Development of Anti-Influenza Virus Drugs I: Improvement of Oral Absorption and *In Vivo* Anti-Influenza Activity of Stachyflin and Its Derivatives

Shigenori Yagi,[1,2] Junko Ono,[1] Jun Yoshimoto,[1] Ken-ichi Sugita,[1] Naohiko Hattori,[1] Toshihiro Fujioka,[1] Tamio Fujiwara,[1] Hirohiko Sugimoto,[1] Koichiro Hirano,[1] and Naofumi Hashimoto[1]

Received September 28, 1998; accepted March 18, 1999

Purpose. Stachyflin and its derivatives which are active against the influenza virus *in vitro*, were studied to improve their reduced *in vivo* activity after oral administration by chemical modification and some vehicles.

Methods. The solubility was examined for different vehicles. The improvement of gastrointestinal absorption was evaluated by the plasma concentration after oral administration to mice or the *in situ* loop method with rats. The *in vivo* anti-influenza activity was examined using mice infected with the influenza virus and evaluated based on the virus titer in the lung by $TCID_{50}$.

Results. PEG 400 showed the highest solubility of Stachyflin and its derivative among the vehicles studied. While no viral inhibition was found in the lung after oral administration of 0.5% HPMC suspension of Stachyflin, *in vivo* anti-influenza virus activity was found with the PEG 400 solution. The absorption of Stachyflin by PEG 400 showed about a fifty-fold increase in AUC compared with that of 0.5% HPMC suspension. Improving the oral absorption of Stachyflin led to an increase in the *in vivo* anti-influenza virus activity. When the Stachyflin derivative in PEG 4000 was administered orally, there was more enhancement of the oral absorption than with PEG 400. When the aqueous solution of the phosphate ester prodrugs of Stachyflin and its derivative was administered orally, the absorption of the parent compound was improved and *in vivo* anti-influenza virus activity was found.

Conclusions. When Stachyflin and its derivatives were administered orally to mice with a solution in PEG and an aqueous solution of their phosphate ester, their oral absorption was improved and *in vivo* anti-influenza virus activity was observed.

KEY WORDS: influenza; oral absorption; PEG; phosphate ester prodrug; anti-influenza virus drug.

INTRODUCTION

Influenza is an infectious disease of the respiratory tract caused by influenza viruses in humans throughout the world. Based on the internal antigens, influenza viruses can be separated into types A, B, and C. Influenza A virus is prevalent on a large scale the world over, while influenza B virus infections, which cause the same condition, many occur regionally. The symptoms caused by influenza C virus are slight and often occur in specific regions or institutions. Influenza A virus has been prevalent on a large scale around the world, because a new type of virus possessing a new surface antigen has appeared every 10–40 years. The situation has become very complex because three types of influenza viruses have been prevalent at the same time as influenza virus of the A/Soviet Russia type in addition to that of A/Hong Kong type, and influenza B virus has been simultaneously prevalent since 1977. Although only humans are the host of influenza B virus, both humans and animals can be infected by influenza A and C viruses, in particular, the host region caused by influenza A virus has spread among avian, porcine, equine, and marine mammals. Recently, a theory which has attracted world attention suggests new types of viruses appear as a result of intermediation of viruses between these animals and human beings. This may be why we have not been able to easily control these viruses. Although vaccines have been used to prevent the infection of influenza virus in the United States, their utility has been considerably limited because of the need for annual revaccination due to frequent changes to the regions of the viral surface glycoproteins. This has let to interest in the development of an antiviral agent for influenza virus therapy.

Stachyflin, which is extracted and refined from the culture of *Stachybotrys sp* RF-7260, is a compound effective against influenza A (H1N1) and A (H2N2) viruses *in vitro* (1). Stachyflin has been found to inhibit fusion between the viral envelope and the endosome composed of the cell membrane, which is the initial step in the entry of influenza into cells (2). This differs from the mechanisms of the antiviral action of amantadine and rimantadine which inhibit replication by interfering with the ion channel activity of the influenza Matrix 2 (M2) protein (3–7). When Stachyflin and its derivative having high *in vitro* anti-influenza virus activity were administered orally to mice infected with influenza virus by nebulization, less *in vivo* activity was observed. The reason for the reduced activity was thought to be the low absorption from the gastrointestinal tract due to their low solubility in water (<1 μg/mL). Many strategies are available for improving the oral absorption of drugs poorly soluble in water. These include reduction of particle size (8–16), addition of surfactants (17), formulation of solid dispersions (18,19), complexation with solubilizing agents such as cyclodextrins (20), and introduction of an ionizable moiety such as a phosphate or carboxyl group to the parent drug. The improvement in the aqueous solubility by the prodrug approach may affect the delivery properties and the *in vivo* behavior of the drug (21–24).

