ANTITUMOR EFFECT OF PIERICIDIN B_1 *N*-OXIDE

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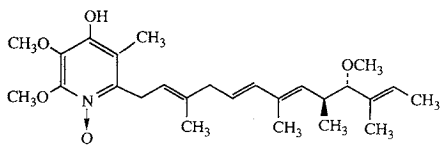
Piericidin B_1 *N*-oxide was isolated from a culture broth of *Streptomyces* sp. as a novel inhibitor of phosphatidylinositol (PI) turnover. Piericidin B_1 *N*-oxide specifically inhibited orthophosphate labeling of PI induced by epidermal growth factor (EGF) without affecting the formation of phosphatidic acid (PA). Like piericidins A_1 and B_1, piericidin B_1 *N*-oxide inhibited ATP synthesis in A431 cells; however, the effect of piericidin B_1 *N*-oxide on PI synthesis was stronger than that of piericidins A_1 and B_1. At the concentration inhibiting PI synthesis, piericidin B_1 *N*-oxide showed no inhibitory effect on DNA, RNA, or protein synthesis. We also demonstrated that piericidin B_1 *N*-oxide reversibly inhibited the growth of A431 cells *in situ* and suppressed the growth of Ehrlich carcinoma *in vivo* when administered to mice by intraperitoneal (ip) injection.

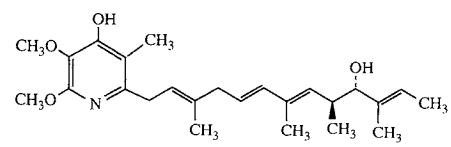
Phosphatidylinositol (PI) turnover has been shown to play a significant role in intracellular pathway elicited by a variety of cellular stimuli such as some types of oncogenes[1,2] and growth factors[3,4]. Phospholipase C (PLC)-γ, a key enzyme of PI turnover, and PI 3-kinase are phosphorylated at tyrosine residues by epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinases[5,6]. Activation of PLC-γ by EGF and PDGF receptors may promote the mitogen signals. Microinjection of exogenous PLC-γ into quiescent NIH3T3 cells induces growth and morphological change[7], while introduction of antibody to PLC-γ or PI 4,5-bisphosphate by same means abolishes oncogene-stimulated DNA synthesis in NIH3T3 cells[8,9]. Thus, inhibitors of PI turnover should help to unravel the physiological function of PI turnover in proliferation and carcinogenesis.

For the past several years we have been screening microbial secondary metabolites for inhibitors of

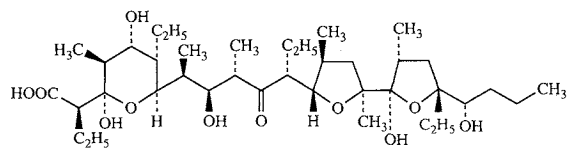
Fig. 1. Structures of piericidin B_1 *N*-oxide, piericidin A_1, and inostamycin.



Piericidin B_1 *N*-oxide



Piericidin A_1



Inostamycin

PI turnover and previously isolated psi-tectorigenin[10], inostamycin[11], piericidin B_1 *N*-oxide[12], and piericidins B_5 and B_5 *N*-oxide[13]. Piericidins are insecticidal substances isolated from *Streptomyces mobaraensis* and *Streptomyces pactum*[14~17]. The structures of piericidins are completely different from those of other PI turnover inhibitors. In the present study we examined the mechanism of action and antitumor effect of piericidin B_1 *N*-oxide.

Materials and Methods

Materials

Piericidin B_1 *N*-oxide[12] and inostamycin[11] were isolated in our laboratory as described previously. Piericidins A_1 and B_1 were kindly supplied by Dr. S. YOSHIDA (The Institute of Physical and Chemical Research, Wako), *Myo*-[^{3}H]inositol, [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, and L-[4,5-^{3}H]leucine were purchased from Amersham; and [^{32}P]orthophosphate (^{32}Pi) was from New England Nuclear. EGF was obtained from Funakoshi Co., Ltd.

