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In vitro effects of E3040, a dual inhibitor of 5-lipoxygenase and thromboxane A_2 synthetase, on eicosanoid production

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Abstract

In vitro pharmacological profiles of E3040, 6-hydroxy-5, 7-dimethyl-2-(methylamino)-4-(3-pyridylmethyl) benzothiazole were investigated. Against the 5-lipoxygenase activity of rat basophilic leukemia cells, E3040 and zileuton (a 5-lipoxygenase inhibitor) had an IC_{50} of 0.23 and 0.93 μM, respectively. Against the thromboxane A_2 synthetase activity of human platelets, E3040 had an IC_{50} of 0.01 μM, which was comparable to that of OKY-1581 (sodium (*E*)-3-[4-(3-pyridylmethyl) phenyl]-2-methylacrylate, a thromboxane A_2 synthetase inhibitor). Against cyclooxygenase activity of sheep seminal vesicles, E3040 showed no inhibition (IC_{50}, > 300 μM). Sulfasalazine and 5-aminosalicylic acid, therapeutic drugs for inflammatory bowel disease, inhibited 5-lipoxygenase activity with an IC_{50} of 293 and 970 μM, respectively. Sulfasalazine inhibited thromboxane A_2 synthetase activity with an IC_{50} of 20 μM. In rat peritoneal leukocytes, E3040 inhibited leukotriene B_4 and thromboxane B_2 production with an IC_{50} of 0.17 and 0.24 μM, respectively. E3040 inhibited leukotriene B_4 production in human neutrophils and thromboxane B_2 production in human platelets (IC_{50} of 0.21 and 0.09 μM, respectively). These results indicated that E3040 potently inhibited 5-lipoxygenase and thromboxane A_2 synthetase and blocked leukotriene B_4 and thromboxane B_2 production in rat peritoneal and human blood cells.

Keywords: E3040; 5-Lipoxygenase; Thromboxane A_2 synthetase

1. Introduction

The inflamed mucosa in inflammatory bowel disease characteristically reveals infiltrating inflammatory cells mainly comprising neutrophils, macrophages and lymphocytes. Studies have shown the excessive mucosal generation of inflammatory mediators, such as leukotriene B_4, thomboxane B_2, prostaglandin E_2 and cytokines (Nielsen and Rask-Madsen, 1996; Lauritsen et al., 1988; Carty et al., 2000) in patients with inflammatory bowel disease. These findings suggest that soluble mediators are involved in inflammation in the digestive tract of patients with inflammatory bowel disease. Conventional therapy for inflammatory bowel disease with sulfasalazine and prednisolone may act by modulating the profile of these mediators, and recent novel agents have been specifically targeted to inhibit the excessive production of leukotriene (Laursen et al., 1990; Roberts et al., 1997), thomboxane (Casellas et al., 1995) and tumor necrosis factor-α (Targan et al., 1997).

Among the inflammatory mediators, leukotriene B_4 is known as a potent mediator of chemotaxis and aggregation of neutrophils. In fact, leukotriene B_4 accounted for most of the chemotactic activity detected in rectal dialysate from colitis patients (Lobos et al., 1987). Thomboxane is known as a proinflammatory mediator that can induce vasoconstriction, which might result in damage to the gastrointestinal and colonic mucosa. In experimental animal models, thomboxane A_2 promoted gastric mucosal ulceration and necrosis (Whittle et al., 1981). Prostaglandin E_2, by contrast, has many proinflammatory actions, contributing to vascular permeability, edema and intestinal fluid secretion, but treatment with cyclooxygenase inhibitors does not improve colonic lesions but rather causes relapse in patients with inflammatory bowel disease (Campieri et al., 1980; Rampton and Sladen, 1981). In animals, exogenous administration of prostaglandin E_2 has been shown to prevent colonic mucosal damage induced by the intracolonic application of necrotizing agents (Wallace et al., 1985; Allgayer et al., 1989). These results suggest that

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prostaglandin E_2 is important for the cytoprotective action in the colonic mucosa (Rampton and Hawkey, 1984).

We have previously reported the structure–activity relationship of 3-pyridylmethyl-substituted 2-amino-6-hydroxybenzothiazole derivatives as novel dual inhibitors of 5-lipoxygenase and thomboxane A_2 synthetase (Hibi et al., 1994) and as potential therapeutic drugs for inflammatory bowel disease. In the present study, we investigated the effects of E3040 (6-hydroxy-5, 7-dimethyl-2-(methylamino)-4-(3-pyridylmethyl)benzothiazole) on 5-lipoxygenase and thromboxane A_2 synthetase in a cell-free system and on leukotriene B_4 and thromboxane B_2 production in rat and human cells, comparing the results with the effects of reference compounds such as zileuton (a 5-lipoxygenase inhibitor) (Carter et al., 1991), OKY-1581 (a thromboxane A_2 synthetase inhibitor) (Feuerstein and Ramwell, 1981), indomethacin (a cyclooxygenase inhibitor), and sulfasalazine and 5-aminosalycylic acid, the last two compounds having been used widely as therapeutic drugs for inflammatory bowel disease.

