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DUAL INHIBITION OF CYCLOOXYGENASE AND LIPOXYGENASE BY 2-ACETYLTHIOPHENE 2-THIAZOLYLHYDRAZONE (CBS-1108) AND EFFECT ON LEUKOCYTE MIGRATION *IN VIVO*

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Abstract—CBS-1108, 2-acetylthiophene 2-thiazolyhydrazone, inhibits 5-lipoxygenase activity in polymorphonuclear leukocytes (PMNs) ($IC_{50} = 2 \times 10^{-6}$M), 12-lipoxygenase ($IC_{50} = 9 \times 10^{-6}$M) and cyclooxygenase ($IC_{50} = 2 \times 10^{-6}$M) in platelets. Inhibition of the two pathways of arachidonic acid cascade could lead to additional beneficial anti-inflammatory activity by comparison with classical aspirin-like drugs. In fact, only inhibitors of both cyclooxygenase and lipoxygenase such as NDGA and CBS-1108 inhibit leukocyte migration in an animal model of acute inflammatory response.

Inhibition of cyclooxygenase has been proposed in 1971 by Vane [1] as a key mechanism of action for the aspirin-like non-steroid antiinflammatory drugs (NSAIDs). However, inflammatory cells such as PMNs‡ also metabolize arachidonic acid via the lipoxygenase pathway into non-prostaglandin inflammatory mediators [2]. In particular leukotriene B_4 (LTB_4) is the most potent chemotactic lipoxygenase product for neutrophil PMNs [3–7]. In the same way, 5-HETE and platelet lipoxygenase product 12-HETE are also chemotactic factors but present a weaker activity [8, 9].

From these data it has been recently proposed that dual inhibitors of cyclooxygenase and lipoxygenase pathways could have additional beneficial activities for the treatment of inflammatory processes. Few products are known to inhibit these enzymes. Biochemical studies concerning only the antioxidant NDGA [10, 11] and new compounds BW 755 C [12, 13] and timegadine [14] have been clearly reported.

The present paper describes the activity of an hydrazone derivative, CBS-1108, on platelet enzymes (cyclooxygenase and 12-lipoxygenase) and on PMNs 5-lipoxygenase in rabbit and in human. Furthermore, the effect of CBS-1108 on leukocyte migration has been investigated.

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‡ Abbreviations: AA, arachidonic acid; ASA, aspirin; CMC, carboxymethylcellulose; DMSO, dimethylsulfoxide; 5-HETE, 5-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraeinoic acid; HHT, 12-hydroxyheptadecatrienoic acid; LTB_4, leukotriene B_4; MEM, minimum essential medium; NDGA, nordihydroguaiaretic acid; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; PhL, phospholipids; PMNs, polymorphonuclear leukocytes; PRP, platelet rich plasma; TG, triacylglycerols; TxB_2, thromboxane B_2.

MATERIALS AND METHODS

Materials. New Zealand male rabbits (2–3 kg) and Wistar male rats (130–170 g) were used. [1-^{14}C] arachidonic acid, 54.9 mCi/mmole, was purchased from New England Nuclear. Standard prostaglandins, NDGA and aspirin were bought from Sigma. Standard LTB_4 was a gift from Merck-Frosst Laboratories, Quebec. HETEs were synthesised in our laboratory.

Preparation of PMNs suspension. Rabbit peritoneal PMNs were elicited as previously described by Borgeat *et al.* [15], filtered and centrifuged (220 *g* for 15 min). Red cell lysis was induced as described [16]. The cells were then washed in MEM Joklik-modified buffer supplemented with Hepes buffer 25 mM, and resuspended in Dulbecco's PBS at the cell concentration required for incubation. The viability of the PMNs was always greater than 96% as determined by Trypan Blue exclusion test [17].

Human blood from normal donors was drawn by venipuncture into 1/10 vol of trisodium citrate–citric acid–dextrose buffer (25 g/l citrate, 13.6 g/l citric acid, 1 g/l dextrose) and diluted with sterile 0.9% NaCl (1:3, v/v). Neutrophils were purified by centrifugation (220 *g* for 40 min) using a Ficoll-Hypaque gradient, followed by dextran sedimentation [18] and hypotonic lysis [16]. Cell pellets were resuspended in PBS solution supplemented with Hepes buffer 30 mM. Observations on smears showed that neutrophils accounted for more than 93% of the total leukocyte content of purified preparation. Viability was always greater than 95%.