In this report, Stachyflin and its derivatives which are active against influenza virus *in vitro* were studied to improve their reduced *in vivo* activity after oral administration. We tried to improve their low solubility by chemical modification and the use of different vehicles. The resulting *in vivo* anti-influenza virus activities were evaluated using mice infected with influenza virus.

MATERIALS AND METHODS

Chemicals

Stachyflin (I) ((6aR, 7S, 9aS, 11S, 13aS)-2, 3, 6, 6a, 7, 8, 9, 9a, 10, 11, 12, 13-dodecahydro-5, 11-hydroxy-6a, 7, 10, 10-tetramethyl-3-oxo-1H-benzo [i] benzopyrano [2,3-e] isoindole,

[1] Shionogi Research Laboratories, Shionogi & Co., Ltd., Sagisu-cho, Fukushimaku, Osaka, 553-0002, Japan.

[2] To whom correspondence should be addressed. (e-mail: shigenori.yagi@shionogi.co.jp)

0724-8741/99/0700-1041$16.00/0

MW = 385.51), its derivative (II) and their phosphate ester prodrugs (I-Phos, II-Phos) were synthesized at Shionogi Research Laboratories. (Fig. 1) Hydroxypropylmethylcellulose (HPMC) was purchased from Shin-etsu Chemical Co., Ltd. (Tokyo, Japan). Polyethylene Glycol (PEG) 400 and 4000 were purchased from Wako Junyaku 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 rpm for 10 min at 25°C (Model H-103RL, Kokusan-enshinki Co., Ltd., Tokyo, Japan), the supernatant was diluted with methanol and the concentration of the compound was measured by high-performance liquid chromatography (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) aqueous solution and acetonitrile (60:40) for parent compounds and 0.3% TFA aqueous solution and acetonitrile (76:24) for phosphate ester prodrugs. The sample was analyzed by UV detection at 220 or 230 nm with a photodiode array UV-VIS detector (SPD-M6A, Shimadzu, Kyoto, Japan).

Animals

Male BALB/c mice (Japan Clea Inc., Osaka, Japan) weighing 22–25 g and male Sprague-Dawley rats (Japan Clea Inc., Osaka, Japan) weighing 250–320 g were used. These animals were used after having been bred for at least a week. They were maintained under a 12-hr light/dark cycle and were fed a standard diet. The animals used were sacrificed by incision of the abdominal main artery under anesthesia with diethyl ether after the experiments.

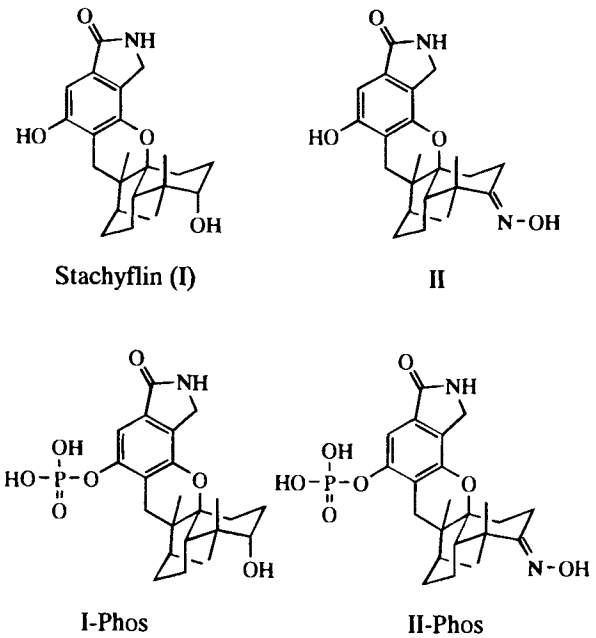

Fig. 1. Chemical structures of anti-influenza virus compounds.

Intraperitoneal and Oral Administration to Uninfected Mice

For the intraperitoneal (i.p.) study, Stachyflin as a 0.5% HPMC suspension was administered (0.1 mL/mouse) to mice who were fed regularly.

For the oral study, Stachyflin and its derivative (II) was administered (0.1 mL/mouse) to unfasted mice as a 0.5% HPMC suspension, a solution in PEG 400 or PEG 4000 (heated at 65°C), and an aqueous solution of their phosphate ester prodrugs (I-Phos, II-Phos).

Blood was collected from the heart with a heparinized syringe under anesthesia with diethyl ether. The blood was centrifuged at 3000 rpm for 10 min at 4°C (Model H-103RL, Kokusan-enshinki Co., Ltd., Tokyo, Japan), and the plasma was stored at −20°C until assay.

