J. Pharm. Pharmacol. 2000, 52: 1247–1255
Received October 18, 1999
Accepted March 31, 2000

© 2000 J. Pharm. Pharmacol.

Development of Anti-influenza Drugs: II. Improvement of Oral and Intranasal Absorption and the Anti-influenza Activity of Stachyflin Derivatives

J. YOSHIMOTO, S. YAGI, J. ONO, K. SUGITA, N. HATTORI, T. FUJIOKA, T. FUJIWARA, H. SUGIMOTO AND N. HASHIMOTO*

Shionogi Research Laboratories, Shionogi & Co. Ltd, Sagisu, Fukushima-ku, Osaka 553-0002, Japan
**Pfizer Pharmaceutical, Taketoyo-cho, Chita-gun, Aichi 470-2393*

Abstract

The in-vivo anti-influenza-virus activity of Stachyflin derivatives (III and its phosphate ester, III-Phos), a new class of haemagglutinin fusion inhibitor, and the improvement of their absorption after oral or intranasal administration were studied in mice, rats, and ferrets.

The absorption of III in PEG 4000 and III-Phos aqueous solution increased about three and four fold in AUC after oral administration to uninfected mice compared with that of 0·5% HPMC (hydroxypropyl-methylcellulose) suspension. Using a mouse influenza virus infection model, significant anti-influenza-virus activity was observed in infected mice treated orally with these compounds dissolved in PEG 4000 or distilled water, respectively, but not in mice treated with 0·5% HPMC. The in-vivo anti-influenza-virus activity in ferrets, a good model for influenza virus infection in man, was also studied. Although the concentration of III in plasma was above the IC50 against the influenza virus strain used for 6 h after the oral administration of III in PEG 400 to uninfected ferrets, no in-vivo anti-influenza-virus activity was observed at the same dosage given 4 times daily for 3 days. The intranasal administration of III-Phos, which was expected to have a more notable in-vivo anti-influenza-virus activity, was examined. III-Phos, whose intranasal absorption had been improved by the modification of III with phosphate ester in rats, inhibited viral replication in the nasal cavity and suppressed influenza-virus-induced fever when administered intranasally to infected ferrets.

This study demonstrates that intranasally administered compounds with anti-influenza-virus activity must permeate the nasal membranes to produce their anti-influenza-virus effect.

Influenza virus is a member of the *Ortbomyxoviridae* family of enveloped RNA viruses, and human influenza A and B viruses cause infectious diseases of the respiratory tract throughout the world. Although vaccines have been used to control influenza virus infection in man, their usage is considerably limited because annual re-vaccination is required due to frequent changes in the viral surface glycoproteins, haemagglutinin and neuraminidase. The recent outbreak of bird H5N1 influenza virus in Hong Kong is being addressed with the utmost urgency (Subbarano et al 1998). This has led to focus on the development of antiviral drugs for treatment and prophylaxis.

Stachyflin, which is extracted and refined from *Stachybotrys* sp. RF-7260, is a compound effective against human influenza A viruses in-vitro (Taishi et al 1998). Recently, the mechanism of the antiviral action of Stachyflin had been elucidated as inhibition of the fusion process between the viral envelope and the host cell membrane, which is an early step in the entry of virus into host cells (Yoshimoto et al 1999). This is quite different from the anti-influenza-virus action of amantadine and

Correspondence: S. Yagi, Shionogi Research Laboratories, Shionogi & Co. Ltd, Sagisu, Fukushima-ku, Osaka 553-0002, Japan.
E-mail: shigenori.yagi@shionogi.co.jp

rimantadine, which inhibit replication by interfering with the ion-channel activity of the influenza Matrix 2 (M2) protein (Pinto et al 1992; Schroeder et al 1994), and that of ribavirin, which inhibits viral RNA synthesis by inhibiting the activity of the multiple enzymes involved in viral RNA synthesis (Gilbert & Knight 1986; Andersen et al 1993).

Recently, we have succeeded in improving in-vivo anti-influenza-virus activity of Stachyflin and its derivatives by the selection of vehicles used or chemical modification (Yagi et al 1999). Unfortunately, these compounds could not be used as therapeutic agents for influenza virus infection because of their in-vitro mutagenicity and lack of activity against fresh human influenza virus clinical isolate (unpublished results).

In this paper, we have described the relationship of oral or intranasal absorption and the in-vivo anti-influenza-virus activity of novel Stachyflin derivatives lacking mutagenicity and having activity against fresh human influenza virus clinical isolate.

Materials and Methods

Materials

Stachyflin ((6a*R*,7*S*,9a*S*,11*S*,13a*S*)-2,3,6,6a,7,8,9,9a,10,11,12,13-dodecahydro-5,11-hydroxy-6a,7,10,10-tetramethyl-3-oxo-1*H*-benzo [i] benzopyrano [2,3-e] isoindole) and its derivatives (III and the phosphate ester of III, III-Phos) were synthesized at Shionogi Research Laboratories (Figure 1). Hydroxylpropylmethylcellulose (HPMC) was purchased from Shin-etsu Chemical Co. Ltd (Tokyo, Japan). Polyethylene glycol (PEG) 400 and PEG 4000 were purchased from Wako Pure Chemical Industries Co. Ltd (Osaka, Japan) and Nacalai Tesque Co. Ltd (Kyoto, Japan), respectively. All other reagents used were of the highest grade available commercially.

