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Inflammation Research

The effect of erdosteine and its active metabolite on reactive oxygen species production by inflammatory cells

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Abstract. *Objective:* We examined the effect of erdosteine (KW-9144), an expectorant, and related compounds on inflammatory cell-derived reactive oxygen species which are involved in airway inflammation.
Methods: Neutrophils were isolated from peritoneal lavages of casein-injected rats and from peripheral blood of healthy human donors. Eosinophils were isolated from peritoneal lavages of horse serum-injected guinea pigs. These cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and the production of reactive oxygen species was measured with luminol-dependent chemiluminescence (LDCL).
Results: M1, an active metabolite of erdosteine, significantly inhibited PMA-induced LDCL of the all cell populations with treatment before stimulation. The effects of S-carboxymethylcysteine (S-CMC), ambroxol and N-acetylcysteine (NAC) on the LDCL response were weaker than those of M1. Furthermore, PMA-induced LDCL was decreased by post-treatment with M1.
Conclusion: These results suggest that M1 (an active metabolite of erdosteine) may exert an antiinflammatory effect by scavenging inflammatory cells-derived reactive oxygen species.

Key words: Erdosteine – Neutrophils – Eosinophils – Reactive oxygen species – Luminol-dependent chemiluminescence

Introduction

In respiratory diseases such as acute and chronic bronchitis and bronchial asthma, failure in expectoration occurs with hypersecretion of the mucus and reduction of mucociliary clearance. There is some evidence that reactive oxygen species produced by inflammatory cells including neutrophils and eosinophils are involved in airway inflammation observed in respiratory diseases [1, 2].

Erdosteine, (±)-[[[(tetrahydro-2-oxo-3-thienyl)carbamoyl] methyl]thio]acetic acid, is a mucolytic and mucoregulator agent which modulates mucus production and viscosity and increases mucociliary transport [3–6]. Erdosteine protected α_1-antitrypsin in rat and human lung from the loss of its elastase inhibitory capacity induced by cigarette smoke [7–9]. It was demonstrated that the protective effect of erdosteine is related to the direct free radical scavenging effect of M1, which is an active metabolite of erdosteine and contains an SH group in the chemical structure (Fig. 1), because α_1-antitrypsin is inactivated by reactive oxygen species.

In order to elucidate whether erdosteine might affect reactive oxygen species produced by inflammatory cells, we investigated the effects of erdosteine and its active metabolite (M1) on luminol-dependent chemiluminescence (LDCL) of rat neutrophils, guinea pig eosinophils and human neutrophils induced by phorbol 12-myristate 13-acetate (PMA). We used S-carboxymethylcysteine (S-CMC), ambroxol and N-acetylcysteine (NAC) as reference drugs.

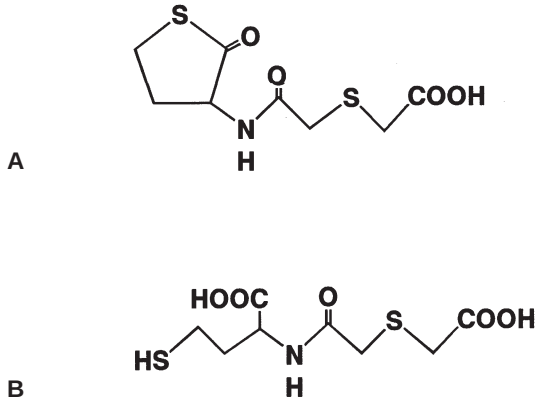

Fig. 1. Chemical structures of erdosteine (A) and M1 (B).

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Materials and methods

Animals

Male Wistar rats (six to eight-week-old) and male Hartley guinea pigs (six-week-old) were purchased from Charles River Japan (Yokohama, Japan) and Japan SLC (Hamamatsu, Japan), respectively. These animals were acclimatized in an animal room maintained at room temperature of 22 ~ 24 °C and a relative humidity of 50 ~ 60% with a 12 h light-dark cycle. Six to nine-week-old rats and nine to ten-week-old guinea pigs were used in experiments. This animal experiment was approved by the Animal Ethical Committee of Kyowa Hakko Kogyo Co., Ltd.