In Vivo Anti-Influenza Assays

Unfasted mice were infected with type A/Kumamoto/5/67(H2N2) influenza virus (5×10^4 $TCID_{50}$/10 mL) by nebulization for 30 min and randomly divided into experimental and control groups after infection. At 30 min after infection, the solution or suspension (0.1 mL/mouse) of compound was intraperitoneally or orally administered to the experimental group under anesthesia with diethyl ether twice (AM 9:00, PM 4:00) daily for 2 days. For the control group, infected mice were only anesthetized with diethyl ether at the same intervals. Mice were sacrificed to assay the virus titer in the lung after 48 hours. The lung was homogenized in a cell culture (Biken, Osaka, Japan) (Eagle's minimum essential medium (E-MEM)) medium (2 mL) and centrifuged at 3000 rpm for 10 min at 4°C (Model H-103RL, Kokusan-enshinki Co., Ltd., Tokyo, Japan). The virus titer in the supernatant was determined from the median tissue-culture infective dose of virus ($\log_{10}$ $TCID_{50}$) per lung by using MDCK (Madin-Darby canine kidney) cell monolayers. The *in vivo* anti-influenza activity of compounds against type A (H2N2) influenza virus was expressed as the ratio (%) of virus titer in the lung homogenate of the experimental to the control groups.

Rat Intestinal Absorption—*In Situ* Loop Method

Rats fasted for 20 hours were anesthetized with pentobarbital sodium (60 mg/kg 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 the parent compound or a solution of the phosphate ester prodrug. The distal end of the loop was secured with silk suture. Sodium heparin diluted with saline (0.5 mL, 10%, v/v) was administered *via* the tail vein prior to the experiment to prevent coagulation in the mesenteric vein. After 0.8 mL of sample (1.5 mg/mL) 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/hr) 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.

Rat Oral Administration

The suspension of the compound or the solution of its phosphate ester prodrug (20 mg/4 mL/kg) was orally administered to the conscious rat with a gastric tube. The blood sample was periodically collected via the jugular vein cannulated with a polyethylene tube (0.58 mm i.d. and 0.96 mm o.d.).

Plasma Sample Analysis

The plasma sample (0.1–0.15 mL) was deproteinized with 1.0 mL methanol for the parent compound and 1.0 mL acetonitrile for the phosphate ester prodrug. After immediately mixed and centrifuging at 12500 rpm for 5 min at 4°C with a refrigerated centrifuge (MR-15A, Tomy Seiko Co., Ltd., Tokyo, Japan), 1.0 mL of the clear supernatant was evaporated and the residue was dissolved with methanol:H_2O (1:1). The HPLC conditions were the same as those given in the Solubility section.

RESULTS AND DISCUSSION

Intraperitoneal and Oral Administration of Stachyflin to Mice

Figure 2 shows the plasma concentration-time profiles after oral (20 mg/0.1 mL 0.5% HPMC/mouse) and intraperitoneal (2 mg/0.1 mL 0.5% HPMC/mouse) administration of Stachyflin to mice. In the case of oral administration, the maximum plasma concentration (C_{max}) was 0.13 ± 0.02 μg/mL (0.34 ± 0.05 μM) and the area under the plasma concentration-time curve (AUC) to 120 min was 7.2 ± 1.1 μg min/mL (Table I). When Stachyflin was administered intraperitoneally, the absorption increased 6–7 times in AUC and 4–5 times in C_{max} at a 1/10 dose compared with oral administration (Table I).

Although no *in vivo* anti-influenza virus activity was observed after oral administration of Stachyflin to infected mice, it was 64% after intraperitoneal administration (Table I). Stachyflin has high *in vitro* anti-influenza virus activity, IC_{50}: concentration for inhibiting 50% of influenza virus, against type A/Kumamoto/5/67/(H2N2) influenza virus of 0.25 ± 0.10 μM (2). The *in vivo* anti-influenza virus activity was, therefore, obtained as the maximum plasma concentration (0.57 μg/mL (1.48 μM)) of Stachyflin rose six times above IC_{50} when Stachyflin was intraperitoneally administered.

Viral replication involves processes of adsorption, invasion, fusion, transcription, protein synthesis, and desorption, but not all viruses simultaneously undergo the same changes.