2. Materials and methods

2.1. Animals

Nine-week-old male Fisher rats (250–275 g) were purchased from Charles River Japan (Kanagawa). Animals were housed in a cage with a wire-mesh bottom in a room at 23°C with a 12-h light/dark cycle. Standard laboratory rat chow and tap water were available ad libitum. Experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals recommended by the Japanese Pharmacological Society and with the recommendations of the Declaration of Helsinki.

2.2. Drugs and chemicals

E3040 (Fig. 1), zileuton, OKY-1581 (sodium (*E*)-3-[4-(3-pyridylmethyl) phenyl]-2-methylacrylate) and BW755C (3-amino-1-[3-(trifluoromethyl) phenyl]-2-pyrazoline) were synthesized at Eisai. The other drugs and chemicals were obtained from commercial sources: Sulfasalazine, indomethacin, bovine serum albumin and dimethyl sulfoxide (DMSO) (Sigma, USA); 5-aminosalicylic acid (Aldrich, USA); calcium ionophore A23187 (Cabiochem, USA); fetal bovine serum, RPMI 1640 containing 25 mM HEPES and L-glutamine, Hanks balanced salt solution (HBSS) free or plus Ca^{2+} and Mg^{2+} (Gibco Laboratories, Life Technologies, USA); Percol (Pharmacia, Sweden); arachidonic acid, prostaglandin H_2, (±)5-hydroxy-(6E,8Z,11Z,14Z)-eicosatetraenoic acid (racemic 5-HETE), (±)13-hydroxy-(9*Z*,11*E*)-octadecadienoic acid (racemic 13-HODE) and enzyme immunoassay (EIA) kit for leukotriene B_4, thromboxane B_2 and prostaglandin E_2 (Cayman Chemical, USA).

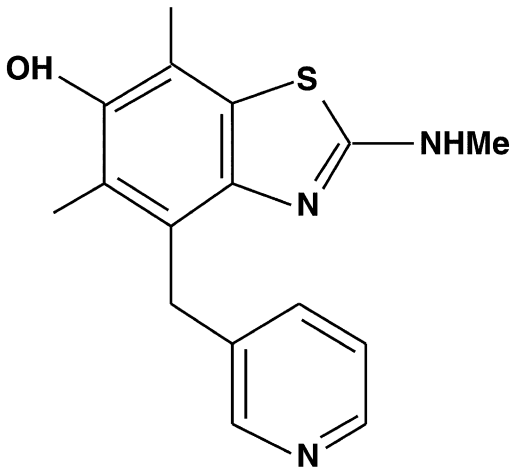

Fig. 1. Chemical structure of E3040.

2.3. RBL-1 cell 5-lipoxygenase

Adherent rat basophilic leukemia (RBL-1) cells were cultured in RPMI 1640 medium containing 25 mM HEPES with 10% fetal bovine serum and were harvested by scraping with a Cell Scraper (32 cm, InterMed, Nunc). The cells were washed with cold saline and suspended in a 50 mM phosphate buffer containing 0.25 M sucrose and 1 mM EDTA, pH 7.4 (approx. 1×10^7 cells/ml). The cell suspension was sonicated using a Branson Sonifier (Cell Disruptor 185, Branson Sonic). The homogenate was centrifuged at 40,000 rpm for 60 min at 4°C (Beckman, L-90K) and the supernatant was stored at −20°C until assayed for 5-lipoxygenase activity. The enzyme solution (0.5 ml) containing 2 mM glutathione was preincubated with test compounds or vehicle (final conc., 0.1% DMSO/0.1% bovine serum albumin/HBSS free of Ca^{2+} and Mg^{2+}) for 5 min at 37°C. The enzyme reaction was initiated by adding arachidonic acid (final conc., 2×10^{-4} M) and $CaCl_2$ (final conc., 2×10^{-3} M) and terminated after 5 min by acidification with ice-cold formic acid (final conc., 0.2 N). 13-HODE in methanol solution was added to each sample as an internal standard for high-performance liquid chromatography (HPLC) analysis. Extraction was carried out by adding two volumes (approx. 1 ml) of ethyl acetate. After centrifugation, the organic phase was removed, evaporated to dryness under a nitrogen stream and dissolved in 200 μl of methanol. The samples were injected (Waters 712 WISP automatic injector, Millipore, USA) onto a Hypersil BDS C18 column (3 μm, 150 × 4.6 mm, Shandon Scientific, UK). A mixture of methanol/water/acetic acid (70:25:0.001, v/v) was used as the mobile phase with a flow rate of 0.9 ml/min (Beckman, 100A). The quantitation of 5-HETE was performed by measuring UV absorbance at 235 nm (Jasco 875-UV Intelligent UV/VIS Detector, Japan Spectroscopic, Unicorder U-228, Nippon Denshi Kagaku, Japan). The level of 5-

HETE in the sample was calculated using an external standard and based on peak height.