Chemicals

Erdosteine (Edmond Pharma., Milano, Italy) was dissolved in distilled water containing $NaCO_3$ whose concentration is the same as erdosteine in molality. Metabolite 1 which is an active metabolite of erdosteine M1; Edomond Pharma, or Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) was dissolved in Hanks' buffer (Nissui Pharmaceutical Co., Tokyo, Japan). Chemical structures of erdosteine and M1 are shown in Fig. 1. Ambroxol hydrochloride (ambroxol; Sigma Chemical Co., St. Louis, MO, USA), NAC (Sigma), glutathione (GSH; Sigma), superoxide dismutase (SOD; Wako Pure Chemical Industries, Osaka, Japan), catalase (Worthington Biochemical Co., Lakewood, NJ, USA) and NaN_3 (Sigma) were dissolved in Hanks' buffer. S-CMC (Sigma) was dissolved in 1 mol/l NaOH and neutralized with 1 mol/l HCl. Luminol (Sigma) was dissolved in distilled water containing triethylamine (Wako Pure Chemical) to make a 1 mg/ml solution. PMA (Research Biochemicals International, MA, USA) was dissolved in ethanol (Kanto Chemical Co., Tokyo, Japan) to make a 1×10^{-3} mol/l solution. These agent solutions were diluted with Hanks' buffer.

Rat peritoneal neutrophils

The peritoneal exudate cells were collected from rats 16 h after intraperitoneal injection of 1% sodium casein solution (120 ml/kg). Neutrophils obtained from the peritoneal exudate cells were suspended in Hanks' buffer in a concentration of 2×10^6 cells/ml. The purity of neutrophils was > 87% as determined by staining with Türk's solution, and the viability was > 97% as measured by trypan blue exclusion. Six separate experiments were performed using a single cell preparation from one rat, and two rats were used for the evaluation of each drug.

Isolation of guinea pig peritoneal eosinophils

Eosinophils were isolated according to the method of Souness et al. [10]. Guinea pigs were given intraperitoneal injections of 0.5 ml horse serum (Gibco Laboratories, MD, USA) twice weekly for 2 weeks, and the cells were collected by peritoneal lavage with heparinized saline 1 week after the last injection. The cells obtained were layered on discontinuous density Percoll (Pharmacia Biotech, Sweden), and eosinophils were separated by centrifugation. The eosinophils were washed several times and suspended in Hanks' buffer in a concentration of 5×10^5 cells/ml. The purity of eosinophils was > 90% as determined by Wright-Giemsa staining, and the viability was > 94% as measured by trypan blue exclusion. One experiment was performed using a cell preparation from several guinea pigs.

Isolation of human neutrophils

Neutrophils were isolated from the heparinized peripheral venous blood of healthy volunteers by centrifugation on mono-poly resolving medium (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), followed by hypotonic lysis of contaminating erythrocytes. The neutrophils were

