding of fMLP, LTB_4, C5a, PAF and IL-8 to their respective receptors stimulates the hydrolysis of phosphatidylinositol 4,5-diphosphate by PLC, resulting in the formation of two intracellular second messengers, IP_3 (52) and diacylglycerol. These receptors belong to the superfamily of guanine nucleotide binding protein (G protein)-linked receptors that contain seven transmembrane spanning domains (53, 54). Schepers and McLeish (55) reported that the activated pertussis toxin-sensitive G protein, coupled with fMLP and LTB_4 receptors, exists in different conformations. Salicylate and piroxicam have been reported to disrupt neutrophil function via their capacities to interfere with GTP/GDP exchange at the α-subunit of a regulatory G protein (56). Further investigations are needed in order to determine whether both KBT-3022 and desethyl KBT-3022 act on G protein and/or PLC.

PMA activates PKC directly and elicits adhesion of neutrophils and O_2^- production without a rise in $[Ca^{2-}]_i$ (57, 58). In this study, PMA accelerated the rate of adhesion of neutrophils to a plate, and higher concentrations of PMA caused the production of O_2^- without a change in $[Ca^{2+}]_i$. KBT-3022 and desethyl KBT-3022 partially but significantly inhibited both PMA-induced adhesion and O_2^- production. KBT-3022 and desethyl KBT-3022 are known not to affect the specific binding of [^{3}H]phorbol ester dibutyrate to a mouse brain membrane (data from the study of Nova Screen®). Furthermore, neither compound scavenged O_2^- produced by a xanthine-xanthine oxidase system (data not shown). Thus, KBT-3022 and desethyl KBT-3022 may disturb the downward signal transduction pathways from PKC involved in neutrophil adhesion and O_2 production.

These results indicate that KBT-3022 and desethyl KBT-3022 may inhibit neutrophil function through disturbance of at least two mechanisms including fMLP, LTB_4 and C5a receptor-mediated elevation of $[Ca^{2-}]_i$ and

downward signal transduction from PKC.

We have previously reported that KBT-3022 and desethyl KBT-3022 (0.1 – 1 μM) inhibit platelet aggregation induced by arachidonic acid and collagen (17). In this study, neutrophil activation induced by fMLP, LTB_4 and PMA was inhibited by both compounds at concentrations similar to those that inhibited platelet aggregation in vitro. Furthermore, the infiltration of polymorphonuclear leukocytes (PMNs) through blood vessels into connective tissue induced by fMLP and LTB_4 was inhibited by oral administration of KBT-3022 at concentrations similar to those that inhibited arachidonic acid-induced pulmonary embolism in mice (59). Therefore, KBT-3022 would be expected to suppress neutrophil activation simultaneously when used as a platelet aggregation inhibitor.

C5a and LTB_4 are produced in PMN-dependent reperfusion injuries (60 – 62) and their soluble receptor, antagonists or inhibitors prevent tissue injury (63, 64). Thus, KBT-3022, which possesses a wider spectrum of inhibition of neutrophil activation than other tested drugs, may be an effective candidate for the prevention of PMN-dependent tissue injury.

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