2.4. Human platelet thromboxane A_2 synthetase

Human platelets were isolated from the blood of healthy volunteers. The platelets were separated from platelet-rich plasma by centrifugation at 2500 rpm for 15 min at 4°C and suspended in phosphate-buffered saline. The platelets were homogenized with a Branson Sonifier (Cell Disruptor 185, Branson Sonic). The homogenate was centrifuged at 40,000 rpm for 60 min at 4°C (Beckman, L-90K) and the pellets were suspended in 50 mM Tris–HCl–saline, pH 8.0 (approx. 1.2 mg of protein/ml) and stored at −80°C until assayed for thromboxane A_2 synthetase activity. The enzyme solution (0.2 ml) containing 10 μM indomethacin was preincubated with test compounds or vehicle for 5 min at 25°C. The enzyme reaction was initiated by adding prostaglandin H_2 (final conc., 10 μg/ml) and terminated after 1 min by acidification with ice-cold citric acid/ethanol solution (final conc., 5.5 mM/10%). After centrifugation at 3000 rpm for 10 min at 4°C, the supernatant was assayed for thromboxane B_2 (a non-enzymatically hydrolyzed product of thromboxane A_2) level by enzyme immunoassay (EIA).

2.5. Sheep seminal vesicle cyclooxygenase

Sheep seminal vesicle gland microsomes were dissolved in 100 mM Tris–HCl, pH 7.4 containing 5 mM tryptophan and 5 mM glutathione (approx. 2.3 mg of protein/ml). The enzyme solution (80 μl) was preincubated with test compounds or vehicle for 10 min at 25°C. The enzyme reaction was initiated by adding arachidonic acid (final conc., 5 μg/ml) and terminated after 20 min by adding indomethacin (final conc., 0.1 mM). After centrifugation at 3000 rpm for 10 min at 4°C, the supernatant was assayed for prostaglandin E_2 level by EIA.

2.6. Eicosanoid production in rat peritoneal leukocytes

Leukocytes were obtained from the peritoneal cavity of rats 20 h after an i.p. injection of 10 ml of a 6% glycogen solution in sterile saline, as reported by Moroney et al. (1990). After the rats had been anesthetized, 60 ml of ice-cold HBSS free of Ca^{2+} and Mg^{2+} was injected intraperitoneally and the peritoneal wash was removed with a syringe and centrifuged at 1000 rpm for 10 min at 4°C. The cells were washed twice with HBSS free of Ca^{2+} and Mg^{2+} and suspended in HBSS containing Ca^{2+} and Mg^{2+} (approx. 5×10^6 cells/ml). Viability, based on Trypan blue exclusion, was better than 95%. The cell suspension (100 μl) was preincubated with test compounds or vehicle for 5 min at 37°C. The reaction was initiated by adding A23187 (final conc., 2 μM) and terminated after 10 min by adding BW755C (a dual inhibitor of 5-lipoxygenase and cyclooxygenase, final conc., 0.1 mM). After the solution had been centrifuged at 3000 rpm for 10 min at 4°C, the supernatant was assayed for leukotriene B_4, thromboxane B_2 and prostaglandin E_2 level by EIA. No cytotoxic effects on the cells were observed after incubation with the test compounds.

