A NOVEL TYPE OF PHOSPHOLIPASE A_2 INHIBITOR, THIELOCIN A1β, AND MECHANISM OF ACTION

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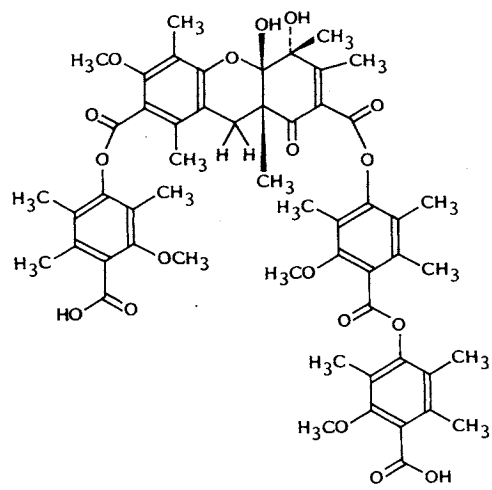
Thielocin A1β, a novel phospholipase A_2 inhibitor, was isolated from *Thielavia terricola* RF-143. It inhibited various phospholipase A_2s in a dose-dependent manner. Among these, group II phospholipase A_2 from rat was most sensitive to thielocin A1β ($IC_{50}=0.0033\,\mu M$). The inhibition of phospholipase A_2 by thielocin A1β was independent of Ca^{2+} and substrate concentration. In addition, the inhibition of rat group II phospholipase A_2 was noncompetitive ($Ki=0.0068\,\mu M$) and reversible. Furthermore, thielocin A1β quenched the relative fluorescent intensity of *Naja naja* venom phospholipase A_2 and in a dose-dependent manner; 50% quench was noted with a molar ratio of thielocin A1β/ enzyme of 2.2. These observations indicated that inhibition of phospholipase A_2 by thielocin A1β may result from direct interaction with the enzyme.

Phospholipase A_2 (PLA_2) is a lipolytic enzyme that specifically hydrolyzes the sn-2 position of a glycerophospholipid[1]. It exists in both extracellular and intracellular forms[2]. The former can be classified into two types, group I (PLA_2-I) and group II (PLA_2-II), based on the primary structure[3]. These enzymes are similar in that they are totally Ca^{2+} dependent, most active at neutral-to-alkaline pH, and have a molecular weight of about 14 kdaltons. Mammalian PLA_2-I is an important enzyme present in abundance in the digestive secretion of the pancreas[4]. On the other hand, mammalian PLA_2-IIs are found in inflammatory regions, such as glycogen-induced ascitic fluid in rabbits[5], casein-induced peritoneal fluid in rats[6], and synovial fluid of patients with rheumatoid arthritis[7]. These findings strongly implicate the importance of mammalian PLA_2-II in promoting inflammatory processes. In fact, some studies have shown the proinflammatory activities of PLA_2-II[8,9]. Recently, we isolated thielocin A1β, a novel PLA_2 inhibitor, from the fermentation broth of *Thielavia terricola* RF-143[10] (Fig. 1). To our surprise, thielocin A1β showed specific inhibition of rat PLA_2-II. In this report, we describe the inhibitory mechanism of PLA_2 and some of its other biological properties.

Fig. 1. Chemical structure of thielocin A1β.



Materials and Methods

Materials

Thielocin A1β was prepared as previously reported[10]. L-α-Phosphatidylethanolamine (from egg yolk), L-α-phosphatidylcholine (from egg yolk), L-α-phosphatidylinositol (from soybean), Triton X-100, mepacrine (quinacrine), *p*-bromophenacyl bromide, bee venom PLA_2, *Naja naja* venom PLA_2,

