THIELOCIN B3, A NOVEL ANTIINFLAMMATORY HUMAN GROUP II PHOSPHOLIPASE A_2 SPECIFIC INHIBITOR FROM ASCOMYCETES

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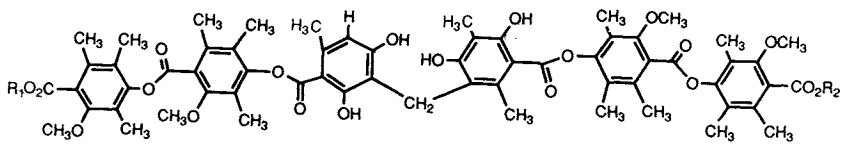
Evidence accumulated to date suggests that extracellular group II phospholipase A_2 (PLA_2-II) is involved in the pathogenesis of inflammatory disease. During screening for PLA_2 inhibitors, we found a novel PLA_2 inhibitor named thielocin B3 in the culture broth of an ascomycetes. Thielocin B3 strongly inhibited human PLA_2-II ($IC_{50}=0.076\ \mu M$) in a reversible and noncompetitive manner ($Ki=0.098\ \mu M$), whereas it inhibited human group I PLA_2 only weakly ($IC_{50}=18\ \mu M$). It also quenched the tryptophan fluorescence of *Naja mocambique* venom PLA_2; almost 100% quenching being attained at a thielocin B3/enzyme molar ratio of 1.0. Its inhibitory activity toward human PLA_2-II and *Naja mocambique* PLA_2 was markedly decreased by methylation of its two carboxyl groups, while the quenching observed for *Naja mocambique* PLA_2 was not altered. These results suggest that the two carboxyl groups do not participate in the binding of thielocin B3 to the enzyme, but play a crucial role in the PLA_2 inhibition. Furthermore, in the rat carrageenan-induced pleurisy model, thielocin B3 significantly reduced both exudate volume and PLA_2 activity in the exudate when coinjected with carrageenan.

Phospholipase A_2 (PLA_2) forms a diverse family of enzymes that catalyze the hydrolysis of sn-2 fatty acyl ester bond of glycerophospholipids[1] and exists in both extracellular and intracellular forms[2]. Much of the work on PLA_2 to date has focused on extracellular PLA_2, because of its relative abundance in venoms and pancreatic juice[3]. Extracellular PLA_2 can be classified into two types, group I (PLA_2-I) and group II (PLA_2-II), based on their primary structures[4]. Mammalian PLA_2-I is abundantly present in the pancreatic digestive secretion[5]. On the other hand, mammalian PLA_2-IIs are found in inflammatory regions, such as casein-induced peritoneal fluid in rats[6], carrageenan-induced pleural exudate in rats[7] and synovial fluid of patients with rheumatoid arthritis[8]. In addition, some inflammatory cytokines and lipopolysaccharide dramatically increased PLA_2-II secretion in several tissues of rat through enhancement of gene transcription[9,10]. These findings strongly implicate the importance of mammalian PLA_2-II in

Fig. 1. Chemical structures of thielocin B3 (**a**), monomethyl esters (**b**, **c**) and dimethyl ester (**d**).



	R_1	R_2
a	H	H
b	CH_3	H
c	H	CH_3
d	CH_3	CH_3

promoting inflammatory processes. In fact, some studies have shown the pro-inflammatory activities of PLA_2-II[11~13].

Recently, we isolated thielocin A1β, a novel PLA_2-II specific inhibitor, from the fermentation broth of *Thielavia terricola* RF-143[14]. It inhibited rat PLA_2-II very strongly with an IC_{50} of 0.0033 μM, but its inhibitory activity toward human PLA_2-II was rather weak with an IC_{50} of 12 μM[15]. In further screening, we isolated several analogues of thielocin A1β from the same fermentation broth. Among them, thielocin B3 (Fig. 1) showed the strongest inhibitory activity toward human PLA_2-II. The present study investigates the mechanism of thielocin B3 inhibition of human PLA_2-II and the anti-inflammatory effect of thielocin B3 in rat carrageenan-induced pleurisy.