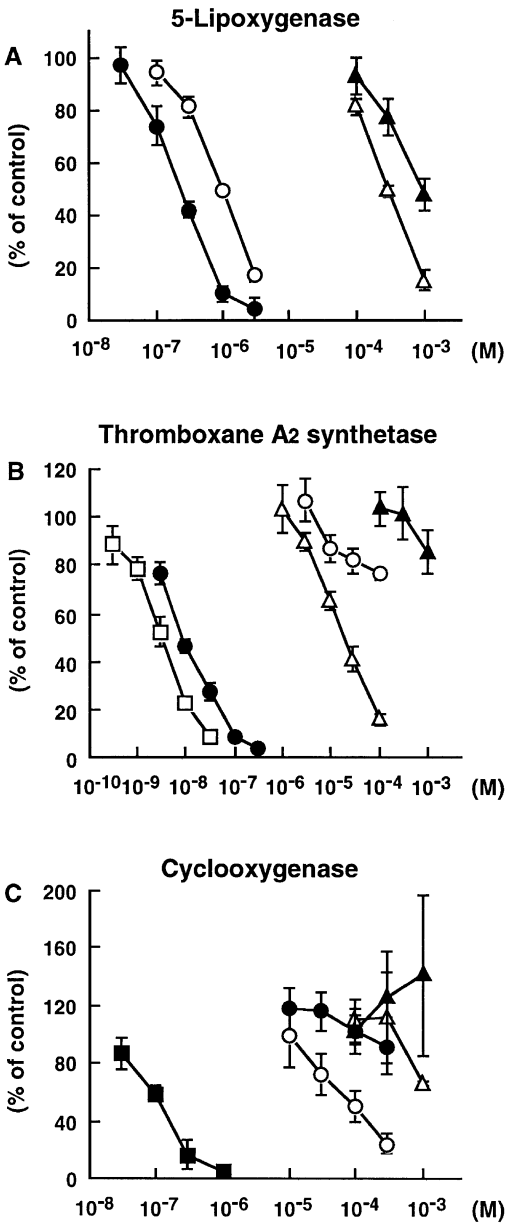

Fig. 2. Effects of E3040, zileuton, sulfasalazine and 5-aminosalicylic acid on 5-lipoxygenase of RBL-1 cells, thromboxane A_2 synthetase of human platelets and cyclooxygenase of sheep seminal vesicles. Test drugs were preincubated with each enzyme solution before arachidonic acid was added. 5-HETE for 5-lipoxygenase activity, thromboxane B_2 for thromboxane A_2 synthetase activity and prostaglandin E_2 for cyclooxygenase activity were measured by HPLC or EIA. Each value represents the mean ± S.D. of three experiments, each performed in duplicate. ●, E3040; ○, zileuton; △, sulfasalazine; ▲, 5-aminosalicylic acid; □, OKY-1581; ■, indomethacin; (A) 5-lipoxygenase; (B) thromboxane A_2 synthetase; (C) cyclooxygenase.

2.7. Leukotriene B_4 production in human neutrophils

Human neutrophils were isolated from the blood of healthy volunteers by dextran sedimentation (final conc., 1.8% dextran and 1.5 U/ml heparin in saline) followed by Percol gradient separation (density: 1.10, 1.08, 1.04, 500 g for 15 min at 25°C). The neutrophils were suspended in HBSS containing Ca^{2+} and Mg^{2+} (approx. 1.2×10^6

