

Anti-cachectic Effect of FK317, a Novel Anti-cancer Agent, in Colon26 and LX-1 Models in Mice

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The effects of FK317 (11-acetyl-8-carbamoyloxymethyl-4-formyl-6-methoxy-14-oxa-1,11-diazatetracyclo[7.4.1.0^{2,7}.0^{10,2}]tetradeca-2,4,6-trien-9-yl acetate), a novel anti-cancer agent, on murine adenocarcinoma colon26- and human lung carcinoma LX-1-induced cachexia were investigated in mice. Mice bearing colon26 or LX-1 s.c. lost weight and became cachectic, associated with tumor growth. FK317 and mitomycin C (MMC) inhibited the growth of both tumors. FK317 ameliorated the weight loss induced by the presence of colon26 or LX-1, while MMC enhanced it. An attenuation of the reduction in the weights of epididymal fat, gastrocnemius muscle and carcass was observed in FK317-treated tumor-bearing mice in both cachexia models, but not in MMC-treated mice. The decreases in the circulating levels of triglyceride, glucose and non-esterified fatty acid, which were induced by the presence of colon26, was partially inhibited by treatment with FK317. Overall, this study revealed that FK317 is a potent anti-cancer drug with anti-cachectic activity, suggesting that FK317 has potential utility for the treatment of cancer.

Key words: FK317 — LX-1 — Colon26 — Cachexia — Weight loss

FK973, a novel substituted dihydrobenzoxazine, is an anti-cancer drug with potent anti-tumor activity.¹⁾ It is a semi-synthetic triacetyl derivative of the natural product FR900482, isolated from the fermentation products of *Streptomyces sandaensis* No. 6897.²⁻⁴⁾ However, during phase I clinical trials of FK973, vascular leak syndrome was observed in patients following FK973 treatment and it was thus withdrawn from development; nevertheless it showed therapeutic anti-tumor activity in some of the cancer patients enrolled in the clinical trial.^{5,6)} We sought a new compound having the same potent anti-tumor activity as FK973, but without the undesirable side effect, and selected FK317 (11-acetyl-8-carbamoyloxymethyl-4-formyl-6-methoxy-14-oxa-1,11-diazatetracyclo[7.4.1.0^{2,7}.0^{10,2}]tetradeca-2,4,6-trien-9-yl acetate) as a candidate from among various derivatives of FK973. FK317 is a deacetylated derivative of FK973. Our subsequent studies have revealed that FK317 shows more potent anti-tumor activity against murine and human tumors *in vitro* and *in vivo* than does the parent compound FK973, and also that FK317 does not induce accumulation of pleural effusion in rats, unlike FK973,⁷⁾ suggesting that FK317 may have therapeutic potential for use in the treatment of cancer. Phase I clinical trials are under way in Japan.

In a previous study to evaluate the anti-tumor effect of FK317 against murine and human solid tumors implanted s.c., no severe decrease in the body weight of host mice was observed following FK317 treatment, although tumor growth was effectively inhibited.⁷⁾ On the other hand, the

reference drug mitomycin C (MMC) induced a severe decrease in the body weight of mice at the doses effective in inhibiting tumor growth. These results led us to hypothesize that FK317 may be an anti-cancer drug with anti-cachectic activity, in contrast to MMC, and that the lower weight loss with FK317 may be attributable to anti-cachectic activity. Cancer cachexia is a complicated syndrome characterized by severe weight loss, tissue wasting, anorexia and anemia.⁸⁻¹⁰⁾ Tissue wasting is mainly caused by the extensive depletion of skeletal muscle and adipose tissue. Cachectic symptoms cause a reduced quality of life in cancer patients, so that treatment of cancer cachexia is of great value.¹¹⁾ Therefore, it is extremely important to clarify whether FK317 shows an effective amelioration of cachectic symptoms. Among experimental models of cachexia, colon26 adenocarcinoma is the most important, since it fulfills the criteria of an early effect with a small tumor mass, and progressive wasting of skeletal muscle and adipose tissue.^{12,13)} In this study, we evaluated the effect of FK317 on weight loss of the host and on cachectic symptoms, as well as on tumor growth in murine adenocarcinoma colon26 and human lung carcinoma LX-1 models in mice, and compared the results with those for MMC.