Preparation of washed platelets. Rabbit blood was mixed with trisodium citrate–citric acid–dextrose buffer (6:1, v/v). Platelet rich plasma (PRP) was prepared and centrifuged (1200 *g* for 15 min). The sedimented platelets were washed twice with a Tris–HCl buffer (pH 7.4) containing glucose (0.1%), gelatin (0.25%) and EDTA (0.2 mM). After centrifuga-

tion platelets were resuspended in the same buffer without EDTA at the initial PRP cell concentration.

Human blood platelets routinely came from the Centre de Transfusion Sanguine de Montpellier in plastic bags containing 40–50 ml of concentrated PRP. The PRP was initially centrifuged at 100 *g* for 10 min to eliminate red blood cells. The supernatant was carefully collected and centrifuged at 1500 *g* for 30 min. The sedimented platelets were washed twice with a Tris–HCl buffer (pH 7.4) containing 20 mM EDTA and suspended in the same Tris-HCl buffer without EDTA in order to obtain 1 mg proteins/ml.

[^{14}C] *arachidonate metabolism.* Rabbit neutrophils were resuspended in a complete PBS preincubated (1 ml, 2–2.5 × 10^7 cells) 15 min at 37° in the presence of 10 μl DMSO (control) or inhibitor then incubated for 3 min at 37° with sodium [^{14}C] arachidonate (1 μCi, 18 μM) and A23187 (1 μM). Incubations were blocked by the addition of water, acidified to pH 3 and extracted twice with ethyl acetate.

Human neutrophils were resuspended in a PBS containing 138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1 mM $MgCl_2$ and glucose 0.1%. The suspension (1 ml, 1.5 × 10^7 neutrophils) was incubated under the same conditions. Incubations were blocked with 2 volumes acetone and the samples were centrifuged (220 *g* for 10 min). The clear supernatants were transferred and acetone was removed under nitrogen. The aqueous supernatants were acidified to pH 3 and extracted twice with diethyl ether [19].

For the measurement or arachidonic acid metabolism in platelets, each reaction mixture (1 ml) containing 1 mg protein/ml was preincubated as described above then incubated in the presence of sodium [^{14}C] arachidonate (0.36 μCi, 6.56 μM). After 10 min, incubations were blocked and suspensions acidified, saturated with NaCl then extracted with ethyl acetate.

The extracts were evaporated to dryness. The residues were dissolved in ethyl acetate and applied on a silica gel G plate. The arachidonic acid, HETEs, LTB_4, HHT, PGE_2 and TxB_2 standards were spotted. The solvent system used for the separation of TxB_2, HHT and 12-HETE in platelet extracts was chloroform : methanol : acetic acid : water (90:8:1:0.8). The solvent system used for 5-lipoxygenase products was diethyl ether : petroleum ether :