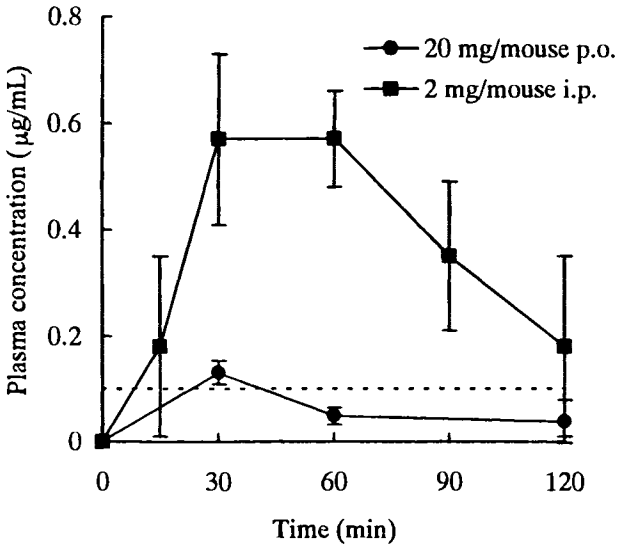

Fig. 2. Plasma concentration-time profiles after oral (20 mg/0.1 mL 0.5% HPMC/mouse) and intraperitoneal (2 mg/0.1 mL 0.5% HPMC/mouse) administration of Stachyflin to uninfected mice. The horizontal line indicates a concentration in plasma of 0.25 μM, the IC_{50} of Stachyflin in the type A/Kumamoto/5/67 (H2N2) influenza virus. Each point represents the mean ± standard deviation (n = 3). $AUC_{(0-2\ hr)}$s were calculated according to the trapezoidal rule.

As it takes 6–8 hours for all of these processes, the most direct attack to display sufficient *in vivo* anti-influenza activity on this problem may be the maintenance of an effective drug concentration in plasma for a long time (high C_{max} and AUC). In the case of Stachyflin, the plasma concentration above IC_{50} in mice would be able to be sustained for at least over 2 hours for effective *in vivo* anti-influenza virus activity.

Figure 3 shows the relationship between intraperitoneal dose and virus inhibition in the lungs. Although no viral inhibition was found at a low dose (<1 mg/twice/day/mouse), it appeared when the dose was raised to 2 mg/twice/day/mouse and became constant at a higher dose. Sudden appearance of the *in vivo* activity is often observed in viral and bacterial infection, because they are rapidly replicating in an order of

Table I. Pharmacokinetic Parameters[a] and the *In Vivo* Anti-Influenza Activity[b] After Oral and Intraperitoneal Administration of Stachyflin to Mice

Route	Dose (mg/mouse)	C_{max} (μg/mL)	$AUC_{(0-2\ hr)}$ (μg min/mL)[b]	Virus inhibition in lung (%)
Oral	20	0.13 ± 0.02	7.2 ± 1.1	N.O.[c]
Intraperitoneal	2	0.57 ± 0.16	45.8 ± 4.1	64

[a] Values are means ± standard deviations for 3 uninfected mice.
[b] Values are means for 6 to 8 mice. Unfasted mice were infected with type A/Kumamoto/5/67 (H2N2) influenza virus by nebulization. After Stachyflin was orally or intraperitoneally administered to infected mice twice (AM 9:00, PM 4:00) daily for 2 days, the mice were sacrificed to assay the virus titer in the lung. The *in vivo* anti-influenza activity was expressed as the ratio of virus titer in lung homogenate of experimental and control groups.
[c] Not observed.

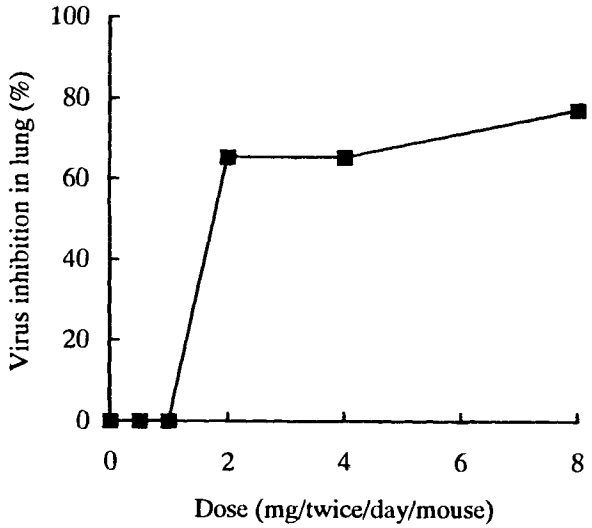

Fig. 3. Relationship between intraperitoneal dose and the *in vivo* virus inhibition in lung of Stachyflin. Values are means for 6 to 8 mice. Mice were infected with A/Kumamoto/5/67 (H2N2) influenza virus by nebulization (n = 6–8). The *in vivo* anti-influenza activity was determined as described in Table I.

index number. Therefore, the plasma concentration of Stachyflin must be kept over a similar level to that after intraperitoneal administration of 2 mg/twice/day/mouse in order to have *in vivo* anti-influenza virus activity.

