

INHIBITORY EFFECT OF FK-565 ALONE AND IN COMBINATION
WITH ZIDOVUDINE ON RETROVIRAL INFECTION
BY FRIEND LEUKEMIA VIRUS IN MICE

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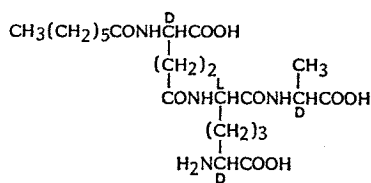
The effects of route and starting time of administration on FK-565 inhibition of splenomegaly by Friend leukemia virus (FLV) were studied in mice, and the concomitant effect of FK-565 in allowing reduction of zidovudine dosage was estimated.

FK-565 inhibited splenomegaly in intravenous and oral doses of 0.01 to 1 mg/kg, but time of initial dosing had little effect on this inhibition. When 0.01 or 1 mg/kg of FK-565 was given intravenously with intraperitoneal doses of 0.63, 2.5, 10 and 40 mg/kg of zidovudine, the inhibition rate of splenomegaly at all doses was markedly and dose-dependently higher than when either drug was given alone, and the concomitant use of FK-565 with zidovudine enabled a 16-fold reduction of the dose of zidovudine. The survival rate and survival time after infection with massive amounts of FLV were higher when FK-565 1 mg/kg and zidovudine 20 mg/kg were given in combination than when either drug was given alone. Inhibition of FLV splenomegaly was reflected in the prolonged survival time of the infected mice.

FK-565, an immunoactive peptide isolated from culture filtrates of *Streptomyces olivaceogriseus* has the chemical structure *N*-[*N*²-(*N*-heptanoyl- γ -D-glutamyl)-*meso*-2-(L), 2'-(D)-diamino-1-pimeloyl]-D-alanine as shown in Fig. 1.¹⁻⁴⁾ The compound induces potent resistance against microbial, fungal and viral infections in normal and immunosuppressed mice,⁵⁻⁷⁾ *in vivo* tumoricidal activity,^{8,9)} inhibitory effect against murine metastases,¹⁰⁾ and a broad spectrum of immunostimulatory activity such as adjuvant effects on humoral and cellular immunity and mitogenesis.¹¹⁾

In this study, we investigated whether FK-565 would inhibit splenomegaly induced by Friend leukemia virus (FLV) and prolong the survival time of mice with FLV infection and whether FK-565 could be used to reduce the dose of zidovudine. Zidovudine has been proven useful against human Acquired Immunodeficiency Syndrome (AIDS) in clinical trials.^{12,13)} Zidovudine effectively inhibits the infectivity and cytopathic effects of human immunodeficiency viruses (HIV) on human T4 cells.¹⁴⁾ However, at therapeutically effective doses, zidovudine can cause anaemia, leukopenia, neutropenia and occasional thrombocytopenia, probably through bone marrow suppression.¹⁵⁾ If FK-565 treatment can allow a lower dose of zidovudine, it should be possible to avoid or reduce in severity zidovudine side effects. Furthermore, the immunostimulatory properties of FK-565 may be useful in protecting patients from opportunistic infections. Because the *in vivo* evaluation of anti-HIV activity must be carried out with chimpanzees, we used FLV which is also a retrovirus and is readily characterized by palpable splenomegaly in susceptible mice.¹⁶⁻¹⁸⁾ This

Fig. 1. Chemical structure of FK-565.



Molecular formula: C₂₂H₃₈N₄O₉
MW: 502.57 daltons

virus attacks erythropoietic cell precursors and causes massive splenomegaly; the infected animals die in 4 to 5 weeks.¹⁹⁻²¹⁾ The short latent period of FLV allows rapid determination of an effective dosage. Therefore, the murine viral model described seems ideally suited as a model system for *in vivo* evaluation of anti-AIDS agents.

Materials and Methods

Drugs

FK-565 and zidovudine (AZT, 3'-azido-3'-deoxythymidine; zidovudine is the recently accepted generic name for azidothymidine) were synthesized at the Product Development Laboratories of Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. The purity of zidovudine was 98.2%.

