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Novel lowly immunosuppressive antitumor fluorouridine derivative, UK-21: antitumor activity and effect on humoral immune response in mice

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Abstract. Our previous studies indicated that a newly synthesized 5-fluorouridine derivative, 2',3',5'- tris-*O*-[*N*-(2-*n*-propyl-*n*-pentanoyl)glycyl]-5-fluorouridine (UK-21), revealed its antitumor activity by being converted to 5-fluorouridine (5-FUR) and showed a low level of immunological side effects. However, the bioavailability of UK-21 given orally did not seem to be good. In the present study, we focused on the antitumor and immunosuppressive activities of UK-21 given i.p. to mice. UK-21 suppressed the growth of L-1210, P388 and EL4 leukemias inoculated i.v. into corresponding syngeneic mice and both the growth of Lewis lung carcinoma transplanted s.c. and its subsequent metastasis to the lung. UK-21 showed antitumor activity at doses almost 10 times lower than those of 5-fluorouracil (5-FU). The side effects of UK-21, especially on immune functions, were examined in comparison with those of 5-FUR, 5-FU, and cyclophosphamide (CY) at doses producing comparable antitumor activity. The suppressive effect of UK-21 on IgM and IgG antibody formation in mice immunized with ovalbumin was clearly weaker than that of 5-FUR, 5-FU, and CY. The suppressive effect of UK-21 on thymus weight was markedly weaker than that of 5-FU and CY. The reduction of WBC counts induced by UK-21 was also lower than that produced by any other agent. The results reported herein suggest the strong possibility of UK-21 being developed as a novel anticancer drug with cytotoxic mechanisms different from those of 5-FU. Our study also points to the chemical modification of 5-FUR as a feasible way of developing new anticancer drugs.

Key words: 5-Fluorouridine derivative – Humoral immune response – Side effect

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Introduction

It is well known that antitumor drugs such as antimetabolites and alkylating agents cause various kinds of side effects, including immunosuppression, injury to bone marrow, and dysfunction of the digestive system. The immunosuppression and injury to bone marrow induced by drugs reduces not only the immunological defense function against microorganisms but also the immunological resistance against tumors. Such suppressive activities of antitumor drugs are paradoxical and critical side effects from the viewpoint of the therapeutic efficacy of the drugs.

Previously, we reported that α-mercaptopropionylglycine and sodium dipropylacetate showed antitumor action through their immunostimulating activity [4, 6–8] and that a related compound, (2-*n*-propyl-*n*-pentanoyl)glycine (KN-539), had host-dependent antitumor activity [9]. Thereafter, in our attempt to search for less immunosuppressive antitumor agents [12–14], we found that 2',3',5'-tris-*O*-[*N*-(2-*n*-propyl-*n*-pentanoyl)glycyl]-5-fluorouridine (UK-21), a conjugate of 5-fluorouridine (5-FUR) and KN-539, and 1-{6-[*N*-(2-*n*-propyl-*n*-pentanoyl)glycyl]amino-*n*-hexylcarbamoyl}-5-fluorouracil (UK-25), a conjugate of 5-fluorouracil (5-FU) and KN-539, exerted potent antitumor activity with a lower level of immunosuppressive side effects.

5-FU is widely used for many types of cancer. It is well known that a metabolite of 5-FU, 5-fluorodeoxyuridine monophosphate (5-FdUMP), effects its antitumor activity mainly through competitive antimetabolic action against thymidylate synthetase [15]. The role of another metabolite of 5-FU, 5-fluorouridine triphosphate (5-FUTP), has been also pointed out as one of the mechanisms of the cytocidal action of 5-FU [1, 3, 5]. Kanamura et al. [3] found that the amount of 5-FU incorporated into the RNA of L1210 cells showed a good correlation with its cytocidal action. Moreover, Akazawa et al. [1] demonstrated that the cytocidal activity of 5-FU occurred through the drug's incorporation into RNA rather than via inhibition of DNA synthesis in their experiment using mutant mouse cells lacking thymidylate synthetase. 5-FUTP might play a

greater role than 5-FdUMP in the therapeutic efficacy of 5-FU against some cancers.

Our previous study suggested that UK-21 revealed its antitumor activity without being converted to 5-FU, in contrast to UK-25. UK-21 suppressed the proliferation of KB tumor cells in vitro [4 day's culture; 50% inhibitory dose(IC_{50}), 3×10^{-11} *M*] at concentrations comparable with those of 5-FUR and more than 1,000 times lower than those of 5-FU (IC_{50}, 1.8×10^{-7} *M*) [12]. It was thought that UK-21 exerted its antitumor activity by being converted to 5-FUR and then to 5-FUTP [12]. It is known that 5-FUR has strong cytocidal activity, but it has not been used clinically because of its serious side effects. Untoward effects including immunosuppression, decreased peripheral leukocyte counts and reduced thymus weights have been found to be low following the administration of UK-21 in comparison with 5-FUR and even other anticancer drugs in clinical use such as 5-FU and tegafur [12–14].

