The Journal of Veterinary Medical Science

NOTE

Virology

Transcriptional inhibition of feline immunodeficiency virus by alpha-amanitin

Taishi TANABE[1)]*, Yurina FUKUDA[1)], Kazuhiko KAWASHIMA[2)], Satomi YAMAMOTO[1)], Takashige KASHIMOTO[3)] and Hisaaki SATO[1)]

[1)]Laboratory of Veterinary Microbiology, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan
[2)]Aino Animal Hospital, Takao 85-5, Fukuroi, Shizuoka 437-0023, Japan
[3)]Laboratory of Veterinary Public Health, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan

J. Vet. Med. Sci.
83(1): 158–161, 2021
doi: 10.1292/jvms.20-0182

Received: 29 March 2020
Accepted: 15 November 2020
Advanced Epub:
27 November 2020

ABSTRACT. Alpha-amanitin, one of the amatoxins in egg amanita, has a cyclic peptide structure, and was reported as having antiviral activity against several viruses. We investigated whether α-amanitin has antiviral activity against feline immunodeficiency virus (FIV). FL-4 cells persistently infected with FIV Petaluma were cultured with α-amanitin. Reverse transcriptase (RT) activity in the supernatant of FL-4 cells was significantly inhibited by α-amanitin. In addition, the production of FIV core protein in FL-4 cells was inhibited by α-amanitin when analyzed by western blotting. Furthermore, α-amanitin inhibited the transcription of FIV in real-time RT-PCR. These data suggested that α-amanitin showed anti-FIV activity by inhibiting the RNA transcription level.

KEY WORDS: amanitin, anti-retroviral drug, feline immunodeficiency virus (FIV), therapy

Feline immunodeficiency virus (FIV) is single-stranded RNA virus that belongs to the genus *lentivirus*, part of the family *Retroviridae* [17]. FIV infection in domestic cats causes clinical manifestations like those of human acquired immunodeficiency syndrome (AIDS). As a precaution, an FIV vaccine called Fel-O-Vax® FIV was released in the United States in 2002 and has since been released around the world [23]. Therapeutic drugs for FIV infection have been studied, but these drugs are not generally used therapeutically in clinics because of their toxicity and induction of resistant viruses [1, 12]. Thus, novel therapeutic drugs to control FIV infection in cats are needed.

Alpha-amanitin is produced from the poisonous mushroom *Amanita phalloides* [10]. It is known to inhibit RNA synthesis by inhibiting DNA-dependent RNA polymerase II [11]. It does not block catalysis directly. It binds to the largest subunits of RNA polymerase II and inhibits their mobility essential to the action of the RNA polymerase [5]. Thus, protein synthesis is inhibited as RNA synthesis is inhibited. At high concentrations, α-amanitin also binds to RNA polymerase III and inhibits RNA synthesis, whereas RNA polymerase III is not inhibited by low concentrations of α-amanitin [19, 25]. Alpha-amanitin was also studied as antiviral effects on several viruses, including adenovirus [13], influenza virus [16, 18], and Rous sarcoma virus (RSV) [8]. Alpha-amanitin activated transcription of the human immunodeficiency virus (HIV) long terminal repeat (LTR) [4], but in RSV of the same Retroviridae was repressed by it [3, 8]. Therefore, we evaluated the antiviral activity of α-amanitin on FIV.

Firstly, to the evaluate the influence of α-amanitin on the proliferation of FL-4 cells, which is an interleukin-2-independent feline lymphoid cell line chronically infected with FIV Petaluma (FIV_{pet}) [26]. Alpha-amanitin (Fujifilm Wako Chemicals Co., Osaka, Japan), was dissolved with ultra-pure water and stored at −30°C until use. The cells were inoculated into a 96-well plate at 2×10^4 cells/well, and two-fold serially diluted α-amanitin with maintenance medium was added to each well. After culturing for 3 days, cell proliferation was measured using CellTiter 96 AQueous One solution reagent (Promega, Madison, WI, USA), which is a colorimetric method using a tetrazolium compound and an electron coupling reagent PMS. Cell proliferation was calculated using the following formula: % reaction=[optical density (OD) of α-amanitin-treated cells/OD of α-amanitin-untreated cells] × 100. The difference in cell proliferation compared with untreated cells was 8.9% or less at ≤2.72 µM α-amanitin (Fig. 1). In addition, in similar experiment using FIV_{pet}-infected Crandell feline kidney (CRFK) cells, the survival rate was 98% or more at ≤0.34 µM α-amanitin (Supplementary Fig. 1).

