ANTITUMOR EFFECT OF PIERICIDIN B\textsubscript{1} N-OXIDE

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Piericidin B\textsubscript{1} N-oxide was isolated from a culture broth of Streptomyces sp. as a novel inhibitor of phosphatidylinositol (PI) turnover. Piericidin B\textsubscript{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\textsubscript{1} and B\textsubscript{1}, piericidin B\textsubscript{1} N-oxide inhibited ATP synthesis in A431 cells; however, the effect of piericidin B\textsubscript{1} N-oxide on PI synthesis was stronger than that of piericidins A\textsubscript{1} and B\textsubscript{1}. At the concentration inhibiting PI synthesis, piericidin B\textsubscript{1} N-oxide showed no inhibitory effect on DNA, RNA, or protein synthesis. We also demonstrated that piericidin B\textsubscript{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\textsuperscript{1,2} and growth factors\textsuperscript{3,4}. Phospholipase C (PLC)-\gamma, 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\textsuperscript{5,6}. Activation of PLC-\gamma by EGF and PDGF receptors may promote the mitogen signals. Microinjection of exogenous PLC-\gamma into quiescent NIH3T3 cells induces growth and morphological change\textsuperscript{7}, while introduction of antibody to PLC-\gamma or PI 4,5-bisphosphate by same means abolishes oncogene-stimulated DNA synthesis in NIH3T3 cells\textsuperscript{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
PI turnover and previously isolated psi-tertogenin\textsuperscript{10)}, inostamycin\textsuperscript{11)}, piericidin \(B_1\) N-oxide\textsuperscript{12)}, and piericidins \(B_2\) and \(B_3\) N-oxide\textsuperscript{13)}. Piericidins are insecticidal substances isolated from \textit{Streptomyces mobaraensis} and \textit{Streptomyces pactum}\textsuperscript{14}~\textsuperscript{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\textsuperscript{12}) and inostamycin\textsuperscript{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), \textit{Myo-}\(^{[3}\text{H}]\)inositol, \([\text{methyl}-^{3}\text{H}]\)thymidine, \([\text{s}-^{3}\text{H}]\)uridine, and \(\text{L-}[\text{4,5-}^{3}\text{H}]\)leucine were purchased from Amersham; and \([^{32}\text{P}]\)orthophosphate (\(^{32}\text{Pi}\)) 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\textsubscript{2}-95% air atmosphere.

**Incorporation of \(^{32}\text{Pi}\) into Phospholipids in A431 Cells**

A431 cells (5 x 10\textsuperscript{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 \(^{32}\text{Pi}\) 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\textsubscript{2}O and transferred to a glass tube prior to extraction with 6.25 ml of H\textsubscript{2}O - MeOH - CH\textsubscript{3}Cl (1 : 2 : 2). The lower organic phase was evaporated to dryness and dissolved in 40 μl of MeOH - CH\textsubscript{3}CO\textsubscript{2}H - H\textsubscript{2}O (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 x 10\textsuperscript{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\textsubscript{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\textsuperscript{10},11). Briefly, A431 cells (3 x 10\textsuperscript{5} cells/well) were grown for 16 hours and then labeled with \textit{myo-}\(^{[3}\text{H}]\)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 15% TCA containing 0.01 mM 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 x 10\textsuperscript{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-3H]thymidine, [5-3H]uridine, or L-[4,5-3H]leucine to measure DNA, RNA, or protein synthesis, respectively. After addition of piericidin B1 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 x 10^4 cells/well) grown in 12-well plates for 16 hours were washed with PBS and exposed to 1 μg/ml of piericidin B1 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 x 10^6 cells/mouse on day 0, and piericidin B1 N-oxide or inostamycin was injected ip daily for 9 days from day 1. Antitumor effect on Ehrlich carcinoma was estimated by weighing of the tumor on day 15. Control animals were injected with vehicle (20% DMSO) alone.

Results
Incorporation of 32Pi into both PI and PA was markedly enhanced by EGF. Piericidin B1 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 B1 N-oxide, B1, and A1 (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 B1 N-oxide at the same concentration was significantly stronger than that by piericidin B1 or A1 (Fig. 3B). Regard-

Fig. 2. Inhibition of PI synthesis by piericidin B1 N-oxide in A431 cells.

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 ± SD of triplicate samples. * P<0.01 vs. B1 or A1.
Fig. 4. Effect of piericidin B1 N-oxide on PI and macromolecular syntheses in A431 cells.

A431 cells (3 x 10^5 cells/well) were labeled with [3H]inositol (○), [3H]thymidine (●), [3H]uridine (△), or [3H]leucine (▲) for 30 minutes. Then, piericidin B1 N-oxide, and EGF were added, and the cells were incubated further for 60 minutes. Values are the mean ± SD of triplicate samples.

Table 1. Antitumor effect of piericidin B1 N-oxide against Ehrlich murine tumor.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Tumor weight (mg±SD)</th>
<th>Inhibition (%)</th>
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</thead>
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<tr>
<td>Control</td>
<td>0</td>
<td>563.0±193</td>
<td>-</td>
</tr>
<tr>
<td>Piericidin B1 N-oxide</td>
<td>0.008</td>
<td>543.8±141</td>
<td>3.4</td>
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<tr>
<td></td>
<td>0.031</td>
<td>316.2±210</td>
<td>43.8</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>323.8±159</td>
<td>42.5</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>243.0±210*</td>
<td>56.8</td>
</tr>
</tbody>
</table>

* P<0.05.

ICR mice (control, n=10; test, n=5) implanted sc with Ehrlich carcinoma on day 0 were treated ip with piericidin B1 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Tumor weight (mg±SD)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1,164±295</td>
<td>-</td>
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<tr>
<td>Inostamycin</td>
<td>0.05</td>
<td>587±296*</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>537±289**</td>
<td>53</td>
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<td></td>
<td>0.78</td>
<td>372±169**</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>3.13</td>
<td>401±137**</td>
<td>65</td>
</tr>
</tbody>
</table>

* P<0.05.

** P<0.01.

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

Fig. 5. Growth inhibition in A431 cells by piericidin B1 N-oxide.

A431 cells were left untreated (○) or exposed to 1 µg/ml of piericidin B1 N-oxide (▲) in 1 ml of DMEM for 4 days. After removal of piericidin B1 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.

ing macromolecular synthesis, piericidin B1 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 B1 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 B1 N-oxide reversively inhibited the growth of the cells. Piericidin B1 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 B1 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 B1 N-oxide showed stronger inhibitory activity on PI turnover than piericidin B1 in A431 cells. The same tendency was recognized between piericidins B2 N-oxide and B5, indicating that the N-oxide is important for inhibition of PI turnover. Piericidin B1 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 PI synthesis without affecting PA production. Therefore, it is likely that the target of piericidin B1 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. Many years ago piericidin A was shown to block electron transport between NADH and coenzyme Q, resulting in a reduced level of intracellular ATP. This phenomenon was similarly demonstrated with other piericidin-group insecticides such as piericidins B1 and B4 N-oxide. However, piericidin B1 N-oxide had a stronger effect on PI synthesis than on ATP reduction. Thus, the inhibition of PI turnover by piericidin B1 N-oxide would not be due to the blocking of electron transport.

Piericidin B1 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 G1 phase (M. IMOTO, unpublished result). In vivo experiments, we demonstrated that piericidin B1 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