Solubility

The compound (5 mg) was weighed in a 10-mL centrifuge tube and 2 mL solvent was added. The suspension was vortexed for 5 min and sonicated for 30 min at room temperature. After centrifugation at 3000 rev min^{-1} for 10 min at 25°C, the supernatant was diluted with methanol and the content of the compound was measured by HPLC. The diluted sample was injected onto a column (4·6 × 150 mm) packed with Nucleosil $5C_{18}$ (Chemico Scientific Co. Ltd, Osaka, Japan). The eluent was a mixture of 0·1% trifluoroacetic acid (TFA) solution and acetonitrile (60 : 40) for Stachyflin and III and 0·3% TFA solution and acetonitrile (72 : 28) for III-Phos. The sample was analysed by UV detection at 220 nm with a photodiode array UV-VIS detector (SPD-M6A, Shimadzu, Kyoto, Japan).

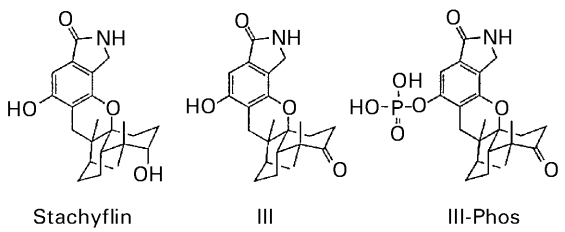

Figure 1. Chemical structures of Stachyflin, its derivative (III) and phosphate ester of III (III-Phos).

Animals

Experiments were conducted using male BALB/c mice (Japan Clea Inc., Osaka, Japan), 22–25 g, male Sprague-Dawley rats (Japan Clea Inc., Osaka, Japan), approximately 300 g and male sable ferrets (Marshall Research Animals, Inc., North, NY), 850–950 g. These mice were allowed to acclimatize for at least a week prior to being enterd into a study. They were maintained under a 12-h light–dark cycle and were fed a standard diet.

In-vitro and in-vivo anti-influenza assay

In-vitro anti-influenza-virus activity of III and III-Phos was evaluated by its inihibition of virus-induced cytopathic effect, described previously (Sladowski et al 1993). For in-vivo anti-influenza-virus assays, unfasted mice were infected with human influenza virus A/Kumamoto/5/67 (H2N2) by nebulization for 30 min (5×10^4 TCID50/10 mL). Infected mice were orally administered III or III-Phos dissolved in PEG 4000 heated at 65°C and distilled water, respectively, twice a day (09 00 h, 16 00 h) for 2 days under ether anaesthesia. As controls, infected mice were anaesthetized with ether twice a day for 2 days. Forty-eight hours after infection, infected mice were killed and virus titres in lung homogenates were assayed by the median tissue-cultured infective dose of virus (TCID50) method (Judd et al 1997; Shigeta et al 1997). Unfasted ferrets were intranasally infected with influenza virus A/Sendai/808/91 (H1N1) fresh clinical isolate (5×10^5 TCID50/0·2 mL/ferret). III in PEG 400 was orally administered 4 times daily to infected ferrets at 6-h intervals (80 mg/20 mL/day/ferret) for 4 days. III-Phos in PBS was intranasally administered 4 times daily to infected ferrets (42·4 mg/0·8 mL/day/ferret) for 4 days. Compounds were first administered 1 h after infection. Influenza virus infection and administration of

compounds were performed under ether anaesthesia. Rectal temperatures of uninfected and infected ferrets were monitored 3–4 times a day for 4–5 days using a thermo senser (Wako Pure Chemical Industries Co. Ltd, Osaka, Japan). Nasal cavities of infected ferrets were washed with 5 mL of saline under ether anaesthesia once a day with 24-h intervals after infection. Virus titres in nasal washings were determined by the TCID50 method, on MDCK cells, as described above.

Estimation of absorption by oral and intranasal administration

Uninfected mice were orally administered with III in PEG 4000 preheated at 65°C or 0·5% HPMC and III-Phos in distilled water using a gastric tube (0·5 mg/0·1 mL/mouse). PEG 4000 solution was confirmed to have solidified in the stomach within 15 min of oral administration. Blood samples were collected from hearts with a heparin-coated syringe under ether anaesthesia.

The oral administration of III to uninfected ferrets was studied with PEG 400 instead of PEG 4000, because PEG 4000 solution became solid during administration. Uninfected ferrets were orally administered with III in PEG 400 using a uretic catheter (20 mg/4 mL/ferret) under ether anaesthesia. Blood samples were collected from conscious ferrets via the jugular vein with a heparin-coated syringe cannulated with a polyethylene tube (0·58 mm i.d., 0·96 mm o.d.).

After sealing the passage of the nasopalatine tract of rats with an adhesive agent (Toa Gousei Co., Tokyo, Japan), preventing drainage of the solution from the nasal cavity to the mouth, III in 0·5% HPMC or III-Phos in distilled water was quickly instilled from the right nostril into the nasal cavity with a micropipette (2 mg/50 μL 0·5% HPMC in PBS/rat). Blood samples were collected via the jugular vein cannulated with a polyethylene tube (0·58 mm i.d., 0·96 mm o.d.). Plasma was separated by centrifugation at 3000 rev min^{-1} for 10 min at 4°C and stored at −20°C. Plasma samples (0·1–0·15 mL) were deproteinized with 1·0 mL methanol (for III) or acetonitrile (for III-Phos). Following the immediate mixing, samples were centrifuged at 10 000 × *g* for 5 min at 4°C. A sample of the clear supernatant (0·95–1·0 mL) was evaporated and redissolved with methanol : H_2O (1 : 1). Samples were quantified for III and III-Phos by HPLC as described above.

