In Vitro Antagonism of ONO-1078, a Newly Developed Anti-Asthma Agent, against Peptide Leukotrienes in Isolated Guinea Pig Tissues

Takaaki Obata, Yutaka Okada, Mariko Motoishi, Naoki Nakagawa, Tamiya Terawaki and Hideki Aishita

Minase Research Institute, Ono Pharmaceutical Co., Ltd., 3-1-1 Sakurai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

Received June 8, 1992 Accepted August 13, 1992

ABSTRACT—We evaluated the antagonist activity of ONO-1078 against peptide leukotrienes (LTs) by a radioligand binding assay and functional experiments in guinea pigs. In the radioligand binding assay, ONO-1078 inhibited [3H]LTD₄ and [3H]LTE₄ bindings to lung membranes (Kᵢ = 0.99 and 0.63 nM, respectively) and was 2,000- to 3,000-fold more potent than FPL55712. Antagonism of ONO-1078 against [3H]LTC₄ binding (Kᵢ = 5640 nM) was approximately twofold more potent than that of FPL55712. The antagonism of ONO-1078 against [3H]LTD₄ binding was competitive. In functional experiments, ONO-1078 showed competitive antagonism against the LTC₄- and LTD₄-induced contractions of guinea pig trachea and lung parenchymal strips with a pA₂ range of 7.70 to 10.71 and was approximately 400- to 3,500-fold more potent than FPL55712. Interestingly, in the presence of an inhibitor of the bioconversion of LTC₄ to LTD₄, ONO-1078 also antagonized the LTC₄-induced contraction of guinea pig trachea (pA₂ = 7.78). ONO-1078 significantly reversed the LTD₄-induced prolonged contraction without effect on the KCl- and BaCl₂-induced contractions of guinea pig trachea. Furthermore, ONO-1078 antagonized the antigen-induced SRS-A mediated contraction of guinea pig trachea. On the other hand, ONO-1078 showed no antagonism against histamine, acetylcholine, 5-hydroxytryptamine, prostaglandin D₂ and U-46619. In addition, ONO-1078 showed little or no effect on the activities of cyclooxygenase, 5-lipoxygenase and thromboxane synthetase. These in vitro studies indicate that ONO-1078 is a highly potent, selective and competitive antagonist of peptide leukotrienes that acts with higher affinity at LTD₄ and LTE₄ receptors than LTC₄ receptors.

Keywords: ONO-1078, Leukotriene antagonist (peptide), Anti-asthma agent, Leukotrienes (peptide) (LTC₄, LTD₄ and LTE₄), FPL55712

Peptide leukotrienes (LTC₄, LTD₄ and LTE₄) are 5-lipoxygenase products of arachidonic acid metabolism (1) and considered to be major constituents of slow reacting substance of anaphylaxis (SRS-A) (2, 3). They induce potent bronchoconstriction (4, 5), increase vascular permeability followed by the formation of mucosal edema (6, 7) and enhance mucus secretion (8). On the basis of their potent biological and pathophysiological effects, it has been hypothesized that peptide leukotrienes may play an important role in bronchial asthma (9). Consequently, peptide leukotriene antagonists or 5-lipoxygenase inhibitors have been suggested to be of therapeutic value in bronchial asthma. FPL55712 (sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate) is the first peptide leukotriene (LT) antagonist with moderate potency and selectivity (10). More recently, highly potent peptide leukotriene antagonists, which appear to act as selective LTD₄/E₄ antagonists with poor or no antagonism against LTC₄ receptors, have been reported (11–17).

ONO-1078 (4-oxo-8-[4-(4-phenylbutoxy)benzoylaminol-2-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate) is a new class of peptide LT antagonist among the series of 8-(benzoylamo)2-tetrazol-5-yl-4-oxo-4H-1-benzopyrans (18). The present investigation describes in vitro antagonism of ONO-1078 against peptide LTs using a radioligand binding assay and functional experiments, and the effects of ONO-1078 were compared with those of FPL55712. The results indicate that ONO-1078 is a highly potent, selective and competitive antagonist of peptide LTs.
MATERIALS AND METHODS

Animals
Male Hartley strain guinea pigs (300–600 g, Keari Co. and Nakajima Experimental Animal Laboratories) were used throughout the experiments.

Drugs and chemicals
ONO-1078 (Fig. 1), FPL55712, OKY-046-HCl, LTC4, LTD4, LTB4, prostaglandin D2 (PGD2) were synthesized by Ono Pharmaceutical Co., Ltd. Other chemicals and drugs used were as follows: [3H]LTC4 (specific activity, 39.3 Ci/mmol), [3H]LTD4 (specific activity, 39.3 and 40.0 Ci/mmol), [3H]LTE4 (specific activity, 39.3 Ci/mmol), [3H]LTB4 (specific activity, 39.3 Ci/mmol), [14C]arachidonic acid (specific activity, 55.0 mCi/mmol) and RIA kit for thromboxane B2 (NEN Research Products and Amersham); indomethacin, carbachol, L-serine, 5-hydroxytryptamine creatinine sulfate (5-HT), phenylmethylsulfonyl fluoride, aprotinin, benzamidine, soybean trypsin inhibitor, glycine, ovalbumin and bovine serum albumin (Sigma Chemical Co.); acetylcholine chloride (Daiichi Pharmaceutical Co.); L-cysteine (Hayashi Pure Chemical Co.); nordihydroguaiaretic acid (NDGA) (Nacalai Tesque Co.); boric acid (Katayama Chemical Co.); assay kit for thromboxane synthetase (Eldan Technologies Co.); U-46619 (Cayman Chemical Co.).

LTs were diluted with either the buffer used in the radioligand binding assay or with 0.1 M phosphate buffer (pH 7.4) used in functional experiments. ONO-1078 was dissolved in ethanol by adding an equimolar amount of NaOH, and then diluted with either distilled water or 10% ethanol/distilled water. FPL55712 was dissolved in ethanol and then diluted with distilled water. Indomethacin, NDGA and OKY-046-HCl were dissolved and diluted with ethanol. Other chemicals were dissolved and diluted with physiological saline.

