# Selective inhibition of 5-lipoxygenase by 5,6-methanoleukotriene $A_4$, a stable analogue of leukotriene $A_4$

Yasuko Koshishara, Sei-itsu Murota, Nicos A. Petasis* and Kyriacos C. Nicolaou*

*Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Sakaecho, Itabashi-ku, Tokyo-173, Japan* and **Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104, USA*

Received 26 March 1982; revision received 1 May 1982

*5-Lipoxygenase* *Leukotriene $A_4$* *5,6-Methanoleukotriene $A_4$, inhibitor* *Mastocytoma* *Lipoxygenase*

## 1. INTRODUCTION

Slow reacting substance of anaphylaxis (SRS-A) produced by basophils or mast cells is a bronchoconstrictive agent believed to be an important mediator of allergic asthma in man. SRS-A has been named leukotriene (LT) $C_4$ and $D_4$ and was formed by 5-lipoxygenase from arachidonic acid which also synthesizes $LTA_4$ and $LTB_4$ [1]. $LTB_4$, $C_4$ and $D_4$ may have important roles in the inflammatory response [2,3]. Therefore, the discovery of specific inhibitors for 5-lipoxygenase could lead to the development of new anti-asthmatic and anti-inflammatory drugs.

The first leukotriene formed in the 5-lipoxygenase pathway is $LTA_4$, which is a relatively unstable intermediate with an epoxide unit of all the following leukotrienes. We found that 5,6-methanoleukotriene $A_4$, which is chemically synthesized and a stable analogue of $LTA_4$ [4], selectively inhibited 5-lipoxygenase in comparison with fatty acid cyclooxygenase (EC 1.14.99.1).

For assaying both lipoxygenase and cyclooxygenase activities simultaneously, we developed a unique in vitro experimental system using a cloned mastocytoma P-815, 2-E-6 cells.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

The sources of the reagents used in these experiments were as follows: [1-$^{14}$C]arachidonic acid (spec. act. 55.5 Ci/mol), Radiochemical Centre (Amersham); foetal bovine serum (mycoplasma- and virus-free), Grand Island Biochemical Co. (NY); sodium *n*-butyrate, Wako Chemical Co. (Osaka); authentic 5-hydroxyeicosatetraenoic acid (HETE) and prostaglandin standards, Ono Pharmaceutical Co. (Osaka); thin-layer silica gel plates ($60F_{254}$; layer thickness 0.25 mm), Merck Co. (Darmstadt).

### 2.2. *Cell culture*

Mastocytoma P-815 cells, originally established as a transformed mast cell line [5], were transferred to a cell culture system as in [6,7]. In brief, cells were harvested from the ascites fluid, cultured without shaking until they became adapted and then cultured in suspension in Eagle's basal medium supplemented with 10% (v/v) foetal bovine serum under $CO_2$/air (1:19) at 37°C. Cells were cloned in a microtitration tray. A cloned cell line, 2-E-6 cells, were adapted to grow in suspension with a gyratory shaker (model G-24, New Brunswick Scientific Co.). Their generation time was 13–14. Cells in the exponentially growing phase were treated with 1 mM sodium *n*-butyrate for 40 h to induce cyclooxygenase activity [7], since the cells had lipoxygenase but little cyclooxygenase activity. Cell numbers were counted in a Coulter Counter (Model G, Coulter Electronics Inc., Hialeah FL).

### 2.3. *Assay of 5-lipoxygenase activity*

*n*-Butyrate treated cells were harvested by centrifugation at 200 × *g* for 5 min, and washed once with phosphate-buffered saline ($Ca^{2+}$- and $Mg^{2+}$-free) (pH 7.4). The washed cells were suspended at $1 \times 10^7$ cells/ml in incubation buffer consisting of 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 0.1% gelatin and $10^{-5}$ M indomethacin and sonicated 5 times for 4 s periods in a

*Published by Elsevier Biomedical Press*
00145793/82/0000–0000/$2.75 © 1982 Federation of European Biochemical Societies 

Branson Sonifier (Model W-185; Ultrasonic, Plainview NY) at a setting of 3. The cell homogenate was centrifuged at 10 000 × *g* for 10 min, and the resulting supernatant fraction was used for assay of lipoxygenase activity. Under the standard assay condition (1.0 ml), the supernatant fraction (0.9 ml) was incubated with 0.2 μCi [$^{14}$C]arachidonic acid and 0.8 mM $CaCl_2$ at 37°C for 5 min with shaking in an open tube. The reaction was terminated by adjusting the mixture to pH 3.0 with HCl. The synthesized HETEs were extracted with 8 vol. ethyl acetate, and the extract was condensed and subjected to thin-layer chromatography in solvent D, consisting of petroleum ether/diethyl ether/acetic acid (50/50/1) as in [8].

Labeled HETEs separated on the plates were scanned with a Berthold Dünnschicht Scanner II. Radioactive zones were scraped off the plate for measurement of radioactivity by a liquid scintillation spectrometer. The activity of 5-lipoxygenase of the cells is expressed as the sum radioactivity of 5-HETE and 5,12-diHETE, i.e., $^{14}$C-labeled HETEs/homogenate of $10^7$ cells.