Materials and Methods

Materials

Thielocin A1β and B3 were prepared as previously reported[14]. *p*-Bromophenacyl bromide (*p*-BPB), l-α-phosphatidylethanolamine (from egg yolk) and *Naja mocambique mocambique* PLA_2 (pI 9.6) were purchased from Sigma (St. Louis, MO). L-3-Phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]linoleoyl (2.18 GBq/mmol) was purchased from Amersham Corp. Human PLA_2-I was purified from human pancreatic juice[16]. Human PLA_2-II was isolated from rheumatoid arthritic synovial fluid[17]. Rat PLA_2-I was isolated from rat pancreas homogenate[18]. Rat PLA_2-II was purified from rat platelets[19]. *Naja mocambique mocambique* PLA_2 (pI 9.6) purchased from Sigma was further purified as described previously[20]. Each of the purified PLA_2s showed a single band of approximately 14 kDa on SDS-polyacrylamide gel electrophoresis (Coomassie brilliant blue staining). Autoclaved [^{3}H]oleic acid-labeled *Escherichia coli* (200,000 cpm containing approximately 1.0 nmol of phosphatidylethanolamine and phosphatidylglycerol) was obtained by the procedure of DAVIDSON *et al.*[21]. All other reagents were of analytical grade or better.

Assay of PLA_2 Activity

PLA_2 activity was measured as described previously[22]. The substrate was prepared by diluting 1-palmitoyl-2-[1-^{14}C]linoleoyl phosphatidylethanolamine with L-α-phosphatidylethanolamine to the specific activity of 2,000 dpm/nmol. The reaction was started by addition of the enzyme. The amount of PLA_2s was adjusted to linear kinetics for quantitation, *i.e.*, less than 20% hydrolysis of the substrate. Thielocin B3 and A1β were added to the assay tubes as a DMSO solution (2% of the final volume), using a DMSO-enzyme control. Control experiments showed that DMSO at this concentration had no effect on enzymatic activities. Inhibition is expressed as the percent of enzyme control. IC_{50} values were determined graphically from plots of percent inhibition versus log concentration of inhibitors.

Fluorescence Measurements

The tryptophan fluorescence was measured in a Hitachi F-3000 fluorescence spectrophotometer. The sample in a total volume of 2.0 ml contained 14 nmol of *Naja mocambique mocambique* PLA_2, 100 mM Tris-HCl buffer (pH 7.4), 3 mM $CaCl_2$ and the indicated concentrations of thielocin B3 was excited at 280 nm and emission was measured at 344 nm.

Carrageenan-induced Pleurisy in Rats and PLA_2 Activity in Pleural Exudate.

Male 8- to 10- week-old Sprague-Dawley rats (weighing 290~370 g) were slightly anesthetized with ether and injected with 0.5 ml of 0.2% carrageenan, dissolved in sterile saline solution, into the right pleural cavity through a blunt-edged 25-gauge needle. Five hours after carrageenan injection, the animals were sacrificed by exsanguination under ether anesthesia. The pleural exudate was harvested after opening the chest, and its volume was measured. The exudate containing blood was removed. The cavity was then washed with 1 ml of sterile saline. This wash was combined with the exudate to measure PLA_2 activity. The combined exudate was centrifuged at 2,000 $\times g$ for 15 minutes at 4°C immediately after collection to

remove cells, and the supernatants were stored at −20°C until analysis. To assay PLA_2 activity in the pleural exudate, [^{3}H]oleic acid-labeled *Escherichia coli* was used as substrate[7].

Results

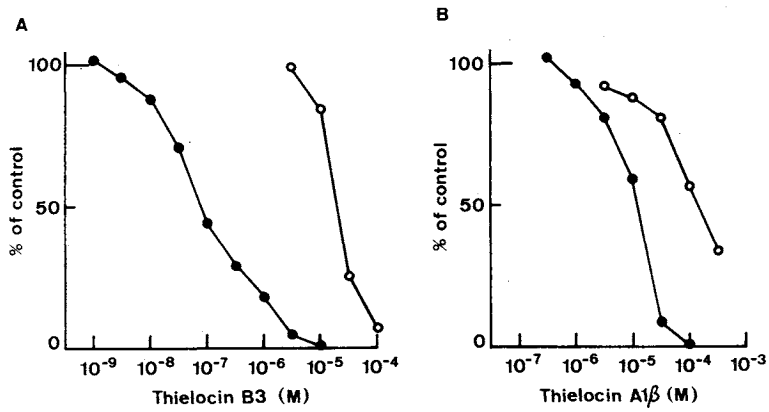
Inhibition of Human Extracellular PLA_2s by Thielocin B3