Animals

Male C3H/HeN strain mice aged 5 weeks were obtained from Charles River Japan, Inc. and used in groups of 10 or 12. The animals were housed in an isolation room at 21~25°C and supplied with food and water *ad libitum*.

Virus and Infection

FLV was prepared as a 20%-suspension of infected C3H/HeN spleen in HANK's balanced salt solution and was originally supplied by Dr. T. AKIYAMA of Kitasato University. After centrifugation at 7,000 rpm for 20 minutes the virus preparation was frozen and stored at -70°C. For experiments of suppression of splenomegaly, 0.2 ml/mouse of 10-fold dilution of FLV was challenged intraperitoneally while in experiments of survival from infection, a 2-fold dilution was used in the same volume and the route.

Drug Schedule

FK-565 in doses of 0.001 to 1 mg/kg were given intravenously or orally, and zidovudine was given in intraperitoneal doses of 0.63 to 40 mg/kg according to the schedule detailed in the footnote of Tables 1~3 and Figs. 2~3. When FK-565 and zidovudine were dosed concomitantly, each drug was given according to the same experimental schedule as when it was used singly.

Evaluation of Inhibition Rate

The tested mice were sacrificed and the spleen was measured 14 or 21 days after FLV challenge. The inhibitory percent of FK-565 and zidovudine alone or in combination, on the development of splenomegaly was calculated as follows:²²⁾

$$(\%) \text{ Inhibition} = \left(1 - \frac{A - A_0}{B - B_0} \right) \times 100,$$

where A and B are the spleen weights treated with and without drug against infected mice, respectively. A₀ and B₀ are the spleen weights treated with and without drug against non-infected mice (control).

Assay of Virus Titer in Plasma

The virus titer in plasma was assayed from standard linear regression curves because spleen weights were correlative with the amount of virus in spleen. In the experiment of Table 3, the blood of mice infected with FLV was drawn and the plasma was stored at -70°C. The plasma was 2-fold serially diluted and 0.2 ml was inoculated in new normal mice. The spleen weights were measured 14 days after inoculation of the plasma. The virus titer in plasma was calculated as a dilution ratio from a standard linear regression curve as a virus titer of 1 when the spleen weight was 200 mg (about 2-fold of spleen weight of normal mice).

Statistical Evaluation

Student's t-test was used to establish whether significant differences existed between control and drug-treated groups in the all experiments.

Results

Effect of Dosing Schedule of FK-565 on Inhibition of Splenomegaly

FK-565 was given intravenously or orally to mice in groups of 10 in doses of 0.001 to 1 mg/kg, 4 hours after challenge with FLV and thereafter once a day for 4 consecutive days (total 5 doses) or 4 hours after challenge with FLV in mice and thereafter intermittently once on days 3, 7 and 11 (total 4 doses) at the same doses used on day one. The spleen weights were measured 14 days after challenge and the rate of inhibition of splenomegaly was calculated (Table 1). The mean spleen weights of the infected controls ranged from 1,455 to 1,480 mg, and were about 15-fold that of the spleen weights of normal mice.

In the animals dosed daily for 5 days after challenge the rates of inhibition for intravenous doses of 0.1 and 1 mg/kg were 30 and 38%, respectively, but for 0.001 and 0.01 mg/kg were less than 20%. The inhibition rates for oral doses of 0.1 and 1 mg/kg were 38 and 36%, respectively, but for 0.001 and 0.01 mg/kg were less than 20%.

In the animals dosed intermittently, the inhibition rates for intravenous doses of 0.01, 0.1 and 1 mg/kg were 36, 37 and 47%, respectively, and for oral doses of 0.1 and 1 mg/kg, 28 and 39%, respectively.

On the other hand, when FK-565 was given orally, intermittent dosing with 0.1 mg/kg gave lower inhibition rates than when given consecutively. This result suggested that consecutive dosing from an early stage is needed when FK-565 is given orally in low doses. This experiment showed that FK-565 alone gave inhibition of splenomegaly both by consecutive dosing and intermittent dosing either intravenously or orally.

Effect of Starting Time on FK-565 Inhibition of Splenomegaly

The inhibitory effects of FK-565 on splenomegaly were examined in the schedules described footnote of Table 2. FK-565 1 mg/kg was given intravenously and zidovudine 20 mg/kg was given intraperitoneally in groups of 10.

