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Fattiviracin A1, a Novel Antiviral Agent Produced by *Streptomyces microflavus* Strain No. 2445

II. Biological Properties

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Fattiviracin A1, showed potent antiviral activities against herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), influenza A virus and human immunodeficiency virus type 1 (HIV-1). It showed no cytotoxicity against Vero cells. Fattiviracin A1 exhibited no significant antibacterial or antifungal activities. Treatment of HSV-1 with fattiviracin A1 decreased its infectivity to Vero cells. The mechanism of its antiviral activity may be due to inactivation of the viral particles and inhibition of viral entry into host cells.

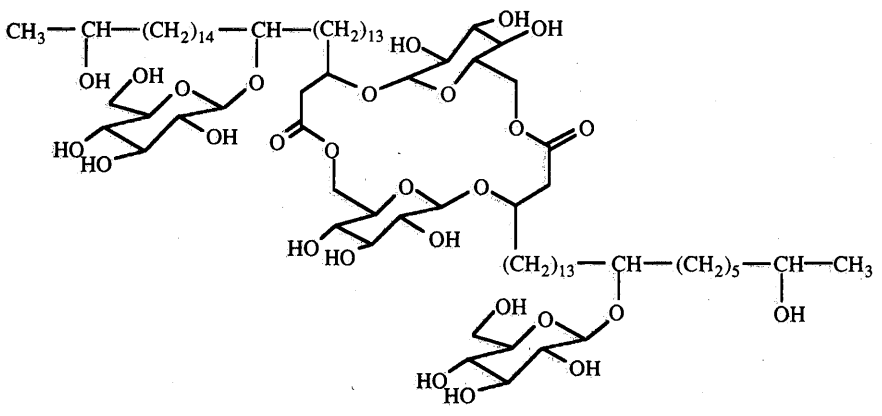
Fattiviracin A1 (Fig. 1) is a novel antiviral agent isolated from the culture filtrate of *Streptomyces microflavus* strain No. 2445. Its taxonomy, production, isolation, physico-chemical properties and structure elucidation have been already reported in the preceding paper[1]. In this paper we describe its biological properties.

Materials and Methods

Antiviral and Cell Growth Assays

The antiviral activities of fattiviracin A1 on HSV-1 (KOS), VZV and influenza A virus (H1N1) were measured by the plaque reduction assay[2]. Confluent monolayers of Vero cells in 6-well plates were infected with 100 PFU of HSV-1 or VZV. Confluent monolayers of MDCK cells were infected with 100 PFU of influenza A virus. After a 1 hour adsorption period, the cultures were overlaid with DULBECCO's modified Eagle minimum

Fig. 1. Deduced structure of fattiviracin A1.



essential medium (DMEM) containing 2% heat-inactivated fetal calf serum (FCS) including various concentrations of the drug. The plates were incubated in the CO_2 incubator for 3 days, then fixed with formalin and stained with crystal violet in methanol. Infectious virus production was quantified by observing the virus-induced cytopathic effect.

The effect of fattiviracin A1 on HIV-1 replication and production from acutely infected cells was tested in MT-4 cells[3]. MT-4 cells (3×10^5 cells) were infected with LAI strain of HIV-1 (12.5 ng of HIV-1 p24 antigen) and incubated in growth medium (10% FCS+RPMI 1640) containing different concentrations of the drug for 3 days. The virus production from the cells was evaluated by measuring p24 amount of the cell-free supernatants using HIV-1 p24 capsid enzyme-linked immunosorbent assay (Abbott).

The anticellular activity was examined as described below. Confluent monolayers of Vero cells were seeded in 6-well plates at 1×10^6 cells per well. After 1 day, the cells were refed with DMEM containing 5% FCS and various concentrations of the drug. After 3 days incubation, cells were fixed with formalin and stained with crystal violet in methanol. Excess dye was washed off, and the dye incorporated by the viable cells was eluted with dimethyl sulfoxide. The optical densities were read at 550 nm.