UK-21 could be expected to be developed as a novel anticancer drug having cytotoxic mechanisms different from those of 5-FU. However, our previous study [14] suggested that the bioavailability of UK-21 given orally was not so good; BALB/c mice were transplanted s.c. with 1×10^6 Meth A cells and given antitumor agents including UK-21, 5-FU, carmofur, and tegafur either i.p. or orally for 10 consecutive days starting on the day of tumor transplantation. The 50% suppressive dose (ED_{50}) of i.p. UK-21, which was calculated from the tumor size on day 10 by the probit method, was 0.015 mmol/kg. On the other hand, the ED_{50} value for oral UK-21 was 0.19 mmol/kg. The ratio of i.p. ED_{50}:oral ED_{50} was 1:12.7. The corresponding ratios found for 5-FUR (0.01:0.029 mmol/kg), carmofur (0.053:0.15 mmol/kg), and 5-FU (0.047:0.15 mmol/kg) were about 1:3, and that noted for tegafur (0.304:0.51 mmol/kg) was 1:1.7. Thus, UK-21 seems to be inferior to the other drugs in terms of oral absorbability. However, it is also possible that the initial bioinactivation of UK-21 in the liver contributes to its poor bioavailability after oral administration.

In the present study, we focused on the antitumor and immunosuppressive activities of UK-21 given i.p. to mice.

Materials and methods

Agents. UK-21 (Fig. 1; mol wt., 812 Da) was synthesized at Ube Laboratories of Ube Industries, Ltd. (Ube, Japan) as follows. First, 9.01 g of dicyclohexylcarbodiimide was added to a suspension of 8.86 g of *N*-(2-*n*-propyl-*n*-pentanoyl)glycine in methylene chloride (380 ml) under cooling on ice and stirring. Then, 1.63 g of 5-FUR, 4.45 g of triethylamine, and 1.63 g of 4-(*N*,*N*-dimethylamino)pyridine were added to the mixture, which was stirred at room temperature for 4 days. Thereafter, the reaction mixture was condensed under reduced pressure and mixed with ethyl acetate. The insolubles were filtered off and the filtrate was condensed under reduced pressure. The residue was separated by silica-gel column chromatography with an eluent consisting of chloroform and methanol (95:5, v/v) to give 4.62 g (yield, 43%) of UK-21 (melting point, 94° C–97° C). The elemental analysis of UK-21 (C, 57.09%; H, 7.72%; N, 8.59%) was in good agreement with the molecular formula $C_{39}H_{62}FN_5O_{12} \cdot 1/2\ H_2O$. [^{1}H]-Nuclear magnetic resonance ([^{1}H]-NMR) and mass spectra supported the structure shown in Fig. 1. 5-FUR (mol. wt., 262 Da) was purchased

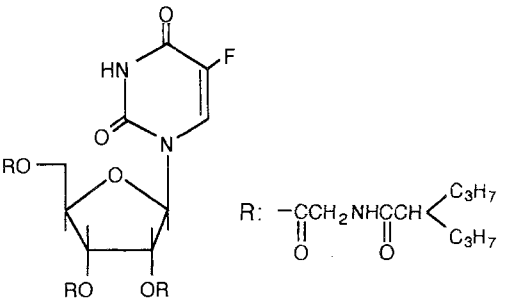

Fig. 1. Chemical structure of UK-21

from Sigma Chemical Co. (St. Louis, Mo., USA). 5-FU (mol. wt., 130 Da) and CY (mol. wt., 262 Da) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Because UK-21 is only slightly soluble in water and it is quite difficult to suspend UK-21 in saline even by using a viscosity-increasing agent such as carboxymethylcellulose sodium (CMC), UK-21 was dissolved in and the other agents were dissolved/suspended in olive oil (Tokai Pharmaceutical Co., Ltd., Nagoya, Japan) for i.p. administration in a volume of 0.2 ml/mouse at an appropriate concentration for the defined dose. A homogenizer (27,000 rpm; Polytron, PTA7 K, Kinematica, Central Kagaku-boueki Inc., Tokyo, Japan) was used to make the fine solution or suspension.

Animals. Female C57BL/6, (BLAB/c × DBA/2)F_1(CDF_1), DBA/2, and ddY mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and used at an age of 7–9 weeks. The mice were maintained with free access to solid rodent chow and water in filtered laminar air-flow isolation cages at 21° C ± 1° C and 60% ± 5% relative humidity.

Tumors. The murine ascitic tumors were maintained by serial i.p. transplantation of an appropriate number of cells every week into the respective syngeneic hosts: 1×10^5 L1210 leukemia cells as well as 1×10^6 P388 leukemia cells were transplanted into DBA/2 mice, and 1×10^6 EL4 T-lymphoma cells were transplanted into C57BL/6 mice. Lewis lung carcinoma (LLC) was maintained as a solid tumor by serial i.m. transplantation of 5×10^6 cells biweekly into the femoral region of C57BL/6 mice. The ascitic tumors were washed well with Hanks' balanced salt solution by centrifugation prior to the transplantation. The viability of the cells as examined by trypan blue dye-exclusion assay was routinely more than 98%. The tumor tissue of LLC growing in the muscle of the femoral region was dissected and cut into fine pieces for preparation of a cell suspension by gentle mechanical means using a glass homogenizer containing 10 ml of RPMI-1640 medium. The cell suspension was passed through a 200-mesh sieve. The viability of the cells as examined by trypan blue dye-exclusion assay routinely ranged from 45% to 60%.

