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FR198248, a New Anti-influenza Agent Isolated from *Aspergillus terreus* No. 13830

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

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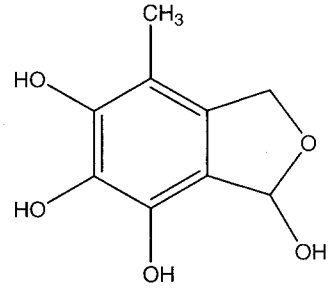
A novel anti-influenza agent, FR198248, was isolated from the cultured broth of a fungal strain No.13830. The strain was identified as *Aspergillus terreus* from morphological characteristics. FR198248, a new type of hydroxyl benzaldehyde compound, showed anti-influenza virus activity in Madin-Darby canine kidney (MDCK) cells *in vitro*. The mode of action of FR198248 against influenza virus A could be ascribed to an inhibitory effect on the stage of virus adsorption. Furthermore, FR198248 possessed potent *in vivo* anti-influenza activity in a murine model of respiratory tract infection.

Influenza viruses have two kinds of spike glycoproteins in their envelope membranes, hemagglutinin (HA) and sialidase (neuraminidase, EC 3.2.1.18), which recognizes sialic acid on host cell glycoconjugates. HA is essential for binding to the cellular sialyloligosaccharides receptors and then mediates the entry of virus into the host cell, while sialidase is a receptor destroying enzyme to cleave the sialic acid from the viral receptors[1~3]. Therefore, sialidase is one of the most important targets for anti-influenza therapy[4~7].

In the course of our screening for the isolation of anti-influenza agents from soil microorganisms, we developed a rapid HPLC method for detection of released sialic acid after sialidase reaction, and discovered FR198248 from the cultured broth of *Aspergillus terreus* No.13830. The structure of FR198248 was elucidated by several spectroscopic experiments as a novel type of hydroxyl benzaldehyde derivative (Fig. 1). FR198248 showed potent antiviral activity against influenza A and B viruses *in vitro*. Furthermore, it possessed potent anti-influenza activity *in vivo*.

In this paper, the taxonomy, fermentation, isolation, physico-chemical properties and biological activities of FR198248 is described. Structure elucidation and structure-

Fig. 1. Structure of FR198248.



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activity relationship studies of FR198248 and its derivatives will be described later.

Materials and Methods

Taxonomy

The fungal strain No. 13830 was originally isolated from a soil sample collected in Mexico. The cultural observations were made after two-weeks of incubation at 25°C. The compositions of Czapek's solution agar and malt extract agar were based on the JCM Catalogue of Strains[8]. The color names used in this study were taken from the Methuen Handbook of Colour[9]. The temperature range of growth was determined on potato dextrose agar. The morphological characteristics were determined principally from the culture on Czapek's solution agar.

HPLC Analysis of FR198248

Detection of FR198248 from the fermentation broth and the fractions under purification were monitored by HPLC using a reverse phase column YMC-ODS-AM(AM 303, 250×4.6 mm i.d., YMC Co., Ltd.). The solvent system was a mixture of 10% aqueous methanol and 10% aqueous acetonitrile. The flow rate was 1.0 ml/minute. The detection wave length was set at 210 nm.

Compounds and Reagents

DMB (1,2-diamino-4,5-methylenedioxy-benzene) and fetuin were purchased from Takara Shuzo Co., Ltd. Ribavirin which purchased it from Viratek, a subsidiary of ICN Pharmaceuticals Inc., through the United Kingdom distributor, Britannia Pharmaceuticals Ltd., Redhill, Surrey. 4-Guanidino-Neu5Ac2en (zanamivir, GG167) was synthesized from Neu5Ac2en in the Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Tsukuba, Japan.