### 2.4. *Assay of prostaglandin synthetase activity*

The 10 000 × *g* supernatant fraction from the above cell homogenate was used for assay of prostaglandin synthetase activity. The supernatant fraction (0.9 ml) was incubated with 0.2 μCi [$^{14}$C]-arachidonic acid at 37°C for 7 min with shaking in an open tube. In this assay condition, HETE was not synthesized (not shown). The reaction was terminated by adjusting the mixture to pH 3.0 with HCl. The prostaglandin synthesized were extracted with 8 vol. ethyl acetate, condensed and subjected to thin-layer chromatography with the upper phase of solvent C, consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11/5/2/10, by vol.) as in [6]. Labeled prostaglandins separated on the plates were scanned and the radioactive zones were each scraped off the plate for measurement of radioactivity. The prostaglandin synthetase activity of the cells is expressed as the sum radioactivity of each prostaglandin synthesized, i.e., cpm of $^{14}$C-labeled prostaglandins/homogenate of $10^7$ cells.

### 2.5. *Addition of inhibitors*

Inhibitors were dissolved in ethyl alcohol of reagent grade. These inhibitors could be dissolved in the solvent up to 2 mg/ml. Inhibitor solution (50 μl) of various concentrations or ethyl alcohol (50 μl) for the control was transferred to the assay tube containing [$^{14}$C]arachidonic acid in toluene. One drop of mixture of propylene glycol/ethyl alcohol (1:3) was added to the above tube. Organic solvent was evaporated completely under $N_2$ gas stream before addition of enzyme and/or others to exclude the effect of the solvent.

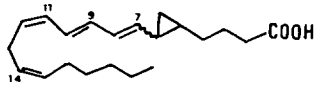
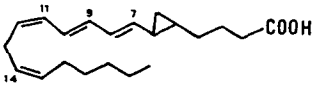
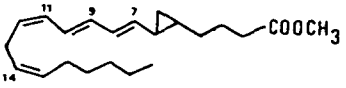
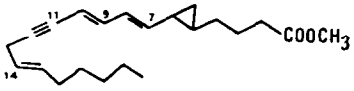
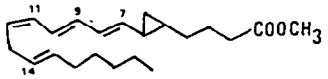
## 3. RESULTS AND DISCUSSION

The cloned mastocytoma P-815, 2-E-6 cells used here can mainly sythesize 5-HETE and 5,12-diHETE (LTB) with a slight production of 12-HETE from arachidonic acid under the culture conditions [9]. The cells have little cyclooxygenase activity unless they are treated with 1 mM *n*-butyrate for 40 h [6,7,9]. *n*-Butyrate treatment can also cause an enhancement of 5-lipoxygenase activity of the cells (submitted). Therefore, *n*-butyrate treatment is favourable to assay for both lipoxygenase and cyclooxygenase activities. Both enzymes were obtained from *n*-butyrate-treated cells and 10 000 × *g* supernatant fractions were prepared from cell homogenates. These assay conditions were fixed at linearly increasing activities of enzymes as in section 2.

Effects of several derivatives of 5,6-methanoleukotriene $A_4$ analogue on both 5-lipoxygenase and cyclooxygenase activities were studied. Various concentrations of the drugs were added separately to the enzyme mixtures and their effects on each enzyme were compared. Fig.1 shows a dose–response curve of 5,6-methanoleukotriene $A_4$ methyl ester. The drug strongly inhibited the synthesis of both 5-HETE and 5,12-diHETE with inhibition of 12-HETE. The concentration of a half inhibition ($ID_{50}$) for 5-lipoxygenase activity was 18 μM. On the other hand, the drug did not inhibit the synthesis of prostaglandins at all up to 150 μM, but slightly inhibited it at 300 μM. These results show that 5,6-methanoleukotriene $A_4$ methyl ester selectively inhibited one of the two main metabolic pathways of arachidonic acid, i.e., leukotriene and prostaglandin synthesis. The effects of other 5,6-methanoleukotriene $A_4$ analogues on both enzymes are summarized in table 1. All these 5,6-methanoleukotriene $A_4$ analogues tested showed selective inhibition for 5-lipoxygenase activity, although the relative potency was different. 5,6-Methanoleuko-

Table 1

Inhibition of 5-lipoxygenase and cyclooxygenase activity by 5,6-methanoleukotriene $A_4$ derivatives

| 5,6-Methanoleukotriene $A_4$ derivatives | | $ID_{50}$ (μM) 5-Lipoxygenase | Cyclooxygenase |
|---|---|---|---|
| KCN-TEI-6090 |  | 31 | 186 |
| KCN-TEI-6127 |  | 44 | *1136 |
| KCN-TEI-6173 |  | 18 | 590 |
| KCN-TEI-6171 |  | 30 | **1219 |
| KCN-TEI-6172 |  | 6 | 484 |
| ETYA [10] | (5,8,11-eicosatriynoic acid) | 50 | 8 |
| BW 755C[11] | (3-amino-1-(m-tri-fluoromethyl)-phenyl-2-pyrazoline | ***7 | 3 |