The residual amounts in the nasal cavity of III-Phos and III were quantified as follows. Thirty minutes after intranasal administration of III-Phos, rats were killed by incision of the abdominal main artery under ether anaesthesia. A polyethylene tube (PE260) was inserted through the oesophagus towards the posterior part of the nasal cavity and ligated. The nasopalatine tract was sealed with an adhesive to prevent drainage of the solution from nasal cavity to mouth. Twenty mL of saline was infused into the nasal cavity from the inserted tube, and the nasal washings exuded from nostril were diluted and deproteinized with methanol. The residual amounts of III and III-Phos in the nasal cavity were quantified by HPLC.

Rat intestinal absorption—in-situ loop method

Rat intestinal absorption of III and III-Phos was evaluated by the in-situ loop method, essentially as has been described previously (Yagi et al 1999). Rats fasted for 20 h were anaesthetized with pentobarbital sodium (60 mg kg^{-1} i.p.) and placed on a warming blanket maintained at about 37°C during the experiment. The small intestine was exposed via a midline incision and a section of the proximal jejunum, about 8 cm long and drained by a single mesenteric vein, was ligated and cannulated at one end with a 23-gauge × 3·2 cm rounded needle (Terumo Co., Tokyo, Japan) attached to a 1-mL plastic syringe containing a suspension of III or a solution of III-Phos. The distal end of the loop was secured with silk suture. Heparin sodium diluted with saline (0·5 mL, 10% v/v) was administered via the tail vein before the experiment to prevent coagulation in the mesenteric vein. After 0·8 mL of sample (1·5 mg mL^{-1}) had been injected directly into the lumen of the ligated intestinal loop, all mesenteric venous blood from the loop was collected continuously from a mesenteric venous cannula (22-gauge needle attached to a silicone tube (0·51 mm i.d., 0·94 mm o.d.)) at 10-min intervals for 60 min. Rat blood was collected for transfusion from the abdominal aorta of three other rats in a 50-mL plastic syringe with 1 mL of 5% heparinized saline. This blood, warmed at 37°C, was infused via the tail venous cannula at the same rate as blood draining from the mesenteric venous cannula (40 mL h^{-1}) using an infusion pump (Model STC-523, Terumo Co., Tokyo, Japan). The intestinal loop was covered with wrapping film to keep it moist. The volume of each blood sample was determined gravimetrically, based on a specific gravity of 1·0. The plasma concentration at each interval and the residual amount in the intestinal loop of the compound after 60 min were determined by HPLC.

Table 1. Solubility of III, III-Phos and Stachyflin.

Solvent	Solubility ($mg\,mL^{-1}$)		
	III	III-Phos	Stachyflin
Distilled water	0·0028	> 100	< 0·001
JP-1[a]	0·0015	N.C.	N.C.
JP-2[a]	0·0016	> 53	N.C.
10% Tween 80	1·29	N.C.	0·54
Ethanol	9·65	N.C.	N.C.
PEG 400	10·6	N.C.	> 3·19

[a]JP-1 is a hydrochloric acid buffer, pH 1·2, and JP-2 is a phosphate buffer, pH 6·8. N.C., not conducted.

Results

Oral absorption of III and III-Phos

The solubility of III and III-Phos in various vehicles is shown in Table 1. For III, the highest solubility was obtained with PEG 400 ($10·6\,mg\,mL^{-1}$), which was comparable to Stachyflin ($> 3·19\,mg\,mL^{-1}$). III-Phos, however, was highly soluble in distilled water ($> 100\,mg\,mL^{-1}$) and JP-2 ($> 53\,mg\,mL^{-1}$).

We have previously reported that the oral absorption of Stachyflin and its phosphate ester was improved when they were administered to mice in PEG or aqueous solution, respectively, instead of 0·5% HPMC suspension (Yagi et al 1999). To confirm the improved oral absorption of III in PEG 4000 and III-Phos in distilled water, the effect of vehicles on oral absorption of III and III-Phos was examined in uninfected mice (Figure 2, Table 2). III in PEG 4000 was administered after preheating the solution at 65°C. As shown in Table 2, the absorption of III in PEG 4000 was higher than that in 0·5% HPMC estimated by the maximum plasma concentration (C_{max}) and the area under plasma concentration-time curve to 120 min ($AUC_{(0-2\,h)}$). The absorption of III-Phos in distilled water was slightly lower than that of III in PEG 4000 but much higher than that in 0·5% HPMC.

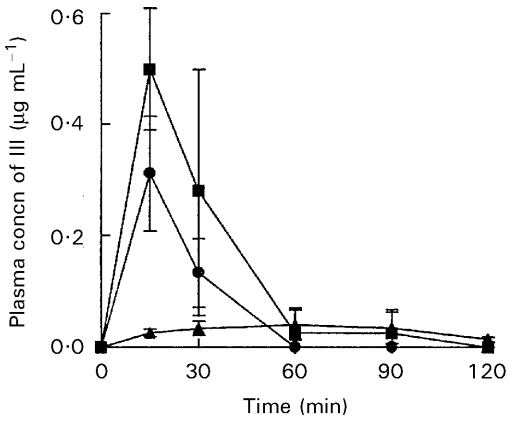

Figure 2. Plasma concentration-time profiles after oral administration of III and III-Phos to mice. The suspension of III in 0·5% HPMC (▲) or solution of III in PEG 4000 (0·5 mg/0·1 mL/mouse) (■), and the aqueous solution of III-Phos (0·625 mg/0·1 mL/mouse) (●) were orally administered with a gastric tube. Each point represents the mean ± s.d., n = 3. $AUC_{(0-2h)}$ values were calculated according to the trapezoidal rule.