Preparation of guinea pig lung membranes
The membrane fractions containing LT receptors were prepared as described by Mong et al. (19) and Aharony (20). Briefly, male Hartley strain guinea pigs (300–600 g) were decapitated and then their lungs were perfused with 20 ml of 0.1 M phosphate-buffered saline (PBS, pH 7.4) and excised. Large blood vessels and hemorrhagic and necrotized tissues were removed. The remaining tissue was minced and washed twice with 25 ml of ice-cold PBS and suspended and centrifuged in 10 mM Tris-HCl/0.25 M sucrose buffer (pH 7.4) containing protease inhibitors: 87 μg/ml phenylmethylsulfonyl fluoride, 10 mM CaCl2, 10 mM MgCl2, 100 μg/ml aprotinin, 157 μg/ml benzamidine, and 100 μg/ml soybean trypsin inhibitor. The suspended tissues were homogenized 4 times on ice by a Phycotocor (Niti-on) at 21,000 rpm for 15 sec. The homogenate was centrifuged at 1,500 × g for 15 min at 4°C, and the supernatant was collected and centrifuged at 40,000 × g for 30 min at 4°C. The resulting pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4), homogenized for 3 strokes by a glass-Teflon motorized homogenizer and centrifuged again at 40,000 × g for 30 min at 4°C. The final pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) to a final protein concentration of 10 mg/ml and stored at −80°C until use. The protein concentration of this crude membrane preparation was determined by the method of Lowry et al. (21) with bovine serum albumin as a standard.

Radioligand binding assay of [3H]LTC4, [3H]LTD4, [3H]LTE4 and [3H]LTB4
The [3H]LT binding studies were carried out in the following buffer: [3H]LTC4 binding, 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM cysteine, 5 mM glycine, 80 mM L-serine borate, 10 mM CaCl2 and 10 mM MgCl2; [3H]LTD4 binding, 10 mM piperazone-N,N'-bis(2-ethanesulfonic acid) buffer (pH 6.5) containing 10 mM CaCl2, 10 mM MgCl2, 5 mM cysteine and 5 mM glycine; [3H]LTE4 binding, 10 mM piperazone-N,N'-bis(2-ethanesulfonic acid) buffer (pH 6.5) containing 10 mM CaCl2, 10 mM MgCl2 and 10 mM cysteine; [3H]LTB4 binding, 50 mM Tris-HCl buffer (pH 7.5). The saturation binding experiments were performed by incubating an aliquot of lung membranes ([3H]LTC4 binding, 200 μg/ml; [3H]LTD4 binding, 400 μg/ml; [3H]LTE4 binding, 400 μg/ml) with various concentrations of [3H]peptide LT ([3H]LTC4, 1 to 50 nM; [3H]LTD4, 0.1 to 5 nM; [3H]LTE4, 0.1 to 5 nM) at 25°C for 30 min. In the competition experiments, an aliquot of the lung membranes ([3H]LTC4 binding, 100 μg/ml; [3H]LTD4 binding, 400 μg/ml; [3H]LTE4 binding, 400 μg/ml) was incubated at 25°C for 30 min with [3H]LT ([3H]LTC4, 0.5 nM; [3H]LTD4, 1.0 nM; [3H]LTE4, 1.0 nM) and then processed as described above for the radioligand binding assay.

Fig. 1. Chemical structure of ONO-1078, 4-oxo-8-[4-(4-phenylbutoxy)benzoylamino]-2-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate.
The binding reaction was terminated by rapid filtration of samples through Whatman GF/C glass microfiber filter strips with a Brandel Cell Harvester followed by four rapid washes with 2.5-ml aliquots of ice-cold 10 mM Tris/100 mM NaCl buffer (pH 7.4). Filters were removed and allowed to dry before assaying filter bound radioactivity by liquid scintillation spectrophotometry. The specific binding was defined as the difference between the amount of \(^3\text{H}\)LT bound in the absence and presence of LT (5 \(\mu\text{M LTC}_4, 1 \mu\text{M LTD}_4, 1\mu\text{M LTE}_4, 1 \mu\text{M LTB}_4\)).

Functional experiments using guinea pig trachea and lung parenchymal strips

Male Hartley strain guinea pigs (250–490 g) were sacrificed by a sharp blow to the head and appropriate tissue sections were removed immediately. Each trachea was cut into zigzag strips. Parenchymal strips, 2-mm wide, were prepared from the left and right lower lobe edges of lungs. The preparations were suspended in 10 ml tissue baths containing modified Krebs’ solution of the following composition: 119 mM NaCl, 4.6 mM KCl, 1.8 mM CaCl\(_2\), 0.5 mM MgSO\(_4\), 24.9 mM NaHCO\(_3\), 1.0 mM KH\(_2\)PO\(_4\) and 11.1 mM glucose. The tissue baths were maintained at 37 ± 1°C and continuously aerated with 95% O\(_2\)-5% CO\(_2\). The resting tension of guinea pig trachea was 2.0 g and that of lung parenchymal strips was 0.5 g. Each preparation was equilibrated for 60 min by washing with fresh physiologic solution every 15 min and pretreated with 10\(^{-6}\) M indomethacin to remove the influence of cyclooxygenase products on the responses to various agonists. Contractile responses were recorded as a change of isometric tension by a force displacement transducer (Nihon Kohden, SB-1T). Concentration-response curves were constructed by cumulative increases in bath concentration of agonists according to the method of Van Rossum (22). Only one concentration-response curve was generated with each tissue. Contractile responses were expressed as a percentage of the response to 10\(^{-5}\) M carbachol for the trachea and 10\(^{-4}\) M carbachol for the lung parenchymal strips. ONO-1078 and FPL55712 were added 30 min before the addition of each agonist. In certain experiments with LTC\(_4\), 45 mM l-serine bo- rate complex, a \(\gamma\)-glutamyl transpeptidase inhibitor, was incubated with the guinea pig trachea for 30 min before the addition of LTC\(_4\) to inhibit the bioconversion of LTC\(_4\) to LTD\(_4\). For the reverse experiment, the tracheal strips were contracted with LTD\(_4\) (3 \(\times\) 10\(^{-8}\) M), KCl (2.5 \(\times\) 10\(^{-2}\) M), or BaCl\(_2\) (10\(^{-4}\) M). When the response reached a steady state, ONO-1078 or FPL55712 was added to the bath.

