# Inhibition of Phosphatidylinositol-specific Phospholipase C Activity by Fluvirucin $B_2$

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We isolated fluvirucin $B_2$ from the culture broth of *Streptomyces* as an inhibitor of phosphatidylinositol-specific phospholipase C (PI-PLC). It inhibited PI-PLC of A431 cell cytosol with an $IC_{50}$ of 1.6 $\mu$g/ml. Fluvirucin $B_2$ also inhibited PI-PLC in cultured A431 cells, whereas it did not inhibit phosphatidylinositol synthesis and macromolecular synthesis markedly. It also inhibited epidermal growth factor-induced rapid rounding of A431 cells, in which PI turnover is involved.

Phosphatidylinositol-specific phospholipase C (PI-PLC) is the rate-limiting enzyme of phosphatidylinositol turnover[1], and catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to produce two second messengers, inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol. The former mobilizes $Ca^{2+}$ from internal stores and the latter stimulates protein kinase C. PI-PLC is activated in response to a wide variety of physiological stimuli such as growth factors and hormones and often shows enhanced activity in transformed cells. Thus, an inhibitor of PI-PLC would be an useful tool for exploring the mechanism of intracellular signal transduction systems. Therefore, we screened microbial secondary metabolites for an inhibitor of PI-PLC.

## Materials and Methods

### Materials

The A431 cell line was kindly supplied by Dr. S. KAWAI, Insitute of Medical Science, University of Tokyo. Calf serum (CS) was purchased from Gibco. Plipastatin A was kindly supplied by Dr. T. AOYAGI, Institute of Microbial Chemistry. Epidermal growth factor (EGF) was obtained from Biomedical Technologies, Inc. [$^3$H]-$PIP_2$ (1.0 Ci/mmol), [$^3$H]*myo*-inositol (2.0 Ci/mmol), [$^3$H]thymidine (49 Ci/mmol), [$^3$H]uridine (30 Ci/mmol), [$^3$H]leucine (58 Ci/mmol), and Amprep SAX column were purchased from Amersham.

### Isolation of Fluvirucin $B_2$

The seed culture of *Streptomyces* sp. MJ677-72F5 was grown in 500-ml Erlenmeyer flasks containing 100 ml of seed medium: 2.0% galactose, 2.0% dextrin, 1.0% soypeptone, 0.5% corn steep liquor, 1.0% glycerol, 0.2% ammonium sulfate, and 0.2% calcium carbonate in water (pH 7.4 before autoclaving). Cultivation was carried out at 27°C for 5 days on a rotary shaker (180 rpm). Fermentation was then performed in 500-ml Erlenmeyer flasks each containing 100 ml of the production medium: 5.0% glucose, 1.0% soya meal, 0.4% polypeptone, 0.1% yeast extract, 0.4% beaf extract, 0.25% sodium chloride, and 0.5% calcium carbonate in water (pH 7.0 before autoclaving). The seed culture (2 ml) was added to each flask, and cultivation was carried out at 27°C for 6 days on a rotary shaker (180 rpm). The broth filtrate (3 liters) was adjusted to pH 2 with HCl and extracted with EtOAc (3 liters). The aqueous phase was then adjusted to pH 8 and extracted with EtOAc (3 liters). The organic residue was dissolved in a $CHCl_3$ - MeOH - EtOAc mixture and applied to a silica gel column (9 mm i.d. × 50 mm). The column was washed with a lower-phase mixture of $CHCl_3$ - MeOH - 29% $NH_4OH$ (100 : 1 : 2, 100 : 2 : 2, 100 : 3 : 2 and 100 : 5 : 2, stepwise), and the active fraction was eluted with the same solvent system (100 : 10 : 2). The crude material (50 mg) was dissolved in a small amount of $CHCl_3$ - MeOH (1 : 2), applied on to a Sephadex LH-20 column (18 mm i.d. × 530 mm) and eluted with $CHCl_3$ - MeOH (1 : 2). The active fraction was concentrated to dryness to give 32 mg of purified compound.

