Characterization of anti-herpes simplex virus type 1 activity of an alkaloid FK 3000 from *Stephania cepharantha*

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(Received December 25, 2001. Accepted March, 29, 2002.)

Abstract

A morphinane alkaloid FK 3000 (6,7-di-*O*-acetylsinococuline) from the root tubers of *Stephania cepharantha* showed antiviral activity against acyclovir (ACV)- and phosphonoacetic acid (PAA)-resistant herpes simplex virus type 1 (HSV-1), influenza virus, measles virus, and poliovirus.

The anti-HSV action of FK 3000 was assessed in comparison with that of PAA that inhibits the activity of HSV DNA polymerase and HSV DNA synthesis. FK 3000 inhibited the growth of thymidine kinase-deficient and ACV and PAA-resistant HSV-1 strains, as well as wild type HSV strains in Vero cells. This compound, as well as PAA, interfered with the synthesis of late viral proteins but not early viral proteins. The analysis of HSV DNA synthesis by slot blot hybridization showed that FK 3000 inhibited the viral DNA synthesis in a dose-dependent manner. However, the viral RNA was partially synthesized in the presence of FK 3000 (even at a dose that HSV DNA synthesis was inhibited) and PAA, indicating that FK 3000, as well as PAA, allowed early viral RNA synthesis but not viral DNA synthesis. Since partially purified HSV DNA polymerase activity was not inhibited by FK 3000, this compound was suggested to inhibit HSV DNA synthesis by a mechanism different from that of PAA.

Key words herpes simplex virus, *Stephania cepharantha*, FK 3000, antiviral activity.

Abbreviations ACV, acyclovir; AP^r, acyclovir- and PAA-resistant; CC_{50}, 50% cytotoxic concentration; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EC_{50}, effective concentration for 50% plaque reduction; HEL, human embryonic lung; HSV, herpes simplex virus; MEM, minimum essential medium; MDCK, Madin-Darby canine kidney; PAA, phosphonoacetic acid; PBS, phosphate-buffered saline; PFU, plaque-forming unit; SDS, sodium dodecylsulfate; TK^-, thymidine kinase deficient.

Introduction

Various anti-herpes simplex virus (HSV) agents have been developed and used for the treatment of HSV infection in humans.[1-5] However, the appearance of drug-resistant HSV strains became evident in immunosuppressed patients, such as organ transplant recipients and patients with AIDS.[6-14] Thus, it is necessary to develop new therapeutic agents with different mode of anti-HSV action from that of known anti-HSV agents.

We previously selected a methanol extract of the root tubers of *Stephania (S.) cepharantha* HAYATA with antiviral activity against HSV type 1 (HSV-1) from 30 Chinese herbal medicines and isolated FK 3000 as the most potent anti-HSV-1 compound in the extract.[15] This compound was effective in delaying the development of heretic skin lesions in mice infected cutaneously with HSV-1 and reducing the mortality.[16] Its therapeutic efficacy was revealed *in vivo*. FK 3000 also showed potent antiviral activity against thymidine kinase-deficient (TK^-) HSV-1 that is resistant to acyclovir (ACV) and wild HSV type 2 (HSV-2) strains, as well as wild HSV-1 *in vitro*.[16] The mode of anti-HSV action of FK 3000 was suggested to be different from that of ACV.

In this study, we have developed a convenient way

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to purify FK 3000 from an ethanol extract of *S. cepharantha.* In order to clarify the inhibitory stage of FK 3000 in a replication cycle of HSV, we characterized its anti-HSV-1 action in comparison with that of phosphonoacetic acid (PAA) that inhibits HSV DNA synthesis. FK 3000 inhibited the growth of TK^- and ACV- and PAA-resistant (AP^r) HSV-1 strain, as well as wild type HSV-1 and -2 strains. This compound, as well as PAA, inhibited the synthesis of HSV DNA and late viral proteins, but not the early viral RNA synthesis. However, it showed no inhibitory effect on HSV-1 DNA polymerase activity. Thus, FK 3000 may be a promising novel anti-HSV agent that possesses different mode of anti-HSV action from that of PAA.