Antigen-induced contraction of guinea pig trachea

Male Hartley strain guinea pigs (300–470 g) were passively sensitized with 1 ml/kg, i.v. of anti-ovalbumin serum (4 hr heterologous PCA titer = 1:8192) produced by the immunization of rabbits with ovalbumin. The trachea from the sensitized animal was removed 24 hr after the passive sensitization and cut into zigzag strips for recording isometric tension as indicated above. Tissues were pretreated with 5 \(\times\) 10\(^{-6}\) M indomethacin and various concentrations of ONO-1078 for 30 min and contracted with the challenge of 10 \(\mu\text{g/ml}\) ovalbumin for 60 min. Contractile responses were expressed as a percentage of the response to 10\(^{-5}\) M carbachol.

Assay of cyclooxygenase, 5-lipoxygenase and thromboxane synthetase activities

Cyclooxygenase activity was measured using sheep seminal vesicular microsomes by the method of Miyamoto et al. (23). 5-Lipoxygenase activity was measured by the method of Ochi et al. (24). Cyclooxygenase and 5-lipoxygenase activities were expressed in terms of the amount of arachidonic acid oxygenated. Thromboxane synthetase activity was measured using a commercially available assay kit. The enzyme activity was expressed in terms of the amount of thromboxane B\(_2\) biosynthesized. Thromboxane B\(_2\) was measured by a commercially available RIA kit.

Statistics

Data from the radioligand binding assay were subjected to computer analysis using programs of Equilibrium Binding Data Analysis (EBDA by McPherson, Elsevier-BIOSOFT, 1983). In functional experiments, the antagonist potency was calculated and expressed as either \(pA_2\) or \(pK_B\). The \(pA_2\) values were obtained by Schild plot analysis (25), whereas \(pK_B\) values were calculated by the method of Furchgott (26). In the enzyme assay, the IC\(_{50}\) values were determined by a linear least square regression analysis. Results were expressed as the mean with standard error of the mean (S.E.M.). Statistical analysis was performed by Student’s paired or unpaired \(t\)-test, with a probability value of \(P < 0.05\) regarded as significant.

RESULTS

Characteristics of \(^3\text{H}\)peptide LT binding to guinea pig lung membranes

The specific bindings of \(^3\text{H}\)LTC\(_4\), \(^3\text{H}\)LTD\(_4\) and \(^3\text{H}\)LTE\(_4\) to guinea pig lung membranes were satur-
able, and Scatchard analysis indicated a single population of binding sites with $K_d$ (equilibrium dissociation constant) values of 25.7 ± 1.3, 0.24 ± 0.02 and 0.62 ± 0.08 nM and $B_{\text{max}}$ (maximum number of binding sites) values of 15,800 ± 700, 254 ± 17 and 178 ± 12 fmol/mg protein (n = 5), respectively. When the data were analyzed by Hill plots, the Hill coefficients were not significantly different from unity ([$^3$H]LTC$_4$, 1.00 ± 0.001; [$^3$H]LTD$_4$, 0.91 ± 0.04; [$^3$H]LTE$_4$, 0.97 ± 0.09; n = 5), indicating that no cooperativity was detected.

Inhibition of [$^3$H]LTC$_4$, [$^3$H]LTD$_4$, [$^3$H]LTE$_4$ and [$^3$H]LTB$_4$ specific bindings to lung membranes by ONO-1078 and FPL55712

ONO-1078 concentration-dependently inhibited [$^3$H]LTC$_4$, [$^3$H]LTD$_4$ and [$^3$H]LTE$_4$ bindings to guinea pig lung membranes at concentration ranges of $10^{-6}-10^{-4}$ M, $10^{-10}-10^{-7}$ M and $10^{-10}-10^{-7}$ M, respectively, whereas ONO-1078 had little effect on the [$^3$H]LTB$_4$ binding at $10^{-6}-10^{-4}$ M (Fig. 2). When compared by $K_i$ values, ONO-1078 selectively inhibited [$^3$H]LTD$_4$ and [$^3$H]LTE$_4$ bindings (Table 1). The rank orders of the inhibition of [$^3$H]LTD$_4$ and [$^3$H]LTE$_4$ bindings were LTD$_4$ > LTE$_4$ > ONO-1078 > FPL55712 and LTD$_4$ > ONO-1078 > LTE$_4$ > LTC$_4$ > FPL55712, respectively. ONO-1078 was approximately 2,000-fold and 3,000-fold more potent than FPL55712 against [$^3$H]LTD$_4$ and [$^3$H]LTE$_4$ bindings, respectively. On the other hand, the inhibition of [$^3$H]LTC$_4$ binding by ONO-1078 was approximately 5,700-fold and 9,000-fold less potent than those of [$^3$H]LTD$_4$ and [$^3$H]LTE$_4$ bindings, respectively. The rank order of the inhibition of [$^3$H]LTC$_4$ binding was LTC$_4$ > ONO-1078 > LTD$_4$ > FPL55712 > LTE$_4$. ONO-1078 was about 2-fold more potent than FPL55712.

To analyze the nature of the interaction of ONO-1078 (3 $\times$ 10$^{-10}$-3 $\times$ 10$^{-9}$ M) with the LTD$_4$ receptors, $K_d$ values and $B_{\text{max}}$ values in the presence of ONO-1078 were determined by cold saturation experiments. As shown in Table 2, Scatchard analysis indicated that ONO-1078 significantly increased the $K_d$ values at 10$^{-9}$-3 $\times$ 10$^{-9}$ M without any influence on the $B_{\text{max}}$ values.