Thielocin B3 inhibited human PLA_2-II very strongly in a dose-dependent manner with an IC_{50} of 0.076 μM (Fig. 2A), whereas the inhibition by thielocin A1β was 160 times weaker (IC_{50} of 12 μM) than that caused by thielocin B3. (Fig. 2B). On the other hand, thielocin B3 and thielocin A1β showed weak inhibitory activity against human PLA_2-I with IC_{50}s of 18 μM and 140 μM, respectively. Thus, thielocin B3 inhibition of human PLA_2-II was 240 times stronger than that of human PLA_2-I. In addition, the group II PLA_2 specific inhibitory activity of thielocin B3 was also conserved in rat extracellular PLA_2s (for rat PLA_2-I; $IC_{50}=2.8$ μM, for rat PLA_2-II; $IC_{50}=0.012$ μM). The inhibition of human PLA_2s by thielocin B3 and thielocin A1β was independent of Ca^{2+} and substrate concentration (data not shown). Furthermore, double reciprocal plot showed that thielocin B3 (Fig. 3A) and thielocin A1β (Fig. 3B) behaved kinetically as noncompetitive inhibitors for human PLA_2-II with mean *Ki* of 0.098 and 12 μM, respectively. Thus, thielocin B3 showed 120 times higher affinity for human PLA_2-II than thielocin A1β.

Reversibility of Thielocin B3 Inhibition against Human PLA_2-II

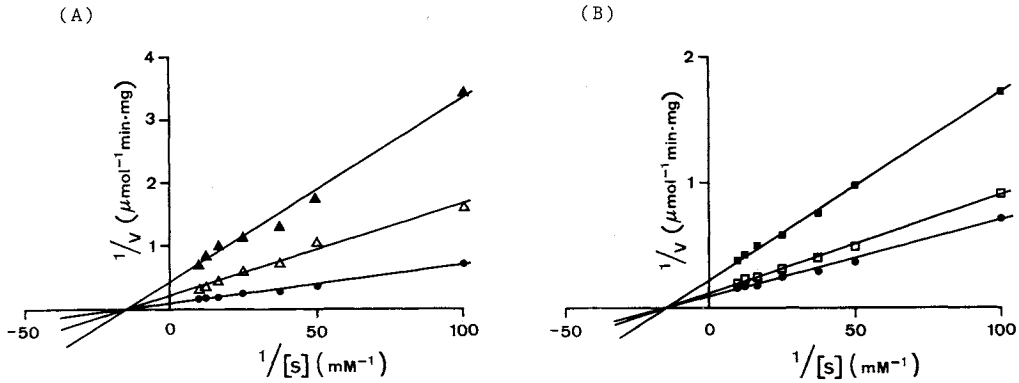
The reversible nature of thielocin B3 was confirmed by the dilution method of LISTER *et al.* (Table 1)[23]. After human PLA_2-II was preincubated with thielocin B3 (37 °C, 20 minutes) at 0.3 μM, a concentration high enough to sufficiently reduce the enzymatic activity, a portion was removed and diluted 30-fold to 0.01 μM with the assay mixture. Slight inhibition was observed, indicating reversible inhibition. Had the inhibition been irreversible, the rate would have been inhibited at least 89%, corresponding to an inhibitor concentration of 0.3 μM. A similar result was observed when thielocin B3 was used at a concentration of

Fig. 2. Inhibition of human extracellular phospholipase A_2 by thielocin B3 (panel A) and thielocin A1β (panel B).



The activities of the enzyme control (*i.e.* 100%) were from 4,000 to 5,900 nmol/minute/mg protein of human PLA_2-I (○) and from 6,100 to 8,200 nmol/minute/mg protein of human PLA_2-II (●). Inhibition is expressed as the percent of enzyme control. Data points are the means of three independent experiments, each performed in duplicate and corrected for no enzymatic hydrolysis (0.5% or less in all experiments). The SD value was 8% or less than the mean for each data point.

Fig. 3. Noncompetitive inhibition of human group II phospholipase A_2 by thielocin B3 (panel A) and thielocin A1β (panel B).



Double reciprocal plot of human PLA_2-II activity toward phosphatidylethanolamine in the presence of thielocin B3 {0.1 μM (△), 0.3 μM (▲); panel A} and thielocin A1β {5 μM (□), 15 μM (■); panel B} or absence of inhibitor (●). Standard assay conditions were employed and the lines were drawn on the basis of regression analysis.

