WA8242A_1, A_2 and B, Novel Secretory Phospholipase A_2 Inhibitors Produced by *Streptomyces violaceusniger*

II. Biological Properties

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WA8242A_1, A_2 and B, novel inhibitors of secretory phospholipase A_2 (PLA_2), were obtained from *Streptomyces violaceusniger* No. 8242. WA8242B inhibited secretory group I and II PLA_2s in a dose-dependent manner. The mode of inhibition of group II PLA_2 by WA8242B was in competitive fashion. WA8242B inhibited group II PLA_2-induced Prostaglandin I_2 (PGI_2) production by human umbilical vein endothelial cells. Furthermore, WA8242B was effective in mouse zymosan writhing model.

Based on their primary structures, three classes of mammalian phospholipase A_2, namely, cytosolic PLA_2, secretory group I and II PLA_2s, have been reported[1,2]. Among them, secretory group II PLA_2 plays some critical roles in the process of inflammation by catalysing the hydrolysis of phospholipids to form arachidonic acid and lysophospholipids used for eicosanoids and platelet-activating factor synthesis. Relatively high level of group II PLA_2 has been detected at inflamed sites and in circulating blood from the patients and experimental models of diseases[3]. In addition, the injection of group II PLA_2 into normal rat joints or normal rat air pouch led to an acute inflammatory responses[4,5]. Consequently, inhibition of secretory group II PLA_2 is likely to be therapeutically beneficial to various inflammatory diseases.

As described in previous paper, we found that the culture of *Streptomyces violaceusniger* No. 8242 produced novel type of secretory PLA_2 inhibitors, WA8242A_1, A_2 and B[6]. This paper describes the biological properties of WA8242B as a representative of these inhibitors.

Materials and Methods

Materials

Porcine pancreatic PLA_2, human synovial PLA_2 and (1-pyreno)decanoic acid labeled substrates were described in the previous paper[6]. [^{14}C] and [^3H]-labeled substrates were purchased (NEN). Human umbilical vein endothelial cells (HUVEC) were grown in MCDB 107 medium supplemented with 10% fetal bovine serum, 10 ng/ml basic fibroblast growth factor and 75 μg/ml kanamycin and incubated at 37°C in a water-saturated atmosphere containing 5% CO_2. All other chemicals were of the analytical grade.

PLA_2 Assay

Porcine pancreatic PLA_2 was used as group I PLA_2 at 100 ng/ml and recombinant human synovial PLA_2 was used as group II PLA_2 at 10 ng/ml. Reaction buffer and standard assay method were described in the previous paper[6].

Three types of substrates were used for the assay of PLA_2. Among the first type were substrates such as (1-pyreno)decanoic acid-labeled phosphatidylcholine and phosphatidylethanolamine as described in the previous paper[6].

The second substrates were [^{14}C]phosphatidylcholine

(L-3-phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl) for group I PLA_2 and [^{14}C]phosphatidylethanolamine (L-3-phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]arachidonyl) for group II PLA_2. PLA_2 assay was performed according to a modification of the method described by K. B. GLASER *et al.*[7]. Each substrate was dried under a gentle stream of nitrogen gas and suspended with reaction buffer without $CaCl_2$ by a handy sonicator for 2 minutes at room temperature.The reaction condition was identical to that cited in the previous paper except that the reaction volume was 800 μl and that the final concentration of substrates were 0.04 μM (0.08 MBq/ml). To determine the release of [^{14}C]arachidonic acid from the substrate, free fatty acid was extracted by the method of NATORI *et al.*[8], and counted in 8 ml of Aquasol II. Polypropylene tube was used for the extraction of free fatty acid because organic solvents was used in this assay.