Table 1. Effect of dosing schedule on inhibitory effect of FK-565 on splenomegaly induced by Friend leukemia virus (FLV) in mice.

Dosing schedule	Dose (mg/kg/dose)	iv		po	
		Weight (mg)	Inhibition (%)	Weight (mg)	Inhibition (%)
Consecutive ^a	1	968 ± 79***	38	986 ± 157*	36
	0.1	1,078 ± 137*	30	964 ± 138**	38
	0.01	1,251 ± 62	17	1,209 ± 146	20
	0.001	1,327 ± 59	12	1,211 ± 115	19
Non-treated control		1,480 ± 59	—	1,468 ± 62	—
Intermittent ^b	1	947 ± 124**	47	984 ± 145**	39
	0.1	1,038 ± 177*	37	1,133 ± 170	28
	0.01	1,047 ± 106**	36	ND	—
Non-treated control		1,455 ± 45	—	1,455 ± 45	—

^a FK-565 was given intravenously or orally 4 hours after challenge with FLV and once a day for 4 consecutive days (total 5 doses).

^b FK-565 was given 4 hours after challenge and once on days 3, 7 and 11 (total 4 doses).

These mice were sacrificed 14 days after challenge and the spleen weights were measured.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show significant difference from non-treated control.

Table 2. Effect of starting time of dosing on inhibitory effect of FK-565 on splenomegaly induced by Friend leukemia virus in mice.

Drug	Dose (mg/kg/dose)	Expt I		Expt II		Expt III	
		Weight (mg)	Inhibition (%)	Weight (mg)	Inhibition (%)	Weight (mg)	Inhibition (%)
FK-565	1	1,158±46**	47	956±89**	56	1,325±139*	31
Non-treated control		2,013±103	—	1,926±35	—	1,805±44	—
Zidovudine	20	576±163**	77	1,051±172**	52	1,120±224*	47
Non-treated control		1,964±73	—	1,964±73	—	1,964±73	—

FK-565 was given in an intravenous dose of 1 mg/kg according the following schedule: Expt I, 4 hours after challenge and once on days 1, 3, 7, 11, 14 and 18 (total 7 doses); Expt II, 24 hours after challenge and once on days 3, 7, 11, 14 and 18 (total 6 doses); Expt III, once on days 3, 7, 11, 14 and 18 (total 5 doses).

Zidovudine was given in an intraperitoneal dose of 20 mg/kg according the following schedule: Expt I, 4 hours after challenge and 3 times a day on days 1 to 4, 7 to 11 and 14 to 18 (total 43 doses); Expt II, 3 times a day on days 1 to 4, 7 to 11 and 14 to 18 (total 42 doses); Expt III, 3 times a day on days 3 and 4, 7 to 11 and 14 to 18 (total 36 doses).

These mice were sacrificed 21 days after challenge and the spleen weights were measured.

* $P < 0.01$, ** $P < 0.001$ show significant difference from non-treated control.

Table 3. Concomitant effect of FK-565 and zidovudine on inhibition of splenomegaly induced by Friend leukemia virus in mice.

Dose (mg/kg/dose)	Alone		Combination			
	Zidovudine		FK-565 (1 mg/kg)		FK-565 (0.01 mg/kg)	
	Weight (mg)	Inhibition (%)	Weight (mg)	Inhibition (%)	Weight (mg)	Inhibition (%)
40	267±52***	81	231±5.3***	89	257±20***	86
10	519±131*	49	272±9.4***	84	256±20***	84
2.5	605±135*	38	352±63***	73	439±83***	60
0.63	633±68**	33	339±60***	72	521±61***	49
0	—	—	487±47***	55	750±119*	20
Non-treated control	894±34	—	894±34	—	894±34	—

FK-565 was given intravenously 0.01 or 1 mg/kg 4 hours after challenge and once a day on days 3, 7 and 11 (total 4 doses). Zidovudine was given intraperitoneally 0.63 to 40 mg/kg, 3 times a day on days 1 to 4 and 7 to 11 after challenge (total 27 doses). Additionally, the drugs were given in combination by the same schedule.