Transplantation i.v. of L1210, P388, and EL4 tumors. L1210 and P388 tumors were transplanted i.v. into CDF_1 mice through the tail vein in numbers of 2×10^3 and 2×10^6 cells, respectively; 2×10^5 EL4 tumor cells were transplanted into C57BL/6 mice. The duration of survival of the host mice was recorded.

Transplantation s.c. of LLC. C57BL/6 mice were injected s.c. with 2×10^5 viable LLC cells into the right hind footpad. The growth of the tumor in the subcutis was evaluated as the volume of the paw measured by a plethysmometer (model TK-101, Unicom Co., Yachiyo, Japan) on days 10, 15, and 20. Mice were killed by bleeding while under deep ether anesthesia on day 21. Then, the lungs were removed and fixed with 10% buffered formalin for counting of the macroscopic metastatic nodules on the surface.

Transplantation i.v. of LLC. C57BL/6 mice were injected i.v. with 1×10^6 viable LLC cells. At 14 days after the transplantation, the macroscopic metastatic nodules on the pulmonary surface were counted as described above.

Table 1. Effect of UK-21 on survival duration in mice transplanted i.v. with L1210, P388, or EL4 tumors

		Survival duration								
		L1210			P388			EL4		
	mmol/kg	*n*	Days	% increase	*n*	Days	% increase	*n*	Days	% increase
Control		8	8.3±0.2	–	7	8.0±0.0	–	9	17.1±0.5	–
UK-21	0.005	8	10.1±0.2**	21.7	8	8.5±0.2*	6.2	9	17.2±0.4	0.6
	0.01	8	12.0±0.0**	44.6	8	9.0±0.2**	12.5	9	19.1±0.6*	11.7
	0.02	8	16.8±1.0**	102.4	8	11.0±0.5**	37.5	9	23.1±0.6**	35.1
5-FUR	0.005	8	9.9±1.0**	19.3	8	8.3±0.2	3.8	8	18.0±0.6	7.0
	0.01	8	12.9±0.7**	55.4	8	8.5±0.2	6.2	8	19.6±0.4**	15.8
	0.02	8	9.1±0.4	9.6	8	9.9±0.3**	23.7	8	16.2±1.9	–5.3
5-FU	0.1	8	10.4±0.2**	25.3	8	9.1±0.3**	13.7	8	18.3±0.4	7.0
	0.2	8	13.0±0.5**	56.6	8	11.5±0.6**	43.8	8	19.8±0.9**	15.8
	0.3	8	12.5±1.2**	50.6	8	12.3±0.6**	53.8	8	21.5±0.4**	26.3
CY	0.15	7	16.7±0.5**	101.2	7	35.3±3.3**	341.2	8	24.6±1.0**	43.9

Female CDF_1 mice were transplanted i.v. with 2×10^3 L1210 cells or 2×10^6 P388 cells. Female C57BL/6 mice were transplanted i.v. with 2×10^5 EL4 cells. The examined agents were given i.p. for 10 consecutive days from the day of transplantation. Data represent mean values ± SE. *n*, number of mice

* $P \leq 0.05$; ** $P \leq 0.01$ vs corresponding control values (ranked multiple-range test)

Immunization with ovalbumin and titration of the antibody in the serum. ddY mice were immunized s.c. with a 0.2-ml emulsion prepared from equal amounts of 10 mg of ovalbumin/ml (Seikagaku kogyo Co., Ltd., Tokyo, Japan) dissolved in saline and Freund's complete adjuvant (Nacalai Tesque, Inc., Kyoto, Japan). Blood was collected from the retroorbital sinus plexus of the mice at 4, 9, and 14 days after the immunization for assay of the WBC count and of the antibody titer in the serum. Mice were killed on day 15 for measurements of thymus and spleen weights.

The serum separated from the blood was subjected to enzyme-linked immunosorbent assay (ELISA) to titrate the anti-ovalbumin IgM and IgG antibodies. General procedures of the ELISA have been described elsewhere [10, 11]. Microtiter plates with 96 flat wells (Immunoplate-1, A/S Nunc Raskikde, Denmark) were coated at 1 μg/100 μl per well with ovalbumin diluted in 0.05 *M* sodium carbonate buffer (pH 9.6) for 1 h at 37° C and then overnight at 4° C. The coated wells were blocked for 1 h at 37° C with phosphate-buffered saline (PBS, pH 7.4) containing 5% normal calf serum, 1% bovine serum albumin, 0.1% Tween-20, and 0.1% NaN_3 (protein-blocking buffer, pH 7.4). Wells were then incubated with 0.1 ml of serum samples diluted with the protein-blocking buffer for 2 h at 37° C. Serum was diluted at 1:100 to titrate the IgM antibody and at 1:1000 to titrate the IgG antibody. The secondary antibodies were horseradish peroxidase-conjugated affinity-purified goat antibodies to mouse IgM (PO-GaM IgM) and IgG (PO-GaM IgG; Cappel, Organon Teknika Co., West Chester, Pa., USA). PO-GaM IgM diluted at 1:5,000 and PO-GaM diluted at 1:2,000 with protein-blocking buffer (not containing NaN_3) were incubated in wells for 1 h at 37° C.

