

## The role of phosphodiesterase 4B in IL-8/LTB<sub>4</sub>-induced human neutrophil chemotaxis evaluated with a phosphodiesterase 4B inhibitor

OSAMU SUZUKI<sup>1,3\*</sup>  
TAIJI GOTO<sup>2</sup>  
TOSHIHARU YOSHINO<sup>2</sup>  
SATOSHI NAKAMURA<sup>3</sup>  
HIROAKI MAEDA<sup>1</sup>

<sup>1</sup> *Biological Research Laboratories III*

<sup>2</sup> *Lead Discovery and Optimization  
Research Laboratories II  
Daiichi Sankyo Co., Ltd., Tokyo  
140-8710, Japan*

<sup>3</sup> *Department of Bioengineering, Tokyo  
Institute of Technology, Kanagawa  
226-8501, Japan*

Accepted December 22, 2014

PDE4B was previously shown to be a dominant PDE4 subtype of neutrophils. However, its physiological role in the neutrophil function has not been evaluated. In this study, the inhibitory effects of a phosphodiesterase 4B (PDE4B)-selective inhibitor (compound A) and subtype non-selective PDE4 inhibitors (roflumilast and cilomilast) were evaluated in human peripheral blood cells. Compound A, roflumilast and cilomilast in a similar manner inhibited TNF- $\alpha$  production by LPS-stimulated human mononuclear cells. However, the inhibitory effect of compound A on IL-8 or LTB<sub>4</sub>-induced chemotactic response of neutrophils was modest even at the highest concentration (10  $\mu\text{mol L}^{-1}$ ), whereas roflumilast and cilomilast inhibited IL-8 or LTB<sub>4</sub>-induced neutrophil chemotaxis. Our results suggest that PDE4B does not play an important role during the chemotactic response of human neutrophils.

*Keywords:* phosphodiesterase 4B, phosphodiesterase 4D, mononuclear cells, neutrophils

Eleven structurally related but functionally distinct gene families (PDE1 to PDE11) have been identified in mammals and comprise the PDE superfamily (1, 2). PDE4 is a cAMP-specific phosphodiesterase and has been shown to play key roles in the regulation of a number of inflammatory processes such as cell tracking, release of inflammatory mediators and immune cell proliferation. The PDE4 family is composed of 4 different genes, PDE4A, B, C, and D, which are distinguished from other PDEs by a unique N terminus, which is involved in intracellular targeting and regulating catalytic activity.

The physiological role of PDE4 subtypes in human blood leukocytes has been investigated mainly in monocytes and T cells but not in neutrophils. It has been suggested that PDE4A and/or PDE4B play key roles in the LPS-induced TNF- $\alpha$  release from monocyte and T cell proliferation using several PDE4 inhibitors with different subtype specificity (3).

\* Correspondence; e-mail: [suzuki.osamu.g5@daiichisankyo.co.jp](mailto:suzuki.osamu.g5@daiichisankyo.co.jp)

† Present address: Cardiovascular-Metabolics Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo 140-8710, Japan

Another study using a gene silencing method has shown that PDE4A, PDE4B and PDE4D have overall non-redundant, but complementary, time-dependent roles in the anti-CD3/CD28 stimulation induced CD4<sup>+</sup> T cell cytokine release and proliferation (4). On the other hand, the physiological role of PDE4 subtypes in human neutrophils has not been investigated. It was reported that the mRNA expression level of each PDE4 subtype in human neutrophil was PDE4B (99.7 %), PDE4D (0.3 %) while PDE4A and C were almost absent (5). The dominant expression of PDE4B in human neutrophils suggested that PDE4B, but not PDE4A, PDE4C or PDE4D, has an important role in cellular control of cAMP in human neutrophils. However, functional studies are necessary to elucidate the physiological role of PDE4B in human neutrophils during the inflammatory process. In order to elucidate the physiological function of proteins, gene knockdown technology using the siRNA method is generally used; however, this method is difficult to apply to neutrophils due to their shortlife nature.