These mice were sacrificed 14 days after challenge and the spleen weights were measured.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show significant difference from non-treated control.

The best effect of FK-565 was obtained when the drug was started 24 hours after challenge, *i.e.* 56% inhibition (Expt II, 6 doses).

In Expts I (7 doses) and III (5 doses) the rates of inhibition were respectively 47 and 31%, although it should be noted that the drug was only given 5 times in Expt III. On the other hand the best effect of zidovudine was obtained when the drug was started on the day of challenge and given 43 doses, *i.e.* 77% inhibition (Expt I), but the effect decreased to 52 and 47%, respectively when started 24 hours (Expt II, 42 doses) and 72 hours (Expt III, 36 doses) after challenge.

Effect of Concomitant FK-565 and Zidovudine on Inhibition of Splenomegaly

We investigated how much the dose of zidovudine could be reduced by using FK-565 in combination. The results are presented in Table 3. When FK-565 was given in intravenous doses of 0.01 or 1 mg/kg 4 hours after challenge and on days 3, 7 and 11 to mice in group of 10, the rates of

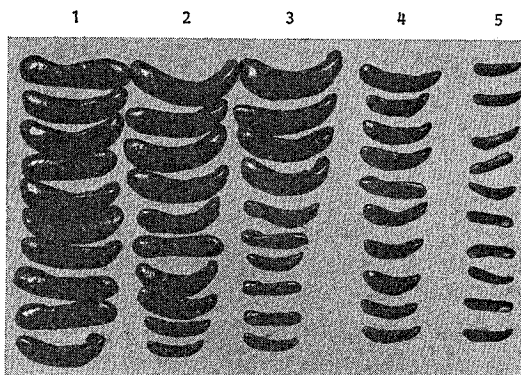
inhibition were 20 and 55%, respectively. When zidovudine was given in intraperitoneal doses of 0.63, 2.5, 10 or 40 mg/kg, 3 times a day, from 24 hours after challenge on days 1 to 4 and 7 to 11 (total 27 doses), the rates of inhibition were 33, 38, 49 and 81%, respectively and these inhibitions were dose-dependent.

Following the same dosing schedule for each test compound, the rates of inhibition for FK-565 0.01 mg/kg and concomitant doses of 0.63, 2.5, 10 or 40 mg/kg of zidovudine were 49, 60, 84 and 86%, respectively. The lowest dose of zidovudine (0.63 mg/kg) in combination with FK-565 0.01 mg/kg gave a similar inhibition to that of zidovudine 10 mg/kg alone, both about 50%. FK-565 therefore, makes possible an approximately 16-fold reduction in the dose of zidovudine required for 50% inhibition. When 1 mg/kg of FK-565 was given with each dose of zidovudine, the rates of inhibition were 72, 73, 84 and 89%, respectively, all higher than 70%.

Photo. 1 shows the spleens of mice given 0.01 mg/kg FK-565 alone, 10 mg/kg zidovudine alone and FK-565 and zidovudine in combination. We also measured the virus titer in the plasma of the tested mice after administration of 0.01 mg/kg FK-565 and 10 mg/kg zidovudine alone and in combination (Fig. 2). When 0.01 mg/kg FK-565 and 10 mg/kg zidovudine were given in combination, the virus titer fell to less than 2, and this plasma could scarcely induce splenomegaly. These results suggest that FK-565 and zidovudine could synergistically kill virus.

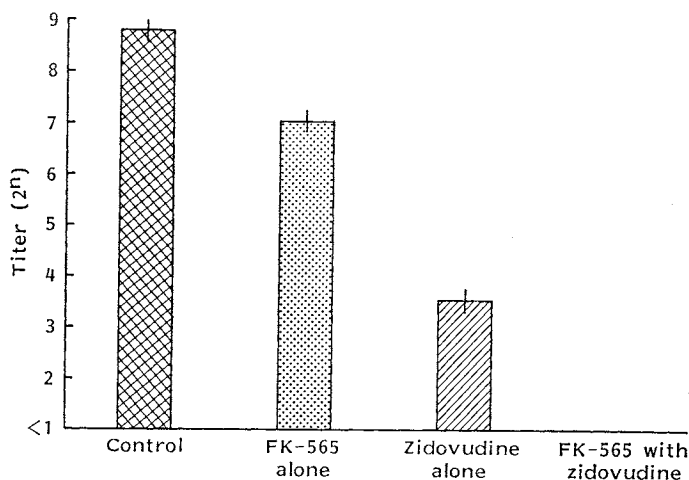
Photo. 1.