Sialidase Assay Using Fluorometric HPLC system

In the course of our screening program for anti-influenza viral agents, we developed a new assay system of influenza virus sialidase activity using HPLC by measuring fluorometric determination of Neu5Ac release after sialidase reaction. 50 μl of the sample solution and 25 μl of diluted virus sialidase solution which were put into each well of 96-well microtiter plate. Then 25 μl of 100 μM fetuin was added to each well as a substrate, and the reaction was initiated at 37°C for 18 hours. Then 100 μl of DMB reagent containing 16.8% acetic acid, 10.5% 2-mercaptethanol and 0.63% $Na_2S_2O_4$ was added to each well and heated at 50°C for 2 hours in the dark to develop the fluorescence of sialic acid labeled with DMB. 10 μl of the resulting solution was monitored by HPLC system using YMC ODS-AM column (100×4.6 mm i.d.), CH_3CN-MeOH-water (9:7:84) as the mobile phase and fluorometric detector (excitation at 370 nm, emission at 450 nm). The percent inhibition relative to positive (no test compound) controls were calculated.

In Vitro Antiviral Activities

MDCK cells, influenza A/PR/8/34 and influenza B/Yamagata/16/88 viruses for plaque assay were supplied from Dr. NEROME, the National Institute of Infectious Diseases, Tokyo, Japan. Plaque assay was performed by a modification of the method described by HAYDEN *et al*[10]. Confluent monolayers of MDCK cells (1.1×10^6 cells/well) were inoculated with the influenza virus diluted in Eagle's modification of minimal essential medium (pH 7.2~7.4) containing 1 μg of TPCK-treated trypsin to give approximately 100 plaques per well (6-well plate) in the absence or presence of varying concentrations of the test compound. Cells were left for 1 hour at 37°C so the virus could adsorb and overlaid with defined cell growth medium containing 1% Noble agar (Sigma Chemical Company), 1 μg of TPCK-treated trypsin per ml, 0.001% DEAE dextran with or without compound. After cells were incubated for 2 days at 37°C (humidified 5% CO_2), plaques were visualized by staining viable cells with neutral red. The antiviral activities of FR198248 on herpes simplex virus type 1 (HSV-1) (miyama strain) and vesicular stomatitis virus (VSV) were also measured by the plaque reduction assay described above. Confluent monolayers of Vero cells in 6-well plates were infected with approximately 100 PFU of HSV-1 and VSV, and then visualized plaques were counted.

In all cases, the IC_{50} was calculated as the concentration required to reduce virus induced plaque formation by 50%.

In Vivo Efficacy

FR198248 was evaluated in a murine model of respiratory tract infection. Mouse-adapted influenza A/PR/8/34 virus was used in this study. Briefly, Balb/c mice (female, four weeks old) were anesthetized by the intravenously administration at 1.0 mg/kg with pentobarbiturate and were inoculated intranasally with 20 μl of virus suspension (1.7×10^2 PFU/mouse). And then mice were anesthetized by the inhalation of ether and were dosed intranasally once daily with test compounds for 3 days after infection. The efficacies of anti-influenza agents were assessed at the 50% effective dose (ED_{50}) calculated on the survival rate at 10 days after infection.

Cytotoxicity Test

The cytotoxic activity of FR198248 against MDCK cells was compared with that of zanamivir and ribavirin. The concentration required to reduce cell viability by 50% (CC_{50}) was measured. The cytotoxicity was colorimetrically determined at 550 nm (and 660 nm as a reference) according to the MTT method[11,12] after 5 days of incubation at 37°C in the presence of the test compounds.

The Mode of Action Experiment

To determine the stage of the influenza virus replication cycle with which FR198248 interferes, the inhibitory effect of FR198248 on virus adsorption was measured by a virus plaque reduction assay for influenza A/PR/8/34 virus in MDCK cells upon different drug treatment periods.