MATERIALS AND METHODS

Drugs FK317 was synthesized in the laboratories of Fujisawa Pharmaceutical Co., Ltd. The chemical structure of FK317 is shown in Fig. 1. MMC was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo. FK317 and MMC

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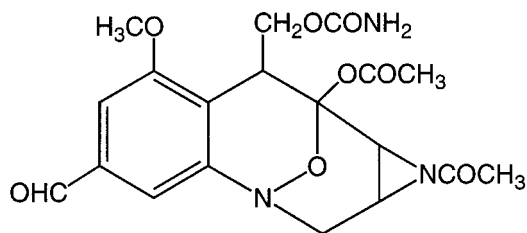


Fig. 1. The chemical structure of FK317.

were dissolved in saline and given i.v. to mice at a volume of 10 ml/kg of body weight.

Animals Male BALB/c mice were purchased from Charles River Japan Inc., Atsugi. Male BALB/c *nu/nu* mice were from CLEA Japan Inc., Tokyo. The mice were kept in conditions of constant temperature and humidity and fed a standard diet and water *ad libitum*.

Tumors Murine adenocarcinoma colon26 was maintained s.c. by 10-day serial passage in BALB/c mice. Human lung carcinoma LX-1 was also maintained s.c. by 21-day serial passage in BALB/c *nu/nu* mice.

Evaluation in colon26 model A fragment (2×2×2 mm) of colon26 tumor was implanted s.c. into the left flank of BALB/c mice. The drug was given i.v. to mice 3 times every 3 days, starting 1 day after tumor inoculation. The body and tumor weights were monitored. The tumor weight was calculated from caliper measurements of the length and width of the tumors, as follows;

$$\text{Tumor weight (mg)} = 1/2 \times a \times b^2$$

where *a* represents the length and *b* represents the width (mm).

Fourteen days after the tumor inoculation, the mice were anesthetized with diethyl ether and blood was collected by cardiac puncture. The epididymal fat and gastrocnemius muscle were removed and weighed. The remaining viscera and the tumors were removed and the carcass wet weight was measured. The carcass was then dried for 3 days in an oven at 80°C and the carcass dry weight was measured.

Serum was harvested after allowing the blood to clot at room temperature for 1 h. Serum was kept frozen until analysis. The levels of glucose, triglyceride, total cholesterol and non-esterified fatty acid (NEFA) were measured by a Chemical Analyzer, Model TBA-80FR (Toshiba, Tokyo).

Evaluation in LX-1 model A fragment (3×3×3 mm) of LX-1 tumor was implanted s.c. into the left flank of BALB/c *nu/nu* mice. The drug was given i.v. to mice 3 times every 3 days, starting 10 days after the tumor inoculation. The body and tumor weights were monitored. Twenty-eight days after the tumor inoculation, the weights

of the epididymal fat, gastrocnemius muscle and carcass dry weight were measured as described above.

Statistical analysis Analysis of variance was performed and Student's *t* test or Dunnett's test was used to determine the significance of differences.

RESULTS

Effect of FK317 and MMC in colon26 adenocarcinoma model

To examine the effect of FK317 and MMC in the colon26 model, three equal doses (1–3.2 mg/kg) of FK317 or MMC were given i.v. to mice on days 1, 4 and 7 after tumor inoculation. As shown in Fig. 2, the tumors grew gradually in the saline-treated mice, while both FK317 and MMC inhibited tumor growth in a dose-dependent manner. The anti-tumor effect of MMC against colon26 was 3 times more potent than that of FK317, when the effects were compared at the same dose. The body weight of saline-treated tumor-bearing mice did not differ from that of normal mice between days 1 and 7, but significantly decreased on day 11 after the inoculation of colon26 adenocarcinoma into the mice, and the decrease continued until the end of the experiment on day 14 (Fig. 3). At the lowest dose (1 mg/kg), FK317 had no effect on the decrease in body weight of the tumor-bearing mice, while the decrease in body weight was dose-dependently ameliorated on days 11 and 14 following FK317 treatment at 1.8 and 3.2 mg/kg. The effects of 1.8 and 3.2 mg/kg FK317 were statistically significant on day 14. On the other hand, treatment with 1.8 and 3.2 mg/kg MMC caused a severe decrease in body weight of the tumor-bearing mice on days 7 and 11, but this was partly reversed on day 14. Thus, the weight loss induced by FK317 was obviously less than that by MMC.

When the mice were killed on day 14, significant reductions in the weights of epididymal fat, gastrocnemius muscle and the dry carcass were observed in the tumor-bearing mice (Fig. 4), demonstrating that mice bearing colon26 became cachectic, in association with the tumor growth. Treatment with FK317 resulted in a dose-dependent attenuation of the reduction in the weights of epididymal fat, gastrocnemius muscle and the dry carcass. The attenuation of tissue wasting observed in 3.2 mg/kg FK317-treated mice was significant. MMC did not show significant attenuation of the tissue wasting induced by colon26 at any dose tested in this study.