Table 1. Distinction between reversible and irreversible inhibition for thielocin B3 and *p*-bromophenacyl bromide (*p*-BPB).

Compound	Concentration (μM)		Phospholipase A_2 activity (% of control)		
	Preincubation[a]	Assay[b]	Predicted irreversible	Predicted reversible	Experimentally[c] found
Thielocin B3	0.3	0.01	11.4	71.2	71.5±10.3
	0.9	0.03	4.0	48.7	37.4± 5.1
p-BPB	300	10	3.0	72.5	2.4± 1.3

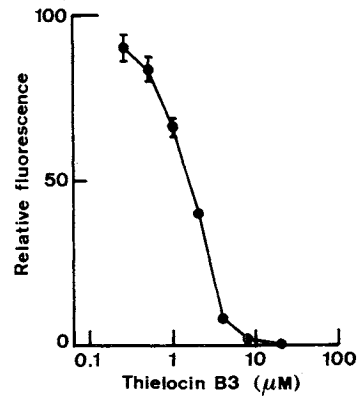
[a] Human PLA_2-II was preincubated with inhibitor at the designated concentration for 20 minutes at 37°C.
[b] Inhibitor concentration after dilution for assay.
[c] Results are mean±SD of triplicate determinations, each performed in triplicate.

0.9 μM during preincubation and then was diluted to 0.03 μM during the assay. On the other hand, *p*-BPB, a reputed irreversible PLA_2 inhibitor, showed similar inhibitory activity before and after dilution, indicating irreversible inhibition.

Effect of Thielocin B3 on the Fluorescence Emission of Snake Venom PLA_2

Direct interaction of thielocin B3 with snake venom PLA_2 (*Naja mocambique*) was examined by monitoring the tryptophan fluorescence of the enzyme in the presence and absence of thielocin B3. Thielocin B3 quenched the relative fluorescence of 7.2 μM snake venom PLA_2 at 344 nm in a dose-dependent manner (Fig. 4). Approximately 100% of the fluorescence was quenched when the molar ratio of thielocin B3/enzyme was 1.0.

Fig. 4. Fluorescence of the snake venom (*Naja mocambique mocambique*) PLA_2 as a function of the concentration of thielocin B3.



Samples were excited at 280 nm and emission was measured at 344 nm. Fluorescence of the enzyme alone=100%. The values indicate averages±SD (n=3).

Table 2. Comparison of PLA_2 inhibition and interaction by thielocin B3 methyl ester derivatives.

Compound		Inhibition (IC_{50}; μM)		Interaction (ED_{50}; μM)
		Human PLA_2-II	Snake venom PLA_2	Snake venom PLA_2
Thielocin B3	**(a)**	0.074	0.0045	1.6
-monomethylester	**(b)**	0.20	0.032	5.2
-monomethylester	**(c)**	0.28	0.31	5.2
-dimethylester	**(d)**	51	>100	7.6

Table 3. Effects of thielocin B3, indomethacin and dexamethasone on exudate volume and PLA_2 activity at 5 hours after the intrapleural injection of carrageenan in rats.

Drug	Dose (mg/kg)	Exudate volume (ml)	PLA_2 activity (pmol/minute/ml)
Control		1.99 ±0.14	6.62±0.61
Thielocin B3[a]	1.0	1.60±0.07*	2.22±0.22**
		(80±4)[c]	(34±3)
	3.0	1.15±0.05**	0.76±0.60**
		(58±3)	(12±9)
Indomethacin[a]	1.0	1.08±0.09**	7.36±0.54
		(54±5)	(111±8)
Dexamethasone[b]	0.1	0.60±0.12**	7.94±0.47
		(30±6)	(120±7)

[a] Thielocin B3 and indomethacin were administered intrapleurally with carrageenan.
[b] Dexamethasone was administered per os 1 hour before the carrageenan injection.
[c] The numbers in the parentheses express the percentage of the control group. Each value represents the mean±SEM obtained from five to six animals.
* $P<0.05$, ** $P<0.01$.