Table 2. Pharmacokinetic parameters[a] following oral administration of III and III-Phos in mice.

Compound and vehicle	Dose (mg/mouse)	C_{max} ($\mu g\,mL^{-1}$)	$AUC_{(0-2\,h)}$[b] ($\mu g\,min\,mL^{-1}$)
III in 0·5% HPMC	0·5	0·06 ± 0·01	3·6 ± 0·7
III in PEG 4000	0·5	0·50 ± 0·11	16 ± 3·3
III-Phos in distilled water	0·625[c]	0·31 ± 0·10	9·7 ± 2·4

[a]Values are means ± s.d. for 3 mice. [b]Calculated according to the trapezoidal rule. Equivalent to 0·5 mg of III.

Table 3. Intestinal absorption of III and III-Phos by an in-situ loop method using the rat small intestine.

	III[a]	III-Phos[a] (as III)
Intestinal loop[b]	29·5 ± 5·30	25·9 ± 17·6
Intestinal wall[b]	45·5 ± 14·5	39·2 ± 15·9
Mesenteric venous blood[c]	0·51 ± 0·02	16·9 ± 2·00
Total	75·5 ± 13·1	82·0 ± 6·10

[a]Percentage of dose. [b]The residual amount after 60 min. [c]The cumulative amount for 60 min.

To understand the mechanism of the effect of the phosphate ester on the oral absorption of III, the absorption characteristics of III were investigated by an in-situ loop method with rat small intestine (Table 3). The cumulative amounts (percentage of dose as III) of III and III-Phos were 0·51 ± 0·02% and 16·9 ± 2·0%, respectively. III-Phos itself was not detected in the mesenteric venous blood and the intestinal wall. The amount of III in the intestinal wall determined as a percentage of dose for III and III-Phos were 45·5 ± 14·5% and 39·2 ± 15·9%, respectively. In the intestinal loop, the amounts of III and III-Phos were 30% and < 1%, respectively. Furthermore, the amount of III in the mesenteric venous blood increased every 10 min (data not shown). These results suggested that III-Phos was rapidly converted to III on the surface of the intestinal membrane and was absorbed as III.

In-vivo anti-influenza-virus activity in infected mice

The antiviral activity of III and III-Phos was evaluated in a mouse influenza virus infection model

Table 4. In-vivo anti-influenza activity after oral administration of III and III-Phos to mice.

Compound and vehicle	Dose (mg/twice daily/mouse)	Virus inhibition in lung (%)
III in PEG 4000	4	85 ± 12[b]
III-Phos in distilled water	4[a]	86 ± 14[b]
	1[a]	59 ± 20

Values are means for 6–8 mice. The in-vivo influenza activity was expressed as the ratio of virus titre in lung homogenate of experimental and control groups. [a]As dose of III. [b]Not significant.

using PEG 4000 and distilled water solutions which showed high oral absorption. Mice infected with influenza virus A/Kumatoto/5/67 (H2N2) virus were orally administered with III in PEG 4000 or III-Phos in distilled water, at a dose of 4 mg/mouse twice daily. The in-vivo anti-influenza-virus activity of compounds was expressed as the ratio (%) of virus titre in the lung homogenate of the experimental to the control groups, whose mice were only anaesthetized with diethyl ether at the same intervals. III and III-Phos significantly inhibited virus replication in lungs of infected mice (Table 4). The finding that lower dosage of III-Phos (1 mg/mouse twice daily) did not affect virus replication in mice lungs suggested that these Stachyflin derivatives inhibited influenza virus replication in a dose-dependent manner.

In-vivo anti-influenza-virus activity using infected ferrets—oral administration

We also studied the in-vivo anti-influenza-virus activity in ferrets, a good model for influenza virus infection in man (Capbell et al 1982; Smith & Sweet 1988). Ferrets are highly susceptible to infection with unadapted influenza virus strains; infection produces a clinical response similar to that observed in influenza-virus-infected humans, with symptoms including a febrile reaction, nasal obstruction and an increase in nasal-wash protein. Using ferrets, we tried to estimate the inhibition of viral replication in the nasal cavity and the suppression of influenza-virus-induced fever after oral administration of the test compounds.

For oral administration in ferrets, III, whose oral absorption in uninfected mice was better than that of III-Phos (Figure 2, Table 2), was estimated. Initially, the pharmacokinetics of III in plasma was examined to determine the dose intervals for the in-vivo anti-influenza experiment. III was administered with PEG 400 instead of PEG 4000, because PEG 4000 solution became solid during administration. Figure 3 shows the plasma concentration–time profile after oral administration of III (20 mg/4 mL/ferret). The half-life calculated from the area under concentration–time curve (AUC) was about 2 h. It was possible to sustain plasma concentrations above the IC50 value (0·08–0·16 μg mL^{-1} for A/Sendai/808/91 (H1N1)) for at least 6 h after oral administration of PEG 400 solution of III to uninfected ferrets, thus providing effective in-vivo anti-influenza-virus activity. Therefore, the in-vivo anti-influenza activity was examined by continuous oral administration to infected ferrets at a dosage of 80 mg/20 mL PEG 400/ferret 4 times daily.