Table I shows the percentage inhibition of the tumor weight and percentage restoration of body and tissue weight caused by treatment with FK317 or MMC, and allows us to compare the anti-tumor and anti-cachectic activities. Tumor weight inhibitions with 1.8 and 3.2 mg/kg FK317 were almost the same as those with 1.0 and 1.8 mg/kg MMC, respectively. However, the degrees of attenuation of cachectic symptoms with 1.8 and 3.2 mg/kg

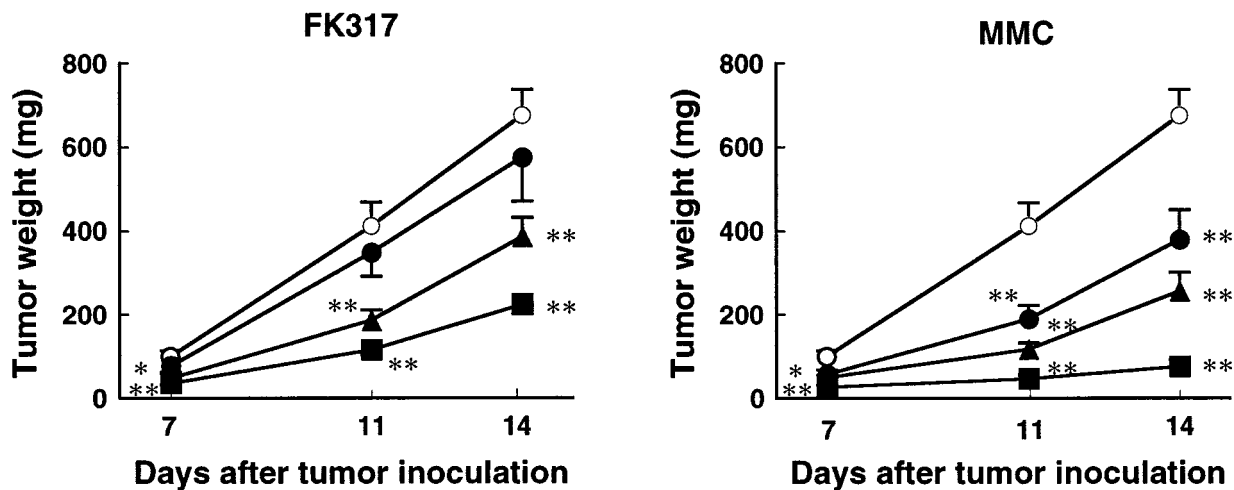


Fig. 2. Effects of FK317 and MMC on the growth of colon26. Colon26 was inoculated s.c. into mice. Mice bearing colon26 were treated with saline (○), 1 (●), 1.8 (▲) or 3.2 (■) mg/kg of FK317 or MMC i.v. 3 times every 3 days, starting 1 day after tumor inoculation. Mice were used in groups of 10. Each point represents the mean and SE. *, ** $P < 0.05$, $P < 0.01$, compared with saline-treated tumor-bearing mice (Dunnett's test).