After the standard enzymatic reaction using *o*-phenylenediamine as a substrate (30 min at 37° C), the optical densities (OD: sample, 492 nm; reference, 690 nm) were measured with an immunoreader (Titertek Multiskan MCC/340, Flow Laboratories, Virginia, USA). All tests were carried out in duplicate. The OD value obtained at the defined condition was considered as the antibody titer.

To determine the WBC count, 20 μl of the blood was mixed with 10 ml of Isoton II and 3 drops of Zap-oglobin II (Coulter Scientific Japan Co., Ltd., Tokyo, Japan). An autohemocytometer (Model MEK-3100, Nihon Kohden kogyo Co., Ltd., Tokyo, Japan) was used for the counting.

Statistical analysis. The results were expressed as mean values ± SE. The data for survival duration (see Table 1) and tumor size (see Table 2) were ranked and subjected to Duncan's multiple-range test (ranked multiple-range test) to analyze the significance of differences between two groups. The dose dependency of drug effects in these data was analyzed by the Kruskal-Wallis test. The significance of differences between the control groups and the other groups in the data for metastasis (see Tables 2, 3) and in the data obtained from mice immunized with ovalbumin (see Figs. 2–6) was analyzed by Student's or Welch's two tailed *t*-test after the *F*-test had been applied to examine the homogeneity of differences between the two groups being compared ($P \leq 0.05$). Two-way analysis of variance (ANOVA) was used to assess the statistical significance of differences between UK-21 and 5-FU in terms of their suppressive effects on IgM or IgG antibody formation (Figs. 3, 4). A value of $P \leq 0.05$ was considered to indicate a significant difference in all statistical analyses.

Results

Antitumor activity of UK-21, 5-FUR, 5-FU, and CY against i.v. transplanted L1210, P388 and EL4 tumors

The antitumor activity of the agents was examined against L1210, P388, and EL4 tumors transplanted i.v. as described in Materials and methods. The agents examined were given i.p. for 10 consecutive days from the day of tumor transplantation and the duration of survival of the mice was recorded (Table 1).

In mice transplanted with L1210 tumor, UK-21 at 0.02 mmol/kg significantly prolonged the survival to almost the same extent as CY at 0.15 mmol/kg and to a greater extent than 5-FUR or 5-FU at any dose (ranked multiple-range test). The prolongation of survival by 5-FUR and 5-FU diminished at the highest dose as compared with the intermediate dose, probably because of their toxic side effects.

In mice transplanted with P388 or EL4 cells, CY showed the most potent activity among the agents tested in prolonging the survival of the mice, and UK-21 showed activity comparable with that of 5-FU.

Table 2. Effect of UK-21, 5-FUR, and 5-FU on the growth of LLC transplanted s.c. and its pulmonary metastasis in C57BL/6 mice

		Tumor size (mm^3)						Metastasis[a]		
		Day 10[b]			Day 20[b]			Day 21[b]		
	mmol/kg	*n*		%	*n*		%	*n*		%
Control		10	58±14	100	10	1,406±182	100	10	19±5	100
UK-21	0.005	8	47±10	82	7	1,097±144	78	7	9±4	49
	0.01	8	35± 9	60	8	686±139**	49	8	5±2*	24
	0.02	8	26± 9*	44	4	659±300*	47	4	3±1*	13
5-FUR	0.005	8	73±12	126	8	1,862±177	132	8	15±6	79
	0.01	8	18± 5**	32	8	428±113**	30	7	2±1*	11
	0.02	8	8± 4**	14	0	Dead		0	Dead	
5-FU	0.05	8	75±25	130	8	1,347±226	96	8	21±8	111
	0.1	8	48± 9	83	8	1,354±236	96	8	7±2*	35
	0.2	8	21± 5*	37	7	363±143**	26	7	5±1*	26

Mice were transplanted s.c. with 2×10^5 LLC cells. The examined agents were given i.p. for 10 consecutive days from the day of transplantation. Data represent mean values ± SE. *n*, Number of mice.
* $P \leq 0.05$; ** $P \leq 0.01$ vs corresponding control values (ranked multiple-range test for tumor size, two-tailed *t*-test for metastasis)
[a] Numer of surface nodules
[b] Days after tumor transplantation

Table 3. Effect of UK-21 and 5-FU on the pulmonary metastasis of LLC transplanted i.v. in C57BL/6 mice

	mmol/kg	*n*	Metastasis[a]	%
Control		7	108± 9	100
UK-21	0.005	6	108±15	100
	0.01	5	72±13*	67
5-FU	0.1	6	107±14	99
	0.2	5	68±15*	63

Mice were transplanted i.v. with 10^6 LLC cells. The examined agents were given i.p. for 8 consecutive days from the day of transplantation. Data represent mean values ± SE. *n*, Number of mice
* $P \leq 0.05$ vs control values (two-tailed *t*-test)
[a] Number of surface nodules at 14 days after tumor transplantation

Antitumor effect of UK-21, 5-FUR, and 5-FU against LLC transplanted s.c. and its pulmonary metastasis

Mice were injected s.c. with LLC cells and the agents were given i.p. for 10 consecutive days from the day of transplantation (Table 2).