Figure 4 shows the area under the curve to 72 h calculated from the virus titre of the nasal washing-

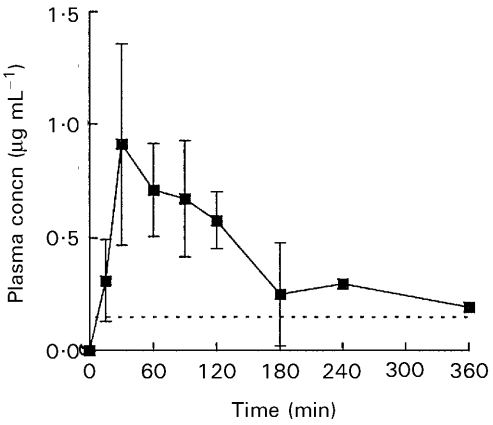

Figure 3. Plasma concentration–time profile after oral administration of III as PEG 400 solution to uninfected ferrets (20 mg/4 mL PEG 400/ferret). The dotted line indicates a concentration in plasma of 0·16 μg mL^{-1}, the mean 50% inhibitory concentration (IC50) of III against type A/Sendai/808/91 (H1N1) influenza virus in-vitro. Each point represents the mean ± s.d., n = 3.

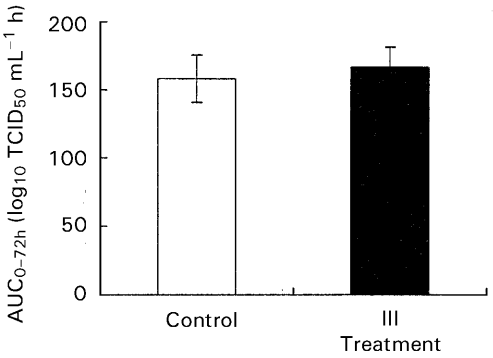

Figure 4. Inhibition of viral replication in nasal cavity induced by influenza A/Sendai/808/91 (H1N1) virus in ferrets following oral administration of III for 3 days (80 mg/20 mL/4 times daily/ferret). The area under the curve ($AUC_{0-72\,h}$) was estimated from the virus titre in nasal washing-time profiles for control and experimental groups. The statistical significance of differences between experimental and control groups were tested by Student's *t*-test.

Table 5. Pharmacokinetic parameters following intravenous and intranasal administration[a] of III and III-Phos in rats.

Compound and vehicle	Route	Dose ($mg\,kg^{-1}$)	AUC[b] ($\mu g\,min\,mL^{-1}$)	Bioavailability (%)
III in PEG 400	i.v.	4·43	44·6 ± 9·44	100
III in 0·5% HPMC	i.n.	6·03	N.D[c]	0
III-Phos in saline	i.v.	10·9	90·6 ± 19·9	100
III-Phos in 0·5% HPMC				
(as III-Phos)	i.n.	6·87	46·8 ± 9·14	82·0
(as III)		5·20	43·9 ± 9·50	83·9

Values are means ± s.d. for 3 rats. [a]Estimated from in-vivo nasal absorption method using conscious rats. [b]Calculated from zero to infinity according to the trapezoidal rule. [c]Not detected in plasma.

time profile ($AUC_{0-72\,h}$) for the control and experimental groups. No inhibitory effect on viral replication in the nasal cavity was observed after oral administration of III, because there was no significant difference in $AUC_{0-72\,h}$ between the control and experimental groups. There was also no significant suppression of fever in the experimental group.

Improvement of intranasal absorption

To study the possibility of successful intranasal administration of Stachyflin derivatives in influenza virus infection, we examined the pharmacokinetic parameters after intravenous and intranasal administration of III and III-Phos in rats (Table 5).

When a 0·5% HPMC PBS suspension of III was intranasally administered to uninfected rats, the absolute bioavailability was 0%. When the aqueous solution of III-Phos was intranasally administered to rats, however, the absolute bioavailabilities determined as III and III-Phos were approximately 84% and 82%, respectively. These results demonstrated that intranasal absorption was improved by the modification of III with phosphate ester.

In-vivo anti-influenza-virus activity using infected ferrets—intranasal administration

In-vivo anti-influenza activity was evaluated in infected ferrets by intranasal administration of III-Phos, which had shown good nasal absorption in rats (Table 5). A/Sendai/808/91 (H1N1) was used to infect ferrets by intranasal administration of the same amount used for oral administration of III (5×10^5 TCID50/0·2 mL/ferret). PBS solution of III-Phos was administered to infected ferrets by intranasal administration of 42·4 mg/0·8 mL PBS/day/ferret, 4 times daily for 4 days at 6-h intervals. For the control group, infected ferrets were anaesthetized with diethyl ether 4 times daily for 4 days. As controls, infected ferrets were anaesthetized with diethyl ether 4 times daily for 4 days at 6-h intervals. Nasal washings of infected ferrets were taken once a day to determine virus titres in the nasal cavity. The rectal temperatures of infected ferrets were recorded three times a day and compared with those of uninfected ferrets.

Figure 5 shows the area under the curve to 96 h calculated from the virus titre of the nasal washing-time profile ($AUC_{0-96\,h}$) for the control and experimental groups. The inhibitory effect on viral replication in the nasal cavity was observed following intranasal administration of III-Phos. There was a significant ($P < 0·05$) difference in $AUC_{0-96\,h}$ between the control and experimental groups. Influenza A virus-infected ferrets displayed a peak temperature of 39·8°C at 72 h after infection in the control group, and the rectal temperature after infection was higher than before infection at 48–96 h (Figure 6A). In contrast, rectal temperatures in infected ferrets treated with intranasal administration of III-Phos were not elevated relative to those before infection (Figure 6B). The index of fever suppression with III-Phos intranasal administration was evaluated by subtracting the area under the curve to 96 h calculated from rectal temperature-time profiles for the control and experimental groups before infection, AUC_{before}, from those after infection, AUC_{after}. Figure 6C shows $AUC_{after} - AUC_{before}$ for the control and experimental groups. The calculated value was $-9·06 \pm 7·64$ for the experimental group and $8·89 \pm 2·25$ for the control group. The difference between $AUC_{after} - AUC_{before}$ was significant ($P < 0·05$). The results described above clearly demonstrated that III-Phos in PBS solution inhibited influenza virus infection in ferrets not only by acting on influenza virus replication in the nasal cavity but also by suppressing influenza-virus-induced fever.