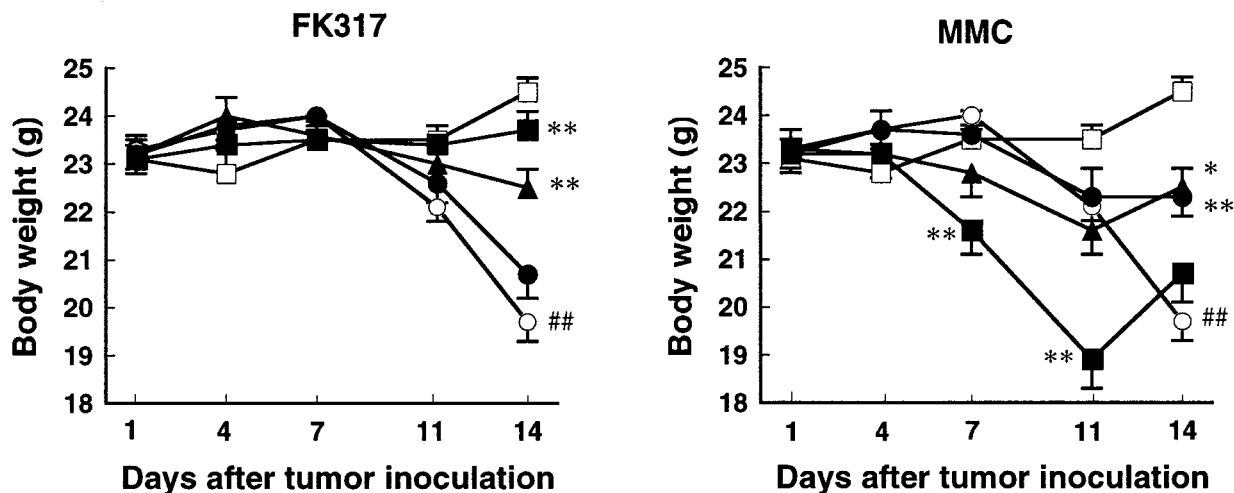


Fig. 3. Effect of FK317 and MMC on body weight of colon26-bearing mice. The method was described in the legend to Fig. 2. Mice bearing colon26 were treated i.v. with saline (○), 1 (●), 1.8 (▲) or 3.2 (■) mg/kg of FK317 or MMC. Normal mice were treated i.v. with saline (□). Mice were used in groups of 10. Each point represents the mean and SE. ## $P < 0.01$, compared with saline-treated normal mice (Student's *t* test). *, ** $P < 0.05$, $P < 0.01$, compared with saline-treated tumor-bearing mice (Dunnett's test).

FK317 were greater than those with 1.0 and 1.8 mg/kg MMC, respectively. Treatment with 3.2 mg/kg MMC was highly effective for inhibiting the growth of colon26 in mice, but worsened the cachectic symptoms. Thus, FK317 was effective in reducing the cachexia induced by colon26 in mice, in contrast to MMC, when administered i.v.

These results suggest that FK317 is an anti-cancer drug with anti-cachectic activity, whereas MMC is not. **Effect of FK317 on blood parameters in mice bearing colon26** Blood parameters in normal and tumor-bearing mice were also measured on day 14 after tumor inoculation, and the results are shown in Table II. Tumor-bearing