Reversibility of Antiviral Activity and the Action of Fattiviracin A1 on the Viral Cycle

For experiments on the reversibility of the antiviral activity of fattiviracin A1, confluent monolayers of Vero cells were infected with 100 PFU of HSV-1. Fattiviracin A1 (10 μg/ml) was added at 0 time infection. The drug was left in the culture for different times (0 to 72 hours) then it was replaced with fresh medium and the cells were incubated for a further 72 hours after infection. The antiviral activities were measured by plaque reduction assay.

To examine the action of fattiviracin A1 on the viral cycle, the drug (10 μg/ml) was added to HSV-1 infected cells at different time (0 to 72 hours) after infection. Cells were incubated for 72 hours after infection. The antiviral activity was measured by plaque reduction assay.

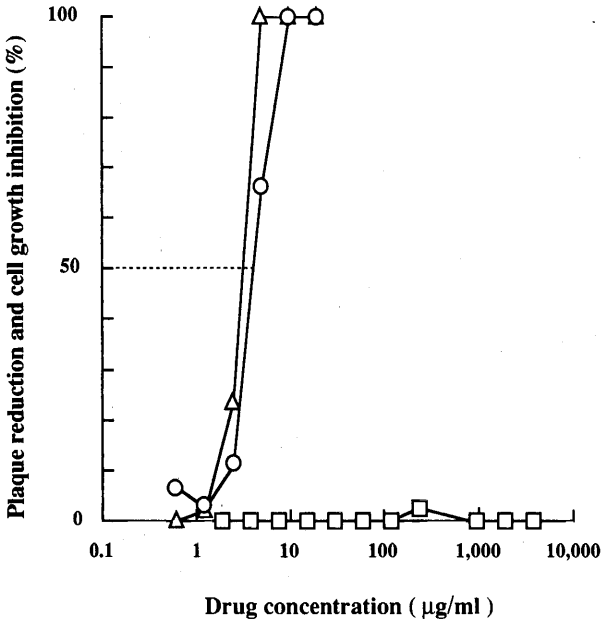
Effect of Fattiviracin A1 on Viral Infection

For experiments on the effect of fattiviracin A1 on viral infection in host cells, confluent monolayers of Vero cells were infected with 0.1 ml of HSV-1 suspension (100 PFU) containing various concentrations of drug. After a 1 hour adsorption period, the cells were overlaid with 2 ml of DMEM containing 5% FCS and incubated for 3 days. The drug was finally diluted 20 times in the culture. The infectious viruses were titrated as plaque forming units.

To examine the effect of treatment time of fattiviracin A1 on viral infection, 100 PFU of HSV-1 suspensions including 5 or 20 μg/ml of the drug were incubated for various times at 37°C. Residual infectious viruses were titrated as plaque forming units.

Fig. 2. Antiviral activities and cytotoxicity of fattiviracin A1.

○ Activity against HSV-1, △ activity against VZV, □ cytotoxicity against Vero cell.



Antimicrobial Activity

Waksman's agar dilution streak method[4] was used for the determination of the antimicrobial spectrum of fattiviracin A1.

Results

Antiviral Activities against HSV-1 and VZV

Confluent monolayer cultures of Vero cells in plates were infected with HSV-1 or VZV. At 1 hour after infection, designated concentrations of fattiviracin A1 were added, and incubation was continued for 3 days. The dose response curve of fattiviracin A1 is shown in Fig. 2. Fattiviracin A1 showed the antiviral EC_{50}'s of 3.88 and 3.37 μg/ml against HSV-1 and VZV, respec-

tively. Cytotoxicity of fattiviracin A1 against Vero cells was not observed even at a concentration of 3.75 mg/ml. Therefore the selectivities of fattiviracin A1 against HSV-1 and VZV were estimated as more than 967 and 1113, respectively.