Cell Culture

A431 cells were maintained in DULBECCO's modified EAGLE's medium (DMEM) supplemented with 5% calf serum (CS). The cells were cultured at 37°C in a 5% CO_2-95% air atmosphere.

Incorporation of ^{32}Pi into Phospholipids in A431 Cells

A431 cells (5×10^5 cells/well) plated in 35-mm plastic wells were grown for 16 hours before use and washed with phosphate-buffered saline (PBS). The cells were incubated in 1 ml of phosphate-free DMEM containing 74 kBq/ml of ^{32}Pi at 37°C for 15 minutes. Then, the inhibitor was added, and the incubation was continued at 37°C for 15 minutes. After addition of EGF (400 ng/ml), the cells were incubated for a further 60 minutes. The assay was terminated by aspiration of the labeled media and replacement of it with 10% trichloroacetic acid (TCA) at 4°C, in which the cells were kept for 15 minutes. The TCA-insoluble material was scraped into 1 ml of H_2O and transferred to a glass tube prior to extraction with 6.25 ml of H_2O-MeOH-$CHCl_3$ (1:2:2). The lower organic phase was evaporated to dryness and dissolved in 40 μl of MeOH-$CHCl_3$ (1:2). Labeled lipids were spotted on silica gel thin-layer chromatography plates and developed with $CHCl_3$-MeOH-CH_3CO_2H-H_2O (25:15:4:2). By comparison with the mobility of authentic markers for phospholipids, the radioactive spots were identified after autoradiography.

ATP Synthesis

A431 cells (3×10^5 cells/well) were seeded into 24-well plates. After 16 hours of incubation, the cells were washed with PBS twice and incubated with 1 ml of CS-free DMEM containing the test chemical for 60 minutes. Washed with PBS twice, the cells were extracted by treatment with 300 μl of 2% TCA at 4°C for 10 minutes. After neutralization of the acid-soluble fraction with 0.5 N NaOH, a 50-μl aliquot of the fraction was added to 3 ml of PBS containing 4 mM $MgSO_4$; and the mixture was reacted with 50 μl of luciferin-luciferase solution. Immediately thereafter, the luminescence was measured in a liquid scintillation counter.

PI Synthesis

Analysis of PI synthesis was accomplished as reported earlier[10,11]. Briefly, A431 cells (3×10^5 cells/well) were grown for 16 hours and then labeled with *myo*-[^{3}H]inositol (37 kBq/ml) in 1 ml of CS-free DMEM at 37°C for 30 minutes. After addition of inhibitor and EGF (400 ng/ml), the cells were incubated further for 60 minutes. Then the incubation was terminated by addition of 1 ml of 10% TCA containing 0.01 M sodium pyrophosphate, and the acid-insoluble fraction was solubilized in 0.5 ml of 0.5 N NaOH. The radioactivity of the labeled PI was measured in a liquid scintillation counter.

Analysis of Macromolecular Synthesis

A431 cells (3×10^5 cells/well) were grown for 16 hours. The cells were incubated for 30 minutes with

1 ml of CS-free DMEM containing 37 kBq/ml of [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, or L-[4,5-^{3}H] leucine to measure DNA, RNA, or protein synthesis, respectively. After addition of piericidin B_1 *N*-oxide and EGF (400 ng/ml), the incubation was continued for an additional 60 minutes. The reaction was stopped by the removal of the medium followed by the addition of 1 ml of 10% TCA. After solubilization with 0.5 ml of 0.5 N NaOH, the acid-insoluble fraction was counted for radioactivity.

Inhibition of Cell Growth

A431 cells (2×10^4 cells/well) grown in 12-well plates for 16 hours were washed with PBS and exposed to 1 μg/ml of piericidin B_1 *N*-oxide in 1 ml of DMEM. After 4 days of incubation, the cell number was counted with a Coulter counter.