The third type of substrate was [^{3}H]arachidonic acid-labeled autoclaved *Eschericia coli* suspension for group I and II PLA_2. PLA_2 assay was performed by a modification of the method of R. B. PEPINSKY *et al.*[9]. The standard reaction buffer of 100 μl contained Tris-HCl buffer (50 mM, pH 7.4), NaCl (100 mM) and $CaCl_2$ (3 mM). The reaction was started by mixing 50 μl of the enzyme solution (20 ng/ml), which had been preincubated with inhibitors for 30 minutes, and 50 μl of substrate solution (1/5 diluted, 0.037 MBq/ml) and incubated for 5 minutes at room temperature in a microtest tube. To terminate the reaction, 300 μl of HCl (2 N) and 400 μl of BSA (10 mg/ml) were added and mixed. After centrifugation of the reaction mixture at 10,000 rpm for 10 minutes, 500 μl of supernatant was transferred to the vial containing 2.5 ml of Aquasol II and free [^{3}H]arachidonic acid was counted.

In the PLA_2 assays, the inhibitor or the vehicle was preincubated with enzyme for 30 minutes at room temperature. Each sample was dissolved in methanol (final 1.25%). All data are the average of at least duplicate determinations.

Group II PLA_2-induced PGI_2 Production by HUVEC

HUVEC were grown to confluence in 24-well plates. Cells were washed twice with Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin (BSA) and 400 μl of HBSS-BSA were added to each well. WA8242B was added and incubated for 20 minutes, then group II PLA_2 (3 μg/ml) and TNF-α (100 unit/ml) were added and incubated for 6 hours. PGI_2 released into the media was determined by enzyme immunoassay kit by measuring the stable metabolite 6-keto prostaglandin $F_{1\alpha}$. Assay was performed in duplicate.

Zymosan Writhing

WA8242B was dissolved in DMSO and diluted 10-fold with 20 mM PBS. The sample was administered intraperitoneally into male mice (ICR, 6 weeks old) 5 minutes before injection of zymosan. After the intraperitoneal administration of 50 mg/kg/10 ml of zymosan, writhing was counted for 30 minutes in a blind fashion. Seven mice were used for vehicle (10% DMSO in 20 mM PBS) and three groups of ten mice were administered with WA8242B. Student's *t*-test was used for statistical analysis of the results.

Results

PLA_2 Inhibition

Biological properties of novel secretory PLA_2 inhibitors were studied using WA8242B as a representative because it was isolated and obtained abundantly. Manoalide, a marine natural product which inhibits some PLA_2s, was used as a reference compound[7,10]. Inhibitory activity of WA8242B against PLA_2 was examined with three types of substrates *in vitro*.

WA8242B inhibited both group I and II secretory PLA_2 in a dose-dependent manner, whatever the types of substrates were used (Fig. 1). The IC_{50} values of WA8242B and Manoalide against secretory PLA_2s are listed in Table 1. The IC_{50} values of WA8242B against each PLA_2 varied with the types of substrates. The effects of WA8242B were stronger against group II PLA_2 than against group I PLA_2 when substrates were prepared as micelle. When the membrane suspension of *E. coli* was used as natural substrate, the IC_{50}s of WA8242B against group I and II PLA_2 were almost the same. Manoalide showed specific inhibitory activity against group II PLA_2, while it accelerated the group I PLA_2 reaction when pyrene-labeled phosphatidylcholine was used. In addition, it didn't inhibit group I PLA_2 even at 2400 nM when [^{14}C]-labeled phosphatidylcholine was used. When the natural substrate was used, maximum inhibition of Manoalide did not exceed 60% at 0.24~2400 nM.

The mixture of WA8242A_1 and A_2 also showed a similar potency with WA8242B against group I and II PLA_2 when pyrene-labeled substrate was used as cited in a previous paper[6].

The mode of action of WA8242B was investigated by kinetic studies (Fig. 2). Group II PLA_2 and pyrene-labeled phosphatidylethanolamine were used for the

Fig. 1. Effect of WA8242B on group I (○) and group II (●) PLA_2.

(1-Pyreno) decanoic acid, [^{14}C] arachidonate and [^{3}H] arachidonate represent the labels of each substrate used.