Solubility Studies

Stachyflin is a lipophilic compound with a calculated partition coefficient (clog P) of 3.76 and is very insoluble in water (<1 μg/mL) at room temperature. The low solubility of Stachyflin was thought to be the main factor of the low inhibitory activity and the low absorption from the intestine when 0.5% HPMC suspension of Stachyflin was administered orally to infected mice. The solubility of Stachyflin was investigated by trying various vehicles (polyethylene glycol, surfactant, soy bean oil, sucrose fatty acid ester, medium chain fatty acid triglyceride, cyclodextrin) to improve the oral absorption. Table II shows the solubility of Stachyflin and II. PEG 400 dissolved both compounds most in all vehicles. PEG of the larger molecular weight was also examined for wider pharmaceutical application. PEG 1000, 1540, and 4000 (PEG 4000 was heated at 60°C) dissolved both compounds more than 10 mg/mL.

Table II. Solubility of Stachyflin and II

Solvent	Solubility (mg/mL)	
	Stachyflin	II
Distilled water	< 0.001	< 0.007
PEG 400	> 3.19	> 2.75
Soy been oil	0.41	0.35
10% HCO-60	0.55	1.9
10% Tween 80	0.54	1.71
ODO[a]	0.81	0.26
γ-CD (20 mg/mL)	0.74	2.19
SBE-β-CD (20 mg/mL)[b]	0.05	0.21
β-CD (20 mg/mL)	0.07	0.49

[a] ODO, Medium chain fatty acid triglyceride.
[b] SBE-β-CD, sulfobutyl ether-β-CD.

Improvement of Oral Absorption by PEG and Phosphate Ester Prodrug and the *In Vivo* Anti-Influenza Activity

Oral absorption of Stachyflin and II was studied with a solution in PEG 400 or PEG 4000 which could be used as a semisolid matrix. The solution heated at 65°C was administered to mice via a gastric tube in the case of PEG 4000. The solubility of Stachyflin and II in PEG 4000 at 65°C was over 20 mg/mL. Stomach incision showed that the administered PEG 4000 solution rapidly solidified in the stomach, reflecting a semisolid matrix. Furthermore, the phosphate ester derivatives (I-Phos and II-Phos) of Stachyflin and II were synthesized to increase their aqueous solubility as a prodrug approach (>20 mg/mL).

Table III shows pharmacokinetic parameters when Stachyflin, II, I-Phos, and II-Phos were administered orally to uninfected mice. The C_{max} and $AUC_{(0-2hr)}$ of both Stachyflin and II increased with PEG in comparison with the suspension in 0.5% HPMC and the improvement of the solubility by PEG contributed to the oral absorption. The absorption of both compounds by PEG 400 increased about fifty-fold in AUC compared with 0.5% HPMC suspension. The same dose of both compounds was administered orally to infected mice to evaluate the *in vivo* anti-influenza activity. While there was no anti-influenza virus activity with 0.5% HPMC suspension (40 mg/twice/day/mouse) for Stachyflin, the *in vivo* anti-influenza virus activity was observed (60 ± 7%) at a lower dose than 0.5% HPMC suspension when PEG 400 solution of Stachyflin was administered as shown in Table III. When PEG 400 solution of II was administered to infected mice (70 ± 9%), the activity

Table III. Pharmacokinetic Parameters[a] and the *In Vivo* Anti-Influenza Activity[b] After Oral Administration of Stachyflin, II and their Phosphate Ester Prodrugs to Mice

Compound (vehicle)	Dose (mg/mouse)	C_{max} (μg/mL)	$AUC_{(0-2\ hr)}$ (μg min/mL)[b]	Virus inhibition in lung (%)
Stachyflin (I)				
(0.5% HPMC)	20	0.13 ± 0.02	7.2 ± 1.1	N.O.[c]
(PEG 400)	10	1.68 ± 0.90	126.9 ± 17.1	60 ± 7
II				
(0.5% HPMC)	20	0.37 ± 0.09	31.0 ± 3.0	43 ± 25
(PEG 400)	2	2.89 ± 0.67	157.2 ± 22.4	70 ± 9
(PEG 4000[d])	2	6.07 ± 1.81	269.4 ± 29.2	67 ± 14
I-Phos				
(water)	2[e]	1.00 ± 0.34	86.6 ± 13.8	68 ± 29
II-Phos				
(water)	2[e]	1.81 ± 0.43	108.0 ± 7.8	70 ± 8

[a] Values are means ± standard deviations for 3 uninfected mice.
[b] Values are means ± standard deviations for 6–8 mice. Unfasted mice were infected with type A/Kumamoto/5/67 (H2N2) influenza virus by nebulization. The *in vivo* anti-influenza activity was determined as described in Table I.
[c] Not observed.
[d] Administered under the condition of solution heated at 65 °C.
[e] As the dose of parent compound.

was enhanced more than in the case of 0.5% HPMC suspension (43 ± 25%). The improvement of oral absorption increased the *in vivo* anti-influenza virus activity of both compounds. PEG 4000 was tried as a semisolid matrix dosage form. When II in PEG 4000 was administered orally, the oral absorption was more enhanced than in PEG 400. Although the mechanism for the increased absorption by PEG 4000 was not clearly known, it was assumed that the PEG 4000 might exert a cosolubilizing effect which enhances the dissolution rate and maintains a higher drug concentration in the gastrointestinal lumen compared with the PEG 400.

