MECHANISM OF ACTION OF ATPENIN B ON RAJI CELLS

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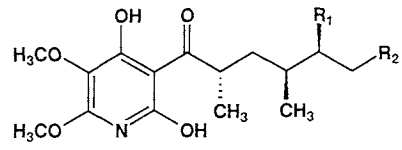
Atpenin B, a new antifungal antibiotic produced by *Penicillium* sp. FO-125, inhibited the growth of Raji cells (IC_{50}, 30 μM). The incorporation of [^{14}C]leucine and [^{3}H]thymidine into Raji cells was inhibited by atpenin B with IC_{50} values of 0.10 and 0.12 μM, respectively. The incorporation of [^{14}C]palmitate into the cells was not inhibited but its incorporation into lipid fractions was inhibited by atpenin B (IC_{50}, 0.13~0.24 μM). Studies on the site of atpenin B action demonstrated that atpenin B decreases the cellular adenosine 5'-triphosphate (ATP) level in Raji cells with IC_{50} value of 0.020 μM, suggesting the inhibition of ATP-generating system by atpenin B.

New antifungal antibiotics named atpenins were isolated from the culture broth of *Penicillium* sp. FO-125[1]. Details of structure determination of atpenins A4, A5 and B (Fig. 1) will be reported in near future[2].

Fig. 1. Structure of atpenins A4, A5 and B.



A4 R_1=Cl R_2=H
A5 R_1=R_2=Cl
B R_1=R_2=H

Atpenins were found to inhibit the growth of Raji cells. Preliminary studies of atpenin action on Raji cells showed that atpenins inhibit the incorporation of free long chain fatty acid into lipid fractions in intact cells. Therefore, this paper deals with the mode of action of atpenin B on Raji cells.

Materials and Methods

Materials

Atpenin B was purified from the culture filtrate of *Penicillium* sp. FO-125 as reported previously[1]. Raji cells were obtained from Dainippon Pharmaceutical Co., Ltd. RPMI-1640 and fetal bovine serum were purchased from Gibco, Co. L-[*U*-^{14}C]Leucine (342 mCi/mmol) and [*U*-^{14}C]palmitate were from Amersham, and [*methyl*-^{3}H]thymidine (85.6 mCi/mmol) was from New England Nuclear. All other reagents used were of analytical grade.

Cell Culture

Raji cells (1.0×10^6 cells/ml) were routinely maintained at 37°C in the culture medium of RPMI-1640 supplemented with 10% fetal bovine serum in a 75-cm^2 disposable tissue culture flask (Corning Co.) under a humidified condition of 5% CO_2/ 95% air. Three-day cultured cells were used for experiments unless otherwise stated. The cell viability was usually >95% as estimated by trypan blue exclusion method.

Assay for Raji Cell Growth

Raji cells (1.0×10^5 cells/ml) were incubated for 3 days with various concentrations of atpenin B (0~1.68 μM) under the same conditions as described above. The cell number and cell viability were determined by trypan blue exclusion method.

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Analysis of Incorporation of Radioactive Precursors into Raji Cells

Various concentrations of atpenin B in 5 μl of ethanol were added to a Raji cell suspension (6.0×10^5 cells) in 300 μl of magnesium- and calcium-free phosphate buffered saline (PBS).

After the mixture was incubated at 37°C for 20 minutes, [^{14}C]palmitate (6.25 nCi) in 5 μl of PBS was added to the cell suspension. After additional 30-minute incubation, 1 ml of PBS was added to the mixture and the cells were harvested on a glass-filter and washed with 3 ml of PBS twice. Cell-associated radioactivity was determined with a liquid scintillation counter (LSC-3100 Aloka, Co.).

Assay for Lipid Synthesis in Raji Cells

Raji cells (6.0×10^5 cells) were incubated with [^{14}C]palmitate in the presence or absence of atpenin B by the same method as described above. After additional 30-minute incubation lipids were extracted by the method of BLIGH and DYER[3]. Total lipid extracts were separated on TLC (F_{254}, E. Merck Co.) using petroleum ether-diethyl ether-acetic acid (70:30:1) or chloroform-methanol-conc ammonia solution (60:35:5) as a developing solvent.

Assay for Cellular ATP Level in Raji Cells

The level of cellular ATP in Raji cells was measured by the method of DELUCA and MCELORY[4] using the kit of ATP bioluminescence HS (Boehringer Mannheim Biochemica).