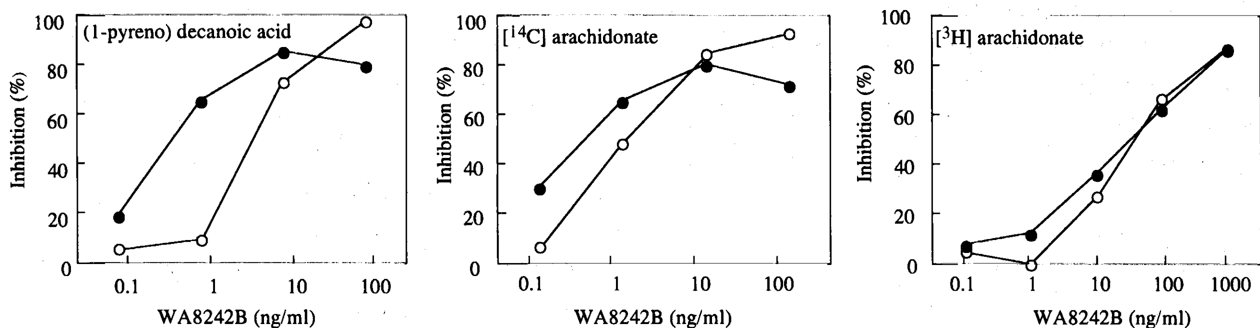


Table 1. IC_{50} values of WA8242B and manoalide against PLA_2s (10^{-9} M).

Group I PLA_2

Substrate	Label	Manoalide	WA8242B
Phosphatidylcholine	Pyrene	—[a]	1.1
Phosphatidylcholine	^{14}C	>2400	7.9
E. coli membrane	^{3}H	260[b]	52

[a] Accelaration at $0.24 \sim 2400 \times 10^{-9}$ M, [b] Maximum inhibition was lower than 60%.

Group II PLA_2

Substrate	Label	Manoalide	WA8242B
Phosphatidylethanolamine	Pyrene	260	0.14
Phosphatidylethanolamine	^{14}C	230	9.4
E. coli membrane	^{3}H	71	49

Dixon plot analysis. Fig. 2 suggests that WA8242B behaved kinetically as a competitive inhibitor against group II PLA_2 with *K*i value of 0.254 ng/ml (3.65×10^{-10} M).

WA8242B didn't induce the lysis of human erythrocyte even at 1×10^{-5} M. To confirm the specific inhibition of WA8242B against group I and II PLA_2, it was evaluated in the phospholipase C assay[11]. WA8242B didn't inhibit the hydrolysis of L-3-phosphatidyl [U-^{14}C]inositol by phospholipase C (*Bacillus cereus*) at 1×10^{-5} M (data not cited).

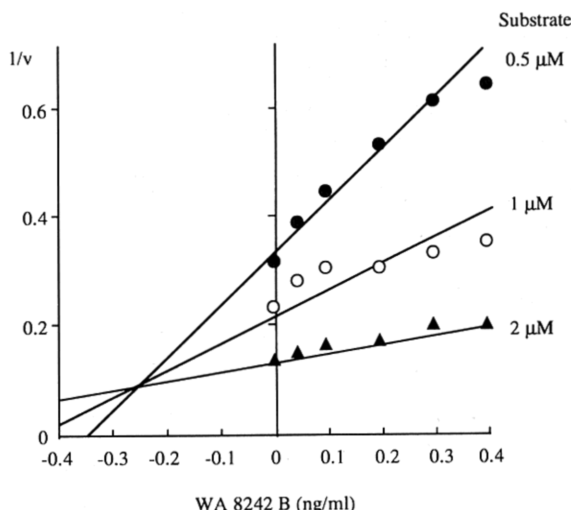
Group II PLA_2-induced PGI_2 Production by HUVEC

Murakami *et al.* reported that PGI_2 production by HUVEC was stimulated by group II PLA_2 and TNF-α[12]. WA8242B was evaluated to determine whether or not it inhibits the secretory group II PLA_2 in intact cell level. Results are shown in Fig. 3.