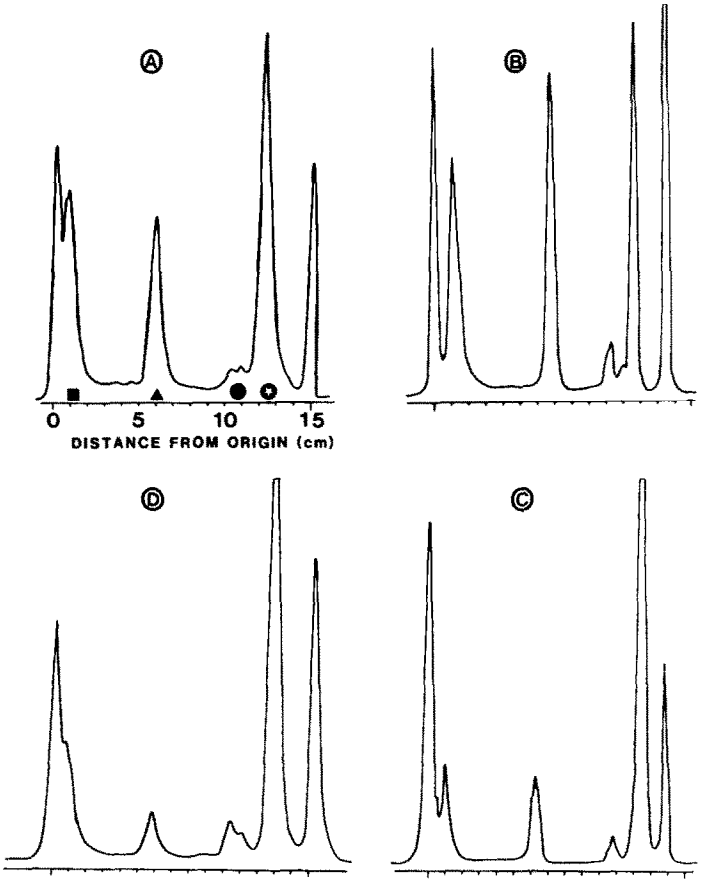

Fig. 1. Metabolites of [^{14}C] arachidonate in rabbit peritoneal PMNs separated by TLC. (A) Control; (B) PMNs were preincubated with ASA (2 × 10^{-4} M); (C) same procedure with NDGA (5 × 10^{-6} M); (D) same procedure with CBS-1108 (2 × 10^{-6} M). Rabbit PMNs were elicited as described in Materials and Methods. Neutrophils (2–2.5 × 10^{-7}) were preincubated with drugs dissolved in DMSO for 15 min at 37° then incubated with [^{14}C] AA (0.6 μCi, 11 μM) and A 23187 (1 μM) for 3 min at 37°. Standards are LTB_4 (■), 5-HETE (▲), 15-HETE (●) and AA (○).

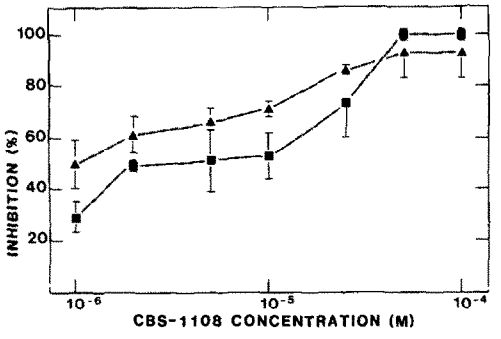

Fig. 2. Inhibition of 5-HETE (▲) and LTB_4 (■) production in rabbit peritoneal PMNs as a function of CBS-1108 concentration. Each point shows the mean value of at least 3 replicates.

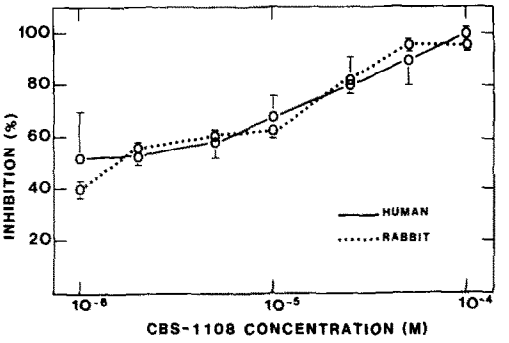

Fig. 3. Inhibition of 5-lipoxygenase pathway (total products) in human and rabbit PMNs as a function of CBS-1108 concentration. Rabbit and human PMNs were elicited as described in Materials and Methods and preincubated ($2–2.5 \times 10^{-7}$) with drugs dissolved in DMSO for 15 min at 37°. Neutrophils were incubated with [^{14}C] AA (11 μM and 18 μM for rabbit and human PMNs, respectively) and A 23187 (1 μM) for 3 min at 37°. Values are mean ± S.D. of at least 7 replicates.

acetic acid (50:50:1) (double migration). The plates were radioscanned (Berthold LB2723) and the standards detected with iodine vapor. The peaks were quantitated as the percentage of total radioactivity.

Acute inflammatory response to carboxymethylcellulose (CMC). Inflammation under dorsal skin of the rat was induced and evaluated as described by Ishikawa *et al.* [20] by injecting CMC in the dorsal air pouch and counting leukocyte number in the pouch fluid. Tested compounds were applied locally in the CMC solution. The inhibitory potency of the agents on leukocyte migration was given by the following equation:

$$\text{Inhibition } (\%) = (1 - C/T) \times 100$$

where C and T were the average values of leukocyte count in the control and treated groups respectively.