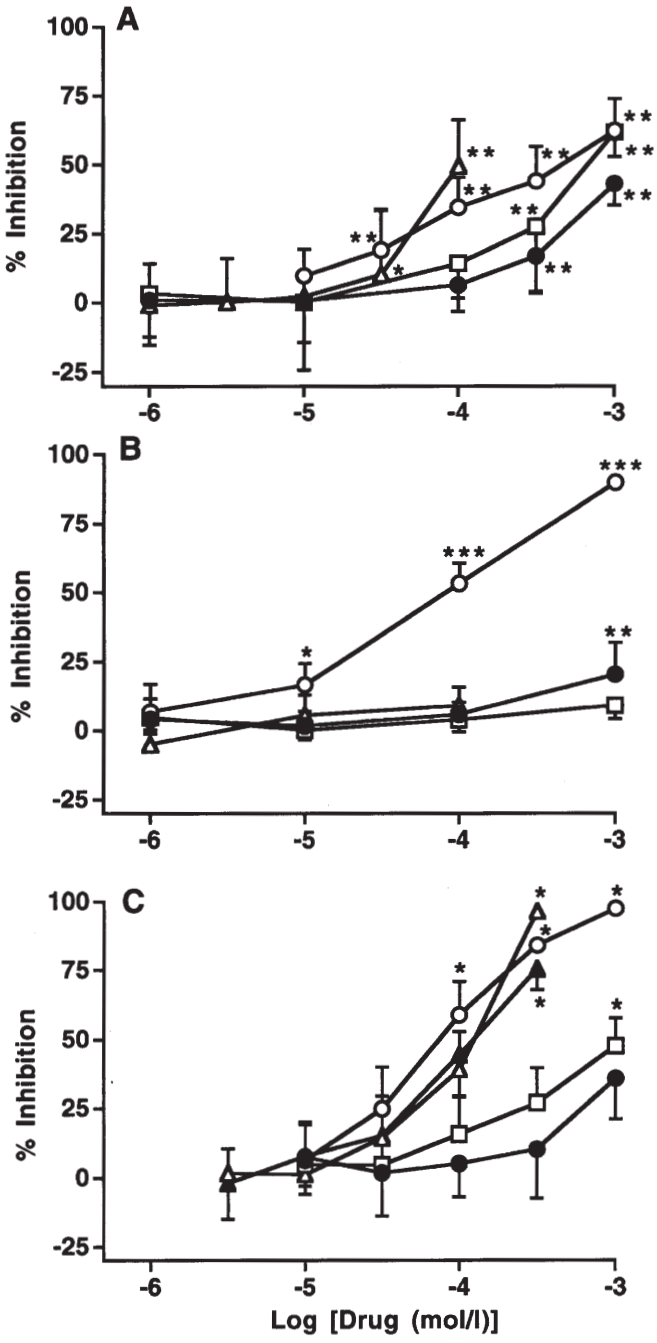

Fig. 2. Inhibitory effects of each drug on LDCL of rat peritoneal neutrophils (A), guinea pig eosinophils (B) and human neutrophils (C) induced by PMA. Cells were preincubated with luminol and Hanks' solution (control) or test drugs (erdosteine (●), M1 (○), S-CMC (□), ambroxol (△), NAC (▲) for 10 min at 37 °C. After the addition of PMA (1×10^{-7} mol/l; A, 1×10^{-6} mol/l; B, 5×10^{-9} mol/l; C), LDCL was measured for 20 min (A, C) or 5 min (B). % Inhibjtion was calculated in each experiment from the integral count of LDCL. The integral count of LDCL in the control was in the range of $2 \sim 6 \times 10^9$ counts in rat, $2 \sim 3 \times 10^9$ counts in guinea pig, $2 \sim 7 \times 10^9$ counts in human. Results are given as the means ± SD (n = 12; A, n = 3 ~ 6; B, n = 6; C). Significantly different from the control response: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

suspended in Hanks' buffer in a concentration of 2×10^6 cells/ml. The purity of neutrophils was > 98% as determined by staining with Türk's solution, and the viability was > 99% as measured by trypan blue exclusion. Each single experiment was performed using cells from separate donors.

Measurement of LDCL

To 480 µl of cell suspension, 5 µl of the test drug solution or Hanks' buffer (control) and 10 µl of the luminol solution were added, and the cell suspension was preincubated for 10 min at 37 °C. After the addition of 5 µl of PMA solution, LDCL was measured with a luminometer (Multi-biolumat LB 9505C, Berthold, Wildbad, Germany) for 5 min in the case of eosinophils or for 20 min in the case of neutrophils. LDCL intensity was determined by integrating the count of LDCL over the measuring time to study the effect of each test drug. The viability of cells determined by trypan blue exclusion was not altered by the treatment with any of the test drugs as compared with control.

In order to evaluate the effect of posttreatment with each test drug, 5 µl of the test drug solution was injected with a microsyringe into the PMA-stimulated cell suspension 10 min after starting to measure LDCL, and the change in the LDCL light curve was observed.

Statistical analysis

All results are expressed as means ± standard deviation (SD). The analysis of variance was evaluated by the Bartlett test. When the variance was equal, statistical differences were examined by the Dunnett multiple comparison test in case the number of samples in each group was the same, and by the Scheffe multiple comparison test in case the number was different. When the variance was unequal and the number of samples in each group was the same, statistical differences were examined by the Steel multiple comparison test. p values less than 0.05 were considered significant. The 50% inhibitory concentrations (IC_{50S}) of the test drugs were calculated from concentration-inhibition curves using probit-plot analysis.