Virus adsorption assays we tested were as follows: Confluent monolayers of MDCK cells in 6-well plates were infected with approximately 100 PFU of influenza A virus under different conditions. In treatment A, the cells were exposed to the influenza virus in the presence of FR198248 or ribavirin and after 1 hour virus adsorption, both compounds and unadsorbed virus were removed, the cells were washed and were further incubated. In treatment B, the cells were exposed to the virus and after the virus adsorption period, the unadsorbed virus was removed and the cells were further incubated with a medium containing 45 μM of FR198248 or 43 μM of ribavirin. In treatment C, FR198248 or ribavirin was present both during and after the adsorption period. After cells were incubated for 2 days, virus induced plaques were visualized by staining viable cells with neutral red and were counted.

Hemagglutination Assay

To determine the affinity of FR198248 to the hemagglutinin (HA), hemagglutination assay based on the inhibition of influenza virus adsorption to erythrocytes was used[13]. 25 μl of influenza A/PR/8/34 virus suspension (2^6 HA titer) were mixed with an equal volume of compound and maintained for 1 hour at room temperature, and then 50 μl of the mixture diluted were incubated with an equal volume of 1% chicken erythrocyte (CRBC) suspension for 1 hour at room temperature for hemagglutination.

Results

Taxonomic Studies

Culture on Czapek's solution agar spread broadly, attaining a diameter of 6.0~6.5 cm in 14 days at 25°C. This colony surface was plane to somewhat sulcate, felty to powdery, and grayish orange. The reverse color was brownish orange, and pale orange soluble pigments were produced. Conidial structures, consisting of conidiophores with biseriate aspergilla and brown conidial heads, were abundantly formed. The conidiophores were erect from vegetative mycelia, macronematous, long, smooth and hyaline. They consisted of foot cells, stipes, vesicles, metulae and phialides. Stipes were straight, aseptate, unbranched, 100~220 μm long and 6~8 μm wide. Vesicles were capitate to spathulate, 12~16.5 μm in diameter, and formed metulae over the upper half of them. The metulae were cylindrical, tightly packed, 5~6.5×1.5~3 μm, with a whole of 2~4 phialides. The phialides were discrete, acrogenous, ampulliform, 5.5~7.5×1.5~2.5 μm, and producing conidia in long chains. Conidial chains aggregated to conidial heads in compact columns. The conidial heads were cylindrical, brown, 65~100 μm long and 40~50 μm in diameter. Conidia were hyaline to pale brown, smooth, one-celled, globose to subglobose, and 2~3 μm in diameter. Vegetative hyphae were hyaline, smooth, septate and branched. The hyphal cells were cylindrical, 1~6 μm in width. Meiosporic structures, sclerotia and chlamydospores were not observed. Colony on malt extract agar grew more rapidly on Czapek extract agar, attaining 7.0~7.5 cm in diameter under the same conditions. The surface was flat, thin, powdery, and grayish orange. The reverse side was the same color. Conidial structures were abundantly observed.

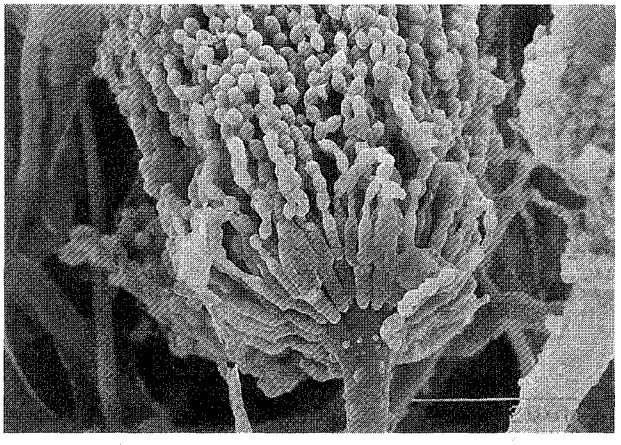
This strain was able to grow at the temperature range from 13 to 42°C, with the growth optimum at 32 to 36°C.

On the basis of the morphological characteristics, strain No. 13830 was considered to belong to the hyphomycete genus *Aspergillus* Link. According to the taxonomic criteria of the genus *Aspergillus* by RAPER and FENNEL[14], this strain resembles *Aspergillus terreus* Thom. Moreover, above characteristics corresponded with the species description of *A. terreus* by RAPER and FENNEL[14] and DOMSCH *et al.*[15] Thus, we identified this isolate as one strain of *A. terreus*, and named it *Aspergillus terreus* No. 13830 (Fig. 2). The strain has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-5371.