RESULTS

Inhibition of 5-lipoxygenase pathway in PMNs. When incubated with rabbit peritoneal PMNs, sodium [^{14}C] arachidonate was metabolized into various products, mainly through the 5-lipoxygenase pathway. Figure 1A shows a typical TLC profile obtained in control cells as previously reported [21]. The major products ($Rf = 0.08$ and $Rf = 0.47$) comigrated with LTB_4 and 5-HETE standards respectively and corresponded to 13 and 16% of the total radioactivity respectively. The chromatographic system used in this experiment did not allow the separation of LTB_4 from its isomers. Aspirin, a specific inhibitor of cyclooxygenase, did not modify the TLC profile (Fig. 1B). In contrast, NDGA (Fig. 1C) and CBS-1108 (Fig. 1D) inhibited the formation of 5-lipoxygenase products, i.e. LTB_4 and 5-HETE at micromolar concentration (Fig. 2). The IC_{50} values of CBS-1108 and NDGA for 5-lipoxygenase pathway were calculated between 2×10^{-6} and 5×10^{-6} respectively (Table 1). In order to compare the inhibitory activity of the compound in rabbit and human PMNs, IC_{50} were determined in neutrophils isolated from venous blood. Figure 3 shows that the enzymes of human and rabbit cells were similarly inhibited by CBS-1108.

Inhibition of cyclooxygenase and 12-lipoxygenase pathways in platelets. Our study was extended to the cyclooxygenase pathway with rabbit and human platelets as source of enzymes. In addition, these cells allowed us to explore inhibition of the 12-lipoxygenase pathway. Figure 4A shows a typical profile of the [^{14}C] arachidonic acid metabolites in rabbit platelets. Thromboxane B_2 and HHT were the major products derived from cyclooxygenase and 12-HETE the only one from platelet lipoxygenase. Aspirin inhibited TxB_2 and HHT production but induced a 2-fold augmentation of 12-HETE formation (Fig. 4B). On the other hand CBS-1108 inhibited both cyclooxygenase and lipoxygenase pathway (Fig.

Table 1. Drugs effect on enzyme activities involved in the metabolism of [^{14}C] arachidonic acid in rabbit peritoneal PMN leukocytes and peripheral blood platelets

Drugs	Inhibition of enzyme activities* 5-lipoxygenase (Rabbit PMNs)	12-lipoxygenase (Rabbit platelets)	Cyclooxygenase (Rabbit platelets)
Aspirin	n.i.	n.i.	$> 10^{-4}$
NDGA	5×10^{-6}	5×10^{-5}	8×10^{-6}
CBS-1108	2×10^{-6}	9×10^{-6}	2×10^{-6}

* IC_{50} (M).
n.i. = not inhibited.