*: no inhibition until 157 μM

**: inhibited less than 15 per cent at 300 μM

***: result on 12-lipoxygenase

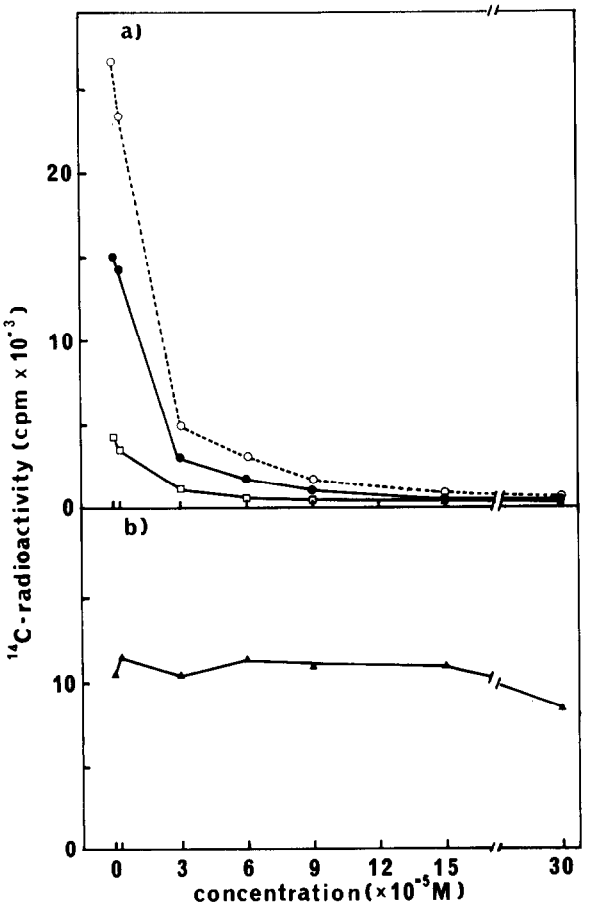



Fig.1. Effect of 5,6-methanoleukotriene $A_4$ on 5-lipoxygenase and cyclooxygenase activity. Supernatant fraction (10 000 × *g*) obtained from cloned mastocytoma P-815, 2-E-6 cells treated with *n*-butyrate was used for assay of 5-lipoxygenase and cyclooxygenase activity as described in the text. Various concentration of 5,6-methanoleukotriene methyl ester (KCN–TEI-6173 in table 1) was added to the assay. (a) HETE syntheses (○—○); 5-lipoxygenase activity (5-HETE and 5,12-diHETE synthesized) (●—●); 5-HETE (□—□); 12-HETE. (b) Prostaglandin syntheses.

triene $A_4$ was much more selective than ETYA (5,8,11-eicosatriynoic acid) and BW 755C (3-amino-1-(*m*-trifluoromethyl)-phenyl-2-pyrazoline), which are well-known as lipoxygenase inhibitors. 15-HETE, which has been reported to be a selective inhibitor of 5-lipoxygenase [8], was not effective as reported in this assay system.

We still do not know the inhibitory mechanism of 5,6-methanoleukotriene $A_4$, but the analogues may act as antagonists to natural $LTA_4$. The inhibitory effects of these drugs on inflammation in vivo are under investigation.

## ACKNOWLEDGEMENTS

We wish to thank Dr Seiji Kurozumi (Teijin Ltd) for the supply of these drugs and Miss Mariko Mizumura for her help in the assay of the enzyme activity.

## REFERENCES

[1] Murphy, R.C., Hammarström, S. and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. USA 76, 4275–4279.
[2] Doig, M.V. and Ford-Hutchinson, A.W. (1980) Prostaglandins 20, 1007–1019.
[3] Koshihara, Y., Nagasaki, I. and Murota, S. (1981) Biochem. Pharmac. 30, 1781–1784.
[4] Nicolaou, K.C., Petasis, N. and Seitz, S.P. (1981) J. Chem. Soc. Chem. Commun. 1195–1196.
[5] Dunn, T.B. and Potter, M. (1957) J. Natl. Cancer Inst. 18, 587–601.
[6] Koshihara, Y., Senshu, T., Kawamura, M. and Murota, S. (1980) Biochim. Biophys. Acta 617, 536–539.
[7] Kishihara, Y., Kawamura, M., Senshu, T. and Murota, S. (1981) Biochem. J. 194, 114-117.
[8] Vanderhoek, J.Y., Bryant, R.W. and Baily, J.M. (1980) J. Biol. Chem. 255, 10064–10066.
[9] Koshihara, Y., Mizumura, M. and Murota, S. (1982) Biochim. Biophys. Acta in press.
[10] Örning, L. and Hammarström, S. (1980) J. Biol. Chem. 255, 8023–8026.
[11] Higgs, G.A., Flower, R.J. and Vane, J.R. (1979) Biochem. Pharmac. 28, 1959–1961.