We have recently identified and reported on a PDE4B-selective inhibitor, compound A. The inhibitory activities of compound A and subtype non-selective PDE4 inhibitors (roflumilast and cilomilast) for PDE4B and PDE4D were directly compared in our previous study (6). The  $IC_{50}$  values for human PDE4B, PDE4D and selectivity ( $IC_{50}$  value for PDE4D/ $IC_{50}$  value for PDE4B) were as follows: compound A (5.5 and 440 nmol L<sup>-1</sup>, 80-fold), roflumilast (0.49 and 0.49 nmol L<sup>-1</sup>, 1-fold), and cilomilast (73 and 21 nmol L<sup>-1</sup>, 0.3-fold) (6).

In the present study, we investigated the physiological role of PDE4B in human neutrophils by evaluating the inhibitory effect of compound A on human neutrophil chemotaxis and comparing it to that of subtype non-selective PDE4 inhibitors.

## EXPERIMENTAL

### *Reagents*

Compound A (2-[4-[[2-(3-fluoro-4-methoxy-phenyl)-7,8-dihydro-6H-thiopyrano [3,2-*d*]pyrimidin-4-yl]amino]phenyl] acetic acid), roflumilast and cilomilast were synthesized at Daiichi Sankyo Chemical Research Laboratories (Japan). LPS (*Escherichia coli* O55:B5), dextran ( $M_r$  = 450,000-650,000), leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (USA). RPMI 1640 without phenol red medium, a human TNF- $\alpha$  ELISA kit and a human IL-6 ELISA kit were purchased from Life Technology (USA). Calcein-AM was purchased from Dojindo Laboratories (Japan) and Ficoll-Paque Plus was purchased from GE Healthcare Japan (Japan). Human IL-8 was purchased from R&D systems (USA).

### *Preparation of cells*

Human peripheral blood (30 mL) was obtained from healthy volunteers (aged 43–44 years, both male and female). The volunteers were forbidden to take any medicine for seven days before blood collection. All experimental procedures were approved by the Institutional Ethical Committee at Daiichi Sankyo and performed in accordance with the guidelines. Mononuclear cells and neutrophils were separated by a Ficoll gradient protocol

according to the manufacturer's instructions. Briefly, erythrocyte sedimentation was performed by adding 3 mL of dextran dissolved in PBS (6 %) to 30 mL of heparinized whole blood and kept for 30 min at room temperature. The supernatant was carefully collected and layered onto 20 mL of Ficoll-Paque Plus and centrifuged at 400 $\times$ g for 30 min at room temperature. After centrifugation, mononuclear cells and neutrophils were obtained in the interface and pellet, respectively. The cells were hemolyzed by adding 2 mL of 0.2 % NaCl solution and then incubation on ice for 1 min, followed by isotonicization. The purity of neutrophils by this sedimentation method was > 90 % pure by morphology. Mononuclear cells and neutrophils were washed twice and re-suspended at 4  $\times$  10<sup>6</sup> cells mL<sup>-1</sup> in culture medium (RPMI 1640 with 2 % FBS).

#### *LPS-induced cytokine release from mononuclear cells*

An LPS-induced cytokine release assay was performed as previously described (7). LPS-containing culture medium (200 ng mL<sup>-1</sup>) with the test compound (2-fold of the final concentration) was added to a 96-well tissue culture plate (100  $\mu$ L per well). Mononuclear cells (2  $\times$  10<sup>6</sup> cells mL<sup>-1</sup>) were added to the well (100  $\mu$ L per well) and incubated for 18 h at 37 °C. After brief centrifugation, the supernatant was collected and kept at -20 °C until use. The concentration of TNF- $\alpha$  and IL-6 in the supernatant was measured using an ELISA kit according to the manufacturer's instructions and the percent concentration of each sample compared to the control samples was calculated.