![Fig. 2.](image-url) Competition of ONO-1078 and FPL55712 for [$^3$H]LTC$_4$, [$^3$H]LTD$_4$, [$^3$H]LTE$_4$ and [$^3$H]LTB$_4$ specific bindings to guinea pig lung membranes. Results are means ± S.E.M. of 5 experiments.  ○: ONO-1078; △: FPL55712; ■: LTC$_4$; ●: LTD$_4$; ▲: LTE$_4$; □: LTB$_4$. 
Table 1. $K_i$ values for competition of [3H]LTC4 and [3H]LTD4 specific bindings and IC$_{50}$ values for competition of [3H]LTB4 specific binding to guinea pig lung membranes

| Compounds     | $K_i$ (nM) [3H]LTC4 | $K_i$ (nM) [3H]LTD4 | $K_i$ (nM) [3H]LTE4 | IC$_{50}$ (nM) [3H]LTB4 |
|---------------|---------------------|---------------------|---------------------|------------------------|
| ONO-1078      | 5640 ± 680          | 0.99 ± 0.19         | 0.63 ± 0.11         | > 100,000              |
| FPL55712      | 10980 ± 820         | 2040 ± 180          | 1910 ± 280          | ---                    |
| LTC4          | 27.7 ± 2.9          | 38.0 ± 5.5          | 32.4 ± 2.6          | ---                    |
| LTD4          | 6220 ± 290          | 0.48 ± 0.04         | 0.28 ± 0.05         | ---                    |
| LTE4          | 34100 ± 1000        | 0.62 ± 0.01         | 1.12 ± 0.11         | ---                    |
| LTB4          | ---                 | ---                 | 5.09 ± 0.44         | ---                    |

Values are means ± S.E.M. of five experiments. ---: Not done.

Table 2. Effect of ONO-1078 on $K_d$ and $B_{\text{max}}$ values for [3H]LTD4 specific binding to guinea pig lung membranes

| ONO-1078 (nM) | $K_d$ (nM) | $B_{\text{max}}$ (fmol/mg of protein) |
|---------------|------------|--------------------------------------|
| 0             | 0.24 ± 0.02| 254 ± 17                             |
| 0.3           | 0.33 ± 0.03| 251 ± 12                             |
| 1             | 0.56 ± 0.05**| 233 ± 12                           |
| 3             | 1.59 ± 0.47*| 228 ± 28                           |

Values are means ± S.E.M. of five experiments. *: Significantly different from the control at P < 0.05; **: Significantly different from the control at P < 0.001.

Antagonism of ONO-1078 and FPL55712 against peptide LT-induced contractions of guinea pig trachea

ONO-1078 produced parallel rightward shifts of the LTC4 and LTD4 concentration-response curves in a concentration-dependent manner at $10^{-11} - 10^{-8}$ M (Fig. 3, A and C). As shown in Table 3, the pK$_B$ values were independent of concentrations of ONO-1078, and Schild plot analysis indicated that the slopes were not significantly different from unity. When compared in terms of pA$_2$ values, ONO-1078 was shown to

Fig. 3. Effect of ONO-1078 on concentration-response curves for contractions to LTC4 in the absence of L-serine borate (in A), LTC4 in the presence of 45 mM L-serine borate (in B), and LTD4 (in C) of guinea pig trachea. Results are expressed as a percentage of the response to $1 \times 10^{-5}$ M carbachol and are means ± S.E.M. of 5 to 23 experiments. ●: control; ○: $1 \times 10^{-11}$ M ONO-1078; ■: $1 \times 10^{-10}$ M ONO-1078; □: $1 \times 10^{-9}$ M ONO-1078; ▲: $1 \times 10^{-8}$ M ONO-1078; Δ: $1 \times 10^{-7}$ M ONO-1078; ◇: $1 \times 10^{-6}$ M ONO-1078.
be approximately 700-fold and 3,300-fold more potent than FPL55712 against the LTC₄ and LTD₄-induced contractions of guinea pig trachea, respectively. In the presence of L-serine borate, which inhibits the bioconversion of LTC₄ to LTD₄, higher concentrations of ONO-1078 (10⁻⁸–10⁻⁶ M) were required to antagonize the LTC₄-induced contractions (Fig. 3, B). ONO-1078 shifted the LTC₄ concentration-response curve to the right in a parallel manner. Schild plot analysis indicated that the slope of ONO-1078 was not significantly different from unity. When compared in terms of the pA₂ values, the antagonist potency of ONO-1078 against LTC₄ receptors was approximately 850-fold less potent than that against LTD₄ receptors (Table 3).

### Table 3. Interactions of ONO-1078 and FPL55712 with LTC₄ and LTD₄ in guinea pig trachea

| Compound | Concen (M) | n  | pKᵦ ± S.E.M. | Schid plot analysis |
|----------|------------|----|--------------|---------------------|
|          |            |    | (pA₂)        | (95% C.L.)          | (95% C.L.)         |
| LTC₄     | 1 x 10⁻¹⁰  | 8  | 10.30 ± 0.25 | 10.47              | 0.86               |
|          | 1 x 10⁻⁹   | 8  | 10.48 ± 0.19 | (9.73 – 11.21)     | (0.48 – 1.23)      |
|          | 1 x 10⁻⁸   | 8  | 10.01 ± 0.31 |                     |                     |
| LTC₄ + L-serine borate | 1 x 10⁻⁸  | 5  | 7.80 ± 0.40  | 7.78               | 0.82               |
|          | 1 x 10⁻⁷   | 5  | 7.67 ± 0.33  | (7.11 – 8.46)      | (0.33 – 1.30)      |
|          | 1 x 10⁻⁶   | 5  | 7.43 ± 0.23  |                     |                     |
| LTD₄     | 1 x 10⁻¹⁰  | 8  | 10.54 ± 0.28 | 10.71              | 0.78               |
|          | 1 x 10⁻⁹   | 8  | 10.38 ± 0.23 | (9.88 – 11.54)     | (0.44 – 1.23)      |
|          | 1 x 10⁻⁸   | 8  | 10.10 ± 0.20 |                     |                     |
| FPL55712 |            |    |              |                     |                     |
| LTC₄     | 1 x 10⁻⁷   | 8  | 7.54 ± 0.17  | 7.64               | 0.78               |
|          | 1 x 10⁻⁶   | 8  | 7.18 ± 0.14  | (7.12 – 8.17)      | (0.55 – 1.00)      |
|          | 1 x 10⁻⁵   | 8  | 7.09 ± 0.15  |                     |                     |
| LTD₄     | 1 x 10⁻⁷   | 6  | 7.51 ± 0.20  | 7.19               | 1.14               |
|          | 1 x 10⁻⁶   | 8  | 6.89 ± 0.15  | (6.71 – 7.66)      | (0.78 – 1.45)      |
|          | 1 x 10⁻⁵   | 8  | 7.58 ± 0.28  |                     |                     |

n: The number of experiments. pA₂ values and slopes are derived from linear least square regression analysis with 95% confidence limits.