HUVEC produced 26.3 pg/ml of PGI_2 spontaneously. WA8242B did not inhibit spontaneous PGI_2 production. TNF-α (100 units/ml) stimulated PGI_2 production up to 42.3 pg/ml. WA8242B inhibited PGI_2 production stimulated by TNF-α. Exogeneous addition of group II PLA_2 (3 μg/ml) stimulated PGI_2 production to 55.0 pg/ml. As shown in Fig. 3, WA8242B dose-dependently inhibited PGI_2 production stimulated by group II PLA_2. When HUVEC was stimulated by TNF-α and group II PLA_2 simultaneously, PGI_2 production was enhanced to 225 pg/ml. The synergic stimulation of PGI_2 production was inhibited by WA8242B dose-dependently with an IC_{50} value of 8.4 μg/ml (1.2×10^{-5} M).

Comparing the IC_{50} values of WA8242B in this assay with those of cell-free assay, potency of WA8242B in this assay was considerably lower. The higher IC_{50} value in the cell test seems to be caused by the low solubility of WA8242B in water. Since WA8242B exists as fine lipid droplet in HBSS at the concentrations tested in this assay,

Fig. 2. Dixon analysis of inhibition by WA8242B of group II PLA_2.



[a] Reaction time was 10 minutes at room temperature.
[b] 1/v: m $mole^{-1}$, min, mg protein.
[c] Each value represents the mean of two determinations.

it may not be able to inhibit enzyme effectively.

Zymosan Writhing

To estimate the effect of WA8242B *in vivo*, the inhibitor was evaluated in a mouse zymosan writhing model[13]. It has been reported that zymosan-induced writhing response is a result of intraperitoneal production of PGI_2 and that cyclooxygenase inhibitors exert analgestic activities in this model. As shown in Fig. 4, WA8242B inhibited writhing responses dose-dependently with the ED_{50} value of 5.6 mg/kg. Single injection of WA8242B without zymosan did not cause any writhing responses even at 10 mg/kg (data not cited).

Discussion

Streptomyces violaceusniger No. 8242 produced novel secretory PLA_2 inhibitors, WA8242A_1, A_2 and B. Biological properties of WA8242B were evaluated both *in vitro* and *in vivo* as a representative.

PLA_2 inhibitory activities of WA8242B *in vitro* were examined with three types of substrates. The three types of substrates were labeled at *sn*-2 position with (1-pyreno)decanoic acid, [^{14}C]arachidonic acid and [^{3}H]arachidonic acid. In the case of pyrene-labeled fluorometric assay and [^{14}C]-labeled radioisotope assay, the substrates were prepared as micelle. These assay

Fig. 3. Effect of WA8242B on group II PLA_2-induced PGI_2 release from HUVEC.