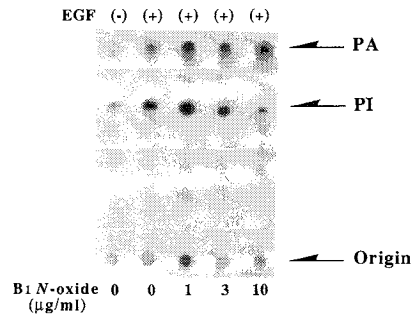
In Vivo Antitumor Activity

Ehrlich carcinoma cells were inoculated subcutaneously (sc) into the flank of ICR mice at 2×10^6 cells/mouse on day 0, and piericidin B_1 *N*-oxide or inostamycin was injected ip daily for 9 days from day 1. Antitumor effect on Ehlrich carcinoma was estimated by weighing of the tumor on day 15. Control animals were injected with vehicle (20% DMSO) alone.

Results

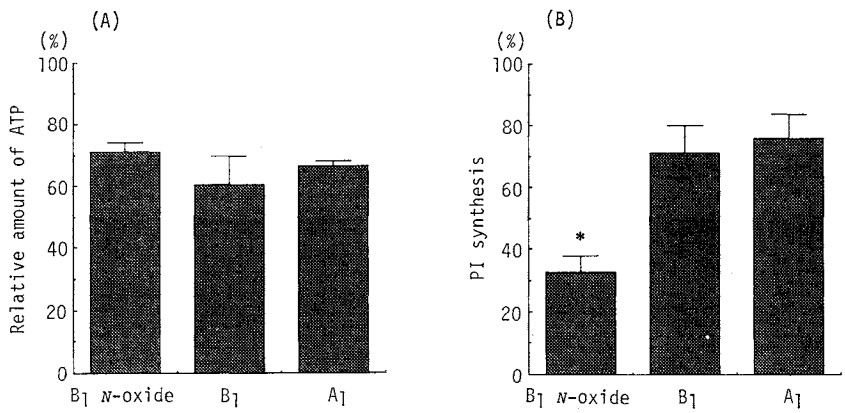
Incorporation of ^{32}Pi into both PI and PA was markedly enhanced by EGF. Piericidin B_1 *N*-oxide, whose structure is shown in Fig. 1, lowered EGF-stimulated PI synthesis dose-dependently; but it did not inhibit PA synthesis, even at 10 μg/ml, as shown in Fig. 2. Piericidins B_1 *N*-oxide, B_1, and A_1 (Fig. 1) at 1 μg/ml all inhibited ATP synthesis about 30~40% in A431 cells (Fig. 3A). In contrast, inhibition of PI synthesis by piericidin B_1 *N*-oxide at the same concentration was significantly stronger than that by piericidin B_1 or A_1 (Fig. 3B). Regard-

Fig. 2. Inhibition of PI synthesis by piericidin B_1 *N*-oxide in A431 cells.



A431 cells were labeled with ^{32}Pi for 15 minutes, and then piericidin B_1 *N*-oxide was added. After 15 minutes EGF was added and incubation was continued for an additional 60 minutes. The result is a representative of duplicate experiments.

Fig. 3. Effects of piericidins on ATP (A) and PI (B) syntheses in A431 cells.



The cells were incubated with 1 μg/ml of each chemical for 60 minutes. Values are the mean $\pm$ SD of triplicate samples. * $P<0.01$ *vs.* B_1 or A_1.

Fig. 4. Effect of piericidin B_1 *N*-oxide on PI and macromolecular syntheses in A431 cells.

A431 cells (3×10^5 cells/well) were labeled with [^{3}H]inositol (○), [^{3}H]thymidine (●), [^{3}H]uridine (△), or [^{3}H]leucine (▲) for 30 minutes. Then, piericidin B_1 *N*-oxide, and EGF were added, and the cells were incubated further for 60 minutes. Values are the mean ± SD of triplicate samples.