### Table 4. Interactions of ONO-1078 and FPL55712 with LTC₄ and LTD₄ in guinea pig lung parenchyma

| Compound | Concen (M) | n  | pKᵦ ± S.E.M. | Schid plot analysis |
|----------|------------|----|--------------|---------------------|
|          |            |    | (pA₂)        | (95% C.L.)          | (95% C.L.)         |
| LTC₄     | 1 x 10⁻⁸   | 7  | 7.66 ± 0.17  | 7.70               | 0.90               |
|          | 3 x 10⁻⁸   | 9  | 7.78 ± 0.17  | (7.45 – 7.94)      | (0.39 – 1.42)      |
|          | 1 x 10⁻⁷   | 7  | 7.57 ± 0.10  |                     |                     |
| LTD₄     | 1 x 10⁻⁸   | 12 | 8.41 ± 0.13  | 8.58               | 0.64               |
|          | 3 x 10⁻⁸   | 8  | 8.11 ± 0.11  | (8.03 – 9.13)      | (0.32 – 0.97)      |
|          | 1 x 10⁻⁷   | 8  | 8.06 ± 0.09  |                     |                     |
| FPL55712 |            |    |              |                     |                     |
| LTC₄     | 3 x 10⁻⁵   | 6  | 4.79 ± 0.16  |                     |                     |
|          | 1 x 10⁻⁴   | 6  | 5.28 ± 0.20  |                     |                     |
| LTD₄     | 3 x 10⁻⁵   | 5  | 5.04 ± 0.13  |                     |                     |
|          | 1 x 10⁻⁴   | 5  | 5.23 ± 0.15  |                     |                     |

n: The number of experiments. —: Not done. pA₂ values and slopes are derived from linear least square regression analysis with 95% confidence limits.
Antagonism of ONO-1078 and FPL55712 against peptide LT-induced contractions of guinea pig lung parenchymal strips

ONO-1078 inhibited the LTC4- and LTD4-induced contractions of guinea pig lung parenchymal strips at $10^{-8} - 10^{-7}$ M. As shown in Table 4, Schild plot analysis indicated that the slopes of ONO-1078 were not significantly different from unity. When compared in terms of $pK_B$ values, ONO-1078 was approximately 400-fold and 1,100-fold more potent than FPL55712 against LTC4 and LTD4, respectively.

Specificity of the action of ONO-1078

ONO-1078 was examined for its ability to reduce contractions induced by a variety of contractile agents. ONO-1078 at $10^{-5}$ M was without effect on responses induced by acetylcholine, histamine, 5-HT, PGD2 and U-46619 (Table 5). ONO-1078 reversed the prolonged contraction induced by $3 \times 10^{-8} - 10^{-6}$ M LTD4 in a concentration-dependent manner, but showed no effect on the prolonged contractions induced by $2.5 \times 10^{-7}$ M KCl and $10^{-3}$ M BaCl2 (Fig. 4). The significant reversal was observed at higher concentrations than $10^{-8}$ M of ONO-1078 and the inhibitions of the LTD4-induced prolonged contraction at 60 min by ONO-1078 were 14.8% at $10^{-8}$ M, 61.5% at $10^{-7}$ M and 85.2% at $10^{-6}$ M. FPL55712 at $10^{-6}$ M also significantly reversed the LTD4-induced contraction with the inhibition of 54.4% at 60 min.

Effect of ONO-1078 on antigen-induced contraction of passively sensitized guinea pig trachea

The challenge with ovalbumin induced a biphasic response: a rapid early phase which reached a maximum at 5–7 min and a second sustained phase which was maintained at 20–60 min after the challenge (Fig. 5). ONO-1078 did not affect the first phase of the contraction at $10^{-7} - 10^{-5}$ M, but significantly reduced the second sustained phase of the contraction to the initial

Table 5. Effect of ONO-1078 on contractile responses of guinea pig trachea induced by various agonists

| Agonists            | EC50 ($- \log [M]$) |
|---------------------|---------------------|
|                     | Control             | ONO-1078 $1 \times 10^{-5}$ M |
| Acetylcholine       | 5.63 ± 0.25         | 5.85 ± 0.45 |
| Histamine           | 4.90 ± 0.09         | 5.07 ± 0.31 |
| 5-Hydroxytryptamine | 7.00 ± 0.15         | 6.48 ± 0.13 |
| Prostaglandin D2    | 6.70 ± 0.31         | 5.98 ± 0.44 |
| U-46619             | 8.49 ± 0.11         | 8.20 ± 0.06 |

Values are means ± S.E.M. of five experiments.

Fig. 4. The reversal effect of ONO-1078 on LTD4 ($3 \times 10^{-8}$ M), KCl ($2.5 \times 10^{-2}$ M), and BaCl2 ($1 \times 10^{-3}$ M)-induced contractions of guinea pig trachea. Results are expressed as a percentage of the initial contraction of each agonist. Results are means ± S.E.M. of 5 to 13 experiments. *, **, ***: Statistically significant difference from the control at P < 0.05, P < 0.01 and P < 0.001 by the unpaired t-test, respectively. •: control; O: $1 \times 10^{-5}$ M ONO-1078; ■: $1 \times 10^{-7}$ M ONO-1078; □: $1 \times 10^{-6}$ M ONO-1078; △: $1 \times 10^{-7}$ M ONO-1078; ▲: $1 \times 10^{-5}$ M ONO-1078; ○: $1 \times 10^{-6}$ M FPL55712.
level before ovalbumin challenge at $10^{-6} - 10^{-5}$ M.

Inhibitory activities for cyclooxygenase, 5-lipoxygenase and thromboxane synthetase

ONO-1078 showed little or no effect on cyclooxygenase, 5-lipoxygenase and thromboxane synthetase activities (IC$_{50}$ = $> 300$, $> 300$ and 44 ± 18 µM, n = 3–5, respectively). Indomethacin, NDGA and OKY-046•HCl as reference compounds showed potent inhibition with respective IC$_{50}$ values of 0.13 ± 0.12, 0.25 ± 0.03 and 0.017 ± 0.003 µM (n = 3–5).