Naja mocambique mocambique PLA_2, *Vipera russeli* PLA_2 and *Crotalus adamanteus* PLA_2 were purchased from Sigma. L-3-Phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]linoleoyl (2.18 GBq/mmol), L-3-phosphatidylethanolamine, 1-acyl-2-[1-^{14}C]arachidonyl (2.21 GBq/mmol), L-3-phosphatidylinositol, 1-stearoyl-2-[1-^{14}C]arachidonyl (2.15 GBq/mmol) and L-3-phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl (2.16 GBq/mmol) were purchased from Amersham Corp. Rat PLA_2-I was isolated from rat pancreas homogenate according to the method of ONO *et al.*[11]. Rat PLA_2-II was purified from rat platelets[12]. Human PLA_2-I was purified from pancreatic juice[13]. Human PLA_2-II was isolated from rheumatoid arthritic synovial fluid according to the method of KANDA *et al.*[14]. Purified PLA_2s showed a single band of approximately 14 kdaltons by Coomassie brilliant blue staining on SDS-polyacrylamide gel electrophoresis. 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.*[15]. All other reagents were of analytical grade or better.

Assay of Phospholipase A_2

PLA_2 activity was measured by the method described previously[16]. 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 lipids were then dried under N_2 and suspended in deionized water with a probe sonicator. The standard reaction mixtures in a total volume of 250 μl contained Tris-HCl buffer (100 mM, pH 7.4), $CaCl_2$ (3 mM), substrate (40 μM) and enzyme. The reaction was started by addition of the enzyme solution. The amount of PLA_2s were adjusted to optimize linear kinetics for quantitation, *i.e.*, hydrolysis of substrate was less than 20% hydrolysis in all experiments. Thielocin A1β was 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. Following incubation at 37°C for 20 minutes, the reactions were terminated by addition of 1.25 ml of DOLE's reagent[17], and released free fatty acid was extracted and subjected to liquid scintillation counting by the method of NATORI *et al.*[18]. Inhibition is expressed as the percent of enzyme control. Data points are the means of two independent experiments, each performed in duplicate and corrected for no enzymatic hydrolysis (0.5% or less in all experiments). The standard error of the mean was 8% or less than the mean for each data point. IC_{50} values were determined graphically from plots of percent inhibition *versus* log concentration of inhibitors.

Fluorescence Measurements

The relative fluorescent intensity of PLA_2 mixtures in a total volume of 2.0 ml contained 7 nmol of *Naja naja* venom PLA_2, 100 mM Tris-HCl buffer (pH 7.4), 3 mM $CaCl_2$, and the indicated concentrations of thielocin A1β were monitored using Hitachi F-3000 fluorescence spectrophotometer.

Results

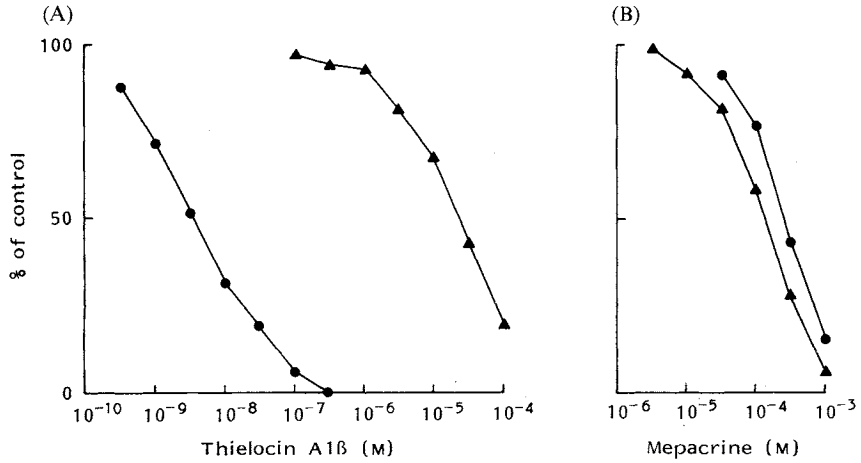
Inhibition of Extracellular PLA_2s by Thielocin A1β

Thielocin A1β showed extremely strong inhibition against rat PLA_2-II in a dose-dependent manner with an IC_{50} of 0.0033 μM (Fig. 2A). However, thielocin A1β showed weak inhibitory activity against PLA_2 purified from rat pancreas, which belongs to group I PLA_2, with an IC_{50} of 21 μM. Thus, thielocin A1β inhibition of rat PLA_2-II was 6.4×10^3 times greater than that of rat PLA_2-I. On the other hand, mepacrine, a reputed PLA_2 inhibitor[19], showed nonspecific inhibitory activity against both rat PLA_2-I ($IC_{50} = 135$ μM) and rat PLA_2-II ($IC_{50} = 240$ μM) (Fig. 2B). These results demonstrate that thielocin A1β specifically inhibits rat group II PLA_2.