UK-21 suppressed the tumor growth dose-dependently on days 10 and 20, although the difference did not reach statistical significance among the dosed groups (Kruskal-Wallis test), and decreased the numbers of metastatic nodules, even at the lowest dose of 0.005 mmol/kg. However, four of eight mice given 0.02 mmol/kg of UK-21 were dead by day 20. 5-FUR suppressed the growth and metastasis of tumors at the dose of 0.01 mmol/kg, but all mice given 0.02 mmol/kg of 5-FUR were dead by day 20. 5-FU suppressed the tumor growth on days 10 and 20 at 0.2 mmol/kg and suppressed the metastasis at 0.1 and 0.2 mmol/kg. The tumor sizes measured in mice treated with 0.02 mmol/kg of UK-21 (26 ± 9 mm^3 on day 10 and 659 ± 300 mm^3 on day 20) were smaller than those measured in mice treated with 0.1 mmol/kg of 5-FU (48 ± 9 and 1,354 ± 236 mm^3), with the differences being statistically significant ($P < 0.05$, ranked multiple-range test), and were larger than those measured in mice treated with 0.2 mmol/kg of 5-FU (21 ± 5 and 363 ± 143 mm^3), although the latter differences did not reach statistical significance. These results indicated that the antitumor activity of 0.02 mmol/kg of UK-21 was stronger than that of 0.1 mmol/kg of 5-FU and comparable with or weaker than that of 0.2 mmol/kg of 5-FU.

Effect of UK-21 on pulmonary metastasis of LLC transplanted i.v.

Mice were injected i.v. with LLC cells and the agents were given i.p. for 8 consecutive days from the day of transplantation. The pulmonary metastasis was examined on day 14 (Table 3). UK-21 at 0.01 mmol/kg suppressed the number of metastases to almost the same extent as 5-FU at 0.2 mmol/kg.

Effect of UK-21 on antibody production in ddY mice immunized with ovalubumin

The agents examined were given i.p. for 15 consecutive days from the day of immunization with ovalubumin. Every group consisted of seven mice at the beginning. One of seven mice treated with 0.02 mmol/kg of UK-21 died on day 13, and all 7 mice treated with 0.02 mmol/kg of 5-FUR died between days 10 and 14. None of the mice in the other groups died during the experiment.

UK-21 did not affect the body weight gain of mice at doses of 0.005 and 0.001 mmol/kg but suppressed it at the highest dose of 0.02 mmol/kg. 5-FUR suppressed it at both doses of 0.01 and 0.02 mmol/kg. 5-FU at 0.3 mmol/kg and