Fermentation

An aqueous seed medium (60 ml) adjusted to pH 6.3~6.4 which is containing sucrose 3%, Pharmamedia (TM: cotton seed flour, Traders Protein) 1%, dried yeast 3%, $CaCO_3$ 0.2%, Adecanol LG-109 (deforming agent, Asahi

Fig. 2. Scanning micrograph of conidial structures of strain No. 13830 on the Czapek's solution agar plate, 25°C, 14 days.



Bar represents 100 μm.

Fig. 3. Isolation procedure of FR198248.

Fermentation broth (1200ml)
- extracted with acetone (1200ml)
- filtered
- concentrated *in vacuo*

Aqueous solution (800ml)
- Sepabeads SP207 column chromatography (250ml)
  - washed with water and 25% aq. MeOH
  - eluted with 50% aq. MeOH (500ml)
- concentrated *in vacuo*

Aqueous solution (250ml)
- Diaion HP20SS column chromatography (100ml)
  - washed with water and 25% aq. MeOH
  - eluted with 50% aq. MeOH (400ml)
- concentrated *in vacuo*

Aqueous solution (100ml)
- extracted with *n*-butanol (400ml)
- concentrated *in vacuo* to dryness

Crude material
- dissolved in 10% aq. MeOH
- YMC gel (ODS-AM 120-S50) column chromatography (180ml)
  - washed with water
  - eluted with 5% aq. CH_3CN containing 0.05% TFA
- concentrated *in vacuo*

Aqueous solution (20ml)
- YMC gel (ODS-AM 120-S50) column chromatography (20ml)
  - washed with water
  - eluted with 50% aq. CH_3CN

Active fraction (100ml)
- concentrated *in vacuo*

FR198248 (cream-colored powder, 47mg)

Denka Co., Ltd.) 0.05% and Silicone KM-70 (deforming agent, Shin-Etsu Chemical Co., Ltd.) 0.05% was poured into a 225-ml Erlenmeyer flask and sterilized at 120°C for 30 minutes. A loopful of fungus strain No.13830 was inoculated from a slant culture into the flask. The flask was cultured on a rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 5 days (S_1). The resultant seed culture (S_1) was inoculated into the flask with sterile seed medium (60 ml). The flask was cultured on a rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 2 days (S_2). The resultant seed culture (S_2) was separately inoculated to 60 ml of sterile production medium consisting of Modified starch 3%, Pine-Dex (TM; starch acid hydrolysates, Mitsuya Chemical Co., Ltd.) 6%, Pharmamedia 1%, Chicken meat bone meal 1%, dried yeast 2%, $(NH_4)_2SO_4$ 0.05%, $Na_2HPO_4 \cdot 12H_2O$ 0.5%, β-Cyclodextrin 1% in a 225-ml Erlenmeyer flask. The flasks were cultured on a rotary shaker at 25°C for 5 days.

Isolation and Purification

The isolation procedure of FR198248 is outlined in Fig. 3. The cultured broth (1200 ml) was extracted with an equal volume of acetone. The acetone extract was filtered with the aid of diatomaceous earth and concentrated *in vacuo* to 800 ml. This extract was passed through a column (250 ml) of Sepabeads SP207 (Mitsubishi Chemical Ind. Co., Ltd.). The column was washed with water and 25% aqueous methanol, and then eluted with 50% aqueous methanol. The eluate (500 ml) was concentrated *in vacuo* to 250 ml. The solution was subjected to a column (100 ml) of Diaion HP20SS (Mitsubishi Chemical Ind. Co., Ltd.). The column was washed with water and 25% aqueous methanol, and then eluted with 50% aqueous methanol. The eluate (400 ml) was concentrated *in vacuo* to 100 ml and the solution was extracted with *n*-butanol (400 ml). The upper layer was concentrated *in vacuo* to dryness. The resultant pale yellowish product was dissolved in 10% aqueous methanol (100 ml) and subjected to a column (180 ml) of YMC gel (ODS-AM 120-S50, YMC Co., Ltd.). The column was washed with water and eluted with 5% aqueous acetonitrile containing 0.05% trifluoroacetate. The eluate was concentrated *in vacuo* to 20 ml, and the solution was applied to a column (20 ml) of YMC gel. After washing the column with water, the active fraction was eluted with 50%