Discussion

In influenza virus infection, one of the virus surface glycoproteins, haemagglutinin, mediates both the

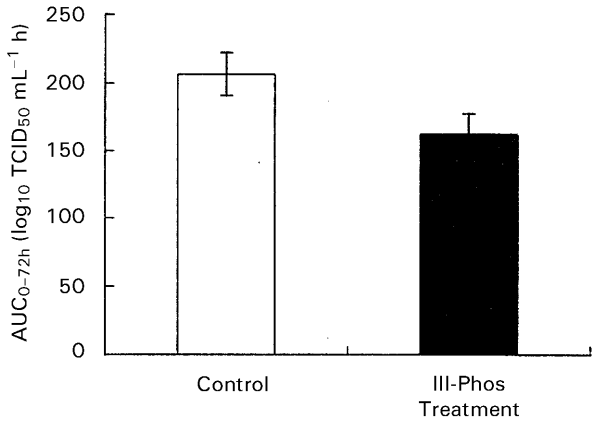

Figure 5. Inhibition of viral replication in nasal cavity induced by influenza A/Sendai/808/91 (H1N1) virus in ferrets following intranasal administration of III-Phos for 4 days (42·4 mg/0·8 mL/4 times daily/ferret). AUC_{0-96h} was estimated from the virus titre of nasal washing-time profiles for control and experimental groups. The statistical significance of differences between experimental and control groups were tested by the Student's *t*-test.

adsorption of virus to host cells and the virus-cell membrane fusion process which is indispensable for the penetration of influenza virus into the host cells. Recently, we have reported a novel anti-influenza-virus compound derived from fungus, which has an inhibitory action on the virus-cell membrane fusion process (Yoshimoto et al 1999). Unfortunately, Stachyflin and its derivative (II) were not appropriate for therapeutic use in influenza-virus infection because of their in-vitro mutagenicity (unpublished results).

In this paper, we have described the absorption and in-vivo anti-influenza-virus activity of novel Stachyflin derivatives (Figure 1), lacking in mutagenicity. In our previous reports, we have shown that an oral administration of Stachyflin in PEG solution improved oral absorption and in-vivo anti-influenza-virus activity in mice (Yagi et al 1999). Using novel Stachyflin derivatives (III and III-Phos), we have studied the oral absorption and anti-influenza-virus activity of III and III-Phos for the development of an anti-influenza drug.

Improved effect, with PEG as vehicle and chemical modification of III, on the oral absorption and anti-influenza-virus activity in mice was confirmed (Tables 2 and 4 , Figure 2). The mechanism of the increased oral absorption of III, by PEG 4000, in mice was not well understood. It could be assumed that the PEG 4000 might exert a co-solubilizing effect which enhances the dissolution rate and maintains a higher drug concentration in the gastrointestinal lumen (Yagi et al 1999). As for the enhancement of oral absorption by chemical modification with phosphate ester, it was suggested that conversion from III-Phos to III on the surface of the

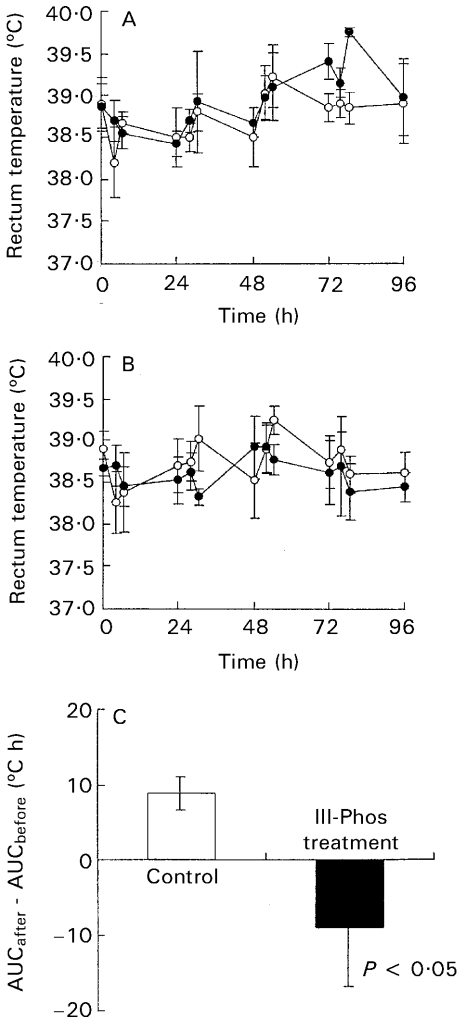

Figure 6. Fever suppression induced by influenza A/Sendai/808/91 (H1N1) virus in ferrets following intranasal administration of III-Phos for 4 days (42·4 mg/0·8 mL/4 times daily/ferret). The index (C) was evaluated by subtracting the area under the curve to 96 h calculated from rectal temperature-time profiles for the control (A) and experimental (B) groups before infection, AUC_{before} (○), from after infection, AUC_{after} (●). Each point represents the mean ± s.d., n = 4. The statistical significance of differences in AUC_{0-96h} between experimental and control groups were tested by the Student's *t*-test.

intestinal membrane by phosphatase resulted in enhancement of its distribution on the surface or apparent solubility due to pulverization and its assuming an amorphous form in precipitation.