Results

LDCL of rat peritoneal neutrophils

The LDCL of rat peritoneal neutrophils induced by PMA (1×10^{-7} mol/l) was significantly inhibited by treatment with erdosteine for 10 min before stimulation at a concentration of 3×10^{-4} mol/l (43% inhibition at 1×10^{-3} mol/l). M1 significantly inhibited the LDCL at 3×10^{-5} mol/l or higher (IC_{50} of 4.0×10^{-4} mol/l). S-CMC and ambroxol, comparative drugs for expectoration, also significantly suppressed this reaction at 3×10^{-4} mol/l and 3×10^{-5} mol/l, respectively (IC_{50} of 6.6×10^{-4} and 1.0×10^{-4} mol/l for S-CMC and ambroxol, respectively) (Fig. 2A).

LDCL of guinea pig peritoneal eosinophils

Erdosteine significantly inhibited the LDCL of guinea pig peritoneal eosinophils induced by PMA (1×10^{-6} mol/l) at a concentration of 1×10^{-3} mol/l (20% inhibition at 1×10^{-3} mol/l), while M1 was effective at 1×10^{-5} mol/l or higher (IC_{50} of 7.2×10^{-5} mol/l). However, S-CMC and ambroxol failed to inhibit at 1×10^{-3} mol/l and 1×10^{-4} mol/l, respectively (Fig. 2B).

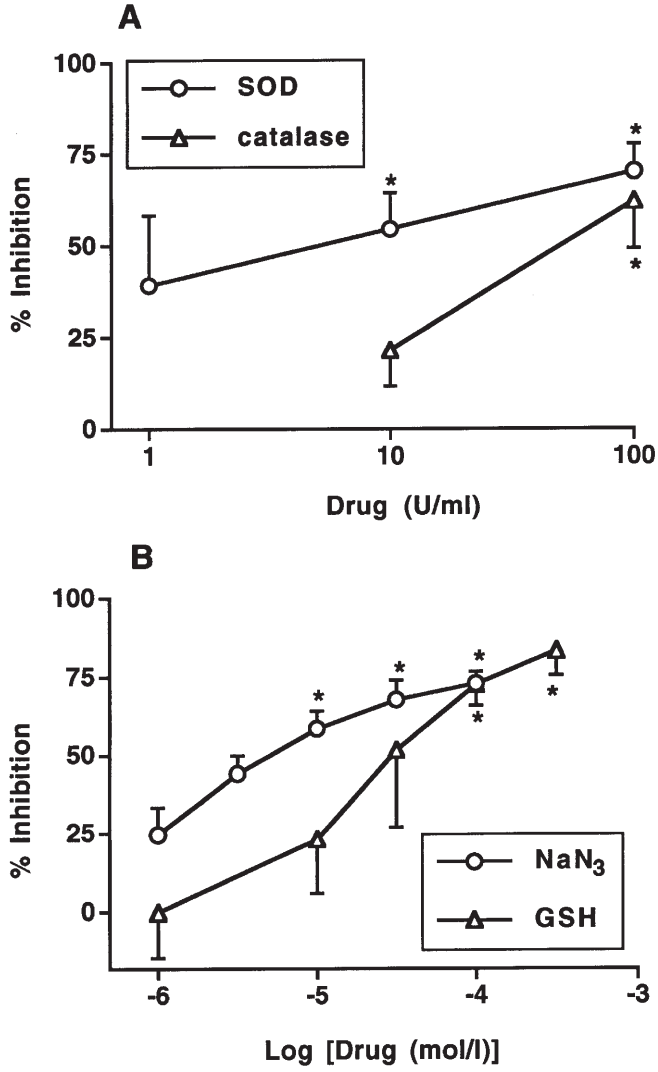

Fig. 3. Effects of SOD, catalase (A), NaN_3 and GSH (B) on LDCL of human neutrophils induced by PMA (5×10^{-9} mol/l). Human neutrophils were preincubated with luminol and Hanks' solution (control) or test drugs for 10 min at 37 °C. After the addition of PMA, LDCL was measured for 20 min. % Inhibition was calculated in each experiment from the integral count of LDCL. The integral count of LDCL in the control was in the range of $2 \sim 8 \times 10^9$ counts. Results are given as the means ± SD (n = 6). Significantly different from the control response: *$p < 0.05$.