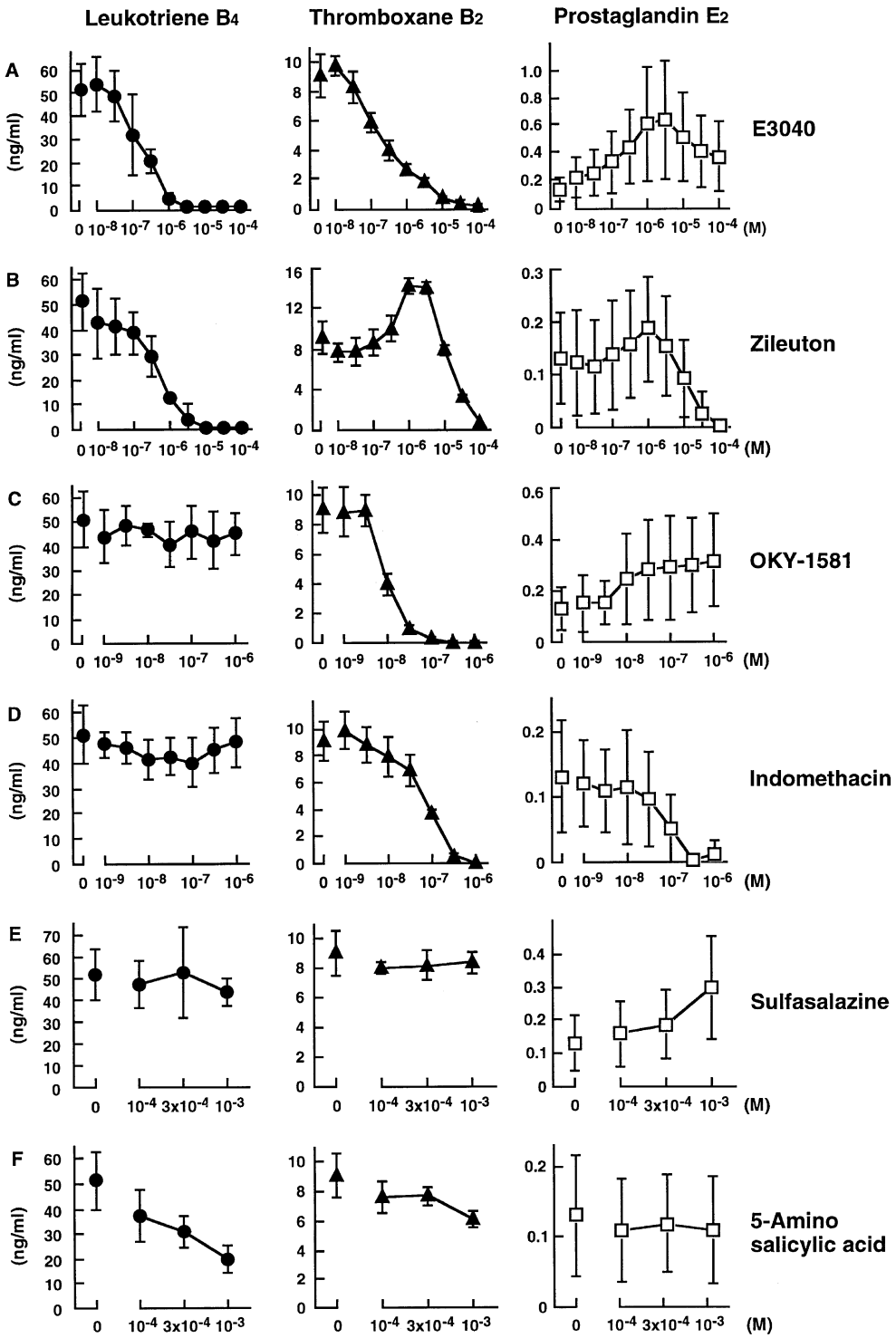

Fig. 3. Effects of E3040, zileuton, OKY-1581, indomethacin, sulfasalazine and 5-aminosalicylic acid on leukotriene B_4, thromboxane B_2 and prostaglandin E_2 production in rat peritoneal leukocytes. Test drugs were preincubated with cell suspensions before A23187 was added. The eicosanoid level in the incubation medium was determined by EIA. Each value represents the mean ± S.D. of three experiments, each performed in duplicate. ●, leukotriene B_4; ▲, thromboxane B_2; □, prostaglandin E_2; (A) E3040; (B), zileuton; (C) OKY-1581; (D) indomethacin; (E) sulfasalazine; (F) 5-aminosalicylic acid.

cells/ml). The cell suspension (160 μl) was preincubated with test compounds or vehicle for 5 min at 37°C. The reaction was initiated by adding A23187 (final conc., 2 μM) and terminated after 10 min by adding BW755C (final conc., 0.1 mM). After the solution had been centrifuged at 3000 rpm for 10 min at 4°C, the supernatant was assayed for leukotriene B_4 level by EIA.

2.8. Thromboxane B_2 production in human platelets

Human platelets were isolated from the blood of the same volunteers as described above. The platelets were suspended in 50 mM Tris–HCl, pH 7.4, containing 10 μM indomethacin (approx. 3×10^8 cells/ml). The cell suspension (200 μl) was preincubated with test compounds or vehicle for 5 min at 25°C. The reaction was initiated by adding prostaglandin H_2 (final conc., 1 μg/ml) and terminated after 3 min by acidification with 0.9 ml of a 0.2 M citric acid/100% ethanol (5:180, v/v) solution. After the solution had been centrifuged at 3000 rpm for 10 min at 4°C, the supernatant was assayed for thromboxane B_2 level by EIA.

2.9. Statistics

Results are expressed as the means ± standard deviation (S.D.). Linear least-squares regression was used to estimate IC_{50} values.

3. Results

3.1. Effect of E3040 and reference compounds on 5-lipoxygenase, thromboxane A_2 synthetase and cyclooxygenase activity

The inhibitory effects of E3040 and reference compounds on the 5-lipoxygenase activity of RBL-1 cell cytosol are shown in Fig. 2A. E3040 inhibited the enzyme in a concentration-dependent manner with an IC_{50} of 0.23 (0.21–0.26) μM. Zileuton, an inhibitor of 5-lipoxygenase, also inhibited the enzyme, with an IC_{50} of 0.93 (0.88–0.99) μM, a potency approximately 4-fold weaker than that of E3040. Sulfasalazine and 5-aminosalicylic acid inhibited the enzyme only at high concentrations with an IC_{50} of 293 (294–313) and 970 (703–1622) μM, respectively.

The inhibitory effects of E3040 and reference compounds on the thromboxane A_2 synthetase activity of human platelet cytosol are shown in Fig. 2B. E3040 potently inhibited the enzyme in a concentration-dependent manner with an IC_{50} of 0.01 (0.008–0.11) μM. OKY-1581, an inhibitor of thromboxane A_2 synthetase, also inhibited the enzyme, with an IC_{50} of 0.003 (0.0029–0.0037) μM, making it approximately 3-fold more potent than E3040. Sulfasalazine inhibited the enzyme with an IC_{50} of 20 (18–23) μM. Zileuton and 5-aminosalicylic acid had no effect at 100 and 1000 μM, respectively.