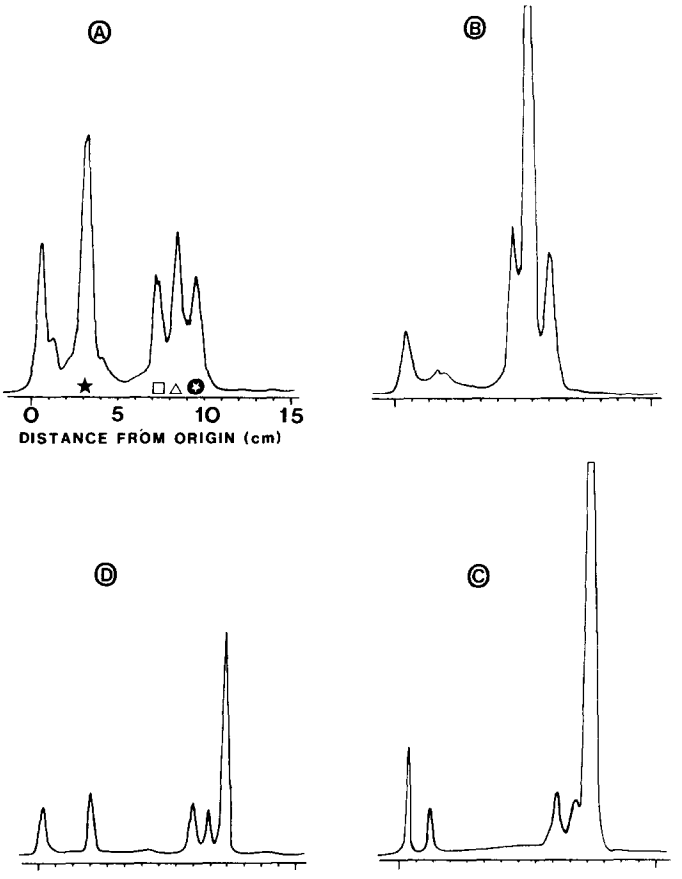

Fig. 4. Metabolites of [^{14}C] arachidonate in rabbit platelets, separated by TLC. (A) Control, (B) platelets were preincubated with ASA (2×10^{-4}), (C) same procedure with NDGA (5×10^{-5} M), (D) same procedure with CBS-1108 (2.5×10^{-5} M). Rabbit platelets were preincubated with drugs dissolved in DMSO for 10 min at 37° and then incubated with [^{14}C] AA (0.36 μCi, 6.56 μM) for 10 min. Standards are TxB_2 (★), HHT (□), 12-HETE (△) and AA (✪).

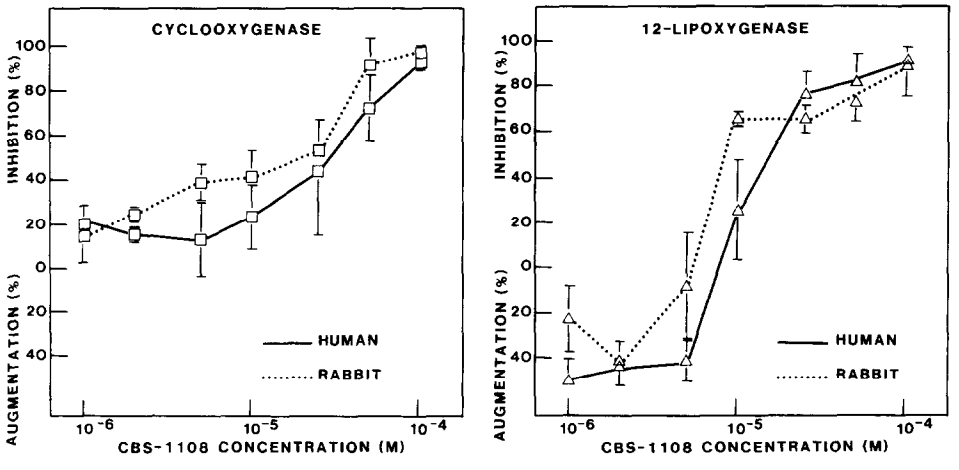

Fig. 5. Inhibition of cyclooxygenase pathway (TxB_2 + HHT) and 12-lipoxygenase pathway (12-HETE) in human and rabbit platelets as a function of CBS-1108 concentration. Values are mean ± S.D. of at least 7 replicates.

4D) as NDGA did (Fig. 4C). IC_{50} of CBS-1108 for platelet enzymes in man and rabbit were of the same order of magnitude (2.5×10^{-5} and 1×10^{-5} M for cyclooxygenase and 12-lipoxygenase respectively Fig. 5 and Table 1).

Inhibition of leukocyte migration in acute inflammatory response to CMC in an animal model. CMC solution injected subcutaneously into an air pouch on the back of rats induced an inflammatory reaction characterized by exudation and leukocyte migration. In the absence of drugs, the CMC-induced exudates contained a variable number of leukocytes from an experiment to another one (3880 ± 700 cells/ml to 26700 ± 2900 cells/ml). When aspirin was injected simultaneously with CMC into the pouch, leukocyte infiltration into the pouch fluid was significantly increased. By contrast both NDGA and CBS-1108 inhibited it in a dose-dependent manner (ED_{50} = 1.5 μmol/100 g BW and 3 μmols/100 g BW respectively) (Fig. 6).

DISCUSSION

The results presented in this paper show that CBS-1108 is a potent inhibitor of 5- and 12-lipoxygenases in rabbit and human blood cells. Moreover, when platelets were used for studying its interaction with cyclooxygenase it was shown that this compound also inhibited prostaglandin synthesis at the same concentrations.