1: Control, 2: FK-565 (0.01 mg/kg), 3: zidovudine (10 mg/kg), 4: combination, 5: normal.



The spleens of mice given FK-565 0.01 mg/kg, zidovudine 10 mg/kg and the combination of FK-565 and zidovudine in the experiment of Table 3.

Fig. 2. Virus titer in plasma after administration of FK-565 and zidovudine alone or in combination in Friend leukemia virus infected mice.



FK-565 0.01 mg/kg and zidovudine 10 mg/kg were given intravenously and intraperitoneally, respectively according the schedule in Table 3. Virus titer in plasma was calculated from the standard linear regression curve as virus titer 1 when the spleen weight was 200 mg.

Effect of Concomitant FK-565 and Zidovudine on Survival
of Mice with Severe Infection

FK-565 had a more potent suppressive effect on splenomegaly induced by FLV in combination with zidovudine than alone. In the following study, we investigated the effect of these two drugs on the survival of mice with severe infection caused by massive inoculation of FLV (Fig. 3).

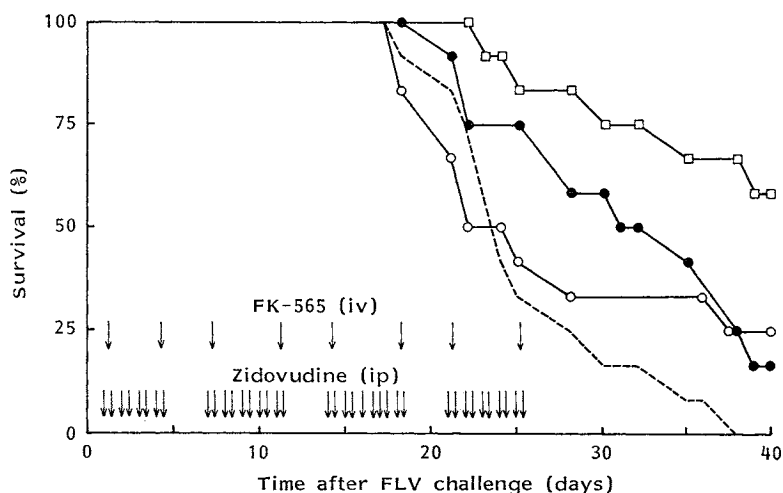
The spleen weights of the non-treated control challenged with FLV intraperitoneally were 1,588 mg 2 weeks and 2,640 mg 3 weeks after challenge, and all the mice died between 18 and 38 days after challenge. The mean survival time was 26 days in the non-treated control group. The effect of FK-565 alone and in combination with zidovudine on the survival rate was investigated for 40 days after challenge and the mean survival time in days was calculated after all remaining animals were sacrificed on day 41.

FK-565 and zidovudine alone or in combination were given mice in groups of 12 from 24 hours after challenge according to the schedule in the footnote. The survival rates were 17% for 1 mg/kg FK-565 alone and 25% for 20 mg/kg zidovudine alone: Mean survival times were 32 and 28 days respectively. Using a combination of 1 mg/kg FK-565 and 20 mg/kg zidovudine, the survival rate (58%) was higher with statistically significant difference from the control, and the mean survival time (37 days) was 9 days longer than when zidovudine was given alone and 11 days longer than that in the control group.

The protective and survival effects, when FK-565 and zidovudine were given in combination were markedly better than when either drug was given alone. These results suggest that the potent suppression of splenomegaly obtained by the use of FK-565 and zidovudine in combination could prolong survival time from death by FLV.

Fig. 3. Protective effect of FK-565 and zidovudine alone and in combination against severe Friend leukemia virus-infection in mice.