In addition, inhibitory activity of thielocin A1β against human PLA_2s were determined (Table 1). Thielocin A1β was active against human group II PLA_2 purified from rheumatoid synovial fluid ($IC_{50} = 12$ μM). On the other hand, thielocin A1β did not inhibit human pancreas PLA_2, which belong to group I PLA_2, up to 100 μM. These results suggest that the specific inhibitory activity of thielocin A1β is

Fig. 2. Inhibition of extracellular phospholipase A_2s by thielocin A1β (A) and mepacrine (B).

The activities of the enzyme control (*i.e.* 100%) were from 12,400 to 16,400 nmol/minute/mg protein of rat PLA_2-I (▲) and from 6,630 to 8,350 nmol/minute/mg protein of rat PLA_2-II (●).



also conserved in human extracellular PLA_2s. Furthermore, thielocin A1β also showed inhibitory activity against various PLA_2s purified from the venoms of bee and snakes. But the dose required for 50% inhibition was varied from 2.0 μM for bee venom PLA_2 to 17 μM for *V. russelli* venom and *C. adamanteus* venom PLA_2.

Table 1. Inhibition of various phospholipase A_2s by thielocin A1β.

Phospholipase A_2	IC_{50} (μM)
Group I	
Human pancreas	>100[a]
Rat pancreas	21
Bee venom	2.0
Naja naja venom	7.1
N. mocambique venom	9.3
Group II	
Human rheumatoid synovial	12
Rat platelet	0.0033
Vipera russelli venom	17
Crotalus adamanteus venom	17

[a] Thielocin A1β exhibited 10% inhibition at 100 μM.

Effect of Ca^{2+} and Substrate Concentration on the Inhibition of Rat PLA_2-II by Thielocin A1β

To begin to examine the mechanism of action, inhibition of rat PLA_2-II by thielocin A1β was measured as a function of Ca^{2+} concentration. The inhibition of rat PLA_2-II by thielocin A1β was independent of Ca^{2+} concentration (Fig. 3A). Therefore, inhibition by thielocin A1β may not be mediated by displacement of catalytically essential Ca^{2+} from enzyme. In addition, we examined the extent of inhibition by thielocin A1β as a function of substrate concentration. Inhibition of rat PLA_2-II by thielocin A1β was independent of the substrate concentration (Fig. 3B). The activity is not due to direct interaction with the substrate of phosphatidylethanolamine.

Independence of the Inhibition of Rat PLA_2-II by Thielocin A1β from Substrate Form and Phospholipid Species

Many inhibitors, including manoalide[20] and acylamino phospholipid analogues[21], depend its inhibitory activity on substrate presented in the physical form (*E. coli* membranes, phospholipids presented as surfactant mixed micelles or sonicated liposomes). Interestingly, thielocin A1β showed similar inhibitory activity against rat PLA_2-II, whatever substrate form was used (Table 2). In addition, thielocin A1β showed similar PLA_2 inhibitory activity even when various phospholipids (phosphatidylethanolamine,

Fig. 3. Effects of Ca^{2+} (panel A) and substrate (panel B) concentration on the inhibition of rat group II phospholipase A_2 activity by thielocin A1β.

(A) Reaction mixtures, contained 5 ng of rat PLA_2-II, were incubated with $CaCl_2$ and 0.01 μM (●), 0.1 μM (■) of thielocin A1β.

(B) Reaction mixtures contained rat PLA_2-II, 1-palmitoyl-2-[1-^{14}C]linoleoyl phosphatidylethanolamine and 0.002 μM (●), 0.005 μM (■), 0.02 μM (▲) of thielocin A1β.