Materials and Methods

Viruses and cells: HSV strains used were a wild type of 7401H HSV-1,[17] TK^- B2006 HSV-1,[18] ACV and PAA-resistant (AP^r) HSV-1,[19] and a wild type of HSV-2 (Ito-1262).[19, 20] A poliovirus type 1 Sabin strain, measles virus Tanabe strain and influenza virus A/PR/8/34 (H1N1) were also used.[17,21,22] These virus stocks were prepared from infected-Madin-Darby canine kidney (MDCK) or Vero cells as reported previously.[19,23] MDCK and Vero cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum and maintained in MEM supplemented with 2% calf serum after virus infection. For preparation of HSV-1 DNA polymerase, human embryonic lung (HEL) cells were grown and maintained in MEM supplemented with 10% and 2% fetal bovine serum, respectively.

Isolation of FK 3000 from roots tubers of S. cepharantha: Chopped dry root tubers of *S. cepharantha* (9.5 kg, Tochimoto Tenkaido Co., Osaka) were extracted with ethanol and the ethanol extract was dried *in vacuo* to give a residue (100 g). The residue was dissolved in water, adjusted to pH 1 with 1 N HCl and extracted with $CHCl_3$. The $CHCl_3$-soluble fraction was evaporated *in vacuo* to give a residue, which was again dissolved in water. The aqueous solution was basified to pH 11 with NH_4OH and extracted with ether. After evaporation of ether, the dried material (1.1 g) was dissolved in a mixed solvent of ethylacetate and hexane, and crystallized. The crystalline material was identified as FK 3000 by various spectroscopic means including proton- and carbon nuclear magnetic resonance, infrared spectrometry and mass spectrometry, and the purity was checked by thin-layer chromatography and high performance liquid chromatography (HPLC) (purity, 99.9%). FK 3000 was dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 20 mg/ml and used for its *in vitro* antiviral experiment. Also, a solution of 50 mg/ml FK 3000 in DMSO was prepared for HSV DNA polymerase assays.

Plaque reduction assay: Duplicate cultures of MDCK cells in 60-mm plastic dishes were infected with 100 plaque-forming units (PFU) of influenza virus for 1 h. The cells were overlaid with 5 ml of nutrient agar (0.8%) medium containing various concentrations of FK 3000 and then cultured at 37°C for 2-3 days.[17] The cells were fixed and stained, and the number of plaques was counted as described previously.[19] The effective concentrations for 50 % plaque reduction (EC_{50}) were determined from a curve of the plaque number to the concentration of FK 3000.

Similarly, duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 PFU of measles virus and poliovirus for 1 h. The cells were overlaid with 5 ml of nutrient methylcellulose (0.8%) medium containing various concentrations of FK 3000 and then cultured at 37°C for 2-5 days.[22] The cells were fixed and stained, and the number of plaques was counted as described above.

Cytotoxicity assay: Cytotoxicity of FK 3000 was examined by a growth inhibition assay of MDCK cells or Vero cells. The cells were seeded at a concentration of 5×10^4 cells/well in 24-well plates and grown at 37°C for 2 days. The culture medium was replaced by fresh medium containing FK 3000 at various concentrations, and the subconfluent cells were further incubated for 2 days. The cells in triplicate wells for each concentration of FK 3000 were treated with trypsin and the number of viable cells was measured by a trypan blue exclusion test. The 50% cytotoxic concentration (CC_{50}) was determined graphically.[19]

Yield reduction assay: Monolayers of Vero cells in 60 mm plastic dishes were infected with a wild HSV-1, AP^r HSV-1, TK^- HSV-1 or wild HSV-2 strain at 4-6 PFU/cell for 1 h. The cells were washed three times with MEM and incubated in maintenance medium containing various concentrations of FK 3000 at 37°C for 24 h. The cultures were frozen and thawed, and centrifuged at

3,000 x g for 15 min. Virus titers in the supernatant were determined by a plaque assay with Vero cells.[19]

Analysis of viral protein synthesis: The cells were mock-infected or infected with HSV-2 and incubated in the presence of various concentrations of FK 3000 or 120 μg/ml PAA at 37℃ for 7 h. HSV-infected and mock-infected cells were labeled with [^{35}S]methionine and [^{35}S]cysteine (43.5 TBq/mmol, NEN) for 1 h post-infection in the presence of FK 3000 or PAA, as described above. The labeled cells were lysed and viral proteins were precipitated with immunoglobulin for human use (Miles Inc., USA).[21] The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography.[20,23] The dried gels were exposed to X-ray films at -80℃.