The in-vivo anti-influenza activity of III in PEG 4000 and III-Phos in infected mice showed about 90% virus inhibition at a dose of 4 mg/mouse twice daily, while no viral inhibition was found in the lung after oral administration of a 0·5% HPMC suspension of III (Table 4). Although the $AUC_{(0-2h)}$ of III in PEG 4000 was about twice that of III-Phos (Table 2), the in-vivo anti-influenza activity of III and III-Phos were almost equal

(Table 4). We have previously reported on the relationship between the intraperitoneal dose of Stachyflin and virus inhibition in the lungs (Yagi et al 1999). Although no viral inhibition was found at a low dose (< 1 mg/mouse twice daily), it was evident when the dose was raised to 2 mg/mouse twice daily and became constant at a higher dose. The reason for this phenomenon was not clear but it is possible that the AUCs of these compounds at this condition might be in constant range in anti-viral activity which was observed in Stachyflin (Yagi et al 1999). For III, an $AUC_{(0-2\,h)}$ of greater than 10 μg min mL^{-1} in mice may be necessary for effective in-vivo anti-influenza-virus activity, and it might become constant at a higher $AUC_{(0-2\,h)}$.

Generally speaking, in man, the influenza virus replicates in the upper respiratory tract. However, in the mouse influenza virus infection model, mouse-adapted influenza virus replicates in the lung and causes pneumonia without fever. From this point of view, the ferret influenza virus infection model is desirable for the evaluation of anti-influenza-virus drugs for humans because of similar symptoms in influenza-virus-infected ferrets to that in influenza-virus-infected humans. Different to the results of the experiments in mice, no in-vivo anti-influenza-virus activity was observed in ferrets after oral administration of III, irrespective of the long-lasting high plasma concentration of compounds which were sufficient to inhibit virus replication in-vitro (Figure 4, data not shown). The reason why III did not inhibit influenza virus replication in ferrets was unclear but it might be due to differences in the way that influenza virus replicates in ferrets compared with in mice. III could be expected to distribute in lungs after oral administration more easily than in the nasal cavity where influenza virus replicates in ferrets. Of course, other factors such as protein binding, intracellular uptake and metabolism to an inactive form cannot be ruled out.

The intranasal administration of III and III-Phos, which was expected to have more notable in-vivo anti-influenza activity, was studied using rats to improve the absorption and infected ferrets were used to examine the effect of the in-vivo anti-influenza virus activity. When III-Phos was intranasally administered to rats, the absolute bioavailabilities as III and III-Phos were 84% and 82%, respectively, while it was 0% in the case of the intranasal administration of III itself (Table 5). The residual amounts in the nasal cavity were measured to investigate the enhancement of the effect by III-Phos. The residual amounts in the nasal cavity of III-Phos and III after 30 min following the intranasal administration of III-Phos were $3{\cdot}9 \pm 1{\cdot}9\%$ and $0{\cdot}28 \pm 0{\cdot}16\%$, respectively (data not shown). Although some of the administered III-Phos was rapidly converted to the parent compound by phosphatase (Werner & Myer 1970) in the nasal secretions, it seemed that most of the III-Phos was absorbed into the blood from the nasal cavity, because the intranasal absorption of III was poor but the absolute bioavailability as III-Phos following intranasal administration of III-Phos was very high (Table 5). It is well known that absorption across biological membranes is generally controlled by factors such as drug-molecule size, solubility and lipophilicity. Corbo et al (1989) reported that the nasal absorption of progestins increased linearly with an increase in the octanol/water partition coefficient. Octanol/pH 7·4 PBS partition coefficients of III-Phos and III were $-2{\cdot}09$ and 3·90, respectively. In this case, the nasal absorption of III-Phos was higher than III despite the high lipophilicity of III. Therefore, the main reason may be their different solubility in water (Table 1). For the nasal transport of water-soluble compounds, it is postulated that the nasal aqueous-pore pathway is one of the mechanisms for nasal absorption (McMartin et al 1987; Fisher et al 1992). However, for PEG, the extent of nasal absorption from the nasal cavity decreases as the molecular size increases and varies very sensitively over a molecular-weight range of less than 300 (Donovan et al 1990). Based on these findings, the nasal absorption enhancement by III-Phos may be due to its contribution to the absorption route through the aqueous-pore pathway in the nasal mucosa in addition to the penetration across the nasal membrane depending upon the high solubility in water of III-Phos from a thermodynamic standpoint.

As shown in Figure 6, III-Phos could inhibit viral replication in the nasal cavity and suppress fever induced on infection by continual intranasal administration for 4 days. With intranasal administration of III-Phos to uninfected ferrets (13·2 mg/0·2 mL PBS/ferret), C_{max} as III and III-Phos was $1{\cdot}67 \pm 0{\cdot}06$ μg mL^{-1} and $1{\cdot}93 \pm 0{\cdot}65$ μg mL^{-1}, respectively, and the area under the plasma concentration–time curve ($AUC_{\phi-\infty}$) from zero to infinity as III and III-Phos was $171{\cdot}0 \pm 47{\cdot}7$ μg min mL^{-1} and $34{\cdot}7 \pm 10{\cdot}7$ μg min mL^{-1}, respectively (data not shown). The residual amount in the nasal cavity after 60 min was about 10 μg for both III and III-Phos (data not shown). C_{max} and $AUC_{\phi-\infty}$ calculated from the plasma concentration–time profile following oral administration of III in PEG 400 (Figure 3) were $0{\cdot}98 \pm 0{\cdot}33$ μg mL^{-1} and $216{\cdot}5 \pm 56{\cdot}5$ μg min mL^{-1}, respectively. There was no significant dif-

ference between C_{max} and $AUC_{\phi-\infty}$ of III following intranasal administration of III-Phos in distilled water and oral administration of III in PEG 400.