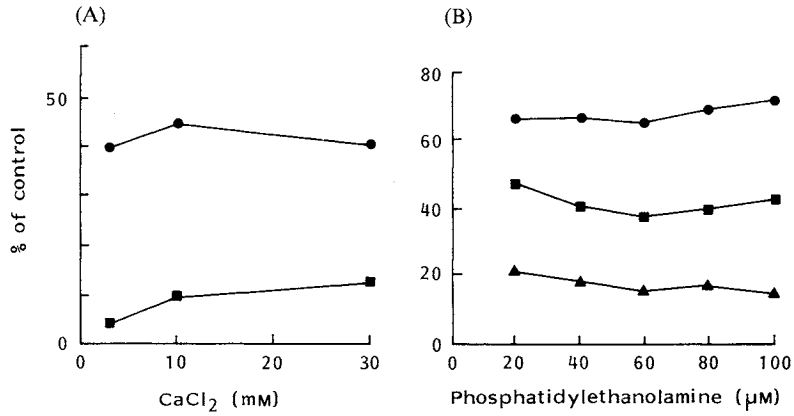


Table 2. Effect of thielocin A1β on the rat group II phospholipase A_2 activities toward various substrates.

Substrate	Specific activity (nmol/minute/mg protein)	IC_{50} (μM)
PE[a]	7,600	0.0033
PE+0.03% Triton X-100[b]	3,400	0.022
Escherichia coli membranes[c]	31,000	0.0090

[a] 1-Palmitoyl-2-[1-^{14}C]linoleoyl phosphatidylethanolamine was used as substrate.

[b] Triton X-100 was suspended in deionized water with 1-palmitoyl-2-[1-^{14}C]linoleoyl phosphatidylethanolamine to the final concentration of 0.03%.

[c] [^{3}H]Oleic acid-labeled *E. coli* phospholipids were used as substrate (background hydrolysis was <3%).

Table 3. Effect of thielocin A1β and cinatrin C_3 on the rat group II phospholipase A_2 activities toward various phospholipids.

Substrate	IC_{50} (μM)	
	Thielocin A1β	Cinatrin C_3
Phosphatidylethanolamine[a]	0.020	230
Phosphatidylinositol[b]	0.019	4.0
Phosphatidylcholine[c]	0.011	23.0

[a] Phosphatidylethanolamine, 1-acyl-2-[1-^{14}C]-arachidonyl.

[b] Phosphatidylinositol, 1-stearoyl-2-[1-^{14}C]-arachidonyl.

[c] Phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]-arachidonyl containing 0.03% Triton X-100 were used as substrates.

phosphatidylcholine and phosphatidylinositol) were used (Table 3). Recently, we isolated cinatrins[22], a novel family of PLA_2 inhibitors. However, cinatrin C_3 showed different inhibitory activity against rat PLA_2-II when various phospholipids were used as substrate (Table 3).

Reversibility of the Inhibition by Thielocin A1β

Further kinetic analysis was carried out to determine the mechanism of rat PLA_2-II inhibition by thielocin A1β. The reversible characteristics of thielocin A1β was confirmed using the dilution method according to LISTER *et al.*[23] (Table 4). Rat PLA_2-II was preincubated with thielocin A1β (37°C, 20 minutes) at 0.3 μM, this concentration is high enough to sufficiently reduce the enzymatic activity (see Fig. 2). After the preincubation, an aliquot was removed and diluted 30-fold to 0.0033 μM in the assay mixture, and there was slight inhibition observed, indicating reversible inhibition. Had the inhibition been ir-

Table 4. Distinction between reversible and irreversible inhibition for thielocin A1β and *p*-bromophenacyl bromide (*p*-BPB).

Compound	Concentration (μM)		Phospholipase A_2 activity (% of control)		
	Preincubation[a]	Assay[b]	Predicted		Experimentally found
			Irreversible	Reversible	
Thielocin A1β	0.1	0.0033	0.75	50	81±11
	0.3	0.01	0	31	25±10
p-BPB	90	3.0	7.5	91	10± 4
	300	10	5.1	30	10± 4

[a] Rat PLA_2-II was preincubated with inhibitor at the designated concentration for 20 minutes.
[b] Inhibitor concentration after dilution for assay.

Fig. 4. Noncompetitive inhibition of rat group II phospholipase A_2 by thielocin A1β.