To determine whether α-amanitin has antiviral activity against FIV, FL-4 cells were plated at 4×10^5 cells/well in 24-well plates in 450 µl of culture medium. Then, 50 µl of appropriately diluted α-amanitin was added to each well. After a 3-day culture

*Correspondence to: Tababe, T.: tanabe@vmas.kitasato-u.ac.jp
(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

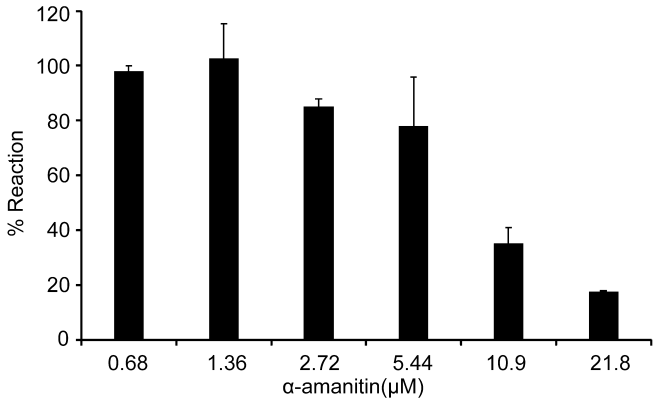

Fig. 1. Anti-proliferation effect of α-amanitin in FL-4 cells. The proliferation of FL-4 cells was measured, and the reaction rate was calculated as described in the main text. The % reaction rates are shown with the untreated group taken as 100%. All experiments were performed in triplicate. A representative result from three independent experiments is shown.

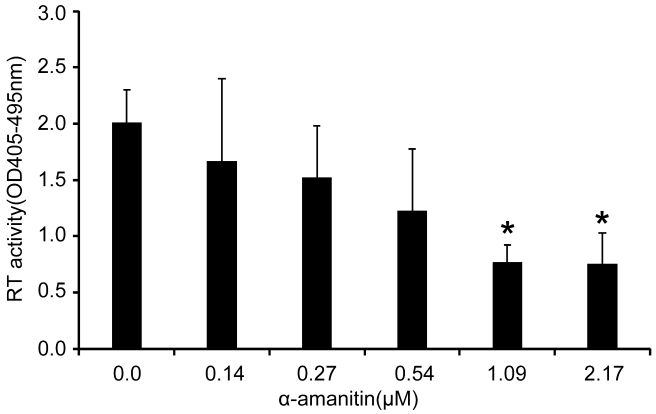

Fig. 2. Antiviral effect of α-amanitin on FL-4 cells. FL-4 cells were treated with α-amanitin, and the reverse transcriptase (RT) activity in the culture supernatant was measured. The results are shown as the mean ± SE. All experiments were performed in triplicate. A representative result from four independent experiments is shown. *$P<0.01$.

period at 37°C with 5% CO_2, the culture supernatant was collected after centrifugation. The supernatant was further centrifuged at 20,400 *g* for 90 min at 4°C. The pellet was recovered and reverse transcriptase (RT) activity was measured using a reverse transcriptase colorimetric assay (Roche Applied Science, Mannheim, Germany). The inhibition rate of α-amanitin was 62.4% for 2.17 μM, 61.5% for 1.09 μM, 38.9% for 0.54 μM, 24.0% for 0.27 μM, and 16.8% for 0.14 μM (Fig. 2). In addition, α-amanitin showed similar results for FIV_{pet}-infected CRFK (Supplementary Fig. 2). Thus, the RT activity of FIV in the culture supernatant, was dose-dependently decreased by α-amanitin. At these concentration of α-amanitin, no cell growth inhibition or cytotoxicity was observed, but antiviral activity was observed.