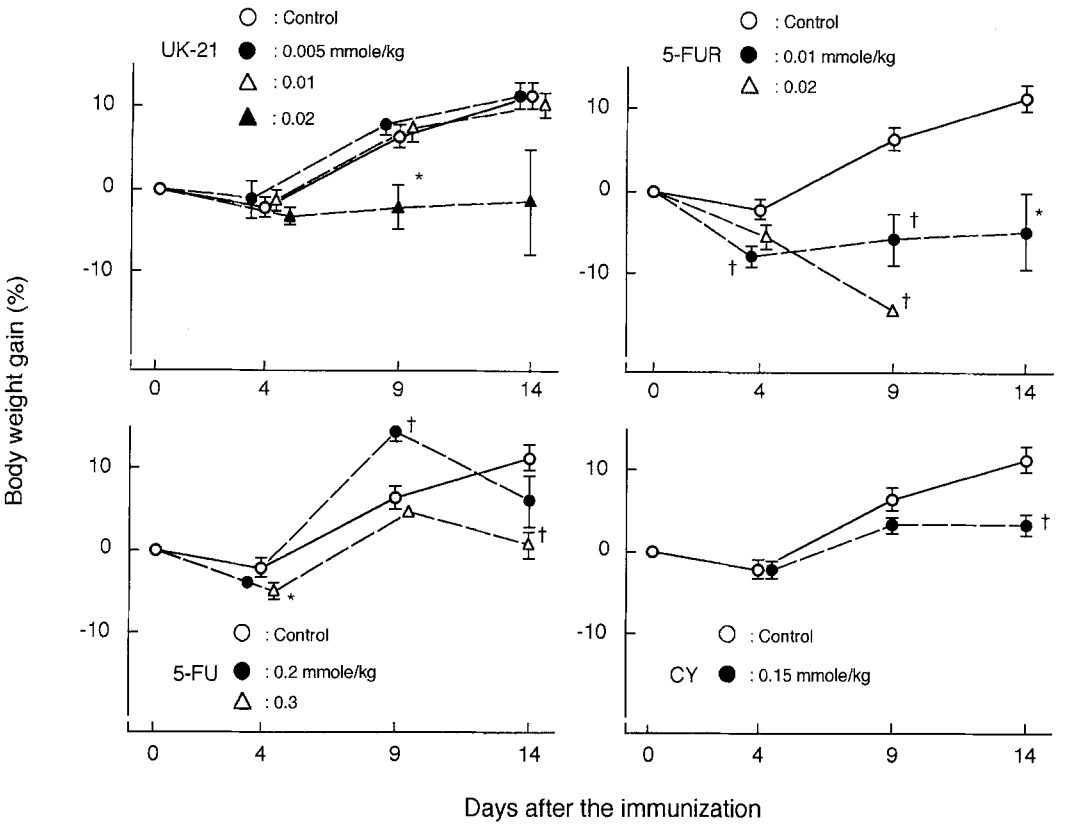

Fig. 2. Effect of UK-21, 5-FUR, 5-FU, and CY on the body weight gain of ddY mice immunized with ovalbumin. Mice were immunized with 1 mg ovalbumin emulsified with Freund's complete adjuvant. Drugs were given i.p. for 15 days from the day of immunization. Every group consisted of seven mice at the beginning. One mouse treated with 0.02 mmol/kg of UK-21 died on day 13, and all mice treated with 0.02 mmol/kg of 5-FUR died between days 10 and 14. Each point represents the mean value ±SE for 6–7 animals. * $P \leq 0.05$; † $P \leq 0.01$ vs control values (two-tailed t-test)

CY at 0.15 mmol/kg also suppressed the body weight gain of the animals (Fig. 2).

UK-21 suppressed IgM antibody formation only at 0.02 mmol/kg on day 4. 5-FUR and 5-FU suppressed it at both doses on day 4, but the suppressive effect of these agents was not so obvious on days 9 and 14. CY suppressed the formation almost completely throughout the experimental period (Fig. 3). The mean IgM antibody titers determined in mice treated with the highest dose of UK-21, 0.02 mmol/kg, were 0.067 on day 4, 0.333 on day 9, and 0.179 on day 14. These values were equal to or higher than those measured in mice treated with 5-FU at doses of 0.2 mmol/kg (0.067 on day 4, 0.155 on day 9, and 0.140 on day 14) or 0.3 mmol/kg (0.047 on day 4, 0.213 on day 9 and 0.178 on day 14). To assess the difference between the suppression induced by UK-21 and that caused by 5-FU, further statistical analysis (two-way ANOVA) was performed on the following combination of the data obtained on days 4, 9, and 14, respectively: 0.01 and 0.02 mmol/kg of UK-21 and 0.2 and 0.3 mmol/kg of 5-FU. The P values obtained for the differences between UK-21 and 5-FU were 0.044 on day 4, 0.077 on day 9, and 0.24 on day 14, indicating that the suppression induced by UK-21 at doses of 0.01 and 0.02 mmol/kg was weaker than that caused by 5-FU at doses of 0.2 and 0.3 mmol/kg, with the differences being statistically significant on day 4 but not reaching statistical significance on day 9.

Against IgG antibody formation, all the agents examined showed suppression (Fig. 4). The IgG antibody titer on day 4 was of undetectable, even in the control animals. The mean IgG antibody titers determined in mice treated with the highest dose of UK-21 were 0.213 on day 9 and 0.368 on day 14. Again, these titers were higher than those measured in mice treated with 5-FU at 0.2 mmol/kg (0.123 on day 9 and 0.237 on day 14) or 0.3 mmol/kg (0.136 on day 9 and 0.261 on day 14). The two-way ANOVA performed on the same combination of data used to assess the suppression of IgM antibody formation disclosed that the suppression of IgG antibody formation induced by UK-21 at 0.01 and 0.02 mmol/kg was weaker than that caused by 5-FU at 0.2 and 0.3 mmol/kg, with the differences reaching statistical significance on day 9 ($P = 0.00080$) and on day 14 ($P = 0.0061$).

A drastic suppression of the WBC count was observed on day 9 following treatment of mice with 5-FUR at doses of 0.01 and 0.02 mmol/kg. 5-FU at 0.03 mmol/kg suppressed the WBC count severely on day 14, and CY at 0.15 mmol/kg suppressed it throughout the experimental period. However, the suppression induced by UK-21 was not so noteworthy at any dose (Fig. 5).

Treatment with 5-FU and CY decreased the thymus weight on day 15 drastically, but treatment with UK-21 did not (Fig. 6). On the other hand, none of the agents examined affected the spleen weight (data not shown).