Analysis of viral DNA synthesis: Monolayers of Vero cells in 60 mm plastic dishes were infected with HSV-2 (6 PFU/cell) for 1 h and incubated in maintenance medium containing various concentrations of FK 3000 or 120 μg/ml PAA at 37℃ for 7 h. The cells were lysed in the presence of 0.5% sodium dodecylsulfate (SDS) and 100 μg/ml of proteinase K, and then DNA was prepared from the lysates as described previously.[20,23] The DNA was blotted to nylon filters (Hybond-N, Amersham) using a slot blot apparatus (Bio-Rad) and fixed by ultraviolet (UV) irradiation. The filters were prehybridized at 65℃ for 4 h and then incubated with denatured radioactive probes at 65℃ overnight. The radioactive probes were synthesized from DNA fragments of an HSV-2 TK gene with a random primer DNA labeling kit (Takara Biochemicals, Japan).[20] The hybridized filters were washed, dried and exposed to X-ray films at -80℃.[23]

Analysis of viral RNA synthesis: Monolayers of Vero cells in 60 mm plastic dishes were infected with HSV-2 (6 PFU/cell) for 1 h and then incubated in maintenance medium containing various concentrations of FK 3000 or 120 μg/ml PAA at 37℃ for 7 h. The cells were collected with a scraper in cold PBS, from which total RNA was prepared by using an SV total RNA isolation system (Promega). The total RNA was blotted to nylon filters, and fixed by UV irradiation. The filters were prehybridized at 65℃ for 4 h and incubated at 65℃ overnight with denatured radioactive probes, which had been prepared from DNA fragments of a HSV-2 TK gene with a random primer DNA labeling kit.[20] The hybridized filters were treated as described above.[23]

Preparation of DNA polymerase and its enzyme activity in the presence of FK 3000: HSV-1 DNA polymerase was partially purified from wild HSV-1-infected HEL cells by phosphocellulose and DNA-cellulose column chromatography as described previously.[22] A reaction mixture (25 μl) containing 50 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 100 mM ammonium sulfate, 80 μM each of dATP, dGTP and dCTP, 0.214 to 3.42 μM of [^{3}H]dTTP (2.04-2.85 TBq/mmol, Moravek Biochemicals, Inc.), 50 μg/ml activated calf thymus DNA (Sigma), 500 μg/ml FK 3000 or 10.4 μg/ml PAA and crude enzyme, was incubated at 37℃ and the acid-insoluble radioactivity was measured 5, 10, 15 and 30 min after the incubation.[22] In this reaction, 1 % DMSO was used as a control.

Results

Isolation of an alkaloid FK 3000

A conventional method for the isolation of a morphinane alkaloid FK 3000 was developed by using a differential extraction technique with organic solvents at different pH followed by crystallization (Fig. 1). The

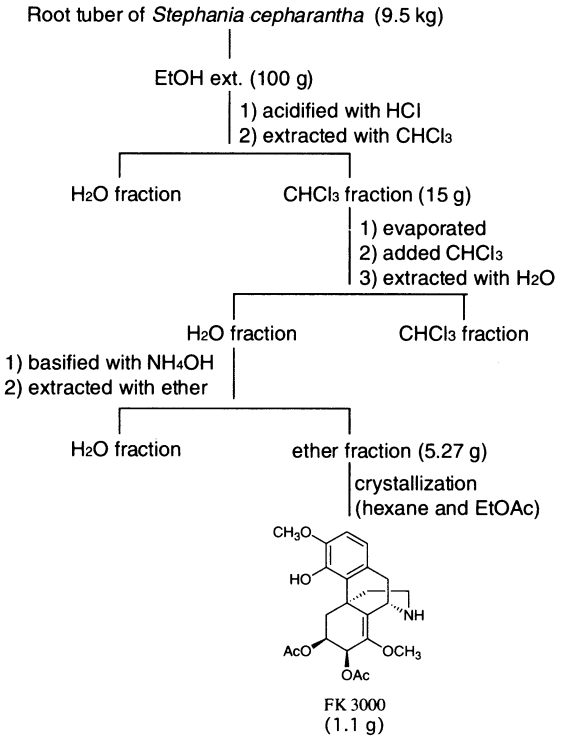

Fig. 1 Isolation of FK 3000 from the root tubers of *S. cepharantha*

crystalline material was obtained from the ethanol extract in a yield of 1.1 % without using column chromatography and identified as FK 3000 by comparison with the spectroscopic data with those of an authentic sample. The purity was determined to be 99.5% by HPLC.