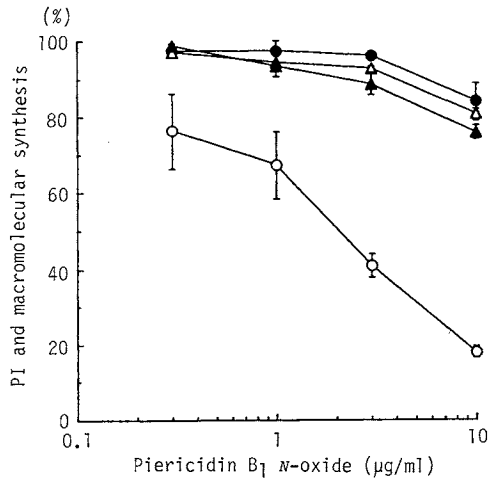


Fig. 5. Growth inhibition in A431 cells by piericidin B_1 *N*-oxide.

A431 cells were left untreated (●) or exposed to 1 μg/ml of piericidin B_1 *N*-oxide (▲) in 1 ml of DMEM for 4 days. After removal of piericidin B_1 *N*-oxide on day 1 (○) or on day 2 (△) the cells were cultured further without the additive. Values are the mean ± SD of triplicate samples.

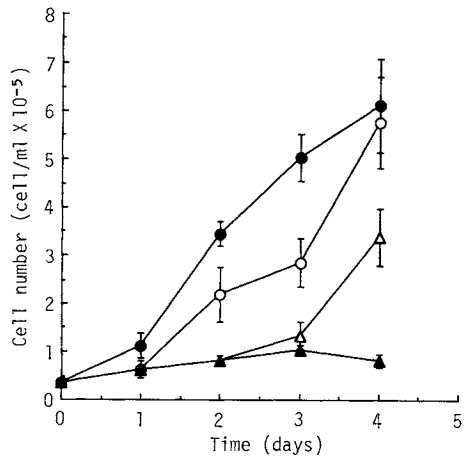


Table 1. Antitumor effect of piericidin B_1 *N*-oxide against Ehrlich murine tumor.

Compound	Dose (mg/kg)	Tumor weight (mg ± SD)	Inhibition (%)
Control	0	563.0 ± 193	—
Piericidin B_1	0.008	543.8 ± 141	3.4
N-oxide	0.031	316.2 ± 210	43.8
	0.125	323.8 ± 159	42.5
	0.500	243.0 ± 210*	56.8

* $P < 0.05$.

ICR mice (control, $n = 10$; test, $n = 5$) implanted sc with Ehrlich carcinoma on day 0 were treated ip with piericidin B_1 *N*-oxide once a day for 9 days beginning on day 1. Tumor weight was measured 15 days after tumor inoculation. Mice were all alive on day 15.

Table 2. Antitumor effect of inostamycin on Ehrlich solid tumor.

Compound	Dose (mg/kg)	Tumor weight (mg ± SD)	Inhibition (%)
Control	0	1,164 + 295	—
Inostamycin	0.05	587 ± 296*	49
	0.20	537 ± 289**	53
	0.78	372 ± 169**	68
	3.13	401 ± 137**	65

* $P < 0.05$.

** $P < 0.01$.

Experimental procedure was the same as in Table 1. Mice were all alive on day 15.

ing macromolecular synthesis, piericidin B_1 *N*-oxide did not inhibit DNA, RNA, or protein synthesis effectively at 0.3 ~ 10 μg/ml, while it did inhibit PI synthesis effectively at these same concentrations (Fig. 4). Piericidin B_1 *N*-oxide inhibited the growth of A431 cells almost completely at 1 μg/ml, as shown in Fig. 5. But after the removal of the chemical the cells began to grow again at the same rate. Therefore, piericidin B_1 *N*-oxide reversively inhibited the growth of the cells. Piericidin B_1 *N*-oxide significantly inhibited the growth of Ehrlich carcinoma about 50% in mice, when injected ip at 0.5 mg/kg once a day for 9 days (Table 1). However, it did not show any antitumor effect on P388 or L-1210 leukemia in mice at a dose of 0.5 mg/kg (data not shown). Piericidin B_1 *N*-oxide at 1 mg/kg was toxic to mice, and 3 out of 5 died on days 8. Inostamycin (Fig. 1), another inhibitor of PI synthesis, also inhibited the growth of Ehrlich carcinoma