DISCUSSION

The antagonist activity of ONO-1078 against peptide LTs was examined in the radioligand binding assay and functional experiments using guinea pig lung and trachea. The radioligand binding assay on guinea pig lung membranes indicated that ONO-1078 selectively interacted with the LTD$_4$ and LTE$_4$ receptors and that the antagonist activity of ONO-1078 against LTD$_4$ and LTE$_4$ was approximately 2,000-fold and 3,000-fold more potent, respectively, than that of FPL55712. In functional experiments, ONO-1078 potently antagonized the LTD$_4$-induced contractions of guinea pig trachea and lung parenchymal strips. The antagonist activity of ONO-1078 against LTD$_4$ in guinea pig trachea and lung parenchymal strips was approximately 3,300-fold and 1,100-fold more potent than that of FPL55712. Data from the radioligand binding assay were in good agreement with those obtained from functional experiments. In particular, ONO-1078 showed potent antagonism against LTD$_4$ with a pA$_2$ value of 10.71 in guinea pig trachea, and ONO-1078 appears to be the most potent LTD$_4$ antagonist so far described (15). On the other hand, ONO-1078 showed antagonism against LTC$_4$ with a K$_i$ value of 5640 ± 680 nM in guinea pig lung membranes and a pA$_2$ value of 7.78 in guinea pig trachea treated with l-serine borate. These results indicated that the interaction of ONO-1078 with LTC$_4$ receptors was approximately 850-fold in functional experiments and approximately 5,700-fold in the radioligand binding assay lower than that with LTD$_4$ receptors. However, it is interesting that ONO-1078 has antagonist activity against LTC$_4$ and that the pA$_2$ value of ONO-1078 against LTC$_4$ is higher than any other peptide LT antagonists so far reported (11–17). The selectivity of the action of ONO-1078 was indicated by the lack of antagonist activity against $[^3]$H$LTB_4$ binding to guinea pig lung membranes and against the contractions of guinea pig trachea induced by a variety of agonists other than peptide LTs. As for the nature of the antagonism of ONO-1078 against peptide LTs, the radioligand binding assay indicated that ONO-1078 was a competitive LTD$_4$ antagonist due to the significant increase in K$_d$ values without any influence on the B$_{max}$ values in the presence of ONO-1078. In addition, as the functional experiments indicated that the slopes in the LTC$_{4r}$ and LTD$_4$-induced contractions obtained by Schild plot analysis were not significantly different from unity, ONO-1078 was a competitive antagonist of peptide LTs. Thus, it was demonstrated that ONO-1078 was a highly potent, selective and competitive antagonist of peptide LTs with higher affinity to LTD$_4$ and LTE$_4$ receptors than to LTC$_4$ receptors. Radioligand binding assay demonstrated the existence of a single class of high affinity and saturable binding sites for peptide LT receptors in guinea pig lung membranes (27–29). Based on the findings that the K$_d$ values for $[^3]$H$LTB_4$ and $[^3]$H$LTE_4$ specific bindings to guinea pig lung membranes were almost identical and that the rank order potency of agonist binding to $[^3]$H$LTE_4$ binding sites was LTD$_4$ > LTE$_4$ > LTC$_4$, identical to that for $[^3]$H$LTB_4$ binding, it has been suggested that LTE$_4$ binds to a subset population of high affinity LTD$_4$ receptors (30, 31). Our results are similar to those findings, and this suggestion would account for the ability of ONO-1078 to inhibit $[^3]$H$LTB_4$ and $[^3]$H$LTE_4$ bindings with almost equal potency. In addition, ONO-1078 showed almost the same affinity to LTD$_4$/E$_4$ receptors as the natural agonists, LTD$_4$ and LTE$_4$. As for the LTC$_4$ receptors, it is reported that LTC$_4$ receptors are biochemically different from LTD$_4$.
receptors in guinea pig lung (29). The saturation experiments indicated that \[^{3}H\]LTC₄ binding sites were 40–70-fold lower in affinity and 40–90-fold higher in density than \[^{3}H\]LTD₄ and \[^{3}H\]LTE₄ binding sites. In addition, the competition experiments indicated that the rank order of potency of agonists for the inhibition of \[^{3}H\]LTC₄ binding was different from those for the inhibition of \[^{3}H\]LTD₄ and \[^{3}H\]LTE₄ bindings. The present results suggest that LTC₄ receptors are different from LTD₄ and LTE₄ receptors.

The existence of multiple LTD₄ receptors has been reported in guinea pig ileum and lung parenchymal strips using FPL55712 (32, 33). In functional experiments, the antagonist activity of ONO-1078 against LTD₄ in guinea pig trachea (pA₂ = 10.71) was approximately 2.1 log degrees higher than that in guinea pig lung parenchymal strips (pA₂ = 8.58). As there existed ONO-1078-sensitive or -less sensitive LTD₄ receptors, it is suggested that LTD₄ receptors in guinea pig lung parenchymal strips may differ from those present in guinea pig trachea.

LTC₄ and LTD₄ have been shown to produce thromboxane A₂ and PGs from guinea pig lung (13, 34) and to induce the contraction of guinea pig parenchymal strips mediated by thromboxane A₂ (35). ONO-1078 antagonized the LTC₄- and LTD₄-induced contractions of guinea pig lung in the presence of indomethacin and moreover, ONO-1078 had little or no effect on cyclooxygenase and thromboxane synthetase activities. Therefore, it appears that the inhibitory activity of ONO-1078 against the LTC₄- and LTD₄-induced contractions of guinea pig lung parenchymal strips is not mediated by the blockade of thromboxane A₂ formation, but is the direct interaction with LTC₄ and LTD₄ receptors.

ONO-1078 reversed the LTD₄-induced sustained contraction of guinea pig trachea in a concentration-dependent manner. Since ONO-1078 showed no reversal effect on the KCl- and BaCl₂-induced contractions of guinea pig trachea, ONO-1078 appears to selectively interact with LTD₄ receptors in guinea pig trachea. This reversal effect of ONO-1078 was approximately 10-fold more potent than that of FPL55712. This result is inconsistent with the finding that ONO-1078 was about 3,300-fold more potent than FPL55712 in inhibiting the LTD₄-induced contraction of guinea pig trachea. This difference in the antagonist activity of FPL55712 against the LTD₄-induced sustained contraction may be due to the potent inhibition of phosphodiesterase (36) in addition to the antagonism against LTD₄.