Antiviral activity of FK 3000 against influenza virus, poliovirus and measles virus

Since FK 3000 has been shown to inhibit the proliferation of HSV-1,[15] we examined antiviral activity of this compound by a plaque reduction assay against other types of viruses such as influenza virus, poliovirus and measles virus, which have different replication cycles from that of HSV-1. The EC_{50} values of FK 3000 were 1.22, 13.5 and 13.9 μg/ml to influenza virus, poliovirus and measles virus, respectively (Table I). On the other hand, the CC_{50} values of FK 3000 in the MDCK and Vero cells were 130 and >300 μg/ml, respectively, 22 to 107-fold higher than the EC_{50} values, indicating that FK 3000 had appreciable antiviral activity against all the viruses examined.

Antiviral activity of FK 3000 against a variety of HSV strains

Anti-HSV activity of FK 3000 was examined against PAA- and/or ACV-resistant HSV strains, as well as wild HSV-1 and HSV-2 strains, by a yield reduction assay with Vero cells. The wild HSV-1, AP^r HSV-1 and TK^- HSV-1 strains were similarly susceptible to FK 3000 (Fig. 2) and their virus yields were reduced about 50-fold in the presence of 20 μg/ml FK 3000, as compared with its absence. The virus yield of the wild HSV-2 strain was reduced 1850-fold in the presence of 20 μg/ml FK 3000, as compared with its absence. This compound was confirmed to have potent antiviral activity against AP^r HSV-1, TK^- HSV-1, wild HSV-2, and HSV-1 strains.

Table I Antiviral activity of FK 3000 from *S. cepharantha*

Virus	EC_{50}[a] μg/ml (CC_{50} μg/ml)
Polio	13.5 ± 0.7 (>300)[b]
Measles	13.9 ± 1.8 (>300)[b]
Influenza	1.22 ± 0.1 (130)[c]

The antiviral activity of FK 3000 was determined by a plaque reduction assay. The EC_{50} values were graphically determined as described in the text. a), mean value ± S.D. of three independent experiments; b), A mean value determined by the two independent growth inhibition assays using Vero cells; c), A mean value determined by the two independent growth inhibition assay using MDCK cells.

Effect of FK 3000 on viral protein synthesis

PAA inhibits the synthesis of HSV DNA and late HSV proteins but permits the synthesis of early HSV proteins before viral DNA synthesis.[24,25] The synthesis of wild HSV-2 proteins was examined in the presence of FK 3000 and PAA to compare their effects on protein synthesis (Fig. 3). In the presence of 120 μg/ml PAA, early viral proteins were mainly synthesized, but the synthesis of late viral proteins was reduced. FK 3000 inhibited dose-dependently the synthesis of viral proteins, such as 51.2, 80.3, 132.7 and 163 kDa proteins, for wild HSV-2. Especially, the pattern of viral proteins synthesized at 10 μg/ml of FK 3000 was similar to that synthesized in the presence of PAA at 120 μg/ml, suggesting that FK 3000, as well as PAA, selectively inhibited the synthesis of late HSV proteins.

Effects of FK 3000 on viral DNA and RNA syntheses

FK 3000 was examined for its inhibitory activity in viral DNA synthesis of a wild HSV-2 strain in Vero cells by slot-blot hybridization. The compound inhibited the DNA synthesis in a dose-dependent manner (Fig. 4). In the presence of 20 μg/ml FK 3000, the viral DNA synthesis was strongly suppressed, and the levels of detected DNA were similar to those in the presence of 120 μg/ml PAA.