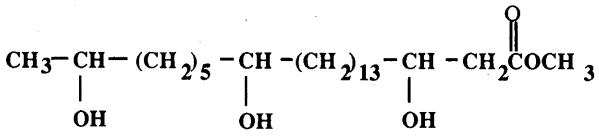
Antiviral Activities against Influenza A Virus and HIV-1

Antiviral activities of fattiviracin A1 against influenza A virus and HIV-1 were examined. Fattiviracin A1 showed antiviral EC_{50}'s of 2.05 and 10.35 μg/ml against influenza A virus and HIV-1, respectively.

Active Site of Fattiviracin A1

Compound **4** (Fig. 3), one of the degradation products of fattiviracin A1, was examined for antiviral activity. It showed antiviral activity (EC_{50}) of 4.35 μg/ml against HSV-1. This result suggests that the long fatty acid chain of fattiviracin A1 may play an important role in its antiviral activity.

Fig. 3. Deduced structure of compound 4.



Effect of Exposure Time of 10 μg/ml Fattiviracin A1 on Infected Cells

As shown in Fig. 4A, an exposure time of less than 12 hour of fattiviracin A1 with HSV-1 infected cells resulted in no inhibition of viral yield. 36 hours of exposure of the drug resulted in 41% inhibition. 48 hours of exposure were sufficient to inhibit viral replication completely. The action of fattiviracin A1 on viral replication was reversible until 12 hours after infection, however it was irreversible after that time.

As shown in Fig. 4B, the virus was completely inhibited by fattiviracin A1 when the drug was added up to 12 hours after infection. However when the drug was added at 24, 36 and 48 hours after infection, the viral inhibition was decreased to 77, 29 and 6%, respectively.

These results suggest that the antiviral action of fattiviracin A1 to HSV-1 occurred from 24 to 48 hours after infection. At this time most steps of the viral cycle may be almost completed, and viral morphogenesis may be still ongoing.

Effect of Fattiviracin A1 on Viral Infection

In order to analyze possible effect of fattiviracin A1 on viral infection steps, the drug was simultaneously added with virus challenge to the host cells and weakened after adsorption period by 20 times dilution. As shown in Fig. 5, treatment of viral particles with fattiviracin A1

Fig. 4. HSV-1 inhibition of fattiviracin A1 in infected Vero cell cultures.

A: After exposure of fattiviracin A1 to the infected cells for different times.
B: After addition of fattiviracin A1 at different times after infection.