To clarify the mechanisms of the inhibition of antiviral activity, we performed western blotting analysis to evaluate the amounts of FIV core protein, Pr50 and p24, in FL-4 cells. FL-4 cells were plated at 2×10^6 cells/well in 6-well plates with or without 1.09 μM α-amanitin in 3 ml of culture media. After culturing for 3 days, the cells were collected and lysed with non-denaturing lysis buffer, i.e., 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, and 1% Protease inhibitor cocktail (Sigma-Aldrich) in 20 mM Tris-HCl (pH 8.0). The lysate was centrifuged at 15,000 *g* for 20 min at 4°C. The collected supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 10 μg and then analyzed by western blotting. An anti-FIV p24 antibody (PAK3-2C1) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), an anti-β actin monoclonal antibody was from Sigma-Aldrich, and peroxidase-conjugated anti-mouse IgG goat serum was from Seikagaku Co. (Tokyo, Japan). Detection was carried out using ECL Prime Western Blotting Detection Reagents (GE Healthcare Life Sciences, Buckinghamshire, UK). The band densities of FIV Gag were normalized to the band density of β-actin, and the α-amanitin-treated group and untreated group were compared. The expression of Pr50 in the FL-4 cells cultured with α-amanitin was reduced by 58% compared with the untreated group (Fig. 3A). Similarly, the expression of p24 in FL-4 cells treated with α-amanitin was reduced by 59% (Fig. 3B). From these results, the reduction in RT activity may be due to the decrease in viral protein production by α-amanitin.

Alpha-amanitin is well known as a DNA-dependent RNA polymerase II inhibitor. Therefore, we examined whether the antiviral effect of α-amanitin was due to the suppression of FIV gene expression. FIV mRNA in FL-4 cells treated with or without α-amanitin was extracted using a High Pure RNA Isolation kit (Roche Applied Science). Real-time RT-PCR was carried out using THUNDERBIRD Probe One-step qRT-PCR Kit (TOYOBO Co., Ltd., Osaka, Japan) according to the method of Bisset *et al.* [2]. The following primers were used as measure the expression level of FIV polymerase (FIV pol): 5′-CCATTCCTCTTGATCCAGATTAT-3′ and 5′-AAATCCAGCCTTGTGGTAGACTACA-3′ [2]. Forward and reverse primer for Feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene comprised of 5′-GCCGTGGAATTTGCCGT-3′ and 5′-GCCATCAATGACCCCTTCAT-3′ [14]. FIV_{pet}- or feline GAPDH-specific probes labeled at the 5′-end with the reporter-dye 6-carboxyfluorescein (FAM) and at the 3′-end with the quencher-dye 5 (6)-carboxytetramethylrhodamine (TAMRA) consisted of the sequences 5′-TACTTTACCTAGGAAGAATAATGCGGGACCAGGAA-3′ and 5′-CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA-3′ [14], respectively. The relative expression of mRNA was calculated by the $2^{-\Delta\Delta CT}$ method after normalizing the Ct values with GAPDH Ct. The relative value of FIV pol was 0.48 ± 0.07, and the expression level was reduced by 52% (Fig. 4). In FIV_{pet}-infected CRFK cells, 0.2 μM amanitin reduced its expression level by 42% (Supplementary Fig. 3). This result suggested that the antiviral effect of α-amanitin was due to the inhibition of FIV replication.

In this study, we showed that α-amanitin suppressed the production of FIV from FL-4 cells due to inhibiting viral transcription. Alpha-amanitin is a DNA-dependent RNA polymerase inhibitor [10]. The concentration of α-amanitin used herein was 1.09 μM. Alpha-amanitin usually inhibits DNA-dependent RNA polymerase II at 1–10 nM and RNA polymerase III at 10–100 μM. Thus,