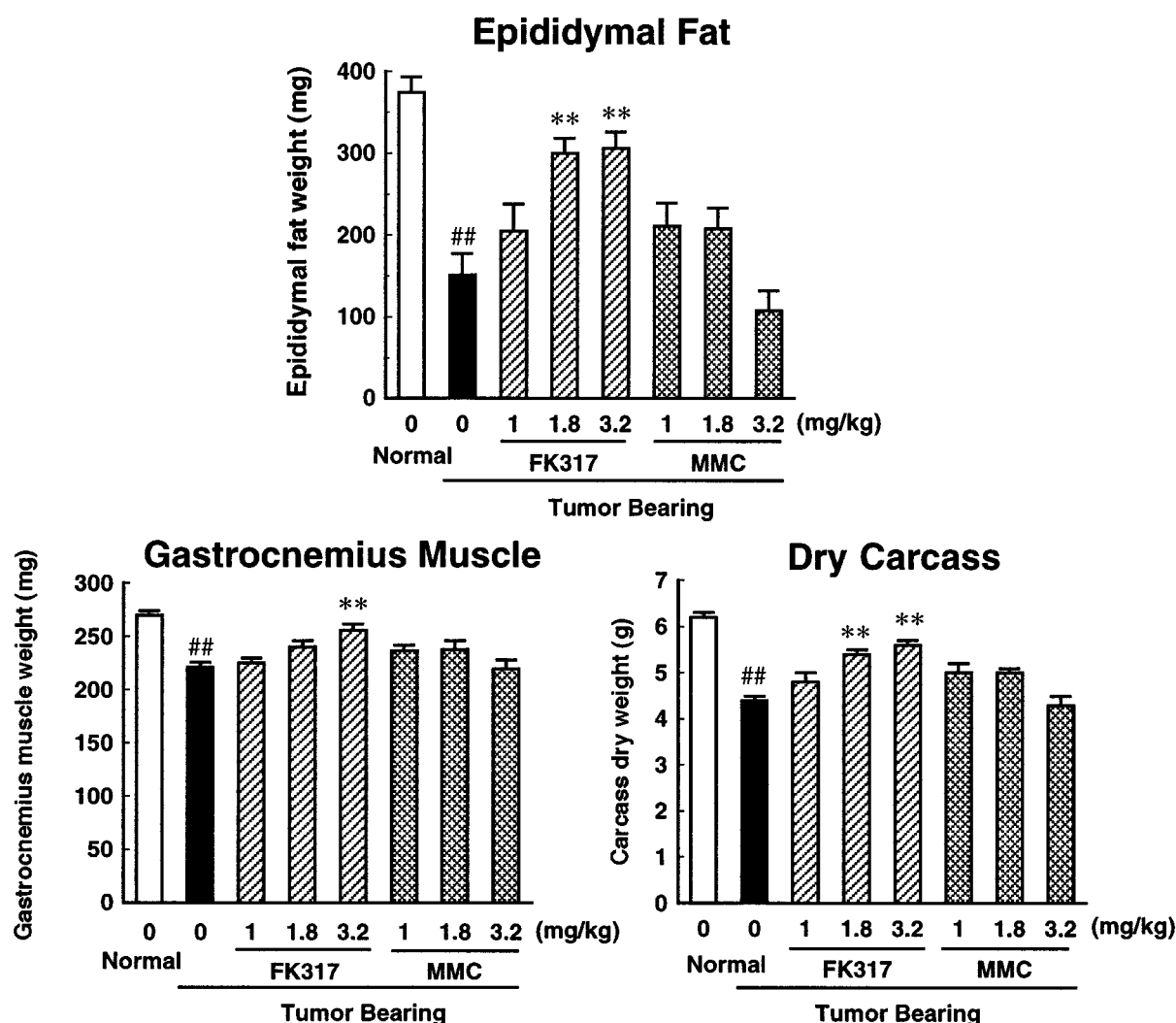


Fig. 4. Effects of FK317 and MMC on the weights of epididymal fat, gastrocnemius muscle and dry carcass of mice bearing colon26. The method was described in the legend to Fig. 2. Mice were used in groups of 10. Each column represents the mean and SE. ^{##} $P < 0.01$, compared with saline-treated normal mice (Student's t test). ^{**} $P < 0.01$, compared with saline-treated tumor-bearing mice (Dunnett's test).

Table I. Effects of FK317 and MMC on Colon26-induced Cachexia in Mice

| Drug | mg/kg | Tumor weight | Body weight | Epididymal fat weight | Gastrocnemius muscle weight | Carcass dry weight |
|-------|-------|------------------|------------------|-----------------------|-----------------------------|--------------------|
| | | % Inhibition | % Restoration | | | |
| FK317 | 1.0 | 15 | 21 | 24 | 8 | 22 |
| | 1.8 | 43 ^{**} | 58 ^{**} | 67 ^{**} | 39 | 56 ^{**} |
| | 3.2 | 67 ^{**} | 83 ^{**} | 70 ^{**} | 71 ^{**} | 67 ^{**} |
| MMC | 1.0 | 44 ^{**} | 54 ^{**} | 27 | 33 | 33 |
| | 1.8 | 62 ^{**} | 58 ^{**} | 26 | 35 | 33 |
| | 3.2 | 89 ^{**} | 21 | -20 | -2 | -6 |

The method is described in the legend to Fig. 2. The percentage restoration is a measure of the degree to which the colon26-induced cachexia symptoms were reversed to the saline-treated normal values by treatment with FK317 or MMC. Mice were used in groups of 10. ^{**} $P < 0.01$, compared with the saline-treated tumor-bearing mice (Dunnett's test).

Table II. Effect of FK317 on Blood Parameters of Colon26-bearing Mice

| Treatment | Glucose (mg/dl) | Triglyceride (mg/dl) | Total cholesterol (mg/dl) | NEFA (mEq/liter) |
|-----------------------|----------------------|------------------------|---------------------------|-------------------------|
| Saline (normal) | 247±18 | 91.3±14.3 | 103±5 | 2.21±0.14 |
| Saline (TB) | 141±12 ^{##} | 58.4±8.7 ^{##} | 122±7 [#] | 1.39±0.14 ^{##} |
| FK317: 1 mg/kg (TB) | 147±13 | 55.1±6.4 | 108±6 | 1.27±0.10 |
| FK317: 3.2 mg/kg (TB) | 179±10 ^{**} | 87.6±8.7 [*] | 97±5 ^{**} | 1.64±0.13 |

The method is described in the legend to Fig. 2. Mice were used in groups of 10. Data are expressed as the mean and SE. #, ## $P < 0.05$, $P < 0.01$, compared with the saline-treated normal mice (Student's *t* test). *, ** $P < 0.05$, $P < 0.01$, compared with the saline-treated tumor-bearing (TB) mice (Dunnett's test).