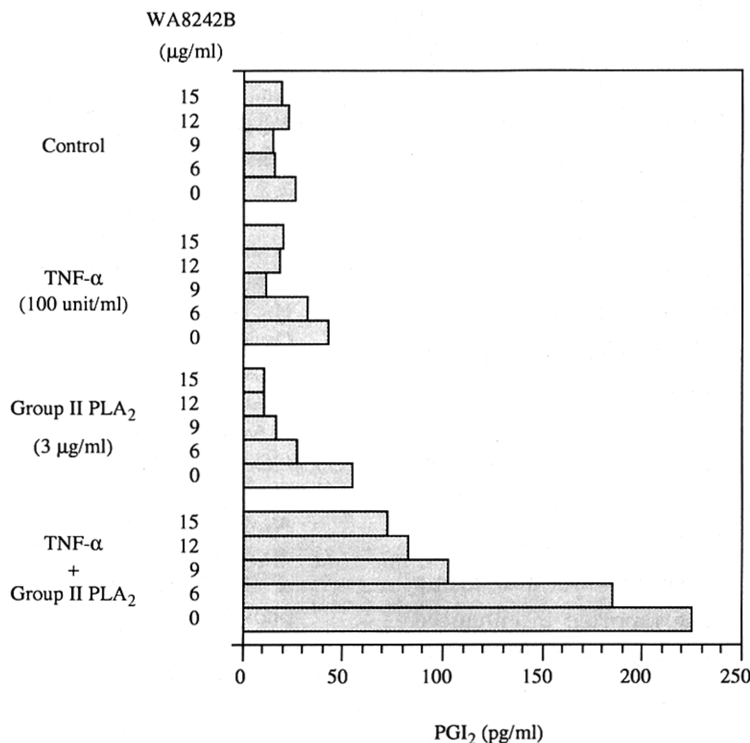
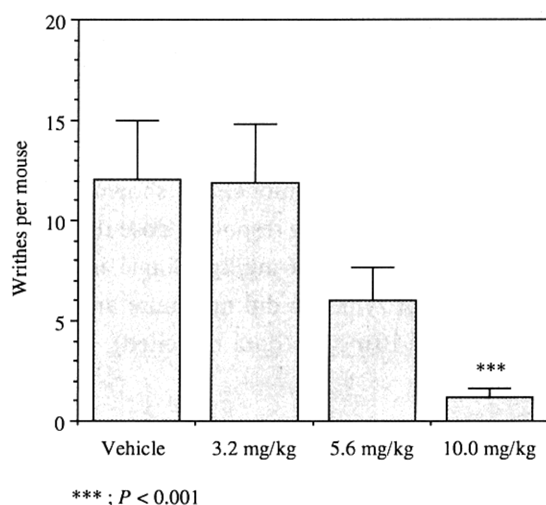


Fig. 4. Effect of WA8242B on zymosan writhing.



methods showed high sensitivities and therefore are desirable for screening. These methods, however, could not discriminate intrinsic inhibitors from nonspecific inhibitors such as detergent. To avoid this risk, [^{3}H]arachidonic acid-labeled *E. coli* membrane suspension was also used as a natural substrate.

WA8242B inhibited both group I and II secretory PLA_2 in a dose-dependent manner, whatever the types of substrates were being used (Fig. 1). Half inhibitory concentrations of WA8242B and Manoalide against secretory PLA_2 were listed in Table 1. The potency of PLA_2 inhibitory activity varied with the types of substrates. The effects of WA8242B were stronger against group II than against group I PLA_2 when the substrates were prepared as micelle. When the membrane suspension of *E. coli* was used as a natural substrate, the IC_{50}s of WA8242B against group I and group II PLA_2 were nearly the same. Manoalide showed specific inhibitory activity against group II PLA_2, but didn't show satisfactory inhibition against group I PLA_2.

Either TNF-α or group II PLA_2 slightly stimulated the release of PGI_2 from HUVEC. In combination, TNF-α and group II PLA_2 showed a marked synergistic effect on the release of PGI_2 from HUVEC. WA8242B inhibited the release of PGI_2 in a dose-dependent fashion when stimulated by group II PLA_2 alone or with TNF-α, though its effect was partial. Involvement of both group II and cytosolic PLA_2 has been reported in enhanced release of PGI_2 from HUVEC by group II PLA_2 and TNF-α stimulation[12]. Considering the partial inhibitory effect of WA8242B in this assay system, WA 8242B may not inhibit cytosolic PLA_2.

The effect of WA8242B on mouse zymosan writhing model was examined because zymosan-induced PGI_2 has been shown to be an important mediator of writhing. As PLA_2 plays a crucial role in the biosynthesis of PGI_2 by supplying arachidonic acid, inhibition of group II PLA_2 should prevent writhing. Though pharmacokinetics behavior of WA8242B has not been studied yet, intraperitoneal injection of WA8242B showed dose-dependent inhibition of writhing. This result supports the role of group II PLA_2 in the production of PGI_2 and proves the usefulness of WA8242B in the treatment of inflammatory diseases.

In conclusion, we have confirmed that WA8242B inhibited secretory group I and II PLA_2 both *in vitro* and *in vivo*. WA8242A_1, A_2 and B including their derivatives are the promising candidates for novel nonsteroidal anti-inflammatory and anti-allergic drugs.

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