Raji cells (6.0×10^5 cells) were suspended in 0.3 ml of PBS and atpenin B at various concentrations in 5 μl of ethanol was added to the cell suspension. After incubation at 37°C for 20 minutes, 1 ml of PBS was added and the cells were collected by centrifugation (800 rpm, 2 minutes). The cells suspended in 0.3 ml of distilled water were cooked at 100°C for 5 minutes to extract cellular ATP. The heat-treated cells were centrifuged at 3,000 rpm for 5 minutes and the supernatant was used to determine the amount of ATP after appropriate dilution. The test sample solution (100 μl) and the reagent (I) (400 μl) of the kit were mixed and the light emission was measured with luminescence reader (BLR-102, Aloka Co.).

Other Enzyme Assays

Acyl-CoA synthetase (from Raji cells and *Pseudomonas aeruginosa*) and glycerol-3-phosphate acyltransferase (GPAT), diacylglycerol acyltransferase (DAT) and acyl-CoA: cholesterol acyltransferase (ACAT) (rat liver microsomal fraction was used as an enzyme source) were assayed according to the methods of TOMODA *et al.*[5,6], KHOKHA *et al.*[7], POLOKOFF and BELL[8], and ROSS *et al.*[9], respectively.

Results

Effect of Atpenin B on Raji Cell Growth

After 3-day cultivation the number of Raji cells increased by five to six times in the absence of atpenin B. Atpenin B inhibited the growth of Raji cells in a dose-dependent fashion as shown in Fig. 2. The concentration of atpenin B requiring 50% inhibition

Fig. 2. Effect of atpenin B on Raji cell growth.

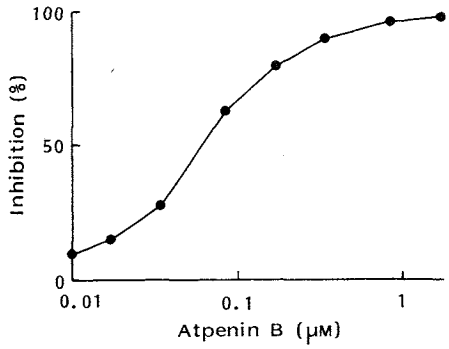
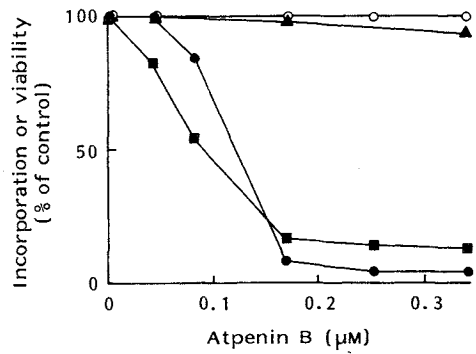


Fig. 3. Effect of atpenin B on the incorporation of labeled precursors into Raji cells and cell viability.

● [^{3}H]Thymidine, ■ [^{14}C]leucine, ▲ [^{14}C]palmitate, ○ cell viability.



of Raji cell growth was measured to be 0.10 μM.

Effect of Atpenin B on Cell Viability and Precursor Incorporation

Raji cells were incubated in PBS with various concentrations of atpenin B at 37℃ for 1 hour. Even at 170 μM of atpenin B, the cell viability was still >95%. The effect of atpenin B on blood cells from rabbits was also tested. The hemolysis of blood cells was not observed at 170 μM of atpenin B after 1-hour incubation at 37℃. These findings indicate that atpenin B does not have any direct effect on the cell membrane.

Then the effect of atpenin B on the incorporations of [^{14}C]leucine, [^{3}H]thymidine and [^{14}C]palmitate into Raji cells was studied (Fig. 3). In this experiment the incorporated radioactivity includes the amount of labeled precursor utilized for higher macromolecule biosyntheses (proteins, DNA or lipids) plus the amount only associated with cells (on the cell surface and inside the cells). Atpenin B inhibited the incorporations of [^{14}C]leucine (IC_{50}, 0.10 μM) and [^{3}H]thymidine (IC_{50}, 0.12 μM) into Raji cells without affecting the cell viability. On the other hand, the incorporation of [^{14}C]palmitate was not inhibited.

Effect of Atpenin B on Lipid Synthesis in Raji Cells

The lipid synthesis from exogenously added [^{14}C]palmitate in Raji cells was extensively studied[6]. According to the results, the incorporation reached a plateau by 20 minutes. [^{14}C]Palmitate was incorporated into phosphatidylcholine (~25%) and triacylglycerol (~20%) and substaintially all the rest (~50%) was recovered from the free fatty acid fraction on TLC. The incorporation into phosphatidylethanolamine was negligible. Effect of atpenin B on the lipid synthesis in Raji cells was studied.

Atpenin B showed a dose-dependent inhibition of [^{14}C]palmitate incorporation into phospholipids and triacylglycerol with IC_{50} values of 0.13 and 0.24 μM, respectively (Fig. 4).