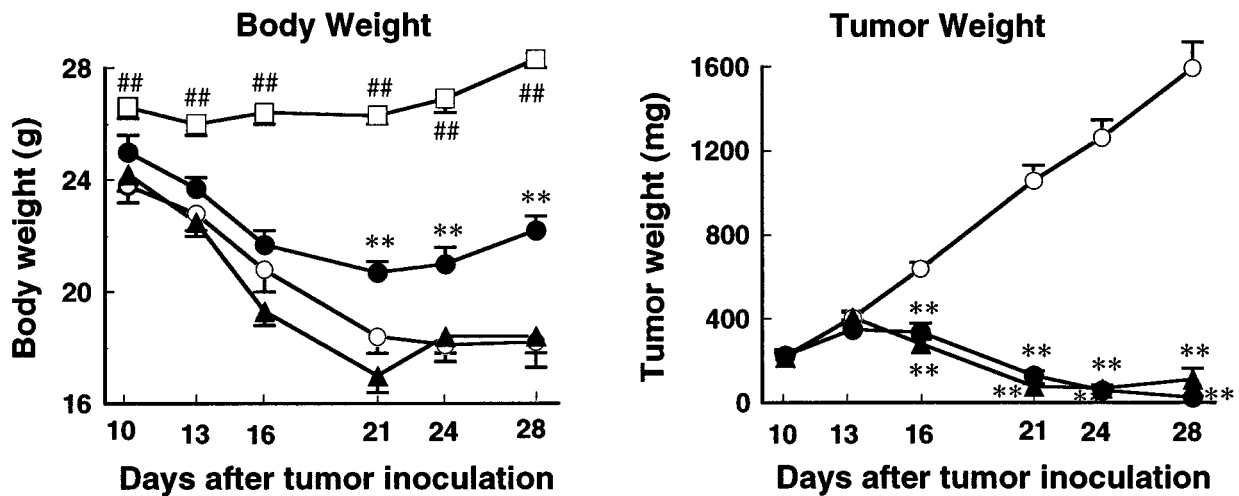


Fig. 5. Effects of FK317 and MMC on the body weight of LX-1-bearing nude mice and the growth of LX-1. LX-1 was inoculated s.c. into mice. Mice bearing LX-1 were treated i.v. with saline (○), 5.6 mg/kg of FK317 (●) or 3.2 mg/kg of MMC (▲) 3 times every 3 days, starting 10 days after the tumor inoculation. Normal mice were treated i.v. with saline (□). Mice were used in groups of 6. Each point represents the mean and SE. ## $P < 0.01$, compared with saline-treated tumor-bearing mice (Student's *t* test). ** $P < 0.01$, compared with saline-treated tumor-bearing mice (Dunnett's test).

mice showed a decrease in the levels of glucose, triglyceride and NEFA, and an increase in total cholesterol, compared to the control mice. Two doses (1 and 3.2 mg/kg) of FK317, ineffective and effective at inhibiting cachexia in this model, respectively, were given to mice 3 times every 3 days, starting 1 day after tumor inoculation. The levels of these four parameters were unchanged in the treatment with 1 mg/kg of FK317. However, following treatment with 3.2 mg/kg of FK317 the levels of these parameters were partially or completely restored to those of normal mice. The restoration of the levels of glucose, triglyceride and total cholesterol was significant. Thus, the attenuation by FK317 of the cachectic symptoms induced by colon26 was accompanied by restoration of blood parameters in mice.

Effects of FK317 and MMC in the LX-1 model in nude mice In the next experiment, 5.6 mg/kg of FK317 or 3.2 mg/kg of MMC was given i.v. to mice 3 times every 3 days, starting 10 days after tumor inoculation. The doses selected in this study were highly effective at inhibiting the growth of MX-1 and LX-1 in nude mice in our previous experiments,⁷ and the maximum tolerable dose of FK317 was estimated as 5.6 mg/kg in nude mice. As shown in Fig. 5, both FK317 and MMC completely inhibited tumor growth until the end of the experiment on day 28, and the anti-tumor activity of FK317 against LX-1 was almost the same as that of MMC. Body weight of vehicle-treated tumor-bearing mice gradually decreased until day 21 and thereafter remained constant. Body weights of drug-treated tumor-bearing mice also decreased