--- Control, ● FK-565, ○ zidovudine, □ combination with FK-565 and zidovudine.



FK-565 1 mg/kg was given intravenously once a day on days 1, 4, 7, 11, 14, 18, 21 and 25 (total 8 doses) after challenge and zidovudine 20 mg/kg was given intraperitoneally twice a day on days 1 to 4, 7 to 11, 14 to 18 and 21 to 25 (total 38 doses). The tested mice were observed for mortalities for 40 days after challenge.

Discussion

In our animal model with splenomegaly induced by inoculation of FLV, we showed that FK-565 had a potent and significant inhibitory activity on splenomegaly in C3H/HeN mice when it was given intravenously or orally, consecutively or intermittently. The FK-565 possibly does not have a direct virucidal activity against FLV, but rather enhances or restores the host defense ability in normal or immunosuppressed conditions by activating natural killer cells,²³⁾ macrophages and the reticuloendothelial system,^{24,25)} and also by inducing colony stimulating factors²⁶⁾ or interleukin 1 production.^{27,28)} Moreover some of these activities are maintained for 5 to 7 days after a single dose.^{24,25)} Therefore, the inhibitory activity of FK-565 against FLV infection appears to be derived from its activation of host defense mechanisms. Unlike FK-565, zidovudine, which has a direct antiviral activity, elicited an excellent suppression in the splenomegaly model by intraperitoneal dosing started 4 hours after viral challenge, but the activity was weaker when the dosing commenced 24 hours after challenge. These results emphasize the importance of starting treatment with zidovudine as soon as possible after exposure to the virus. FK-565, however, with its different mode of action, was not much affected by starting time of dosing, and even when started 24 hours after challenge gave a rate of inhibition of about 50%, similar to that of zidovudine. What's more, this effect was obtained with a total dose of 0.12 mg/mouse of FK-565, whereas a total dose of 16.8 mg/mouse of zidovudine was needed.

RUPRECHT *et al.*¹⁹⁾ reported in an animal model of splenomegaly with Rauscher murine leukemia virus in Balb/c mice that zidovudine gave 94% inhibition when given intraperitoneally 20 mg/kg 4 hours after challenge and thereafter every 8 hours for 20 days. Suramin, gave intravenously 40 mg/kg 4 hours after challenge and thereafter twice weekly, gave 35% inhibition.²⁰⁾

In the study on concomitant dosing with FK-565 and zidovudine, the rates of inhibition were markedly higher than when either drug was given alone. For example, the inhibition rates of zidovudine alone were 33 to 49% with 0.63 to 10 mg/kg; concomitantly with FK-565 0.01 mg/kg, the inhibition rates rose to 49 to 84% and with FK-565 1 mg/kg, 72 to 84%, and no free virus could be detected in the plasma at this rate (84%) of inhibition. Additionally, to obtain 50% inhibition with zidovudine alone, a dose of 10 mg/kg was needed, but when FK-565 0.01 mg/kg was given with zidovudine, the dose of the latter could be decreased to 0.63 mg/kg. That is, concomitantly with FK-565, the 50% inhibitory dose of zidovudine was reduced to about 1/16 that of the dose of the drug alone. A similar concomitant effect was also observed with oral doses of FK-565 although the data is not shown in this paper. These potent suppressive effects could prolong survival time in mice with severe FLV infection longer than could either drug alone.

Effective drugs are needed which possess a high therapeutic index and few adverse effects such as depression of bone marrow-derived cells, especially granulocytes. Clearly, breaking the virus replication cycle by therapeutic intervention could be a major step toward stemming retroviral epidemics. Pharmacological agents designed to interfere with such retroviral function as reverse transcription could be an important therapeutic means, possibly together with immunological agents. Presently the main cause of death in AIDS patients is severe infection due to the lack of cellular immunity. In the United States, many studies suggest that most of the known HIV positive patients, more than 1.5 million, cannot be cleared of the virus and that these patients will ultimately go on to develop clinical disease.²⁹⁾ If the results of this experiment can be extrapolated to patients with AIDS-related complex (ARC) or/and AIDS, prognosis could be improved, survival time prolonged, and the adverse effects of zidovudine reduced by such therapy.

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