Effect on Cellular ATP Level in Raji Cells

Cellular ATP level in Raji cells was measured to be 3.0×10^{-15} mol/cell in the absence of atpenin B. This value was within almost the same level as those of chick embryo fibroblasts (2.4×10^{-15} mol/cell)[10] and of mouse leukemia (5.06×10^{-15} mol/cell)[11]. After 20-minute treatment of Raji cells with atpenin B the cellular ATP level was decreased dramatically in a dose-dependent fashion (Fig. 5). Fifty percent decrease in ATP pool was observed at 0.020 μM of atpenin B. Under the same conditions, 50% decrease was at 0.018 μM of

Fig. 4. Effect of atpenin B on [^{14}C]palmitate incorporation into lipid fractions in Raji cells.

▲ Phospholipids, ● triacylglycerol.

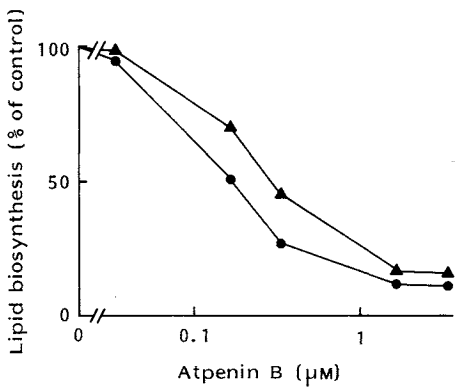
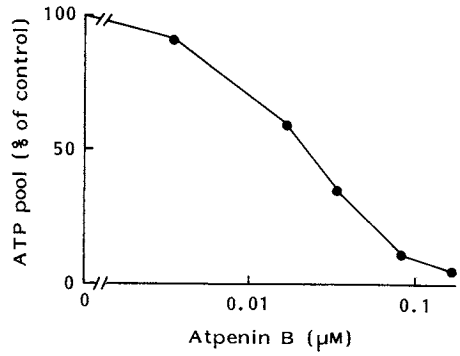


Fig. 5. Effect of atpenin B on cellular ATP level of Raji cells.



antimycin A for comparative purpose.

Effect of Atpenin B on Other Enzyme Activities Involved in Lipid Synthesis

Long chain acyl-CoA synthetase activity was not inhibited by atpenin B even at the concentration of 170 μM when Raji cell membrane fractions[6] or a purified synthetase from *P. aeruginosa*[5] was used as an enzyme source. Effect of atpenin B on other acyltransferase activities such as GPAT, DAT and ACAT in a rat liver microsomal fraction was studied. Atpenin B exhibited no inhibitory activity against GPAT nor DAT but showed very weak inhibitory activity against ACAT (50% inhibition at 340 μM).

Discussion

Atpenins were originally discovered as inhibitors of lipid metabolism, because atpenin B inhibited [^{14}C]palmitate incorporation into lipid fractions such as phospholipids and triacylglycerol in Raji cells (Fig. 4). Accordingly the following three possibilities were reasoned for possible inhibition sites of atpenin B: 1) Long chain acyl-CoA synthetase, 2) the common steps for phosphatidylcholine and triacylglycerol biosyntheses such as GPAT, monoacylglycerol phosphate acyltransferase and diacylglycerol phosphate hydrolase and 3) ATP-generating system. Studies were carried out to examine these possibilities.

Atpenin B did not inhibit long chain acyl-CoA synthetase activity even at 170 μM when Raji cell membrane fractions or a synthetase purified from *P. aeruginosa* was used as an enzyme source. Among enzymes involved in lipid biosynthesis, acyltransferases such as GPAT and DAT with a rat liver microsomal fraction were not inhibited by atpenin B, either. Therefore the third possibility was studied using intact Raji cells.

The cellular ATP level in Raji cells decreased dramatically in the presence of atpenin B (Fig. 5), suggesting that atpenin B inhibits the ATP-generating system. This conclusion was supported by the inhibition of the incorporation of [^{14}C]leucine and [^{3}H]thymidine into the cells by atpenin B (Fig. 3), because the active transport of these precursors requires ATP. However, the incorporation of [^{14}C]palmitate into Raji cells was not inhibited by atpenin B. This result suggested that the transport system of long chain fatty acids into animal cells does not appear to require ATP, though the system is still unclear. The inhibition of [^{14}C]palmitate incorporation into lipid fractions in intact Raji cells could be also explained as follows. The reaction of acyl-CoA synthetase requires ATP as substrate. In the presence of atpenin B the cellular ATP level decreased, leading to the decrease of the cellular acyl-CoA level and eventually acyl-CoA-dependent reactions such as acyltransferases might be blocked.

Inhibitory activity of atpenins against filamentous fungi[1] such as *Piricularia oryzae, Trichophyton mentagrophytes* and *Microsporum gypseum* might be also due to the inhibition of ATP-generating system by atpenins in such fungi. Further investigation will be necessary to clarify this point.

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