Compounds presenting such an activity have already been described [22]. In particular BW 755 C [12, 13] and timegadine [14] have been studied. Using the same reference compound, NDGA, Ahnfelt-Rønne and Arrigoni-Martelli [14] have compared the inhibitory activity of those drugs: the NDGA IC_{50} for lipoxygenase was 2×10^{-6} M, equal to the value reported in the present paper. According to these authors, timegadine and BW 755 C are 15-fold weaker inhibitors of 5-lipoxygenase pathway.

In our study, CBS-1108 inhibits this enzyme at a lower concentration than NDGA and therefore appears as the more potent product. In addition CBS-1108 equally inhibits both cyclooxygenase and lipoxygenases in the same range of concentration whereas timegadine is more dissociated.

At low concentrations (1 to 5×10^{-6} M), CBS-1108 stimulates the production of 12-HETE. A similar stimulation is usually observed with inhibitors of cyclooxygenase which block the formation of TxB_2 and HHT and consequently increase the substrate availability for lipoxygenase [23]. This simple explanation does not seem valuable in the case of CBS-1108 since the drug only weakly inhibits cyclooxygenase at concentrations where 12-HETE formation is stimulated. The reasons for such an effect may be more complex. Another possibility could be the prevention by the drug of the self-catalysed destruction of lipoxygenase as previously reported for cyclooxygenase with other compounds [24]. Selective stimulation of platelet 12-HETE production has been observed with drugs known to interfere with intracellular calcium [25]. We cannot exclude that CBS-1108 also interacts with calcium, independently of its effect in the oxidative metabolism of arachidonic acid.

In vivo, prostaglandins, leukotrienes and HETEs mediate allergic and inflammatory reactions [26]. Vasodilatation in inflammatory site is decreased by inhibitors of prostaglandin synthesis [27]. On the other hand, leukotrienes and HETEs play an important role in inflammatory processes by increasing vascular permeability and by stimulating leukocyte functions (i.e. adhesion, migration and degranulation) [5, 28, 29].

Classical NSAIDS which inhibit only cyclooxygenase can deviate arachidonic acid metabolism to lipoxygenase products and increase chemotaxis as reported in the present paper and previously [23, 30]. By contrast, CBS-1108 which is a dual inhibitor of cyclooxygenase and lipoxygenase could be an anti-inflammatory drug with a wider spectrum of activity than selective inhibitors of prostaglandin synthesis. In fact, CBS-1108 is a very potent inhibitor of chemotaxis *in vivo* when applied into the site of inflammation. However, the control of the enzymatic activity of the migrated cells has not been done since the little number of collected PMNs did not allow further metabolic (*ex vivo*) studies.

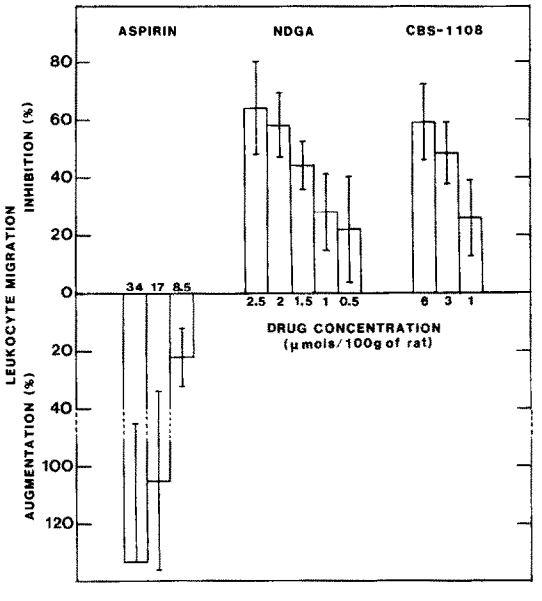

Fig. 6. Inhibition of leukocyte migration in acute inflammatory response to CMC rats. CMC solution (5 ml) is injected subcutaneously on the dorsum of the rats with the tested compound. The CMC fluid is collected 7.5 hr later, the leukocytes stained with Brilliant cresyl blue and counted. Values are mean ± S.D. of 6 rats per dose in each assay × duplicate experiments.

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