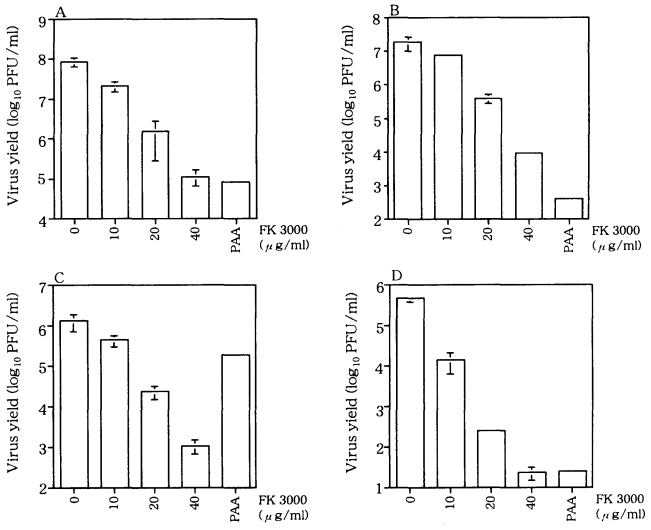

Fig. 2. Inhibition of the growth of four HSV strains by FK 3000
FK 3000 was examined for its antiviral activity against wild HSV-1 (A), TK^-HSV-1 (B), AP^r HSV-1 (C) and wild HSV-2 (D) strains in a yield reduction assay. Vero cells were infected with the HSV strains and incubated in the presence of various concentrations of FK 3000 or 120 μg/ml PAA as described in the text. Virus titers in the culture supernatants were determined by a plaque assay. The titers represent the average values with standard errors for triplicate samples.

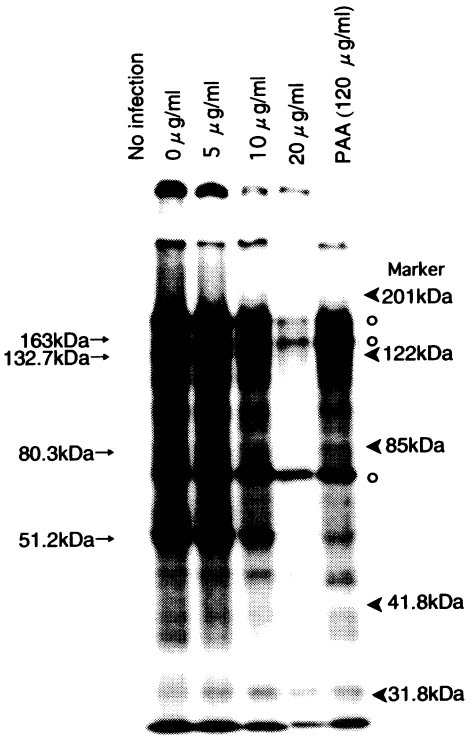

Fig. 3 Inhibition of protein synthesis of a wild HSV-2 strain by FK 3000

FK 3000 was examined for its effect on viral protein synthesis in Vero cells infected with HSV-2. The cells were mock-infected (no infection)- or infected with HSV-2 and incubated in the presence of 0, 5, 10 and 20 μg/ml of FK 3000 or 120 μg/ml of PAA at 37℃. The infected and mock-infected cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 7 h post infection as described in the text. Immunoprecipitates from HSV-2-infected cells were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. Marker proteins (31.8, 41.8, 85, 122 and 201 kDa) were co-electrophoresed in a gel. Arrows indicate late viral proteins (163, 132.7, 80.3, and 51.2 kDa) whose production was reduced in the presence of FK 3000, as well as PAA. Circles indicate early viral proteins whose production was not reduced in the presence of FK 3000, as well as PAA.

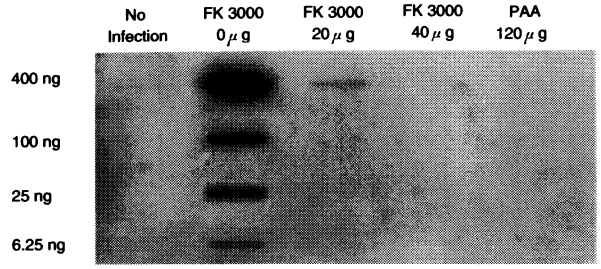

Fig. 4 Inhibition of DNA synthesis of HSV-2 by FK 3000

The cells were infected with HSV-2 and incubated in the presence of 0, 20 and 40 μg/ml of FK 3000 and 120 μg/ml of PAA for 7 h. The cells were lysed, from which DNA was prepared. The DNA was blotted to nylon filters at 6.25, 25, 100 and 400 ng/slot and fixed by UV irradiation. The DNA was also prepared from mock-infected cells (no infection). The filters were hybridized with denatured radioactive probes, as described in the text. The hybridized filters were washed and the dried filters were exposed to X-ray films at -80℃.