From the above findings, it was suggested that there is no relationship between plasma drug concentration and in-vivo anti-influenza-virus activity in ferrets, and that the direct effects, the accumulation of III and III-Phos on the surface of nasal membrane and good nasal absorption of III-Phos, contribute to the in-vivo anti-influenza-virus activity after intranasal administration of III-Phos to infected ferrets.

Conclusion

When III was orally administered in PEG 4000 solution and as phosphate ester in aqueous solution, the oral absorption was improved and an in-vivo anti-influenza-virus effect was observed in mice infected with A/Kumamoto/5/67 (H2N2). When III-Phos was administered intranasally to ferrets infected with A/Sendai/808/91 (H1N1), it limited viral replication in the nasal cavity and prevented fever. This study showed that compounds active against influenza virus must be present in the nasal cavity and permeate the nasal membrane to display in-vivo anti-influenza-virus activity.

References

Andersen, D. O., Murray, B. K., North, J. A. (1993) Effect of rivabilin on HeLa cells expressing E. coli xanthine guanine phosphoribosyl transferase. Antivir. Chem. Chemother. 4: 249–252

Capbell, D., Sweet, D. C., Hay, A. J., Douglas, A., Skehel, J. J., Mason, T. J., Smith, H. (1982) Genetic composition and virulence of influenza virus: differences in facets of virulence in ferrets between two pairs of recombinants with RNA segments of the same parental origin. J. Gen. Virol. 58: 387–398

Corbo, D. C., Liu, J. C., Chien, Y. W. (1989) Drug absorption through mucosal membranes. Effect of mucosal route and penetrant hydrophilicity. Pharm. Res. 6: 848–852

Donovan, M. D., Flynn, G. L., Amidon, G. L. (1990) Absorption of polyethylene glycols 600 through 2000: the molecular weight dependence of gastrointestinal and nasal absorption. Pharm. Res. 7: 863–868

Fisher, A., Illum, L., Davis, S., Schacht, E. (1992) Di-iodo-L-tyrosine labelled dextrans as molecular size markers of nasal absorption in the rat. J. Pharm. Pharmacol. 44: 550–554

Gilbert, B. E., Knight, V. (1986) Biochemistry and clinical applications of ribavilin. Antimicrob. Agents Chemother. 30: 201–205

Judd, A. K., Sanchez, A., Bucher, D. J., Huffman, J. H., Bailey, K., Sidwell, R. W. (1997) In vivo anti-influenza virus activity of a zinc finger peptide. Antimicrob. Agents Chemother. 41: 687–692

McMartin, C., Hutchinson, L. E. F., Hyde, R., Peters, G. E. (1987) Analysis of structural requirements for the absorption of drugs and macromolecules from the nasal cavity. J. Pharm. Sci. 76: 535–540

Pinto, L. H., Holsinger, L. J., Lamb, R. A. (1992) Influenza virus M2 protein has ion channel activity. Cell 69: 517–528

Schroeder, C., Ford, C. -M., Wharton, S. -A., Hay, A. J. (1994) Functional reconstitution in lipid vesicles of influenza virus M2 protein expressed by baculovirus: evidence for proton transfer activity. J. Gen. Virol. 75: 3477–3484

Shigeta, S., Mori, S., Watanabe, J., Soeda, S., Takahashi, K., Yamase, T. (1997) Synergistic anti-influenza virus A (H1N1) activities of PM-523 (polyoxometalate) and ribavirin in vitro and in vivo. Antimicrob. Agents Chemother. 41: 1423–1427

Sladowski, D., Steer, S. J., Clothier, R. H., Balls, M. (1992) An improved MTT assay. J. Immunol. Methods 157: 203–207

Smith, H., Sweet, C. (1988) Lessons for human influenza from pathogenicity studies with ferrets. Rev. Infect. Dis. 10: 56–75

Subbarano, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Awayne, D., Bender, C., Huang, J., Hemphill, M., Rowa, T., Shaw, M., Xu, X., Fukuda, K., Cox, N. (1998) Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279: 393–396

Taishi, T., Takechi, S., Mori. S. (1998) First total synthesis of (±)-Stachyflin. Tetrahedron Lett. 39: 4347–4350

Werner, E., Myer, P. (1970) Localisation von lipiden und hydrolasen in der regio respiratoria der normalen nasenschleimhaut des menschen. Acta Histochem. Bd. 35: S61

Yagi, S., Ono, J., Yoshimoto, J., Sugita, K., Hattori, N., Fujioka, T., Fujiwara, T., Sugimoto, Y., Hashimoto, N. (1999) Development of anti-influenza drugs: I. Improvement of oral absorption and *in vivo* anti-influenza activity of stachyflin and its derivative. Pharm. Res. 16: 1041–1046

Yoshimoto, J., Kakui, M., Iwasaki, H., Fujiwara, T., Sugimoto, H., Hattori, N. (1999) Identification of a novel HA conformation change inhibitor of human influenza virus. Arch. Virol. 144: 865–878