LDCL of human neutrophils

Erdosteine caused a slight and nonsignificant decrease in the LDCL of human neutrophils induced by PMA (5×10^{-9} mol/l) even at a concentration of 1×10^{-3} mol/l (36% inhibition at 1×10^{-3} mol/l). On the other hand, M1 significantly inhibited this reaction at 1×10^{-4} mol/l or higher (IC_{50} of 7.5×10^{-5} mol/l). S-CMC, ambroxol and NAC, comparative drugs for expectoration, also significantly suppressed this reaction, but these drugs needed higher concentration than M1 to inhibit the reaction (48% inhibition at 1×10^{-3} mol/l of S-CMC, IC_{50} of 9.8×10^{-5} and 1.2×10^{-4} mol/l for ambroxol and NAC, respectively) (Fig. 2C).

Since M1 is a racemic compound containing a chiral carbon atom, the effect of each enantiomer of M1 on this reaction was investigated. Both (+) and (–) isomers significantly inhibited this reaction at a concentration of 1×10^{-4} mol/l or higher, and these results were similar to those of the racemate (data not shown).

Moreover, PMA-induced LDCL was blocked by SOD (O_2^- scavenger), catalase (H_2O_2 scavenger), NaN_3 (myeloperoxidase (MPO) inhibitor) and GSH (a physiological reducing agent) (Fig. 3).

Effect of posttreatment of M1 on LDCL of human neutrophils

To test whether or not M1 has a direct scavenging effect, the change in the LDCL of human neutrophils by the addition of M1 after stimulation with PMA was observed. By the addition of 3×10^{-5} or 1×10^{-4} mol/l of GSH at the plateau of PMA-induced LDCL, a rapid decrease in LDCL was observed (Fig. 4 A). The change in LDCL by posttreatment with M1 was similar to that in GSH (Fig. 4 B).

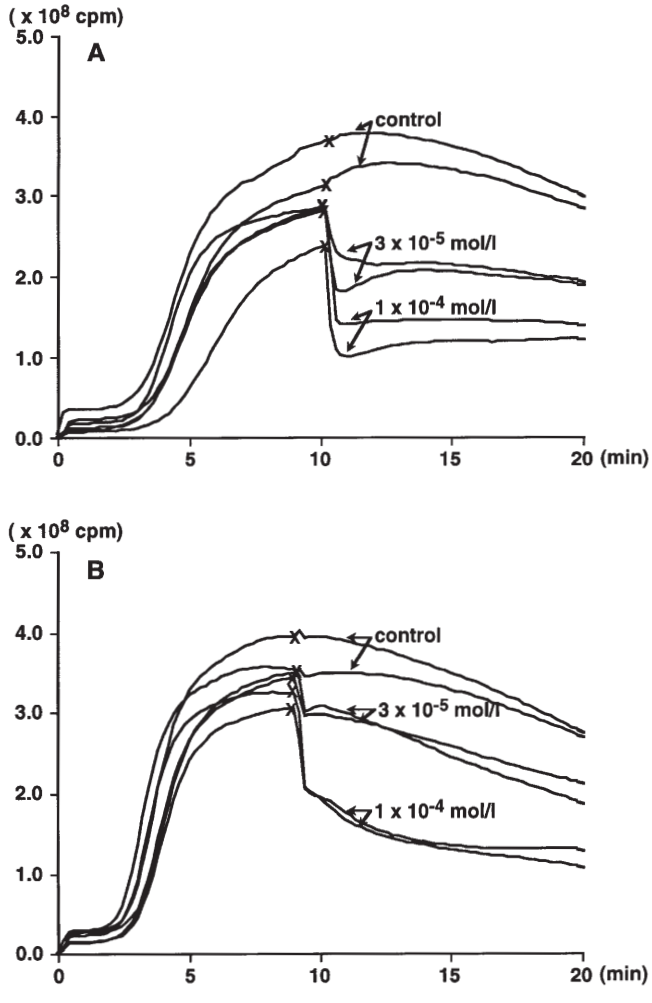

Fig. 4. Changes in PMA-induced LDCL of human neutrophils by the addition of GSH (A) or M1 (B) after stimulation. Human neutrophils were preincubated with luminol for 10 min at 37 °C. Neutrophils were stimulation with PMA (5×10^{-9} mol/l) at 0 min. Hanks' solution (control) or test drug solution was added to the cell suspension at the moment indicated by crosses.