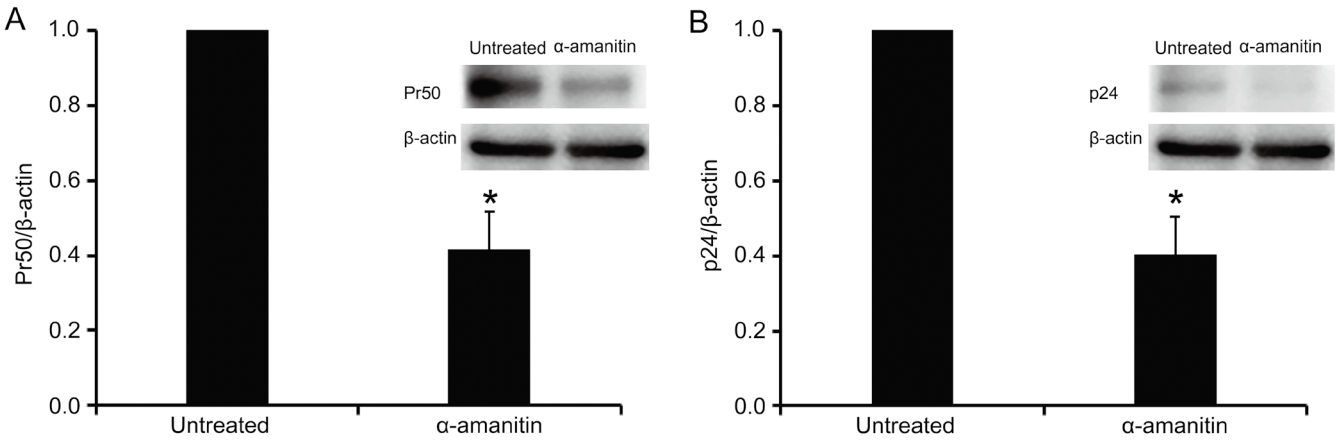

Fig. 3. Effect of α-amanitin on feline immunodeficiency virus (FIV) Gag production in FL-4 cells. FL-4 cells were treated with or without α-amanitin (1.09 μM) for 3 days. The levels of FIV Gag protein in the cells were determined by measuring the band density of the precursor Gag Pr50 (A) or p24 (B) by western blotting. The band density of FIV Gag was normalized to the band density of β-actin in α-amanitin-treated or untreated cells. All experiments were performed in triplicate. A representative result from three independent experiments is shown. **P*<0.05.

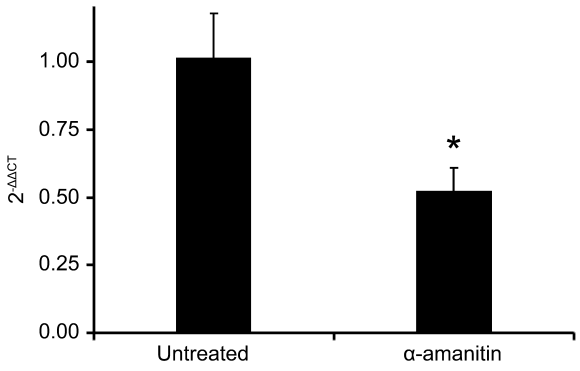

Fig. 4. The suppression of feline immunodeficiency virus (FIV) replication by α-amanitin. RNA was extracted from FL-4 cells, which were treated with or without α-amanitin (1.09 μM) and subjected to real-time RT-PCR amplification for FIV polymerase (FIV pol) with feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. All experiments were performed in triplicate. The results of three experiments are summarized. **P*<0.01.

our results suggested that this antiviral activity is for RNA polymerase II. However, α-amanitin activated the transcription of HIV-1 LTR [4], whereas Rous sarcoma was inhibited. HIV replication is controlled by Tat and NFκB, which significantly enhance the transcription elongation process, including viral RNA [6, 9]. Cassé *et al.* [4]. reported that their treatment of mammalian cells with actinomycin D and α-amanitin led to an enhancement of transcription directed by HIV LTR promoters. This enhancement was observed when a high drug concentration was used, i.e., 10 μM of α-amanitin. We were not able to use such a high concentration of α-amanitin in the present study because of its toxicity. In addition, FIV has orfA instead of Tat [7, 20, 24]. The differences in our present findings and the results reported by Cassé *et al.* [4] may be due to the concentration of α-amanitin used, the difference in cells used, and the structural difference in LTRs. Although we showed that α-amanitin has antiviral activity at 1.09 μM against FIV, Magdalan *et al*. [15] reported that α-amanitin at 1 μM induced apoptosis in primary cultured dog hepatocytes. It was also reported that α-amanitin shows toxic to the liver and kidney of dogs and cats [10, 21, 22]. The concentrations used in this study may cause hepatotoxicity in cats. Therefore, it is necessary to study its usable dose and toxicity more carefully before considering treatment in FIV-infected cats. Combinations of antiretroviral agents have been effective in HIV treatment in humans, and similarly in FIV-infected cats, we speculate that better antiviral activity could be achieved by using a drug combination rather than a single-drug treatment. To establish drug combination therapy for FIV infection, the effectiveness of various drugs needs to be further verified.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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