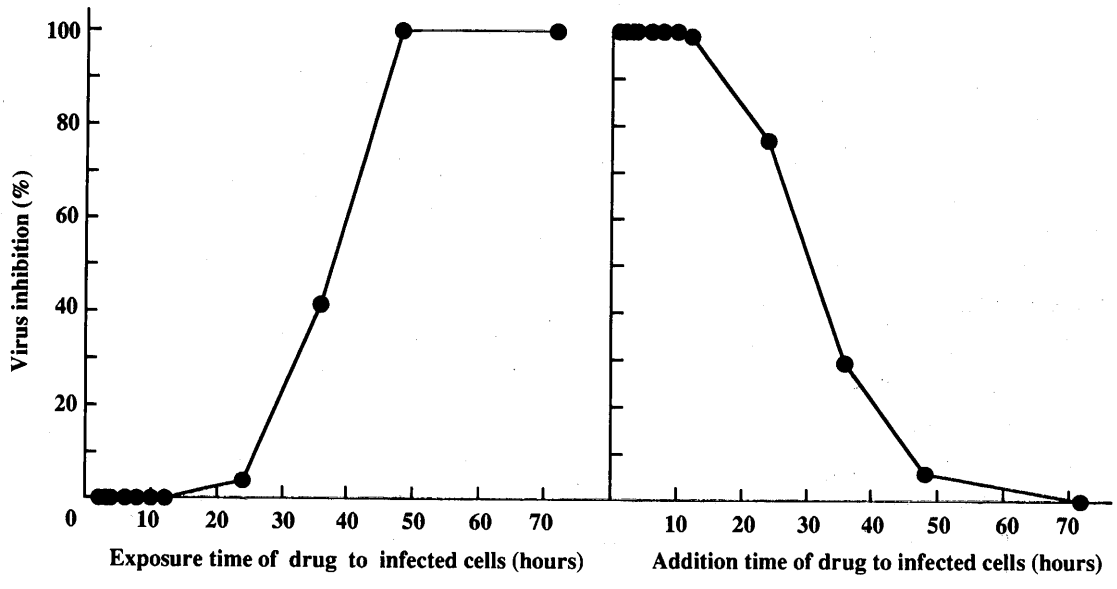


Fig. 5. Effect of fattiviracin A1 on viral infection.

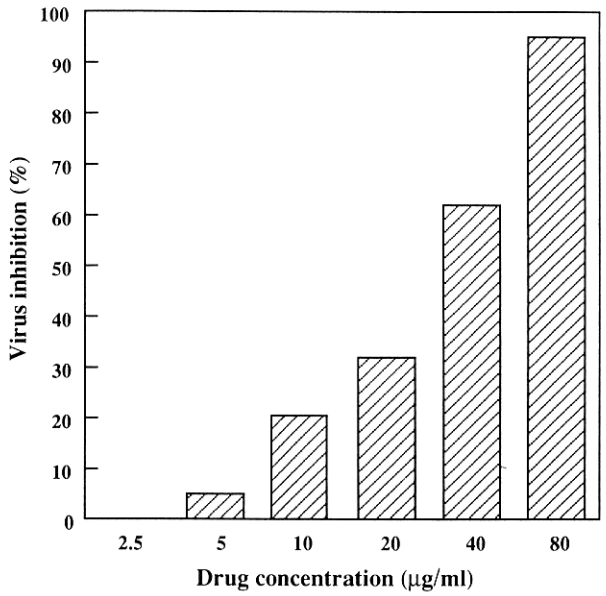
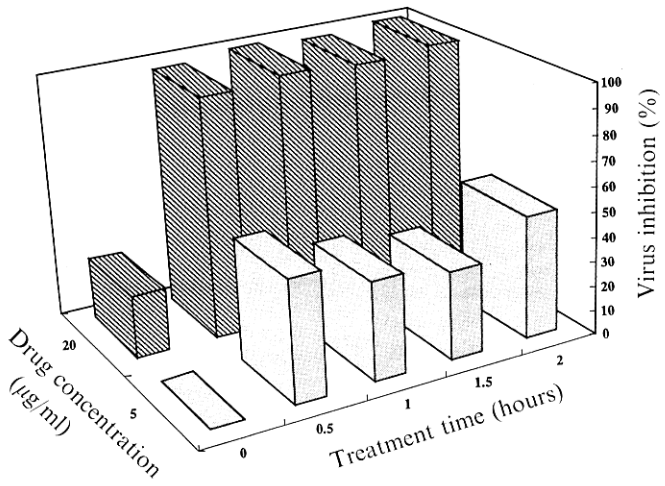


Fig. 6. Effect of treatment time of fattiviracin A1 on viral infection.



decreased the viral infection. In this experiment, diluted drug may remain during the incubation of cultures for three days, and affect viral replication as described above. However the observed viral inhibitions were higher than those expected from the remaining agent in cultures. For example, treatment with 40 μg/ml of drug during infection caused 61% inhibition, while presence of 2 μg/ml (the expected remaining concentration after dilution) during culture inhibited less than 12 % of viruses (the data were obtained from Fig. 2). This result suggests that, on the infection step, fattiviracin A1 may inactivate the viral particles and prevent viral entry into the host cells.