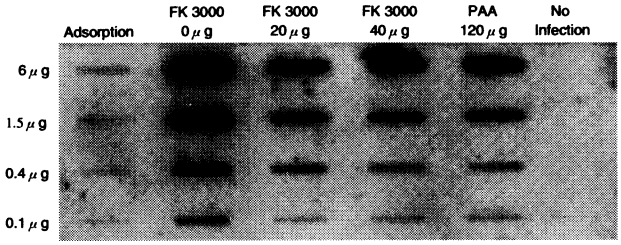

Fig. 5 Inhibition of RNA synthesis of HSV-2 by FK 3000

The cells were infected with HSV-2 and incubated with 0, 20 and 40 μg/ml of FK 3000 and 120 μg/ml of PAA for 7 h. RNA was prepared from the infected cells and blotted to nylon filters at 0.1, 0.4, 1.5 and 6 μg/slot. The RNA was also prepared from infected cells immediately after virus adsorption for 1 h (adsorption) and uninfected cells as a control (no infection). The filters were hybridized with denatured radioactive probes as described in the text. The hybridized filters were washed and the dried filters were exposed to X-ray films at -80℃.

Similarly, the effect of FK 3000 on viral RNA synthesis was investigated with a wild HSV-2 strain in Vero cells by slot-blot hybridization. The viral RNA was found to be synthesized in the presence of 20 and 40 μg/ml FK 3000, but their levels were similar to that in the presence of 120 μg/ml PAA, where HSV DNA synthesis was inhibited (Fig. 5), indicating that FK 3000 and PAA partially inhibited the RNA synthesis of wild HSV-2 in comparison to the absence of them.

Effect of FK 3000 on DNA polymerase activity

For the purpose of investigating the inhibitory mechanism of DNA synthesis mediated by FK 3000, HSV-1 DNA polymerase was partially purified from wild HSV-1-infected HEL cells and the effects of FK 3000 and PAA on the polymerase activity were compared (Fig. 6). Both DNA polymerase activities of HSV-1 and HSV-2 have been shown to be inhibited by PAA.[20] In this experiment, we used partially purified HSV-1 DNA polymerase instead of HSV-2 DNA polymerase, because we could not prepare HSV-2 DNA polymerase with high activity from infected HEL cells. PAA markedly inhibited the activity at a concentration of 50 μM (10.4 μg/ml), but FK 3000 did not inhibit the activity at 500 μg/ml.

Discussion

FK 3000 has been isolated and purified from an ethanol extract of *S. cepharantha* by time-consuming chromatographic separation.[15] However, in the present

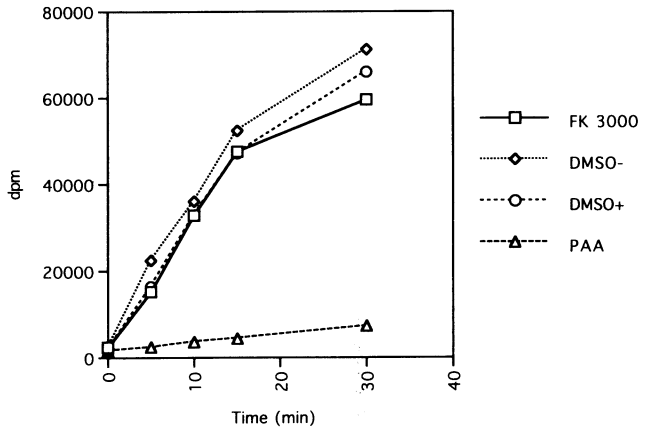

Fig. 6. Effect of FK 3000 on the activity of HSV DNA polymerase. Effects of FK 3000 (500 μg/ml, open square) and PAA (120 μg/ml, open triangle) on HSV DNA polymerase activity were examined in the reaction mixture as described in the text. The reaction mixture with 1 % DMSO (DMSO+) or without DMSO (DMSO-) was used as negative controls.[21]

experiment, we adopted more convenient procedures (differential extraction method) to purify FK 3000 without any chromatographic fractionations, and obtained pure FK 3000 with an amount comparable to that reported previously by a chromatographic method.