#### *Neutrophil chemotaxis assay*

A neutrophil chemotaxis assay was performed as previously described (8). Neutrophil suspensions (4  $\times$  10<sup>6</sup> cells mL<sup>-1</sup>) were incubated with 5  $\mu$ mol L<sup>-1</sup> calcein-AM for 30 min at 37 °C. The cells were washed twice with ice-cold culture medium and re-suspended at 2  $\times$  10<sup>6</sup> cells mL<sup>-1</sup>. Test compounds (roflumilast 0.001, 0.01, 0.1 and 1.0 mmol L<sup>-1</sup>, compound A and cilomilast 0.01, 0.1, 1.0 and 10 mmol L<sup>-1</sup> in DMSO) were added (1/1,000 volume) to each cell suspension and 75  $\mu$ L of the cell suspension with test compounds was loaded into the upper well of each transwell, followed by pre-incubation at 37 °C for 30 min. IL-8 (10 ng mL<sup>-1</sup>) or LTB<sub>4</sub> (1 ng mL<sup>-1</sup>) was loaded into the lower well of the transwell plate (275  $\mu$ L) and pre-incubated at 37 °C for 30 min. A chemotaxis assay was started by carefully placing the upper well onto the lower well and the transwell plate was incubated for 40 min at 37 °C. The chemotaxis assay was terminated by separating the upper well from the lower well. The cells in the lower well were completely lysed by adding 10  $\mu$ L of 10 % SDS aqueous solution. The migrated cells were quantified by measuring calcein fluorescein of the cell lysate (excitation 485 nm/emission 535 nm) using a microplate reader (PerkinElmer Inc., USA). Vehicle-treated wells served as controls and the percent inhibitions of test compound-treated wells were calculated after correction with blank wells.

#### *Data analysis*

The mean and the standard error (SE) were calculated. The IC<sub>50</sub> value was calculated and a variable-slope sigmoidal dose-response curve was fitted using the GraphPad Prism version 5.04 (GraphPad Software).

## RESULTS AND DISCUSSION

### *Inhibitory effect of compound A on LPS-induced TNF- $\alpha$ release from mononuclear cells*

First, we tested the inhibitory effect of compound A on human mononuclear cells. It was previously reported that subtype non-selective PDE4 inhibitors strongly suppressed LPS-stimulated TNF- $\alpha$  release but IL-1 $\beta$  or IL-6 release were partial or absent from human monocytes (9, 10). Roflumilast and cilomilast inhibited the TNF- $\alpha$  production in a concentration-dependent manner (Fig. 1a) while the inhibitory effect on IL-6 production was absent (Fig. 1b). Similarly, compound A inhibited TNF- $\alpha$  production by LPS-stimulated mononuclear cells in a concentration-dependent manner (Fig. 1a) but did not inhibit IL-6 production (Fig. 1b). Compound A showed the same inhibitory profiles on LPS-stimulated cytokine release from mononuclear cells as subtype non-selective PDE4 inhibitors.

### *Inhibitory effect of compound A on human neutrophil chemotaxis*

Next, we investigated the inhibitory effect of compound A on neutrophil migration. Roflumilast and cilomilast inhibited IL-8-induced neutrophil chemotaxis in a concentration dependent manner (Fig. 2a). In contrast, compound A slightly inhibited the IL-8-induced chemotactic response of neutrophil cells and the inhibitory effect was modest even at the highest concentration (10  $\mu\text{mol L}^{-1}$ ) (Fig. 2a). In order to determine whether this weak inhibitory effect of compound A on neutrophil chemotaxis is specific for IL-8-induced chemotaxis or not, we subsequently tested the inhibitory effect of compound A on LTB<sub>4</sub>-induced neutrophil migration. The inhibitory effect of compound A on LTB<sub>4</sub>-induced chemotaxis was limited while roflumilast and cilomilast inhibited neutrophil migration effectively (Fig. 2, Table I).

It was a surprise that the inhibitory effect of compound A on IL-8 or LTB<sub>4</sub>-induced neutrophil migration was weak. It has been suggested that the inhibitory effect of subtype non-selective PDE4 inhibitors on neutrophils is mainly due to an inhibitory effect on

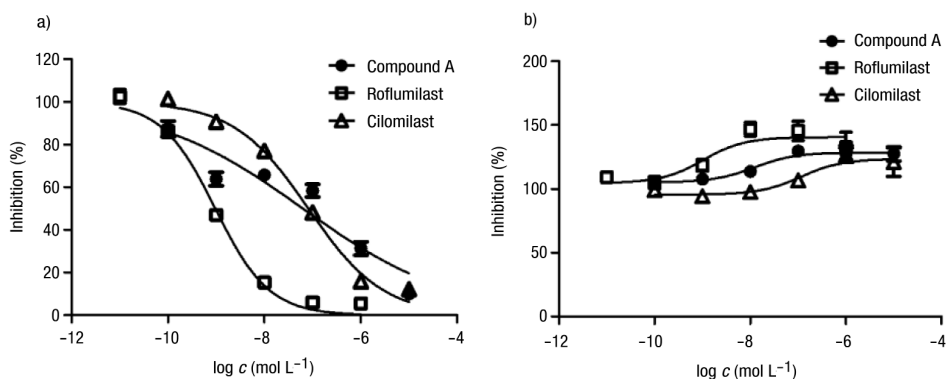


Fig. 1. Percent inhibition by compounds in LPS-induced cytokine release from human mononuclear cells *vs.* controls: a) TNF- $\alpha$ , b) IL-6. Data is shown as mean  $\pm$  SE ( $n = 3$ ).