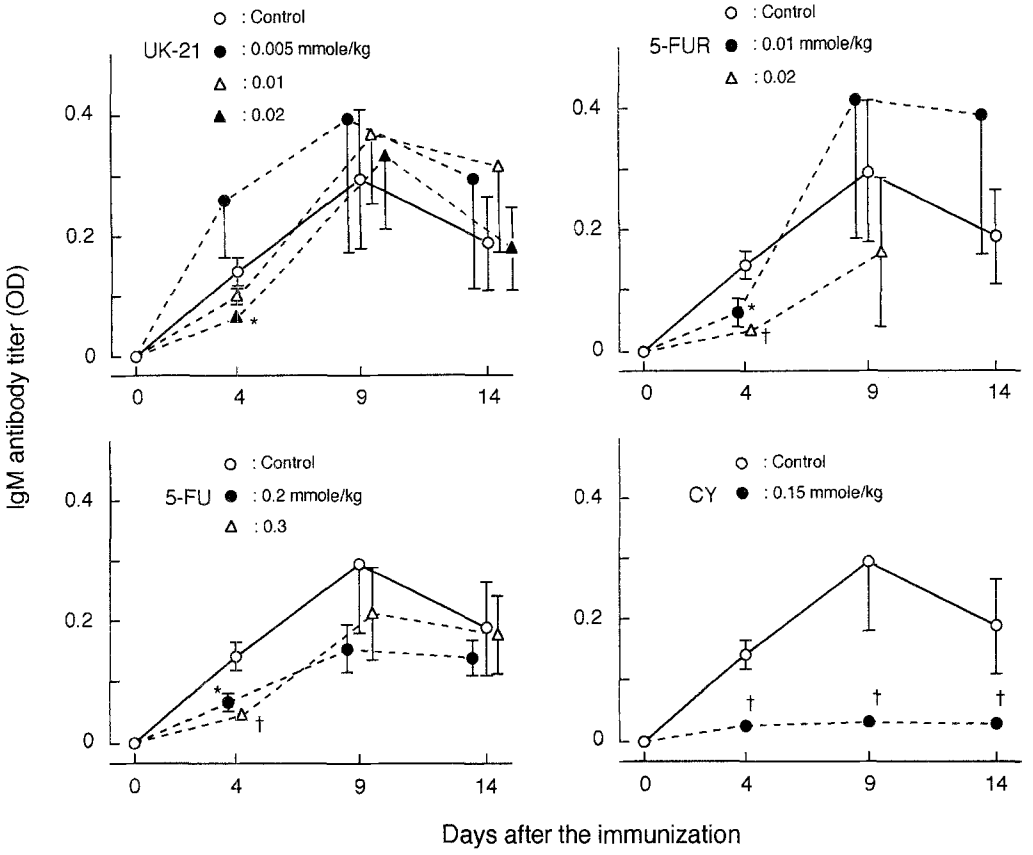

Fig. 3. Effect of UK-21, 5-FUR, 5-FU and CY on IgM antibody production in ddY mice immunized with ovalbumin. Each point represents the mean value ±SE for 6–7 animals. *$P \leq 0.05$; † P 0.01 vs control values (two-tailed t-test). See the legend to Fig. 2 for the experimental protocol

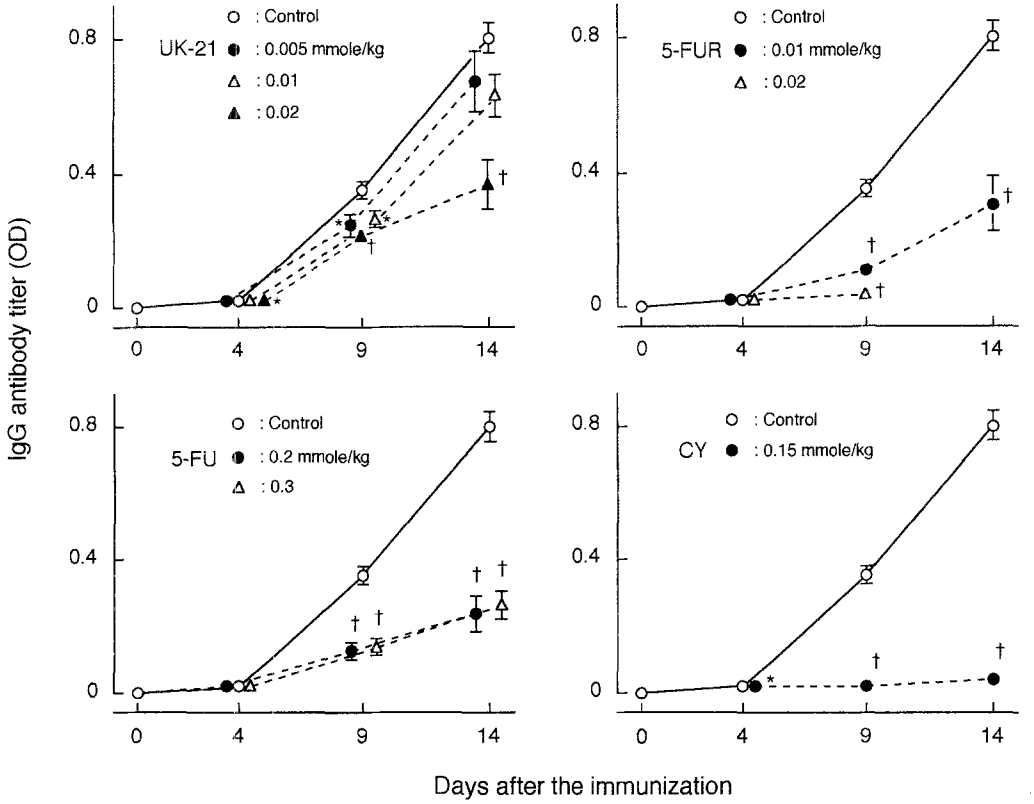

Fig. 4. Effect of UK-21, 5-FUR, 5-FU, and CY on IgG antibody production in ddY mice immunized with ovalbumin. Each point represents the mean value ±SE for 6–7 animals. *$P \leq 0.05$; † $P \leq 0.01$ vs control values (two-tailed t-test). See the legend to Fig. 2 for the experimental protocol