### Preparation of PI-PLC

A431 cells were cultured in a 145-mm dish with DULBECCO's modified EAGLE's medium (DMEM) containing 5% CS. The cells were collected, and homogenized in 400 $\mu$l of buffer A (20 mM Hepes; pH 7.2, 30 mM sodium pyrophosphate, 50 mM sodium chloride, 5 mM $\beta$-glycerophosphate, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 10 $\mu$g/ml of leupeptin), and the cytosol fraction was prepared by ultracentrifugation at 100,000 × $g$ for 30 minutes. The cytosol fraction was diluted with buffer A and used in assays.

### *In Vitro* PI-PLC Assay

PI-PLC activity was assayed by the method of WAHL *et al.*[2] with slight modifications. The reaction buffer consisted of 7.5 $\mu$l of buffer A, 10 $\mu$l of 50 mM sodium phosphate (pH 6.8) - 1 mM EGTA - 100 mM potassium chloride, 2.5 $\mu$l of 8 mM calcium chloride, 2.5 $\mu$l of

[$^3$H]$PIP_2$ (2.08 nCi/assay), and 2.5 $\mu$l of test sample. The reaction was started by the addition of 2.5 $\mu$l of the enzyme solution. The reaction mixture was incubated for 10 minutes at 37°C, and the reaction was terminated on ice with 50 $\mu$l of 1% bovine serum albumin and 250 $\mu$l of 10% trichloroacetic acid. The precipitate was removed by centrifugation, and the radioactivity of the soluble fraction containing [$^3$H]$IP_3$ was measured with a liquid scintillation counter.

Inositol Phosphate Formation

A431 cells ($3 \times 10^5$/well) grown for 24 hours beforehand in 24-well plates were prelabeled with [$^3$H]*myo*-inositol (1 $\mu$Ci/ml) for 16 hours in inositol-free DMEM containing 10% dialyzed serum. Then, the medium was removed and the cells were pre-incubated for 30 minutes in the presence of a test sample in 0.5 ml of inositol-free DMEM containing 30 mM LiCl. Next, after addition of 400 ng/ml of EGF, the cells were further incubated for 15 minutes at 37°C. The reaction was terminated by addition of ice-cold 10% $HClO_4$, and the mixture was then neutralized by 1.53 M KOH in 75 mM Hepes. The solution was kept on ice for 15 minutes, after which it was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was applied onto an Amprep SAX column, and the column was then washed with water and eluted with 0.17 M $KHCO_3$. The eluate was counted in a liquid scintillation counter.

EGF-stimulated Rapid Rounding of A431 Cells

A431 cells ($2.5 \times 10^4$/well) grown for 16 hours beforehand in 48-well plates were washed twice with 0.5 ml of $Ca^{2+}$, $Mg^{2+}$-free DULBECCO's phosphate-buffered saline (PBS−) and pre-incubated with a test sample in PBS− at 37°C for 15 minutes. Then, the cells were further incubated with 100 ng/ml of EGF. After 10 minutes, the cells were examined by phase-contrast microscopy.

## Results and Discussion

After screening of about 3,000 samples, the culture broth of *Streptomyces* sp. MJ677-72F5 was found to inhibit the PI-PLC activity. The active principle was purified and identified as fluvirucin $B_2$ (Fig. 1) by spectral analysis. Fluvirucins have been isolated from *Actinomadura* sp. as antibiotics active against influenza A virus[3] and fungi[4]. Fluvirucin $B_2$ inhibited the PI-PLC from A431 cells with an $IC_{50}$ of 1.6 $\mu$g/ml, as shown in Fig. 2; and the inhibitory activity was similar to that of plipastatin A, a non-specific inhibitor of phospholipases[5]. Fluvirucin $B_2$ did not inhibit protein-tyrosine kinase, protein-tyrosine phosphatase, protein kinase C, or phosphatidate phosphatase at 100 $\mu$g/ml. On the other hand, it did inhibit the formation of inositol phosphates in cultured A431 cells with an $IC_{50}$ of 9.4 $\mu$g/ml (Fig. 3). This microbial metabolite did not inhibit EGF-induced PI synthesis in A431 cells, but rather activated the synthesis at 30 ~ 100 $\mu$g/ml (data not shown). Though the mechanism of this phenomenon is not known, it is interesting that another PI-PLC inhibitor, Q12713, showed the same effect[6].