Double reciprocal plot of rat PLA_2-II activity toward phosphatidylethanolamine in the presence of (0.005 μM, △ and 0.015 μM, □) or absence (●) of thielocin A1β. Standard assay conditions were employed and the lines were drawn on the basis of regression analysis.

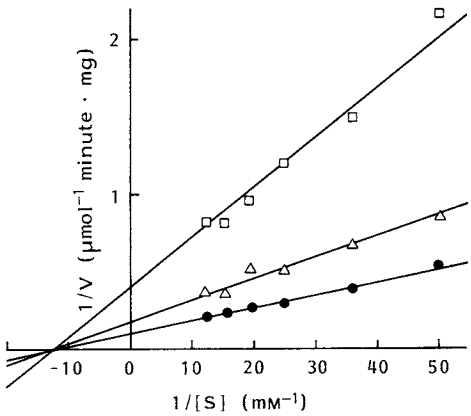


Fig. 5. Effect of thielocin A1β on the fluorescence of snake venom phospholipase A_2.

Samples were excited at 280 nm and the emission intensity was measured from 300 to 460 nm; ——, enzyme alone; – – –, enzyme + thielocin A1β; —·—, thielocin A1β alone.

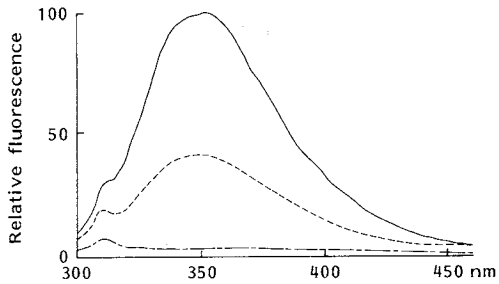
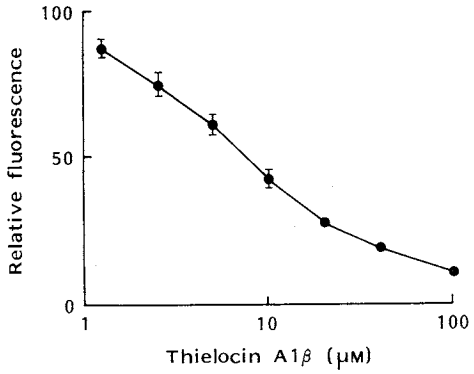


Fig. 6. Fluorescence of the snake venom phospholipase A_2 as a function of the concentration of thielocin A1β.

Samples were excited at 280 nm and emission was measured at 348 nm. Fluorescence of the enzyme alone = 100%. The values indicate averages ± SD (n = 3).



reversible, the rate should have been inhibited at least 99.2%, corresponding to an inhibitor concentration of 0.1 μM. A similar result was observed when the thielocin A1β concentration during the preincubation was set at 0.3 μM and then diluted to 0.01 μM during the assay. On the other hand, *p*-bromophenacyl bromide (*p*-BPB), a putative irreversible PLA_2 inhibitor, showed similar inhibitory activity before and after dilution, indicating irreversible inhibition. Furthermore, double reciprocal plot showed that thielocin A1β behaves kinetically as a noncompetitive inhibitor for rat PLA_2-II with *Ki* of 0.0068 μM (Fig. 4).

Effect of Thielocin A1β on the Fluorescence Emission of Snake Venom PLA_2

Direct interaction of thielocin A1β with snake venom PLA_2 (*N. naja*) was examined by monitoring tryptophan fluorescence of the enzyme in the presence and absence of thielocin A1β. Excitation of this PLA_2 at 280 nm resulted in a broad fluorescent peak ranging from 300 to 420 nm with maximal emission at 348 nm (Fig. 5). Furthermore, Fig. 5 also demonstrated the addition of 12.5 μM thielocin A1β to PLA_2 markedly quenches tryptophan fluorescence as evidenced by the diminished relative emission in the wide spectral region. Thielocin A1β quenched the relative fluorescence of 3.5 μM snake venom PLA_2 at 348 nm in a dose-dependent manner (Fig. 6). Fifty % of the fluorescence was quenched when the molar ratio of thielocin A1β/PLA_2 was 2.2. These fluorescence studies indicate that thielocin A1β interacts with *N. naja venom* PLA_2.