50~70% in mice at 0.05~3.13 mg/kg (Table 2). Inostamycin at 12.5 mg/kg was toxic to mice and killed 3 out of 5 on day 2. It also showed no antitumor effect on mouse leukemia models *in vivo*.

Discussion

Piericidin B_1 *N*-oxide showed stronger inhibitory activity on PI turnover than piericidin B_1 in A431 cells[12]. The same tendency was recognized between piericidins B_5 *N*-oxide and B_5[13], indicating that the *N*-oxide is important for inhibition of PI turnover. Piericidin B_1 *N*-oxide did not inhibit PI 4-kinase, PLC, protein kinase C, EGF receptor tyrosine kinase, or EGF-induced inositol phosphate formation at the concentration that inhibited PI turnover in A431 cells (data not shown), whereas it significantly inhibited PIsynthesis without affecting PA production. Therefore, it is likely that the target of piericidin B_1 *N*-oxide may be CDP-DG: inositol transferase, the enzyme that catalyzes PI synthesis from CDP-DG and inositol. Inostamycin is known to inhibit this enzyme activity[18]. Many years ago piericidin A was shown to block electron transport between NADH and coenzyme Q[19], resulting in a reduced level of intracellular ATP. This phenomenon was similarly demonstrated with other piericidin-group insecticides such as piericidins B_1 and B_1 *N*-oxide. However, piericidin B_1 *N*-oxide had a stronger effect on PI synthesis than on ATP reduction. Thus, the inhibition of PI turnover by piericidin B_1 *N*-oxide would not be due to the blocking of electron transport.

Piericidin B_1 *N*-oxide reversively inhibited the growth of A431 cells. Inhibition of PI synthesis might block the growth at a specific point in the cell cycle. Inostamycin was suggested to inhibit the cell cycle at G_1 phase (M. IMOTO, unpublished result). In *in vivo* experiments, we demonstrated that piericidin B_1 *N*-oxide and inostamycin both had an antitumor effect on Ehrlich solid tumors. On the other hand, they did not show any antitumor effect on P388 and L-1210 leukemia models, suggesting that the anticancer activities of these substances are observed only on developed tumors with a slower growth rate. Whether these antitumor activities are due to inhibition of PI synthesis remains to be studied.

Acknowledgments

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References

1) FLEISCHMAN, L. F.; S. B. CHAHWALA & L. CANTLEY: *Ras*-transformed cells: altered levels of phosphatidylinositol-4,5-bisphosphate and catabolites. Science 231: 407~410, 1986
2) KATO, M.; S. KAWAI & T. TAKENAWA: Altered signal transduction in erbB-transformed cells. J. Biol. Chem. 262: 5696~5704, 1987
3) PIKE, L. J. & A. T. EAKES: Epidermal growth factor stimulates the production of phosphatidylinositol monophosphate and the breakdown of polyphosphoinositides in A431 cells. J. Biol. Chem. 262: 1644~1651, 1987
4) KAMATA, T. & H. KUNG: Effects of *ras*-encoded proteins and platelet-derived growth factor on inositol phospholipid turnover in NRK cells. Proc. Natl. Acad. Sci. U.S.A. 85: 5799~5803, 1988
5) NISHIBE, S.; M. I. WAHL, S. M. T. HERNANDEZ-SOTOMAYOR, N. K. TONKS, S. G. RHEE & G. CARPENTER: Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. Science 250: 1253~1256, 1990
6) AUGER, K. R.; L. A. SERUNIAN, S. P. SOLTOFF, P. LIBBY & L. C. CANTLEY: PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell 57: 167~175, 1989
7) SMITH, M. R.; S. RYU, P. SUH, S. RHEE & H. KUNG: S-phase induction and transformation of quiescent NIH 3T3 cells by microinjection of phospholipase C. Proc. Natl. Acad. Sci. U.S.A. 86: 3659~3663, 1989
8) SMITH, M. R.; Y. LIU, H. KIM, S. G. RHEE & H. KUNG: Inhibition of serum- and ras-stimulated DNA synthesis by antibodies to phospholipase C. Science 247: 1074~1077, 1990
9) FUKAMI, K.; K. MATSUOKA, O. NAKANISHI, A. YAMAKAWA, S. KAWAI & T. TAKENAWA: Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. Proc. Natl. Acad. Sci. U.S.A. 85: 9057~9061, 1988
10) IMOTO, M.; T. YAMASHITA, T. SAWA, S. KURASAWA, H. NAGANAWA, T. TAKEUCHI, Z. BAO-QUAN & K. UMEZAWA: Inhibition of cellular phosphatidylinositol turnover by psi-tectorigenin. FEBS Lett. 230: 43~46, 1988