FK 3000 has anti-HSV activity *in vitro* and *in vivo*.[15,16] In the present study, the compound inhibited the growth of wild HSV-1, TK^- HSV-1, AP^r HSV-1 and wild HSV-2 strains in the yield reduction assay (Fig. 2). In this assay, we used FK 3000 at 10, 20 and 40 μg/ml. These concentrations of FK 3000 were higher than its EC_{50} values (9.2 μg/ml against HSV-1 and 8.7 μg/ml against HSV-2) as described previously.[15] However, they were much less than the CC_{50} values (700 μg/ml[15] and >300 μg/ml in Table I) to Vero cells, indicating that FK 3000 at 10, 20 and 40 μg/ml was not cytotoxic. Actually, viral RNA was synthesized in the presence of FK 3000 at 20 and 40 μg/ml, as well as PAA at 120 μg/ml, that is not cytotoxic (Fig. 5). Thus, the anti-HSV activity of FK 3000 at 10, 20 and 40 μg/ml were not due to its cytotoxicity.

FK 3000 exhibited antiviral activity against a TK^- HSV-1 strain, as well as wild HSV-1 and HSV-2 strains.[17] We confirmed the anti-HSV activity of FK 3000 against an ACV-resistant HSV strain, as shown in Fig. 2. Furthermore, the growth of AP^r-HSV-1 strain was inhibited by FK 3000 (Fig. 2). The AP^r HSV-1 and TK^- HSV-1 strains are resistant to ACV and/or PAA. This indicates that FK 3000 has a mode of anti-HSV action different from that of ACV and PAA. FK 3000 also exhibited antiviral activity against influenza virus, measles virus and poliovirus (Table I), indicating that this compound shows the broad spectra of antiviral activity. These viruses are RNA viruses and have different replication cycles from that of HSV. It would be interesting to clarify some factor that is inhibited by FK 3000 and relates to the different virus growth. FK-3000 inhibited HSV DNA synthesis but not HSV RNA synthesis (Figs. 4 and 5). The synthesis of late HSV proteins was inhibited in the presence of 20 μg/ml FK 3000, as well as 120 μg/ml PAA, where the growth of HSV and synthesis of HSV DNA were almost completely inhibited (Figs. 3 and 4). Since PAA specifically inhibits the synthesis of HSV DNA but permits the syntheses of early HSV proteins and RNA,[24,25] FK 3000 was suggested to have similar anti-HSV action as PAA. Therefore, a major site of its inhibitory action may be concluded to be viral DNA synthesis. However, FK 3000 did not appreciably inhibit HSV DNA polymerase activity in contrast with PAA (Fig. 6). Since Underwood *et al.* showed that cytomegalovirus DNA maturation was inhibited by a benzimidazole ribonucleoside,[24] we examined HSV DNA maturation in the presence of FK 3000 using a contour-clamped homogeneous electric field (CHEF) gel electrophoresis. However, FK 3000 did not interfere with the maturation of HSV DNA (data not shown). Since the process of HSV DNA synthesis involves many factors, such as helicase, primase, uracil-DNA glycosylase, ribonucleotide reductase *etc.*, which are coded in the HSV genome,[25,26] a further study is now in progress in our laboratories to identify the inhibitory mechanism of FK 3000 in the HSV DNA synthesis.

Acknowledgments

We wish to express our gratitude to Ms. T. Okuda and Mr. Y. Yoshida, Faculty of Medicine, Toyama Medical and Pharmaceutical University, for their excellent technical assistance.

和文抄録

Stephania cepharantha（タマザキツヅラフジ）から得たモルフィン骨格を有するアルカロイド FK 3000 は acyclovir や phosphonoacetic acid (PAA) 抵抗性を有す

る HSV-1, influenza virus, measles virus, polio virus に対しても抗ウイルス作用を有していた。この抗 HSV 作用を HSV DNA polymerase を阻害することにより HSV DNA 合成を阻害することが知られている PAA との比較から検討した。HSV に感染した Vero 細胞においてFK 3000は，PAA と同様に後期ウイルス蛋白の合成を阻害したが初期ウイルス蛋白には影響しなかった。Slot blot hybridization 法で HSV DNA 合成を調べると，FK 3000 は 濃度依存的にウイルス DNA 合成を阻害することが判明した。しかし，ウイルス RNA 合成は HSV DNA 合成が阻害される濃度でも FK 3000 および PAA によって部分的にのみ阻害された。このことは PAA と同様に FK 3000 はウイルス DNA 合成は阻害するが初期ウイルス RNA の合成は許容することを示している。FK 3000 は粗精製した HSV-DNA polymerase 活性を阻害しないことから，PAA と異なった機構で HSV DNA 合成を阻害していることが示唆された。

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