When an aqueous solution of I-Phos and II-Phos was orally administered to uninfected mice at a dose of 2 mg/mouse as a parent compound, the C_{max} and $AUC_{(0-2hr)}$ were much higher than those of the 0.5% HPMC suspension of each parent compound and the *in vivo* anti-influenza activity of I-Phos and II-Phos were 68 ± 29% and 70 ± 8% (4 mg/twice/day/mouse), respectively (Table III). Figure 4 shows the plasma concentration of the parent compound-time profile after oral administration of I-Phos and II-Phos to rat at a dose of 20 mg/4 mL/kg as a parent compound. When an aqueous solution of I-Phos or II-Phos was orally administered, the C_{max} and the AUC to 4 hours were much higher than those of the 0.5% HPMC suspension of each parent compound.

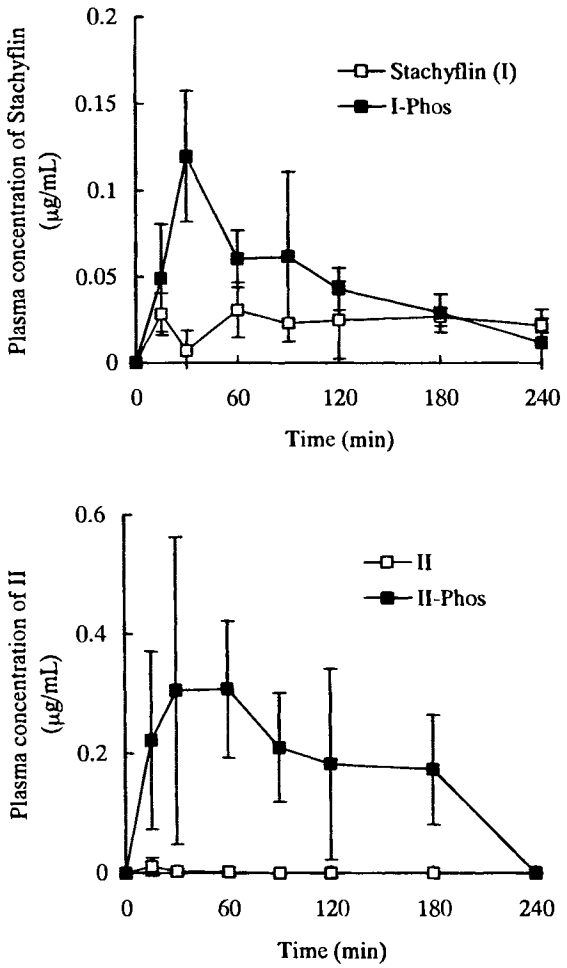

Fig. 4. Plasma concentration-time profiles after oral administration of Stachyflin, II, and their phosphate ester prodrugs (I-Phos, II-Phos) to rats. Each point represents the mean ± standard deviation (n = 3). The suspension (Stachyflin and II) or the aqueous solution (I-Phos and II-Phos) was orally administered to the conscious rat with a gastric tube (20 mg/4 mL/kg). The blood sample was periodically collected via the jugular vein cannulated with a polyethylene tube.

Intestinal Absorption *In Situ* Loop Method

The absorption characteristics of the parent compound and its phosphate ester prodrug were evaluated to study the effect enhanced by phosphate ester prodrug by an *In Situ* loop method using the rat small intestine. Figure 5 shows the cumulative amount transported into mesenteric venous blood every 10 min. The cumulative amounts (% of dose as parent compound) for 60 min of I-Phos and II-Phos were 5.4 ± 1.3% and 10.0 ±