11) IMOTO, M.; K. UMEZAWA, Y. TAKAHASHI, H. NAGANAWA, Y. IITAKA, H. NAKAMURA, Y. KOIZUMI, Y. SASAKI, M. HAMADA, T. SAWA & T. TAKEUCHI: Isolation and structure determination of inostamycin, a novel inhibitor of phosphatidylinositol turnover. J. Nat. Prod. 53: 825~829, 1990
12) NISHIOKA, H.; T. SAWA, K. ISSHIKI, Y. TAKAHASHI, H. NAGANAWA, N. MATSUDA, S. HATTORI, M. HAMADA, T. TAKEUCHI & K. UMEZAWA: Isolation and structure determination of a novel phosphatidylinositol turnover inhibitor, piericidin B_1 *N*-oxide. J. Antibiotics 44: 1283~1285, 1991
13) NISHIOKA, H.; T. SAWA, Y. TAKAHASHI, H. NAGANAWA, M. HAMADA, T. TAKEUCHI & K. UMEZAWA: Isolation and structure determination of novel phosphatidylinositol turnover inhibitors, piericidin B_5 and B_5 *N*-oxide, from *Streptomyces* sp. J. Antibiotics 46: 564~568, 1993
14) TAMURA, S.; N. TAKAHASHI, S. MIYAMOTO, R. MORI, S. SUZUKI & J. NAGATSU: Isolation and physiological activities of piericidin A, a natural insecticide produced by *Streptomyces*. Agric. Biol. Chem. 27: 576~582, 1963
15) TAKAHASHI, N.; A. SUZUKI, Y. KIMURA, S. MIYAMOTO, S. TAMURA, T. MITSUI & J. FUKAMI: Isolation, structure and physiological activities of piericidin B, natural insecticide produced by a *Streptomyces*. Agric. Biol. Chem. 32: 1115~1122, 1968
16) YOSHIDA, S.; K. YONEYAMA, S. SHIRAISHI, A. WATANABE & N. TAKAHASHI: Isolation and physical properties of new piericidins produced by *Streptomyces pactum*. Agric. Biol. Chem. 41: 849~853, 1977
17) YOSHIDA, S.; K. YONEYAMA, S. SHIRAISHI, A. WATANABE & N. TAKAHASHI: Chemical structures of new piericidins produced by *Streptomyces pactum*. Agric. Biol. Chem. 41: 855~862, 1977
18) IMOTO, M.; Y. TANIGUCHI & K. UMEZAWA: Inhibition of CDP-DG: inositol transferase by inostamycin. J. Biochem. 112: 299~302, 1992
19) HORGAN, D. J.; T. P. SINGER & J. E. CASIDA: Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. J. Biol. Chem. 243: 834~843, 1968