The effect of fattiviracin A1 treatment time on viral particles was examined to see if the drug inactivates the infectious viruses. Viral particles were pretreated with the drug for the indicated times before infection to the host cells, and the drug was weakened after adsorption period by 20 times dilution. As shown in Fig. 6, treatment times of more than 0.5 hours increased the viral inhibition. For example, 20 μg/ml fattiviracin A1 without preteatment showed about 26% viral inhibition, while 97% viral inhibition was observed after 0.5 hours incubation at the same concentration.

From these results, it is suggested that the antiviral mechanism of fattiviracin A1 is due to inactivation of viral particles and inhibition of viral entry into the host cells.

Table 1. Antimicrobial activities of fattiviracin A1.

Strain used		MIC (μg/ml)
Bacillus subtilis	IFO 3007	>100
Staphylococcus aureus	IFO 3060	>100
Micrococcus luteus	IFO 3232	>100
Escherichia coli	IFO 3301	>100
Pseudomonas aeruginosa	IFO 3167	>100
Proteus vulgaris	IFO 3448	>100
Saccharomyces cerevisiae	IFO 0305	>100
Candida albicans	IFO 0583	>100
Aspergillus niger	IFO 4066	>100
Aspergillus oryzae	IFO 4075	>100

Antimicrobial Activities of Fattiviracin A1.

Table 1 shows the minimum inhibitory concentrations (MIC) of fattiviracin A1 against various microorganisms. No effect on bacteria, yeasts and fungi was observed at concentrations up to 100 μg/ml.

Discussion

Streptomyces microflavus strain No. 2445 produces a novel antiviral agent, named fattiviracin A1 (originally named AH-2445 A1). Fattiviracin A1 has potent activities against two enveloped viruses of the herpes family, HSV-1 and VZV, and two enveloped RNA viruses, influenza A virus and HIV-1. It has no cytotoxicity against Vero cells, so its selectivities against HSV-1 and VZV are remarkably high as much as that of acyclovir[5]. The structure of fattiviracin A1 is similar

to those of cycloviracins B_1 and B_2[6,7], which were reported to have anti-HSV-1 activity. However fattiviracin A1 differs from these known compounds in the length of its side chain and the kind and number of sugar molecules.

Some fatty alcohols such as linolenyl alcohol, elaidyl alcohol[8,9], 1-docosanol[10], some fatty acids such as linolenic acid, elaidic acid, and their monoglycerides[11] have been reported to be effective inhibitors of enveloped viruses including HSV-1, HSV-2 and respiratory syncytial virus. Antiviral fatty acids were found to affect viral envelope, causing leakage of viral envelope and at higher concentrations, a complete disintegration of the envelope and the viral particles[12]. Fattiviracin A1 has fatty acid moieties in its molecule which may play an important role for the inhibitory activity, and the antiviral activity of its degradation compound supports this idea. The antiviral mechanism of fattiviracin A1 is clearly different from acyclovir in that the latter inhibits herpesvirus DNA polymerase activity. Fattiviracin A1 directly inactivates virus since preincubation of virus with this compound diminishes infectivity. Although its mechanism of action has to be precisely defined, fattiviracin A1 inhibits plaque formation in infected cell cultures. Since removal of the drug in the early stage results in no inhibition, the compound may not inhibit viral DNA and protein synthesis in the first infected cell, and it may not be incorporated into infected cells. Fattiviracin A1 probably acts on the mature viruses which bud from the first infected cells, and inactivates the virus particles and inhibits the infection of neighboring cells.

An advantage of fattiviracin A1 is its solubility. Fattiviracin A1 is easily soluble in water, differing from fatty acids and fatty alcohols, mentioned above. Even though it has two long fatty acid chains, the presence of four sugar molecules in its structure may increase its water solubility. Fattiviracin A1 could become a significant new antiviral drug with a potentially broad spectrum for several enveloped viruses including HIV. The detailed anti-HIV mechanisms of fattiviracin A1 will be reported in the future.

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