As seen in Table 1, fluvirucin $B_2$ inhibited the growth of various cultured cells. It inhibited the growth of A431 cells with an $IC_{50}$ of 0.7 $\mu$g/ml, but did not inhibit

Fig. 1. Structure of fluvirucin $B_2$.

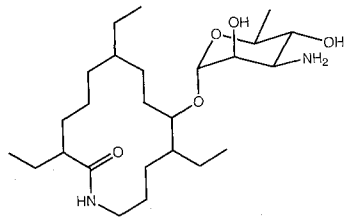



Fig. 2. Inhibition of phospholipase C by fluvirucin $B_2$.
PI-PLC solution was incubated with various concentrations of fluvirucin $B_2$(○) or plipastatin A (●) for 10 minutes. Values are means ± SD of quadruplicate samples.

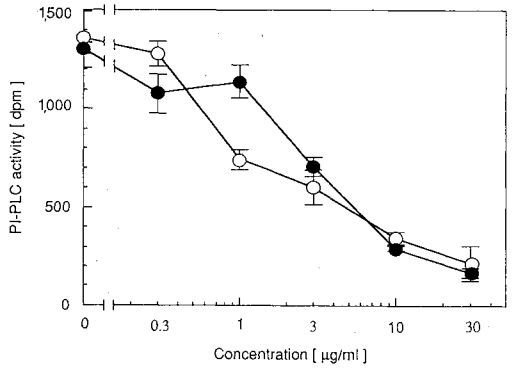



Fig. 3. Inhibition of inositol phosphate formation by fluvirucin $B_2$ in cultured A431 cells.
After pre-treatment of A431 cells with [$^3$H]inositol for 16 hours, the cells were treated with the indicated concentrations of fluvirucin $B_2$ for 30 minutes. Then, the cells were further incubated with 400 ng/ml of EGF for 15 minutes. Formation of total inositol phosphates ($IP_t$) was measured as described under "Materials and Methods". Values are means ± SD of quadruplicate samples.

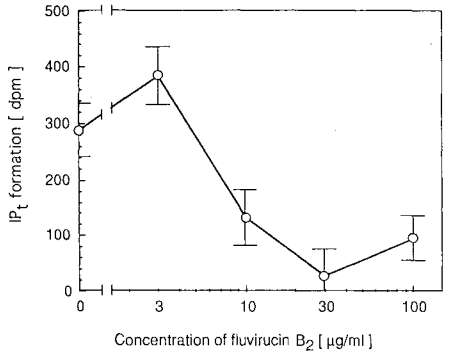


Table 1. Growth inhibition of cultured cells by fluvirucin $B_2$.

| Cells | Growth inhibition* $IC_{50}$ ($\mu$g/ml) |
|---|---|
| A431 | 0.7 |
| ER12** | 1.5 |
| NIH3T3 | 5.8 |
| *erb* B2/NIH3T3 | 2.9 |
| H-*ras*/NIH3T3 | 0.9 |
| K-*ras*/NIH3T3 | 2.9 |
| *fos*/NIH3T3 | 19.2 |
| Jurkat T lymphoma | 0.5 |
| NRK | 26.2 |
| K-$ras^{ts}$/NRK (33°C) | 8.5 |
| (39°C) | 11.7 |
| $RSV^{ts}$/NRK (33°C) | 21.5 |
| (39°C) | 24.0 |

* Each cell line was incubated for 3 days with fluvirucin $B_2$.

** ER12 cells are NIH3T3 cells overexpressing human EGF receptors.

Fig. 4. Effect of fluvirucin $B_2$ on macromolecular synthesis in A431 cells.