Discussion

The data from these studies show that thielocin A1β inhibits a broad spectrum of extracellular PLA_2. Thielocin A1β exhibits extremely strong inhibitory activity against rat PLA_2-II with an IC_{50} of 0.0033 μM (Table 1). Some alkaloids and non-steroidal anti-inflammatory agents displace Ca^{2+} and thus the inhibition by these agents appear to be dependent on Ca^{2+} concentration[24]. However, we found the inhibition by thielocin A1β to be independent of the Ca^{2+} content. Many non-specific PLA_2 inhibitors have been thought to affect the "quality of the interface" by modifying phospholipid bilayer properties that render phospholipid inaccessible to the enzyme[25]. For example, DAVIDSON *et al.*[15] found that lipocortin, which is thought to be an important steroid inducible inhibitor, inhibits PLA_2 by sequestering the phospholipid substrate; the inhibition can be overcome by high phospholipid substrate concentrations. Moreover, we have recently reported that the inhibitory activity of duramycin, a polypeptide PLA_2 inhibitor[26], exclusively depends on the substrate concentration, and its activity is probably due to direct interaction with the substrate of phosphatidylethanolamine[16]. On the other hand, our data suggest that thielocin A1β interacts directly with the protein to inhibit PLA_2 activity since the inhibitory activity was independent of the substrate concentration (Fig. 3B). More, experiments (Fig. 6) monitoring the fluorescence of *N. naja* venom PLA_2 show that thielocin A1β quenches the fluorescence of this protein supporting direct interaction of thielocin A1β with the protein. DE HAAS and co-workers[27] reported that a substrate-derived PLA_2 inhibitor, (*R*)-2-dodecanoyl-amino-1-hexanol-phosphoglycol, competitively inhibits porcine pancreas PLA_2. However, thielocin A1β inhibits rat PLA_2-II noncompetitively, and the *Ki* value of 0.0068 μM corresponded well to the IC_{50} value of 0.0033 μM (Fig. 4). Recently, we reported the mechanism of inhibition by cinatrins[16] which showed variable inhibitory activity against rat PLA_2-II when different phospholipids were used as substrate (Table 3). It appeared that this discrepancy was due to the amphipathic property of cinatrin C_3. In contrast, thielocin A1β inhibits PLA_2 regardless of substrate form and phospholipid substrate. Therefore, thielocin A1β may serve as valuable tool for revealing the substrate-function relationship of extracellular PLA_2.

Manoalide, which is a potent inhibitor of bee venom ($IC_{50}=0.05\,\mu M$)[28] and cobra venom ($IC_{50}=2\,\mu M$)[20] PLA_2, also inhibits phosphatidylinositol-specific phospholipase C purified from guinea pig uterus ($IC_{50}=1.5\,\mu M$)[29]. Thielocin A1β did not inhibit phosphatidylinositol-specific phospholipase C purified from human platelets at concentrations up to 50 μM (Y. NOZAWA; personal communication). These results taken together suggest that thielocin A1β is a specific inhibitor for extracellular PLA_2.

Several studies have implicated PLA_2-II in the pathogenesis of inflammation[19]. ARITA and co-workers have recently found that some inflammatory factors dramatically increased PLA_2-II secretion from several tissues of rat *via* enhancement of gene transcription[30~34]. Recently, MURAKAMI *et al.*[35] demonstrated that the effect of rat PLA_2-II on PGD_2 generation by mast cells obtained from the peritoneal cavity of rats was abolished by pretreatment of the enzyme with thielocin A1β. Thus, PLA_2-II may function in the process of inflammation by acting on IgE-antigen-primed mast cells to generate eicosanoids. Importantly, thielocin A1β inhibited PLA_2-II reversibly. Hence, it may be possible to control extracellular PLA_2 activity in inflammatory regions with thielocin A1β.

Studies are in progress to develop a kinetic model for PLA_2 inhibition by thielocin A1β, and to define,

the physiological role of extracellular PLA_2 in the progression of inflammatory diseases.

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