Table 1. Physico-chemical properties of FR198248.

Appearance		Cream-colored powder
Nature		Acidic
Molecular formula		$C_9H_{10}O_5$
EI-MS (*m/z*)		180[M^+-H_2O]
Anal	Calcd.	: C 54.55, H 5.09
	Found	: C 54.22, H 5.07
UV λ_{max} in MeOH		235, 270, 310 nm
IR ν_{max} cm^{-1}		3440, 3230, 1640, 1510, 1490, 1430, 1400, 1300, 1110, 1010, 940, 920
Solubility : soluble		MeOH, DMSO
insoluble		$CHCl_3$, H_2O
TLC, RP-18WF_{254} (E.Merck) (10% aq. CH_3CN-10% aq. MeOH containing 0.05% TFA= 10 : 1)		Rf 0.42
mp.		112~117℃ (dec.)

aqueous acetonitrile. The eluate was concentrated *in vacuo* to give FR198248 substance as cream-colored powder (47 mg).

Physico-chemical Properties of FR198248

Physico-chemical properties of FR198248 are summarized in Table 1. FR198248 was readily soluble in methanol and dimethyl sulfoxide but insoluble in chloroform and water. The color reaction of FR198248 was negative to ninhydrin reagent and was positive to iodine vapor, ceric sulfate and $FeCl_3$. It showed UV absorption at 235, 270 and 310 nm. It showed IR absorptions at 3440, 3230 and 1620 cm^{-1} due to phenolic hydroxyl and carbonyl groups, respectively. The EI-MS spectrum showed a molecular ion peak at *m/z* 180 ($M-H_2O$). The structure elucidation studies of FR198248 and its derivatives will be described elsewhere.

Sialidase Assay Using Fluorometric HPLC Method

Before starting this screening system, we tested the inhibitory activity of zanamivir as positive control. Zanamivir showed inhibitory activity (IC_{50} : 0.051 μM), and it was correlated nearly with the IC_{50} value (0.093 μM) we formerly evaluated by usual method[16]. On the other hand, the activity of FR198248 was weak (151 μM). It is speculated that the reason why FR198248 could be found is that high productivity of the fungus strain screened.

Table 2. *In vitro* anti-influenza activity of FR198248, zanamivir and ribavirin by plaque reduction assay.

Compound	IC_{50} (μM)
FR198248	11.0
Zanamivir	0.10
Ribavirin	8.50

In Vitro Anti-influenza Activity

The *in vitro* anti-influenza virus activities of FR198248 were compared with that of ribavirin using plaque reduction assay. In this study, the antiviral effect of inhibition has been quantified by reduction in plaque numbers.

The results of inhibition of plaque formation in MDCK cells against influenza A/PR/8/34 virus are shown in Table 2. The IC_{50} value of FR198248 was 11.0 μM. FR198248 showed almost equal activity of ribavirin (IC_{50} of 8.50 μM).

Spectra of Antiviral Activities of FR198248

Also investigated was the antiviral activities of

Table 3. Spectra of antiviral activity of FR198248 by plaque reduction assay.

Virus	IC_{50} (μM)
Influenza A/PR/8/34 virus	11.0
Influenza B/Yamagata/16/88 virus	16.1
Herpes simplex virus type1 (HSV-1) Miyama	>100
Vesicular stomatitis virus(VSV)	>100

Table 4. *In vivo* anti-influenza activity of FR198248 and ribavirin in a murine model.