The exposure of the sensitized guinea pig trachea to specific antigen produced a rapidly developing and prolonged contraction. It is suggested that histamine and SRS-A are involved in this response, with histamine mediating the early phase of the contraction and SRS-A primarily mediating the late protracted phase of the contraction (37). ONO-1078 selectively reduced the prolonged phase of antigen-induced contraction of guinea pig trachea without effect on the histamine-mediated early phase. Since ONO-1078 had no effect on 5-lipoxygenase activity and it is reported that a classical SRS-A antagonist, FPL55712, selectively reduced the prolonged phase of the antigen-induced contraction of guinea pig trachea (37), it appears that selective reduction of the late phase contraction by ONO-1078 is due to the antagonism of peptide LTs. Recently, it is reported that selective antagonists of LTD₄ and LTE₄ only partially inhibited the antigen-induced contraction of guinea pig trachea, suggesting that this contraction may be partly mediated by the activation of LTC₄ receptors in guinea pig trachea (38, 39). On the contrary, ONO-1078 completely reduced the SRS-A mediated contraction of guinea pig trachea. This result could be explained by the antagonism of ONO-1078 against LTC₄ in addition to the potent antagonism against LTD₄. These results suggest that ONO-1078 potently antagonizes not only exogenous peptide LTs but also endogenously generated peptide LTs and it may be efficacious to possess antagonist activity against LTC₄ as well as LTD₄ and LTE₄.

In conclusion, ONO-1078 is a novel, highly potent, selective, and competitive antagonist of peptide LTs. Furthermore, ONO-1078 also antagonizes endogenously generated peptide LTs. From these in vitro properties, ONO-1078 may have therapeutic value in the treatment of bronchial asthma and peptide LTs-related respiratory disorders.

REFERENCES

1 Samuelsson, B., Hammarström, S., Murphy, R.C. and Borgeat, P.: Leukotrienes and slow reacting substance of anaphylaxis (SRS-A). Allergy 35, 375–381 (1980)
2 Lewis, R.A., Austen, K.F., Drazen, J.M., Clark, D.A., Marfat, A. and Corey, E.J.: Slow reacting substances of anaphylaxis: identification of leukotriene C-1 and D from human and rat sources. Proc. Natl. Acad. Sci. U.S.A. 77, 3710–3714 (1980)
3 Murphy, R.C., Hammarström, S. and Samuelsson, B.: Leukotriene C₄, a slow reacting substance (SRS) from mouse mastocytoma cells. Proc. Natl. Acad. Sci. U.S.A. 76, 4275–4279 (1979)
4 Drazen, J.M., Austen, K.F., Lewis, R.A., Clark, D.A., Goto, G., Marfat, A. and Corey, E.J.: Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. Proc. Natl. Acad. Sci. U.S.A. 77, 4354–4358 (1980)
5 Dahlén, S.E., Hedqvist, P., Hammarström, S. and Samuelsson, B.: Leukotrienes are potent constrictors of human bronchi. Nature 288, 484–486 (1980)
6 Hau, X.Y., Dahlén, S.E., Lundberg, J.M., Hammarström, S. and Hedqvist, P.: Leukotrienes C₄, D₄ and E₄ cause widespread and extensive plasma extravasation in the guinea pig. Naunyn Schmiedebergs Arch. Pharmacol. 330, 136–141 (1985)

7 Rinkema, L.E., Bemis, K.G. and Fleisch, J.H.: Production and antagonism of cutaneous vascular permeability in the guinea pig in response to histamine, leukotrienes and A23187. J. Pharmacol. Exp. Ther. 230, 550–557 (1984)

8 Marom, Z., Shelhamer, J.H., Bach, M.K., Morton, D.R. and Kaliner, M.: Slow-reacting substances of anaphylaxis, leukotrienes C₄ and D₄, increase the release of mucus from human airways in vitro. Am. Rev. Respir. Dis. 126, 449–451 (1982)

9 Samuelsson, B.: Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. Science 220, 568–575 (1983)

10 Augstein, J., Farmer, J.B., Lee, T.B., Sheard, P. and Tattersall, M.L.: Selective inhibition of slow reacting substance of anaphylaxis. Nature New Biol. 245, 215–217 (1973)

11 Fleisch, J.H., Rinkema, L.E., Haich, K.D., Swanson-Bean, D., Goodson, T., Ho, P.K.P. and Marshall, W.S.: 1,2-hydroxy-3-propyl-4-(4-(1H-tetrazol-5-yl)butoxy) phenyl ethanone, an orally active leukotriene D₄ antagonist. J. Pharmacol. Exp. Ther. 233, 148–157 (1985)

12 Hay, D.W.P., Muccitelli, R.M., Tucker, S.S., Vickery-Clark, M.A. and Torphy, T.J.: Pharmacologic profile of SK&F 104353: a novel, potent and selective peptidoleukotriene receptor antagonist in guinea pig and human airways. J. Pharmacol. Exp. Ther. 243, 474–481 (1987)

13 Mong, S., Wu, H.-L., Miller, J., Hall, R.F., Gleason, J.G. and Crooke, S.T.: SKF104353, a high affinity antagonist for leukotrienes C₄ and D₄, binds specifically to LTD₄ receptors in guinea pig lung membranes. Eur. J. Pharmacol. 182, 301–312 (1990)

14 Aharony, D., Falcone, R.C. and Krell, R.D.: Characterization of leukotriene D₄ receptor in guinea pig lung membranes by the novel leukotriene antagonist ICI198,615. J. Pharmacol. Exp. Ther. 243, 921–926 (1987)

15 Snyder, W.D., Giles, R.E., Keith, R.A., Yec, Y.K. and Krell, R.D.: In vitro pharmacology of ICI198,615: a novel, potent and selective peptide leukotriene antagonist. J. Pharmacol. Exp. Ther. 243, 548–556 (1987)