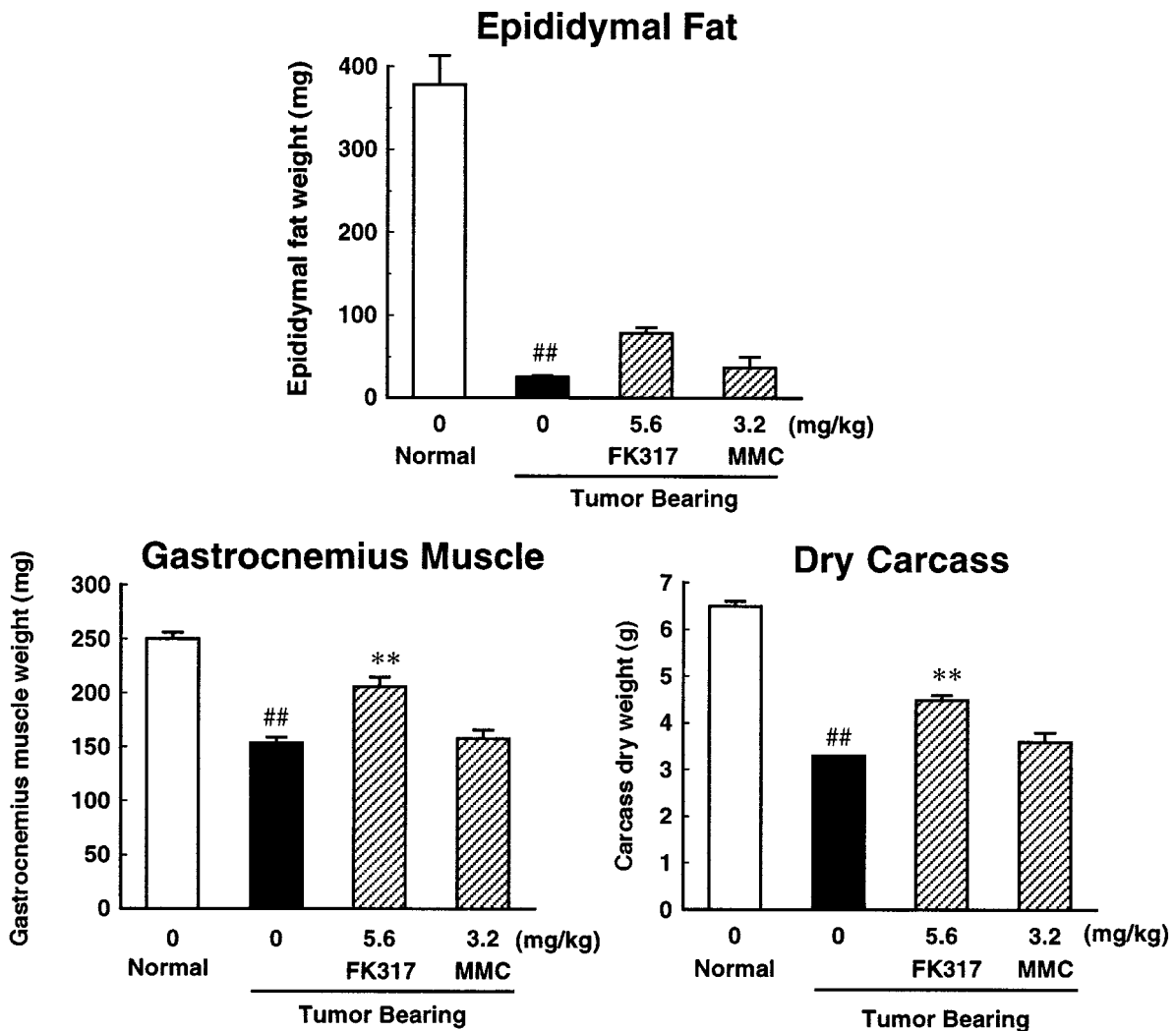


Fig. 6. Effects of FK317 and MMC on the weights of epididymal fat, gastrocnemius muscle and dry carcass of mice bearing LX-1. The method was described in the legend to Fig. 5. Mice were used in groups of 6. Each column represents the mean and SE. ^{##} $P < 0.01$, compared with saline-treated normal mice (Student's t test). ^{**} $P < 0.01$, compared with saline-treated tumor-bearing mice (Dunnett's test).

during the drug treatment period between days 10 and 16. After the completion of drug treatment, the body weight was constant or slightly increased in the FK317-treated tumor-bearing mice, while a further decrease in body weight was observed in the MMC-treated tumor-bearing mice on days 16 and 21, but some recovery was seen on days 24 and 28. Body weights of FK317-treated mice on days 21, 24 and 28 were significantly higher than those of vehicle-treated tumor-bearing mice. Thus, weight loss induced by FK317 was obviously less than that by MMC.

When mice were killed on day 28, significant reductions in the weights of epididymal fat, gastrocnemius

muscle and the dry carcass were observed in LX-1-bearing nude mice (Fig. 6), indicating a cachectic effect, associated with tumor growth. Treatment with FK317 resulted in a significant attenuation of weight reduction in the gastrocnemius muscle and dry carcass, but the weight of epididymal fat was not restored following FK317 treatment. MMC did not show any effective attenuation of the tissue wasting induced by LX-1. Thus, FK317 was effective for reducing cachectic symptoms induced by human lung carcinoma LX-1 in nude mice, in contrast to MMC, suggesting that FK317 shows an anti-cachectic activity in the LX-1 model, but MMC does not.