Compound	ED_{50} (mg/kg) [a]
FR198248	1.98
Ribavirin	2.30

Tested compound was intranasally administered to Balb/c mice (n=7) infected with influenza A /PR/8/34 virus.

[a] The ED_{50} was assessed at 10 days after infection.

Table 5. Cytotoxicity against MDCK cells *in vitro*.

Compound	CC_{50} (μM)	SI[a]
FR198248	> 500	>45.5
Zanamivir	>300	>3000
Ribavirin	64.0	7.52

[a] SI (Selectivity index) was calculated by the formulation of CC_{50}/(IC_{50} in plaque reduction assay)

FR198248 against influenza B/Yamagata/16/88 virus, HSV-1 and VSV. FR198248 selectively showed antiviral activities against influenza A and B viruses (IC_{50}: 11.0 μM and 16.1 μM, respectively). On the other hand, no activity was observed against HSV-1 and VSV at the dose of 100 μM (Table 3).

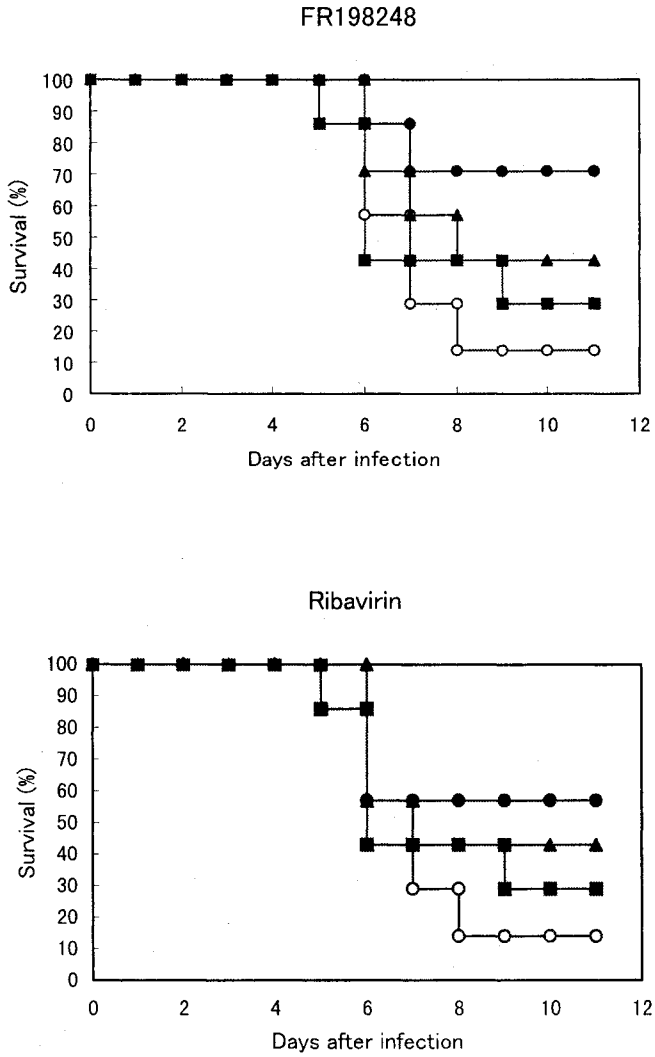
In Vivo Activity

The protective efficacy of FR198248 and ribavirin was examined in a murine model of respiratory tract infection with influenza A/PR/8/34 virus (Fig. 4). These compounds prolonged the survival of infected mice. The ED_{50} of FR198248 and ribavirin at day 10 after infection was 1.98 and 2.30 mg/kg, respectively (Table 4). Thus, FR198248 showed potent protective effect in a murine model of influenza viral infection.

Fig. 4. Protective effect of FR198248 and ribavirin in intranasal infection of influenza A/PR/8/34 virus in mice.

The test compound was administered intranasally once daily for 3 days.