Discussion

It has been suggested that reactive oxygen species produced by inflammatory cells have the capacity to cause inflammation and tissue injury in respiratory diseases [1, 2]. Although it has been reported that erdosteine exhibits inhibitory activity against the effects of free radicals produced by cigarette smoke [7–9], it remains unclear whether erdosteine affects inflammatory cells-derived reactive oxygen species. We found that erdosteine was rapidly metabolized to an active metabolite, M1 (unpublished data). Therefore, we investigated the effect of M1 on the LDCL of rat peritoneal neutrophils induced by PMA to elucidate its effect on reactive oxygen species. M1 significantly inhibited this reaction at 3×10^{-5} mol/l or higher, demonstrating that M1 has scavenging effects on free radicals. Moreover, erdosteine also inhibited this reaction, but its effect was weaker than that of Ml.

Eosinophils produce reactive oxygen species, but the mechanism of production is different from that of neutrophils [11]. In the present experiment, guinea pig peritoneal eosinophils were used as they were easy to collect. As a result, it was evident that M1 was effective on eosinophil-derived reactive oxygen species.

Because it was found that M1 has an inhibitory effect on the LDCL of animal cells, we carried out a further experiment using human neutrophils to elucidate the efficacy of M1 in humans. Considering that M1 significantly inhibited the PMA-induced LDCL of human neutrophils at a concentration of 1×10^{-4} mol/l or higher, M1 may regulate reactive oxygen species in humans. It has been shown that the main reactive oxygen species from activated human neutrophils is HOCl, produced by MPO using O_2^-/H_2O_2 and Cl^- [12]. Since PMA-induced LDCL was blocked by SOD (O_2^- scavenger), catalase (H_2O_2 scavenger) and NaN_3 (MPO inhibitor) in the present experiment, it was confirmed that the production of reactive oxygen species in this reaction was catalyzed by MPO. It has been reported that GSH, a physiological agent, and NAC, a cysteine-derivative expectorant, scavenge H_2O_2 and HOCl by the reducing effect of the SH group [13, 14]. In this study, pretreatment with either drug significantly inhibited the PMA-induced LDCL of human neutrophils. M1 which has a SH group inhibited the LDCL of human neutrophils, and the rapid decrease in LDCL was observed by the addition of M1 after stimulation. From these observations, it was concluded that the inhibitory effect of M1 on LDCL might be based on free radical scavenging activity by the reducing effect of the SH group.

A comparative evaluation was carried out using S-CMC and ambroxol which are expectorants without a free SH group. As a result, both drugs needed higher concentrations than M1 to inhibit LDCL. Although ambroxol does not have a scavenging effect on H_2O_2, it has a scavenging effect on HOCl as strong as GSH. Its effect may be related to the amino group of the molecule [14]. Although the effect of S-CMC on reactive oxygen species is unknown, S-CMC has an amino group. Furthermore, a rapid decrease in LDCL was observed by the addition of S-CMC after stimulation (data not shown). Therefore, it was considered that S-CMC may scavenge free radicals as does ambroxol.

There are differences in the effects of the drugs on the different cell population tested. The differences between cells prepared by different methods and the different reactive oxygen species produced by neutrophils and eosinophils might account for the result. We have confirmed that the peak plasma concentration (C_{max}) of M1 was 1.3×10^{-5} mol/l with oral administration of erdosteine at 300 mg/man (data not shown). In this study, M1 failed to inhibit PMA-induced LDCL of human neutrophils at 1×10^{-5} mol/l. However, the effect of antioxidant is dependent on the amount of reactive oxygen species produced. Thus, it is considered that M1 may show the inhibitory effect on the reaction induced by physiological stimulant such as FMLP at a concentration equivalent to that in plasma.

In conclusion, M1 which is an active metabolite of erdosteine regulated reactive oxygen species produced by rat neutrophils, guinea pig eosinophils and human neutrophils. By the scavenging effect of M1, erdosteine might exert an inhibitory effect on inflammation in which neutrophils and eosinophils are involved.

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