A431 cells ($5 \times 10^5$/well) grown for 16 hours beforehand were labeled with 1 $\mu$Ci/ml [$^3$H]thymidine (○), [$^3$H]uridine (●), or [$^3$H]leucine (▲) for 1 hour in the presence of fluvirucin $B_2$ in serum-free DMEM. Then, the radioactivity of TCA-insoluble fraction was counted. The 100% values were 18669±400, 2625.0±131.2 and 1487.5±57.6 dpm for DNA, RNA and protein syntheses, respectively. Values are means±SD of quadruplicate samples.

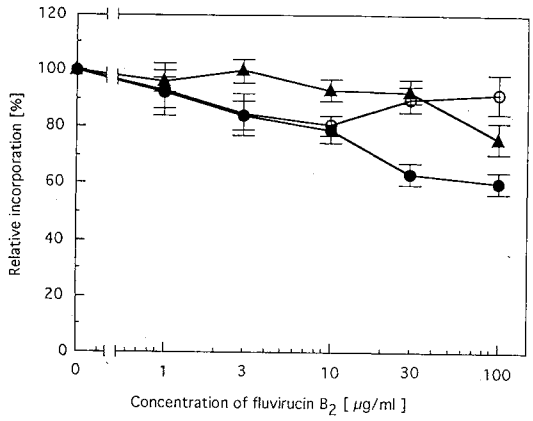
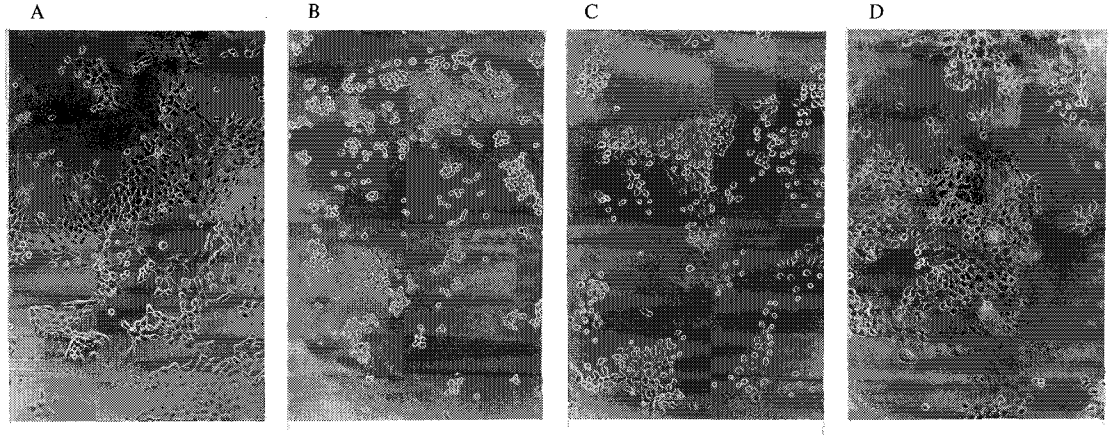



Fig. 5. Inhibition by fluvirucin $B_2$ of EGF-induced rapid rounding of A431 cells.

A431 cells were pre-incubated without (A, B) or with fluvirucin $B_2$ (C, 30 $\mu$g/ml; D, 100 $\mu$g/ml) for 15 minutes, then incubated without (A) or with (B, C, D) 100 ng/ml of EGF for 10 minutes.




macromolecular synthesis markedly in these cells (Fig. 4). Therefore, fluvirucin $B_2$ would inhibit growth without inhibiting DNA, RNA and protein syntheses.

PI turnover was suggested to be involved in the mechanism of EGF-induced rapid rounding of A431 cells[7]. Fluvirucin $B_2$ inhibited the rounding completely at 100 $\mu$g/ml, as shown in Fig. 5. The rapid rounding is also inhibited by inhibitors of oxidative phosphorylation[8], and such inhibition is reversed by glucose. However, the inhibition of rapid rounding by fluvirucin $B_2$ was not reversed by addition of 1 mg/ml glucose.

Thus, fluvirucin $B_2$ inhibited PI-PLC both *in vitro* and *in situ*. Though its effect on other phospholipases is now under investigation, fluvirucin $B_2$ will be an useful tool for the mechanistic study of intracellular signal transduction.

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