The inhibitory effects of E3040 and reference compounds on the cyclooxygenase activity of sheep seminal vesicle microsomes are shown in Fig. 2C. E3040 had no effect on the enzyme at 300 μM. Indomethacin, an inhibitor of cyclooxygenase, potently inhibited it with an IC_{50} of 0.11 (0.087–0.14) μM. Zileuton inhibited the enzyme at a high concentration with an IC_{50} of 92 (64–130) μM. Sulfasalazine and 5-aminosalicylic acid had little or no effect even at 1 mM (about 30% and 0% inhibition, respectively).

3.2. Effect of E3040 and reference compounds on eicosanoid production in rat peritoneal leukocytes

Rat peritoneal leukocytes produced leukotriene B_4, thromboxane B_2 and prostaglandin E_2 after stimulation with A23187. The amount of each mediator in the assay medium was 51.29 ± 11.72, 9.07 ± 1.51 and 0.13 ± 0.09

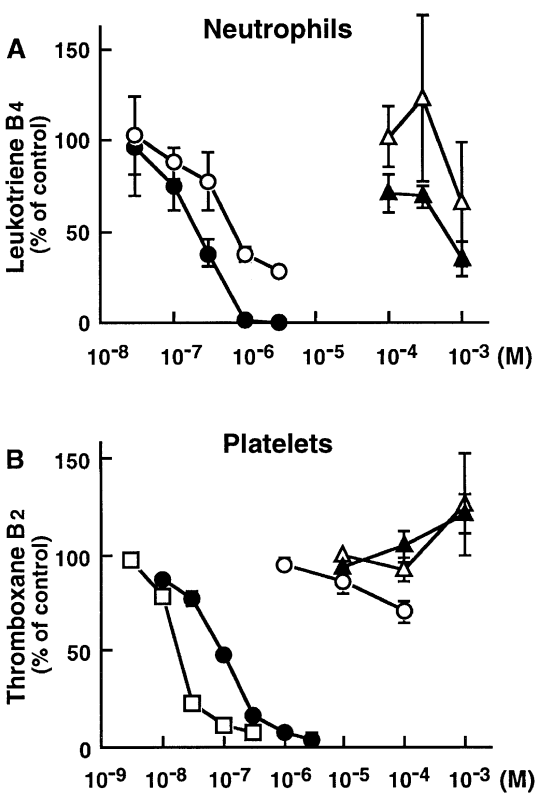

Fig. 4. Effects of E3040, zileuton, sulfasalazine and 5-aminosalicylic acid on leukotriene B_4 and thromboxane B_2 production in human neutrophils and platelets. Test drugs were preincubated with cell suspensions before the addition of A23187 for leukotriene B_4 assay or prostaglandin H_2 for thromboxane B_2 assay, respectively. Leukotriene B_4 and thromboxane B_2 levels were determined by EIA. Each value represents the mean ± S.D. of three experiments, each performed in duplicate. ●, E3040; ○, zileuton; □, OKY-1581; △, sulfasalazine; ▲, 5-aminosalicylic acid; (A) neutrophils; (B) platelets.

Table 1
Effects of E3040 and reference compounds on 5-lipoxygenase, thromboxane A_2 synthetase, cyclooxygenase activity and eicosanoid production

	E3040	Sulfasalazine	5-Amino salicylic acid	Zileuton	OKY-1581	Indomethacin
Enzymes						
5-Lipoxygenase	0.23	293	970	0.93	–	–
Thromboxane A_2 synthetase	0.01	20	> 1000	> 100	0.003	–
Cyclooxygenase	> 300	> 1000	> 1000	92	–	0.11
Rat peritoneal cells						
Leukotriene B_4	0.17	> 1000	510	0.35	> 1	> 1
Thromboxane B_2	0.24	> 1000	> 1000	25	0.01	0.07
Prostaglandin E_2	> 100	> 1000	> 1000	13	> 1	0.06
Human blood cells						
Leukotriene B_4	0.21	> 1000	522	0.69	–	–
Thromboxane B_2	0.09	> 1000	> 1000	> 100	0.02	–

The values in this table represent IC_{50} in μM. Linear least-squares regression was used to estimate IC_{50} from the mean of dose–response curve of three experiments, each performed in duplicate. –: No determination.