DISCUSSION

Our present study revealed a beneficial effect of FK317 on weight loss of the host and cachectic symptoms, as well as on tumor growth, in the colon26 and LX-1 models in mice, compared to those of MMC. Both drugs inhibited the growth of colon26 and LX-1 in mice (Figs. 2 and 5), suggesting that FK317 is a potent anti-cancer drug, similar to MMC. Nevertheless, the weight loss induced by FK317 was obviously less than that induced by MMC in both the colon26 and LX-1 tumor models (Figs. 3 and 5). Almost all anti-cancer drugs currently used induce weight loss in the host, and this in turn often interferes with therapy. Therefore, FK317 seems to be a promising anti-cancer drug candidate.

To understand further why FK317 did not induce weight loss in the host, the anti-cachectic effect of FK317 was next investigated in the murine adenocarcinoma colon26 model, and compared with that of MMC. Colon26 is an undifferentiated tumor induced by the carcinogen N-nitroso-N-methylurethan, and mice bearing colon26 develop progressive body weight loss and tissue wasting, as previously reported.^{12,13} Therefore, colon26 is a suitable model to evaluate the anti-cachectic effect of a drug. It is of particular importance that treatment with FK317 resulted in alleviation of cachectic symptoms induced by colon26, whereas treatment with MMC did not (Figs. 3 and 4, Table II). Human lung carcinoma LX-1 was also used as a second model in this study; it is often used as a xenograft in nude mice to evaluate the anti-tumor activity of drugs.^{14,15} Although it is not generally recognized as a cachexia model, our present study demonstrated that nude mice with transplanted LX-1 lung carcinoma showed severe weight loss, associated with tumor growth (Fig. 5), and that cachectic symptoms, such as a reduction in the weights of epididymal fat, gastrocnemius muscle and carcass, appeared (Fig. 6). This indicates that human lung carcinoma LX-1 became cachectic, in association with tumor growth, in mice. In tumor-bearing mice, FK317 partially attenuated the decrease in the weights of body and the epididymal fat, gastrocnemius muscle and carcass, but MMC did not (Figs. 5 and 6). The results obtained in the two tumor models suggest that FK317, a novel anti-cancer drug, shows an anti-cachectic activity. Cancer cachexia is a serious clinical problem.⁸⁻¹⁰ Anti-cancer therapies including chemotherapy and radiation are usually focused on treatment of the tumor itself, but treatment of cancer cachexia is also important, since the exhausted state of cancer patients with cachexia is not only an obstacle to anti-cancer therapy, but also reduces

the quality of life.^{8,11} Therefore, inhibition of cachectic symptoms is likely to be extremely beneficial. Accordingly, FK317 may be useful in the treatment of cancer patients.

It is not yet clear why FK317 alleviates cachectic symptoms. In the case of colon26, it has been suggested that interleukin (IL)-6 derived from the tumor plays an important role in induction of cachexia in mice.¹⁶⁻¹⁸ Furthermore, we have recently indicated that a cytokine-suppressive agent, and synthetic double-stranded oligodeoxynucleotides targeting the transcriptional factor NF- κ B that binds *cis*-elements, inhibit cachexia induced by colon26 in mice, suggesting that cachexia can be attenuated by inhibiting the action of IL-6.^{19,20} Therefore, it is possible that FK317 selectively inhibits the growth of the tumor, which produces IL-6 at a high level, thereby leading to a reduction in circulating IL-6, and alleviation of cachexia. However, it is not clear whether the inhibition of LX-1-induced cachexia by FK317 in nude mice can be explained by selective inhibition of IL-6 producing tumors, since there are no data on the involvement of IL-6 in the LX-1 nude mice model. Another possible explanation is that FK317 may be a prodrug which is activated only in the tumors. This idea may be partially supported by our recent observations that cytotoxic, active metabolites of FK317 were more abundant in tumors, as compared to other normal tissues such as liver and kidney, while the concentration of non-cytotoxic, inactive ones was higher in various normal tissues than in tumors,²¹ thereby resulting in effective tumor inhibition and no cachexia symptoms. Thus, we speculate that a difference in the metabolic pathways of FK317 in tumors and normal tissues contributes to its potent anti-tumor activity without weight loss.

In conclusion, the present study demonstrated that weight loss with FK317 was less than with MMC in both colon26 and LX-1 models, and also that the cachectic symptoms induced by the presence of these tumors in mice were alleviated by FK317, but not by MMC, suggesting that FK317 is a potent anti-cancer drug with anti-cachectic activity and thus is a promising candidate for use in the treatment of cancer.

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