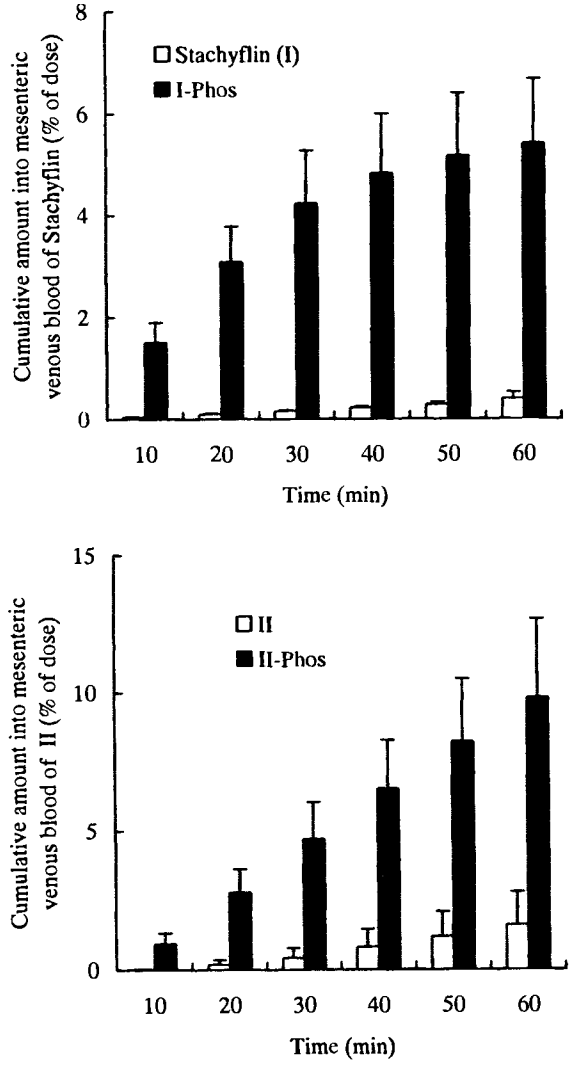

Fig. 5. The cumulative amount transported into mesenteric venous blood of Stachyflin, II, and their phosphate ester prodrugs every 10 min. The cumulative amount was measured by the rat *in situ* loop method. The suspension of parent compounds or the aqueous solution of their phosphate ester prodrugs (1.5 mg/mL) was injected directly into the lumen of the ligated intestinal loop (0.8 mL). All mesenteric venous blood from the loop was collected continuously at 10-min intervals for 60 min from mesenteric venous cannula. Each value represents the mean ± standard deviation (n = 3).

2.8%, respectively. They were about 1/3–2/3 of ceftibuten (orally well-absorbed antibiotic with a bioavailability of 74% in healthy humans and 57% in rat (25)) whose cumulative amount was 16% (20). The phosphate ester prodrug itself was not detected in the mesenteric venous blood and in the intestinal wall after the experiments, and only the parent compound was detected. The amounts (% of dose) as the parent compound in the intestinal wall by 60 min were 19.8 ± 7.8% and 29.4 ± 7.7% for I-Phos and II-Phos, respectively. Furthermore, for both I-Phos and II-Phos, the residual amount as phosphate ester prodrugs was less than 1% and that as parent compound was about 30% in the intestinal loop at 60 min, and the amount of parent compound in the mesenteric venous blood increased every 10 min. I-Phos and II-Phos were rapidly converted to the parent compound on the surface of the intestinal membrane and was absorbed as the parent compound.

Both I-Phos and II-Phos were stable in JP-1 and JP-2 at 37°C up to 2 hours and their octhanol/pH7.4 PBS partition coefficients were about −2.5. Although the mechanism for the increased oral absorption on phosphate ester prodrug was not clearly known, we assumed that conversion to the parent compound on the surface of the intestinal membrane by the phosphatase resulted in enhancement of its surface area due to pulverization by an order of several micrometers in precipitation.

In conclusion, when Stachyflin and its derivative were administered orally to uninfected mice as a solution in PEG 400, PEG 4000 which was planned as a semisolid-matrix-capsule, and an aqueous solution of their phosphate ester prodrugs, the oral absorption was improved and *in vivo* anti-influenza virus activity was observed.