ng/ml (Means ± S.D., $n = 3$), respectively. E3040 inhibited leukotriene B_4 and thromboxane B_2 production in a concentration-dependent and well-balanced manner with an IC_{50} of 0.17 (0.06–0.26) and 0.24 (0.20–0.28) μM, respectively (Fig. 3A). Zileuton inhibited leukotriene B_4 production in a concentration-dependent manner with an IC_{50} of 0.35 (0.25–0.51) μM (Fig. 3B). This compound inhibited thromboxane B_2 and prostaglandin E_2 production at a higher concentration with an IC_{50} of 25 (20–31) and 13 (10–17) μM, respectively. OKY-1581 potently inhibited thromboxane B_2 production with an IC_{50} of 0.01 (0.008–0.012) μM (Fig. 3C). Indomethacin inhibited both prostaglandin E_2 and thromboxane B_2 production with an IC_{50} of 0.06 (0.038–0.081) and 0.07 (0.065–0.079) μM, respectively (Fig. 3D). Sulfasalazine did not inhibit leukotriene B_4 and thromboxane B_2 production at 1 mM (Fig. 3E). 5-Aminosalicylic acid inhibited leukotriene B_4 production with an IC_{50} of 510 (329–1172) μM and had almost no effect on thromboxane B_2 and prostaglandin E_2 production at 1 mM (Fig. 3F).

3.3. Effect of E3040 and reference compounds on leukotriene B_4 and thromboxane B_2 production in human neutrophils and platelets

E3040 inhibited leukotriene B_4 production in a concentration-dependent manner with an IC_{50} of 0.21 (0.17–0.26) μM in human neutrophils (Fig. 4A). Zileuton also inhibited leukotriene B_4 production with an IC_{50} of 0.69 (0.45–1.69) μM. Sulfasalazine had no effect at 1 mM and 5-aminosalicylic acid inhibited leukotriene B_4 production with an IC_{50} of 522 (317–1610) μM. E3040 inhibited thromboxane B_2 production in a concentration-dependent manner with an IC_{50} of 0.09 (0.080–0.094) μM in human platelets (Fig. 4B). OKY-1581 also inhibited thromboxane B_2 production with an IC_{50} of 0.02 (0.003–0.17) μM. Zileuton, sulfasalazine and 5-aminosalicylic acid had no effect at 0.1 and 1 mM, respectively.

IC_{50} values of E3040 and the reference compounds are summarized in Table 1.

4. Discussion

The present in vitro experiments on the activity of 5-lipoxygenase, thromboxane A_2 synthetase and cyclooxygenase in the arachidonic acid pathway characterized E3040 as a dual inhibitor of 5-lipoxygenase and thromboxane A_2 synthetase. E3040 produced a concentration-dependent inhibition of 5-lipoxygenase in the supernatant of RBL-1 cell homogenates, with an IC_{50} of 0.23 μM. The inhibitory effect of E3040 was about 4-fold more potent than that of zileuton, a 5-lipoxygenase inhibitor which has been clinically tested for the treatment of inflammatory bowel disease (Laursen et al., 1990). The chemical structure of zileuton contains a modified hydroxamic acid moiety (*N*-hydroxyurea), and it is speculated that this moiety interacts with the putative iron atom at the active site of the enzyme. However, E3040 may inhibit 5-lipoxygenase through a redox mechanism by virtue of its 6-hydroxybenzothiazole moiety, which has previously been shown to inhibit 5-lipoxygenase (Abe et al., 1988). This inhibitory mechanism is well known in other 5-lipoxygenase inhibitors such as nordihydroguaiaretic acid (NDGA) and BW755C. E3040 also potently inhibited thromboxane A_2 synthetase in the supernatant of human platelets with an IC_{50} of 0.01 μM. The 3-pyridyl moiety contained in the compound is known to selectively bind to the iron residue of thromboxane A_2 synthetase (Tanouchi et al., 1981). The inhibitory potency of E3040 is nearly the same as that of OKY-1581, a selective thromboxane A_2 inhibitor. Comparison of the inhibitory potency of E3040 on 5-lipoxygenase and on thromboxane A_2 synthetase showed that the inhibition of thromboxane A_2 synthetase was about 20-fold more potent than that of 5-lipoxygenase. E3040 had no effect on sheep seminal vesicle cyclooxygenase in concentrations up to 300 μM.