To explore in more detail the inhibitory mechanism of thielocin B3 on PLA_2, we examined the effect of thielocin B3 methyl ester derivatives (see Fig. 1) on human PLA_2-II. Two thielocin B3 monomethyl esters (**b**, **c**) and thielocin B3 dimethyl ester (**d**) showed inhibitory activity against human PLA_2-II. However, the dose required for 50% inhibition increased on methylation; 0.20 μM and 0.28 μM for thielocin B3 monomethyl esters (**b** and **c**), and 51 μM for thielocin B3 dimethyl ester (**d**). Thus, thielocin B3 showed a marked decrease (*ca.* 690 times) of its inhibitory activity against human PLA_2-II on dimethylation. Similar results were obtained with *Naja mocambique* venom PLA_2 (Table 2). On the other hand, quenching of relative fluorescent intensity of *Naja mocambique* venom PLA_2 by thielocin B3 was not markedly altered on methylation (Table 2).

Effect of Thielocin B3 on Exudate Volume and PLA_2 Activity in Rat Carrageenan-Induced Pleurisy

Recently, we have reported that PLA_2-II activity in the exudate of pleural cavity was increased up to 24 hours after the intrapleural injection of carrageenan. In this model, thielocin A1β correspondingly reduced both exudate volume and PLA_2-II activity in the exudate in a dose-dependent manner when coinjection with carrageenan[7]. Thielocin B3 had the maximal inhibitory activity among the members against human PLA_2-II, and this led us to examine the effect of thielocin B3 on the volume of pleural exudate in rat carrageenan-induced pleurisy (Table 3). At 5 hours after the injection of carrageenan, the exudate volume in the pleural cavity was dose-dependently decreased by thielocin B3 in a significant manner to 58±3% ($p<0.01$) at 3 mg/kg, 80±4% ($p<0.05$) at 1 mg/kg. In addition, thielocin B3 significantly

reduced the PLA_2-II activity in the pleural exudate dose-respondingly to $12\pm9\%$ at 3 mg/kg and $34\pm3\%$ at 1 mg/kg. For reducing the exudate volume, both indomethacin, a cyclooxygenase inhibitor, and dexamethasone, a steroidal antiinflammatory drug, were more potent than thielocin B3. However, neither indomethacin nor dexamethasone administration resulted in significant reduction of the PLA_2-II activity in the pleural exudate.

Discussion

These results demonstrated that thielocin B3, a novel PLA_2 inhibitor isolated from fungi, inhibits both human PLA_2-I and PLA_2-II in a dose-dependent manner. In addition, the inhibitory activity of thielocin B3 is rather specific against human PLA_2-II (Fig. 2). Recently, human PLA_2-II enriched in rheumatoid synovial fluid was purified from human platelets, and its gene was cloned and overexpressed[24,25]. Cloning and sequencing showed that the primary structure of human PLA_2-II has about 35% homology with that of human pancreatic PLA_2, which belongs to group I PLA_2. However, the sequence of human PLA_2-II preserves the core of residues found to be invariant or highly conserved among extracellular PLA_2s[26]. Furthermore, the X-ray crystal structures of human PLA_2-II in the presence and absence of a transition state analogue, L-1-*O*-octyl-2-heptylphosphonyl-sn-glycero-3-phosphoethanolamine, have been reported[27,28]. SCOTT *et al.* reported that the backbone conformation of the homologous core in the inhibited form of human PLA_2-II is virtually superimposable on that found in the crystal structures of the pancreatic PLA_2. On the contrary, the conformation of the homologous core in the uninhibited crystal form of human PLA_2-II differs slightly from that described for other enzymes. The main contributor to this change is the amino-terminal helix, which provides side chains to the substrate-binding site and forms a substantial portion of the interfacial recognition surface[28]. Whether thielocin B3 interacts with this site remains to be clarified, we are now conducting further studies to determine the structure of the complex between human PLA_2-II and thielocin B3.

In a recent paper[15], we reported the inhibitory mechanism of rat PLA_2-II by thielocin A1β. It exhibits extremely strong inhibitory activity with an IC_{50} of 0.0033 μM. However, against human PLA_2-II, it shows rather weak activity with an IC_{50} of 12 μM. It is noteworthy that thielocin B3 exhibits strong inhibitory activity against human PLA_2-II ($IC_{50}=0.076$ μM). Furthermore, the double reciprocal plot shows that both thielocin B3 and thielocin A1β behave kinetically as noncompetitive inhibitors for human PLA_2-II with *Ki* of 0.098 μM and 12 μM, respectively (Fig. 3). Therefore, thielocin B3 has 120 times higher affinity for human PLA_2-II than thielocin A1β. In addition, the ability of thielocin B3 to reversibly inhibit human PLA_2-II was confirmed using the dilution method according to LISTER *et al.*[23] (Table 1). These results distinguish thielocin B3 from agents such as manoalide and *p*-BPB, which inhibit PLA_2 by covalent modifications of lysine and histidine residues, respectively[29,30].