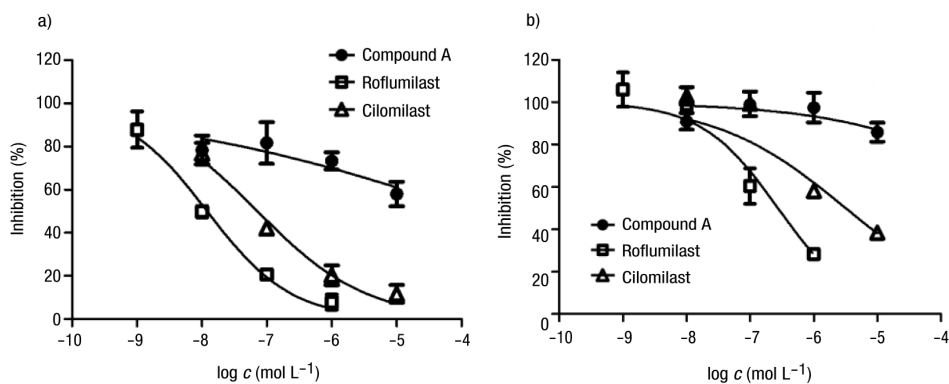


Fig. 2. Percent inhibition by compounds in: a) IL-8 or b) LTB<sub>4</sub>-induced human neutrophil chemotaxis vs. controls. Data is shown as mean  $\pm$  SE ( $n = 3$ ).

Table I. Summary of *in vitro* profiles of compound A

Subject	IC <sub>50</sub> (nmol L <sup>-1</sup> ) <sup>a</sup>		
	Compound A	Roflumilast	Cilomilast
LPS-induced TNF- $\alpha$ release from mononuclear cells	34 $\pm$ 14	0.66 $\pm$ 0.16	55 $\pm$ 14
IL-8-induced neutrophil chemotaxis	> 10,000	32 $\pm$ 25	110 $\pm$ 67
LTB <sub>4</sub> -induced neutrophil chemotaxis	> 10,000	250 $\pm$ 78	2,700 $\pm$ 450

<sup>a</sup> Mean  $\pm$  SE,  $n = 3$ .

PDE4B considering the predominant expression of PDE4B in human neutrophils over other PDE4 subtypes (5). As PDE4D is expressed in neutrophils, even if small, it might be possible that a small amount of PDE4D, not PDE4B, may control neutrophil migration. This discrepancy between the expression level and functional importance of PDE4 subtypes might be explained by the subcellular compartmentalized regulation of cAMP concentration controlled by localized PDE subtype enzymes. It was recently reported that cells regulate their intracellular levels of cAMP in a highly compartmentalized manner (1, 11). It is possible that the PDE4D-controlled cAMP level in a specific compartment is critical for the regulation of neutrophil chemotaxis.

In order to elucidate the functional importance of PDE4 subtypes in human neutrophil functions, evaluation of neutrophil function by a pharmacological method using PDE4A, C and D-selective inhibitors may be required. However, highly subtype-selective inhibitors are not currently available. It is also important to investigate whether other functions of neutrophils such as superoxide generation or elastase release are suppressed by compound A or not.

The clinical importance obtained from the present study is that PDE4B-selective inhibitors such as compound A could be anti-inflammatory drugs that do not inhibit neutrophil function but have a strong inhibitory effect on inflammatory cytokine such as IL-1 $\beta$  or TNF- $\alpha$  from mononuclear cells, thus having a reduced risk of opportunistic infection.

## CONCLUSIONS

The present study has shown for the first time that PDE4B-selective inhibitors do not inhibit IL-8 or LTB<sub>4</sub>-induced neutrophil chemotaxis. This evidence strongly suggests that PDE4B does not play an important role during the IL-8 or LTB<sub>4</sub>-induced chemotactic response of human neutrophils. Further studies are required to address the physiological role of PDE4 subtypes during human leukocyte activation.