We further clarified the characteristics of E3040 as a dual inhibitor in rat peritoneal cells. Moroney et al. (1990) have reported that glycogen-elicited rat peritoneal leukocytes serve as a convenient and useful system to investigate inhibition of the 5-lipoxygenase and cyclooxygenase pathway of arachidonate metabolism. The mixed peritoneal leukocytes, which contained approximately 75% polymorphonuclear leukocytes and 25% mononuclear leukocytes, produced leukotriene B_4, thromboxane B_2 and prostaglandin E_2 on stimulation with the calcium ionophore A23187 in a different regulatory manner. Consistent with the effects on the cell-free enzyme activity, E3040 inhibited both leukotriene B_4 and thromboxane B_2 production (IC_{50} of 0.17 and 0.24 μM, respectively) but not prostaglandin E_2 production in the cells. To ascertain the inhibitory effects of E3040 in human cells, human peripheral neutrophils and platelets were used to assay eicosanoid production. Neutrophils, which substantially infiltrate the inflamed colonic mucosa, are considered to metabolize arachidonic acid mainly via the 5-lipoxygenase pathway to produce leukotriene B_4, whereas platelets exclusively produce thromboxane B_2. The present data indicated that E3040 also had inhibitory effects on both leukotriene B_4 and thromboxane B_2 production in neutrophils and platelets with an IC_{50} of 0.21 and 0.09 μM, respectively. The IC_{50} values of E3040 on leukotriene B_4 production in rat and human leukocytes were almost the same as that on 5-lipoxygenase activity in RBL-1 cell homogenates; however, the IC_{50} values for thromboxane B_2 production in the cells were much higher than that for thromboxane A_2 synthetase activity in human platelet homogenates. The reason for the differences in the inhibitory potency of E3040 for thromboxane A_2 synthetase between cell-free and cell systems is not clear, but might be related to the assay conditions: arachidonic acid, A23187 and prostaglandin H_2 were used as enzyme substrate in the cell homogenates, the rat peritoneal cells and human platelets, respectively.

Zileuton inhibited leukotriene B_4 production (IC_{50} of 0.35 μM) in rat peritoneal leukocytes. However, the compound at higher concentrations also inhibited thromboxane B_2 and prostaglandin E_2 production (IC_{50} of 25 and 13 μM, respectively). In the enzyme assays, zileuton inhibited cyclooxygenase activity with a relatively high IC_{50} (92 μM) but had no effect on thromboxane A_2 synthetase activity. While this IC_{50} value for cyclooxygenase was slightly lower than that previously reported (Carter et al., 1991; Fruchtmann et al., 1993), these findings indicated that zileuton had also an inhibitory effect on the cyclooxygenase pathway in rat leukocytes at higher concentrations. OKY-1581 selectively inhibited thromboxane B_2 production and indomethacin inhibited both thromboxane B_2 and prostaglandin E_2 production in rat peritoneal leukocytes. Sulfasalazine and 5-aminosalicylic acid are widely used for the treatment of inflammatory bowel disease. Several mechanisms of these drugs have been postulated (Greenfield et al., 1993), and the inhibitory effects on eicosanoid production have been investigated. In the present study, sulfasalazine and 5-aminosalicylic acid showed weak inhibitory effects on 5-lipoxygenase (IC_{50} of 293 and 970 μM, respectively) and cyclooxygenase (about 30% inhibition and no inhibition at 1 mM, respectively). However, sulfasalazine inhibited thromboxane A_2 synthetase at a relatively low concentration (IC_{50} of 20 μM), as reported by Stenson and Lobos (1983). In the cell assay systems, sulfasalazine and 5-aminosalicylic acid showed weak or no inhibition of leukotriene B_4, thromboxane B_2 and prostaglandin E_2 production, even at 1 mM. These compounds have been reported to exert their inhibition of eicosanoid production at higher concentrations (Sharon and Stenson, 1984; Stenson and Lobos, 1982; Pescar et al., 1987).

An imbalance in the intestinal immune system has been reported in the pathogenesis of inflammatory bowel disease (Sartor, 1994), and sulfasalazine or 5-aminosalicylic acid has been shown to affect cytokine production (Mahida et al., 1991; Fujiwara et al., 1990; Liptay et al., 1999). In studies using peripheral blood mononuclear cells from patients with inflammatory bowel disease, E3040 showed inhibitory activity against interleukin1-β, interleukin1-ra and tumor necrosis factor-α production from lipopolysaccharide-stimulated cells (Mitsuyama et al., unpublished observation). It is not clear whether the inhibitory action against cytokine production caused by E3040 is related to its inhibition of eicosanoid production (Cominelli et al., 1989; Spangelo et al., 1991). Further studies are necessary to clarify the inhibitory effects of E3040 on cytokine production.

In conclusion, E3040 potently inhibited cell-free 5-lipoxygenase and thromboxane A_2 synthetase but not cyclooxygenase. The compound inhibited both leukotriene B_4 and thromboxane B_2 production in a well-balanced manner in rat peritoneal leukocytes, human neutrophils and platelets. The properties of E3040, a novel dual inhibitor of 5-lipoxygenase and thromboxane A_2 synthetase, may make it a potential new therapeutic drug for inflammatory bowel disease.

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