REFERENCES

1. T. Taishi, S. Takechi, and S. Mori. First total synthesis of (±)-Stachyflin. *Tetrahedron Lett.* **39**:4347–4350 (1998).
2. J. Yoshimoto, N. Hattori, M. Kakui, H. Iwasaki, H. Sugimoto, and T. Fujiwara. Identification of a phenotypically irreversible HA conformational change inhibitor. *Arch. Virol.* (in press).
3. L. H. Pinto, L. J. Holsinger, and R. A. Lamb. Influenza virus M2 protein has ion channel activity. *Cell* **69**:517–528 (1992).
4. C. Schroeder, C.-M. Ford, S.-A. Wharton, and A. J. Hay. Functional reconstitution in lipid vesicles of influenza virus M2 protein expressed by baculovirus: Evidence for proton transfer activity. *J. Gen. Viol.* **75**:3477–3484 (1994).
5. R. J. Sugrue and A. J. Hay. Structural characteristics of the M2 protein of influenza A viruses: Evidence that it forms a tetrameric channel. *Virology* **180**:617–624 (1991).
6. A. J. Hay. Potential targets and actions of antiviral agents against influenza viruses. *Chem. Scr.* **26**:77–81 (1986).
7. A. J. Hay. The action of adamantamines against influenza a viruses: inhibition of the M2 ion channel protein. *Semin. Virol.* **3**:21–30 (1992).
8. R. M. Atkinson, C. Bedford, K. J. Child, and E. G. Tomich. Effect of particle size on blood griseofulvin-levels in man. *Nature* **193**:588–589 (1962).
9. K. B. Choudhari and N. M. Sanghavi. Dissolution behavior and characterization of diazepam-pullulan coground mixtures. *Int. J. Pharm.* **89**:207–211 (1993).
10. A. B. Straughn, M. C. Meyer, G. Raghow, and K. Potenberg. Bioavailability of microsize and ultramicrosize griseofulvin products in man. *J. Pharmacokinet. Biopharm.* **8**:347–362 (1980).
11. W. E. Barrett and J. R. Bianche. The bioavailavility of ultramicrosize griseofulvin (GRIS-PEG) tablets in man. *Curr. Ther. Res.* **18**:501–509 (1980).
12. J. H. Fincher. Particle-size of drugs and its relationship to absorption and activity. *J. Pharm. Sci.* **57**:1825–1835 (1968).
13. F. Nimmerfall and J. Rosenthaler. Dependence area under the curve on proquazone particle size and in vitro dissolution rate. *J. Pharm. Sci.* **69**:605–607 (1980).
14. H. E. Paul, K. J. Hayes, M. F. Paul, and A. R. Borgmann. Laboratory studies with nitrofurantoin. *J. Pharm. Sci.* **56**:882–885 (1967).
15. A. S. Ridolfo, L. Thompkins, L. D. Bechtol, and R. H. Carmichael. Benoxaprofen, a new anti-inflammatory agent: particle size effect on dissolution rate and oral absorption in humans. *J. Pharm. Sci.* **68**:850–852 (1979).
16. S-L Lin, J. Menig, and L. Lachman. Interdependence of physiological surfactant and drug particle size on the dissolution behavior of water-insoluble drugs. *J. Pharm Sci.* **57**:2413–2148 (1968).
17. R. L. Oberle and T. L. Moore. Evaluation of nonionic surfactants to improve solubility and oral absorption of CGS-9896. *Pharm. Res.* **10**:Suppl., S222 (1993).
18. A. B. Straughn, M. C. Mayer, G. Raghow, and K. Rotenberg. Bioavailability of microsize and ultramicrosize griseofuluvin products in man. *J. Pharmacokin. Biopharm.* **8**:347–362 (1980).
19. I. Sugimoto, A. Kuchiki, H. Nakagawa, K. Togo, S. Kondo, I. Iwane, and K. Takahashi. Dissolution and absorption of nifedipine from nifedipine-polyvinylpyrrolidone coprecipitate. *Drug Der. Ind. Pharm.* **6**:137–160 (1980).
20. N. Hashimoto, T. Fujioka, K. Hayashi, K. Odaguchi, T. Toyoda, M. Nakayama, and K. Hirano. Renin inhibitor: Relationship between molecular structure and oral absorption. *Pharm. Res.* **11**:1443–1447 (1994).
21. S. A. Varia and V. J. Stella. Phenytoin prodrugs V: *In vivo* evaluation of some water-soluble phenytoin prodrugs in dogs. *J. Pharm. Sci.* **73**:1080–1087 (1984).
22. S. A. Varia and V. J. Stella. Phenytoin prodrugs VI: *In vivo* evaluation of a phosphate ester prodrug of phenytoin after parenteral administration to rats. *J. Pharm. Sci.* **73**:1087–1090 (1984).
23. D. Fleisher, K. C. Johnson, B. H. Stewart, and G. L. Amidon. Oral absorption of 21-corticosteroid esters: A function of aqueous stability and intestinal enzyme activity and distribution. *J. Pharm. Sci.* **75**:934–939 (1986).
24. C. Sessa, M. Zucchetti, T. Cerny, O. Pagani, F. Cavalli, M. De Jong, D. Gentili, C. McDaniel, C. Prins, L. Schacter, B. Winograd, and M. D'Incalci. Phase I clinical and pharmacokinetic study of oral etoposide phosphate. *J. Clin. Oncol.* **13**:200–209 (1995).
25. N. Muranushi, T. Yoshikawa, M. Nishiuchi, T. Oguma, K. Hirano, and H. Yamada. Characteristics of the intestinal absorption of 7432-S, a new orally active cephalosporin. *18th Symposium on Drug Metabolism and Action. Abstr.* 55–58 (1986).