*Acknowledgements.* – We thank Dr. Kiyoshi Mizukami, Dr. Hiroshi Tsuchida, Dr. Yoshitaka Sogawa, Ms. Maki Etori, Ms. Nana Takagi, Dr. Akiko Shiina, Dr. Kiyoshi Morimoto and Dr. Kazuki Hirahara for their contribution to this work.

## REFERENCES

1. D. H. Maurice, H. Ke, F. Ahmad, Y. Wang, J. Chung and V. C. Manganiello, Advances in targeting cyclic nucleotide phosphodiesterases, *Nat. Rev. Drug Discov.* **13** (2014) 290–314; DOI: 10.1038/nrd4228.
2. S. H. Francis, M. A. Blount and J. D. Corbin, Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions, *Physiol. Rev.* **91** (2011) 651–690; DOI: 10.1152/physrev.00030.2010.
3. C. D. Manning, M. Burman, S. B. Christensen, L. B. Cieslinski, D. M. Essayan, M. Grous, T. J. Torphy and M. S. Barnette, Suppression of human inflammatory cell function by subtype-selective PDE4 inhibitors correlates with inhibition of PDE4A and PDE4B, *Br. J. Pharmacol.* **128** (1999) 1393–1398; DOI: 10.1038/sj.bjp.0702911.
4. D. Peter, S. L. Jin, M. Conti, A. Hatzelmann and C. Zitt, Differential expression and function of phosphodiesterase 4 (PDE4) subtypes in human primary CD4<sup>+</sup> T cells: predominant role of PDE4D, *J. Immunol.* **178** (2007) 4820–4831; DOI: 10.4049/jimmunol.178.8.4820.
5. P. Wang, P. Wu, K. M. Ohleth, R. W. Egan and M. M. Billah, Phosphodiesterase 4B2 is the predominant phosphodiesterase species and undergoes differential regulation of gene expression in human monocytes and neutrophils, *Mol. Pharmacol.* **56** (1999) 170–174; DOI: 10.1124/mol.56.1.170.
6. O. Suzuki, K. Mizukami, M. Etori, Y. Sogawa, N. Takagi, H. Tsuchida, K. Morimoto, T. Goto, T. Yoshino, T. Mikkaichi, K. Hirahara, S. Nakamura and H. Maeda, Evaluation of the therapeutic index of a novel phosphodiesterase 4B-selective inhibitor over phosphodiesterase 4D in mice, *J. Pharmacol. Sci.* **123** (2013) 219–226; DOI: 10.1254/jphs.13103FP.
7. I. Clöz-Tayarani, A. F. Petit-Bertron, H. D. Venters and J. M. Cavillon, Differential effect of serotonin on cytokine production in lipopolysaccharide-stimulated human peripheral blood mononuclear cells: involvement of 5-hydroxytryptamine<sub>2A</sub> receptors, *Int. Immunol.* **15** (2003) 233–240; DOI: 10.1093/intimm/dxg027.
8. G. Vacca, G. W. J. Randerath and A. Gillissen, Inhibition of granulocyte migration by tiotropium bromide, *Respir. Res.* **12** (2011) article 24; DOI: 10.1186/1465-9921-12-24.

9. U. Prabhakar, D. Lipshutz, J. O. Bartus, M. J. Slivjak, E. F. Smith, J. C. Lee and K. M. Esser, Characterization of cAMP-dependent inhibition of LPS-induced TNF alpha production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor, *Int. J. Immunopharmacol.* **16** (1994) 805–816; DOI: 10.1016/0192-0561(94)90054-x.
10. T. Yoshimura, C. Kurita, T. Nagao, E. Usami, T. Nakao, S. Watanabe, J. Kobayashi, F. Yamazaki, H. Tanaka and H. Nagai, Effects of cAMP-phosphodiesterase isozyme inhibitor on cytokine production by lipopolysaccharide-stimulated human peripheral blood mononuclear cells, *Gen. Pharmacol.* **29** (1997) 633–638; DOI: 10.1016/S0306-3623(96)00580-0.
11. M. D. Houslay, Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown, *Trends Biochem. Sci.* **35** (2010) 91–100; DOI: 10.1016/j.tibs.2009.09.007.