16 Krell, R.D., Aharony, D., Buckner, C.K., Keith, R.A., Kasner, E.J., Snyder, D.W., Bernstein, P.R., Matassa, V.G., Yec, Y.K., Brown, F.J., Hesp, B. and Giles, R.E.: The preclinical pharmacology of ICI204,219. Am. Rev. Respir. Dis. 141, 978–987 (1990)

17 Jones, T.R., Zamboni, R., Kelley, M., Champion, E., Charette, L., Ford-Hutchinson, A.W., Frenette, R., Gauthier, J.-Y., Leger, S., Masson, P., McFarlane, C.S., Pichuta, H., Rokach, J., Williams, H., Young, R.N., DeHaven, R.N. and Pong, S.S.: Pharmacology of L-660,711 (MK-571): a novel potent and selective leukotriene D₄ receptor antagonist. Can. J. Physiol. Pharmacol. 67, 17–28 (1989)

18 Nakai, H., Konno, M., Kosuge, S., Sakuyama, S., Toda, M., Arai, Y., Obata, T., Katsube, N., Miyamoto, T., Okegawa, T. and Kawasaki, A.: New potent antagonists of leukotrienes C₄ and D₄. 1. Synthesis and structure-activity relationships. J. Med. Chem. 31, 84–91 (1988)

19 Mong, S., Wu, H.-L., Scott, M.O., Lewis, M.A., Clark, M.A., Weichman, B.M., Kinzig, C.M., Gleason, J.G. and Crooke, S.T.: Molecular heterogeneity of leukotriene receptor antagonists: correlation of smooth muscle contraction and radioligand binding in guinea pig lung. J. Pharmacol. Exp. Ther. 234, 316–325 (1985)

20 Aharony, D.: Assessment of leukotriene D₄ receptor antagonists. Methods Enzymol. 187, 414–421 (1990)

21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L.: Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)

22 Van Rossum, J.M.: Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. Arch. Int. Pharmacodyn. Ther. 143, 299–330 (1963)

23 Miyamoto, T., Ogin, N., Yamamoto, S. and Hayashi, O.: Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. J. Biol. Chem. 251, 2629–2636 (1976)

24 Ochi, K., Yoshimoto, T., Yamamoto, S., Taniguchi, K. and Miyamoto, T.: Arachidonate 5-hydroxyxygenase of guinea pig peritoneal polymorphonuclear leukocytes. Activation by adenosine 5'-triphosphate. J. Biol. Chem. 258, 5754–5758 (1983)

25 Arunlakshana, O. and Schild, H.O.: Some quantitative uses of drug antagonists. Br. J. Pharmacol. 14, 48–58 (1959)

26 Furchgott, R.F.: The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In Handbook of Experimental Pharmacology, Vol. 33, Edited by Blaschko, H. and Muscholl, E., p. 283–335, Springer-Verlag, New York (1972)

27 Bruns, R.F., Thomsen, W.J. and Pugsley, T.A.: Binding of leukotrienes C₄ and D₄ to membranes from guinea pig lung: regulation by ions and guanine nucleotides. Life Sci. 33, 645–653 (1983)

28 Pong, S.S. and DeHaven, R.N.: Characterization of a leukotriene D₄ receptor in guinea pig lung. Proc. Natl. Acad. Sci. U.S.A. 80, 7415–7420 (1983)

29 Hogaboom, G.K., Mong, S., Wu, H.-L. and Crooke, S.T.: Peptidoleukotrienes: distinct receptors for leukotriene C₄ and D₄ in the guinea-pig lung. Biochem. Biophys. Res. Commun. 116, 1136–1143 (1983)

30 Aharony, D., Cataneo, C.A. and Falcone, R.C.: Kinetic and pharmacologic analysis of [³H]leukotriene E₄ binding to receptors on guinea pig lung membranes: evidence for selective binding to subset of leukotriene D₄ receptors. J. Pharmacol. Exp. Ther. 248, 581–588 (1989)

31 Mong, S., Scott, M.O., Lewis, M.A., Wu, H.-L., Hogaboom, G.K., Clark, M.A. and Crooke, S.T.: Leukotriene E₄ binds specifically to LTD₄ receptors in guinea pig lung membranes. Eur. J. Pharmacol. 109, 183–192 (1985)

32 Norman, P., Abram, T.S., Cuthbert, N.J. and Gardiner, P.J.: The inhibition of [³H]leukotriene D₄ binding to guinea-pig lung membranes. The correlation of binding affinity with activity on the guinea-pig ileum. Eur. J. Pharmacol. 182, 301–312 (1990)

33 Fleisch, J.H., Rinkema, L.E. and Baker, S.R.: Evidence for multiple leukotriene D₄ receptors in smooth muscle. Life Sci. 31, 577–581 (1982)
34 Folco, G., Hansson, G. and Graström, E.: Leukotriene C₄ stimulates TxA₂ formation in isolated sensitized guinea pig lungs. Biochem. Pharmacol. 30, 2493–2495 (1981)
35 Weichman, B.M., Muccitelli, R.M., Osborn, R.R., Holden, D.A., Gleason, J.G. and Wasserman, M.A.: In vitro and in vivo mechanisms of leukotriene-mediated bronchoconstriction in the guinea pig. J. Pharmacol. Exp. Ther. 222, 202–208 (1982)
36 Chasin, M. and Scott, C.: Inhibition of cyclic nucleotide phosphodiesterase by FPL55712, an SRS-A antagonist. Biochem. Pharmacol. 27, 2065–2067 (1978)
37 Adams, G.K., III and Lichtenstein, L.: In vitro studies antigen-induced bronchospasm: effect of antihistamine and SRS-A antagonist on response of sensitized guinea pig and human airways to antigen. J. Immunol. 122, 555–562 (1979)
38 Snyder, D.W., Cho, H.L. and Gilmore, J.: Endogenously formed leukotriene C₄ activates LTC₄ receptors in guinea pig tracheal strips. Eur. J. Pharmacol. 195, 209–215 (1991)
39 Redkar-Brown, D.G. and Aharony, D.: Inhibition of antigen-induced contraction of guinea pig trachea by ICI198,615. Eur. J. Pharmacol. 165, 113–121 (1989)