Symbols: ●, 32 mg/kg; ▲, 3.2 mg/kg; ■, 0.32 mg/kg; ○, Vehicle.



Cytotoxic Activity

As shown in Table 5, the cytotoxic activity of FR198248 against MDCK cells was weaker than ribavirin. The selective index value (CC_{50}/IC_{50} in plaque assay) of FR198248 was 6-fold and over higher than ribavirin.

Table 6. Influence of various treatment periods on the anti-influenza activity of FR198248 and ribavirin.

	FR198248 or Ribavirin present				Plaque inhibition (%)	
	During virus adsorption		After virus adsorption			
Treatment	FR198248	Ribavirin	FR198248	Ribavirin	FR198248	Ribavirin
No	−	−	−	−	0	0
A	+	+	−	−	95	2
B	−	−	+	+	5	92
C	+	+	+	+	91	89

MDCK cells were infected with 100 PFU of influenza virus. After 1hr virus adsorption with (+) or without (-) test compounds (FR198248 ; 45 μM, Ribavirin ; 43 μM), unabsorbed virus and the compound were removed. The cells were overlaid with medium with or without test compounds, and further incubated for 2 days, whereafter the number of plaques was counted.

The Mode of Action

FR198248 was found to exert its anti-influenza activity by interference with an early stage of the influenza virus replication cycle, since replication was blocked only when the compound was present during the virus adsorption period.

The compound was expose to the cell either: (A) during the virus adsorption period only; (B) after the virus adsorption only; (C) both during and after the virus adsorption. As shown in Table 6, FR198248 lost completely all antiviral activity when not present during the virus adsorption period. The presence of only FR198248 during virus adsorption was as effective as the presence of the compound during the whole incubation period (pre and post adsorption). On the other hand, the target of action of ribavirin was the step after virus entry into the cell as it has been known to inhibit viral RNA polymerase.

Hemagglutination Assay

The mode of action experiment showed that the stage of the influenza virus replication cycle with which FR198248 interferes was the step of virus adsorption (Table 6). Furthermore, it was investigated whether FR198248 directly inhibit hemagglutination of influenza A virus. However, FR198248 did not inhibit it at the dose of 125 μM.

Discussion

The structure of FR198248 was determined as a novel hydroxyl benzaldehyde. FR198248, the metabolite produced by the strain of *Aspergillus terreus* No.13830, selectively inhibited the multiplication of influenza A and B viruses in MDCK cells. However, FR198248 did not inhibit viral proliferation of HSV-1 and VSV. Furthermore, FR198248 showed a potent protective efficacy in a murine model of respiratory tract infection with influenza virus.

There are some reports concerning hydroxyl benzaldehyde derivatives from fungus origin such as fomecin A, B and flavipin[17,18]. To the best of our knowledge, this is the first report concerning hydroxyl benzaldehyde which are known to have anti-influenza activity.

In this present study, the mode of action of FR198248 by virus adsorption inhibition assay were investigated. Full inhibitory activity was achieved only when the compound was present during the virus adsorption period. The mechanism of action of FR198248 could thus be ascribed to inhibition of virus adsorption. To determine the affinity of FR198248 to the virus hemagglutinin (HA), hemagglutination inhibition assay was tested. However, FR198248 did not directly inhibit hemagglutination at the dose of 125 μM.

An interesting issue that remained to be elucidated is why FR198248 has potent anti-influenza activity *in vitro* and *in vivo* in spite of its weak activity for viral sialidase

inhibition. It is speculated that an answer to the anti-influenza activity of FR198248 is that it had a low affinity for active site of viral sialidase but it might inhibit the interaction between the host cell receptor and viral sialidase protein by masking sialylsugar chains of the host cell receptor. However, there needs further investigation to determine the precise mechanism of action for FR198248.