Direct, but non-covalent, interactions of inhibitory agents that modulate the structure of PLA_2 have been described in only rare instances. Several non-covalent PLA_2 inhibitors were examined for their direct interaction with the enzyme by the fluorescent method, and in this study, venom or pancreatic PLA_2 was used because of enzyme availability. FRANSON and ROSENTHAL[31] reported that PGBx, oligomers of PGB_1, directly interacted with *Naja mocambique* PLA_2 and 50% quenching was noted with a molar ratio of PGBx/enzyme of 1.5. In our experiments, approximately 100% of the fluorescence of *Naja mocambique* PLA_2 was quenched with the molar ratio of thielocin B3/enzyme at 1.0 (Fig. 4). These observations indicated that inhibition of extracellular PLA_2 by thielocin B3 may result from direct interaction with the enzyme.

BALLOU and CHEUNG[32] reported that unsaturated fatty acids inhibit human PLA_2-II. Methylation of unsaturated fatty acids caused complete loss of inhibitory activity, while subsequent demethylation restored it, suggesting that a free carboxyl group is necessary. As thielocin B3 possesses two carboxyl groups at both ends, we examined the effect of two monomethyl esters and a dimethyl ester of thielocin B3 on human PLA_2-II (Table 2). The inhibitory activity of thielocin B3 decreased with methylation and markedly decreased with dimethylation (690 times). On the other hand, quenching of the relative fluorescent intensity of *Naja mocambique* PLA_2 by thielocin B3 was not markedly altered by methylation. These results

indicate that the two carboxyl groups of thielocin B3 may not participate in the interaction with the enzyme, but play a crucial role in the PLA_2 inhibition.

Several investigators reported that inactivation of purified PLA_2 with *p*-BPB before injection resulted in attenuation of the subsequent inflammatory reaction[11]. We recently found that in the rat carrageenan-induced pleurisy model, thielocin A1β correspondingly reduced both exudate volume and PLA_2-II activity in the pleural exudate in a dose-dependent manner when coinjected with carrageenan. These results suggest that thielocin A1β shows antiinflammatory activity due to inhibition of PLA_2-II activity[7]. However, thielocin A1β showed rather weak inhibitory activity against human PLA_2-II (IC_{50} = 12 μM) as compared to rat PLA_2-II (IC_{50} = 0.0033 μM). On the other hand, thielocin B3 showed extremely strong inhibition activity against both human PLA_2-II (IC_{50} = 0.074 μM) and rat PLA_2-II (IC_{50} = 0.012 μM). Interestingly, thielocin B3 administered intrapleurally at a dose of 3.0 mg/kg (Table 3) and intraperitoneally at a dose of 50 mg/kg (unpublished data) decreased the exudate volume in the pleural cavity to 58 $\pm$ 3% and 66 $\pm$ 9%, respectively. NAKANO and ARITA reported[33] that intravenously injection of endotoxin in rat increased both PLA_2-II activity in the plasma and the levels of PLA_2-II mRNA in the several tissues (aorta, spleen, lung and thymus) at 24 hours after endotoxin challenge. Moreover, accumulation of PLA_2-II mRNA in the tissues of endotoxin-treated rats was suppressed by dexamethasone administration. Hence, it may be possible that PLA_2-II activity in that pleural exudate is reduced by administration of dexamethasone. Nevertheless, dexamethasone did not significantly decrease the PLA_2-II activity in the pleural exudate, in spite of it remarkably reduced the exudate volume in the pleural cavity at 5 hours after the injection of carrageenan (Table 3). Therefore, it may take longer period (over 5 hours) to reduce the PLA_2-II activity in pleural exudate by dexamethasone administration in the rat carrageenan-induced pleurisy model.

In conclusion, thielocin B3 showed the specific inhibitory activity against human PLA_2-II and it also showed reduction of exudate volume in the rat carrageenan-induced pleurisy model. Therefore, further studies are now in progress to investigate the antiinflammatory activity of thielocin B3 and the involvement of PLA_2-II in the pathogenesis of rat carrageenan-induced pleurisy.

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