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References

1) PALESE, P. & R. W. COMPANS: Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA). J. Gen. Virol. 33: 159~163, 1976
2) SUZUKI, Y.; M. KISO & A. HASEGAWA: New ganglioside analogs that inhibit influenza virus sialidase. Glycoconjugate J. 7: 349~356, 1990
3) MURTI, K. G. & R. G. WEBSTER: Distribution of hemagglutinin and neuraminidase on influenza virions as revealed by immunoelectron microscopy. Virology 149: 36~43, 1986
4) VON ITZSTEIN, M.; W.-Y. WU, G. B. KOK, M. S. PEGG, J. C. DYASON, B. JIN, T. VAN PHAN, M. L. SMYTHE, H. F. WHITE, S. W. OLIVER, P. M. COLMAN, J. N. VARGHESE, D. M. RYAN, J. M. WOODS, R. C. BETHELL, V. J. HOTHAM, J. M. CAMERON & C. R. PENN: Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature 363: 418~423, 1993
5) JACQUELINE, M. W.; R. C. BETHELL, J. A. V. COATES, N. HEALY, S. A. HISCOX, B. A. PEARSON, D. M. RYAN, J. TICEHURST, J. TILLING & C. R. PENN: 4-Guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid is a highly effective inhibitor both of the sialidase and of growth of a wide range of influenza A and B viruses *in vitro*. Antimicrob. Agents Chemother. 37: 1473~1479, 1993
6) MCCAULEY, J. W.; L. A. PULLEN, M. FORSYTH, C. R. PENN & G. P. THOMAS: 4-Guanidino-Neu5Ac2en fails to protect chickens from infection with highly pathogenic avian influenza virus. Antiviral Research 27: 179~186, 1995
7) RYAN, D. M.; J. TICEHURST & M. H. DEMPSEY: GG167 (4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid) is a potent inhibitor of influenza virus in ferrets. Antimicrob. Agents Chemother. 39: 2583~2584, 1995
8) NAKASE, T.: JCM Catalogue of Strains, 6th edition. Japan Collection of Microorganisms, the Institute of Physical and Chemical Research (RIKEN), Toppan, 1995
9) KORNERUP, A. & J. H. WANSCHER: Methuen Handbook of Colour, 3rd edition. Methuen, London, 1978
10) HAYDEN, F. G.; K. M. COTE & R. G. DOUGLAS, Jr.: Plaque inhibition assay for drug susceptibility testing of influenza viruses. Antimicrob. Agents Chemother. 17: 865~870, 1980
11) Mosmann, T.: Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assay. J. Immunol. Methods 65: 55~63, 1983
12) PAUWELS, R.; J. BALZARINI, M. BABA, R. SNOECK, D. SCHOLS, P. HERDEWIJN & E. DE CLERCQ: The colorimetric assay method for cell cytotoxicity. J. Virol. Methods 20: 309, 1988
13) KELM, S.; J. C. PAULSON, U. ROSE, R. BROSSMER, W. SHMID, B. P. BANDGAR, E. SHREINER, M.HARTMANN & E. ZBIRAL: Use of sialic acid analogues to define functional groups involved in binding to the influenza virus hemagglutinin. Eur. J. Biochem. 205: 147~153, 1992
14) RAPER, K. B. & D. I. FENNEL: The Genus *Aspergillus*, Williams and Wilkins, Baltimore, 1965
15) DOMSCH, K. H.; W. GAMS & T.-H. ANDERSON: Compendium of Soil Fungi. Vol. 1. Academic Press, London, 1980
16) POTIER, M.; L. MAMELI, M. BELISLE, L. DALLAIRE & S. B. MELANCON: Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-*N*-acetylneuraminate) substrate. Analytical Biochemistry 94: 287~296, 1979
17) MCMORRIS, T. C. & M. ANCHEL: Fomecin A and B, phenolic aldehydes from the *basidiomycete fomes juniperinus*. Canadian J. Chem. 42: 1595~1598, 1964
18) HAYASHI, K.; K. TOKURA, K. OKABE, K. YAMAMOTO & K. TAWARA: Synthesis and antimicrobial activities of fomecins A and B, asperugin and related compounds. Chem. Pharm. Bull., 30: 2860~2869, 1982