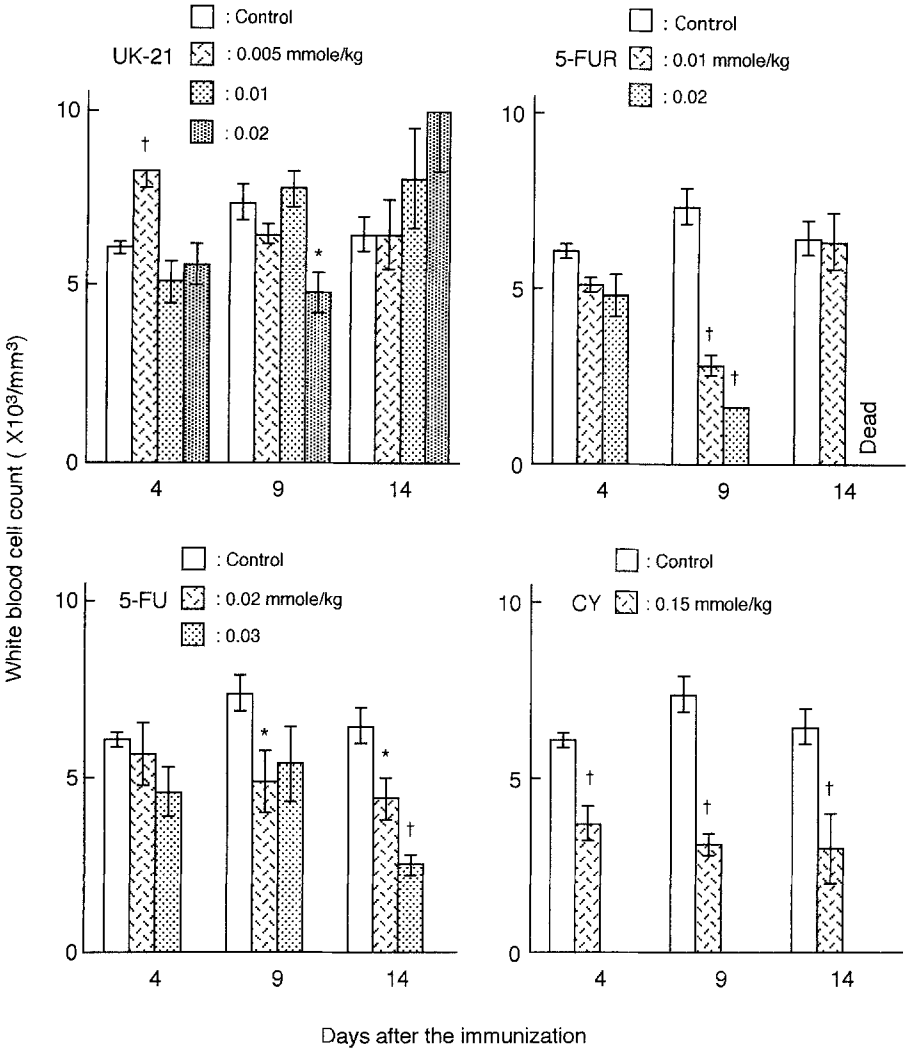

Fig. 5. Effect of UK-21, 5-FUR, 5-FU, and CY on the white blood cell count of ddY mice immunized with ovalbumin. Each column represents the mean value ± SE for 6–7 mice. *$P \leq 0.005$; † $P \leq 0.01$ vs control values (two-tailed t-test). See the legend to Fig. 2 for the experimental protocol

Discussion

First, we compared the anticancer activity of UK-21, 5-FUR, 5-FU, and CY against leukemic cells (including L1210, P388, and EL4 cells) inoculated i.v. into corresponding syngeneic mice. The agents examined were given by i.p. injection, a route different from that used for tumor cell inoculation (Table 1). Against these tumors, UK-21 at doses of 0.01–0.02 mmol/kg showed antitumor activity comparable with that of 5-FU at doses of 0.2–0.3 mmol/kg. CY, an alkylating agent, is known to show excellent antileukemic activity. CY at a dose of 0.15 mmol/kg showed the strongest antitumor activity against P388 leukemic cells among the agents examined and almost the same activity as UK-21 at 0.02 mmol/kg against L1210 and EL4 tumors.

Then, against LLC cells, UK-21 at a dose of 0.02 mmol/kg showed activity comparable with that of 5-FU at a dose range of 0.1–0.2 mmol/kg (Table 2). UK-21 was also effective in minimizing the metastasis of LLC. These results indicate that UK-21 shows antitumor activity at doses almost 10 times lower than those of 5-FU.

Finally, the side effects of the agents, especially on immune functions, were examined at doses producing comparable antitumor activity. It was strongly suggested that the suppressive effect of UK-21 on IgM and IgG antibody formation was weaker than that of 5-FU and CY (Figs. 3, 4). It is known that immune function is not always suppressed even by thymectomy, because the function is supported by peripheral mature T-lymphocytes for a while after the thymectomy. However, the lack of thymus function will result in gradual immunodeficiency associated with a reduction in the number of peripheral T-lymphocytes. Therefore, the toxicity of an anticancer agent to the thymus should be considered a serious immunological side effect along with its toxicity to peripheral lymphocytes. The suppressive effect of UK-21 on the thymus weight of mice was markedly weaker than that of 5-FU and CY, although the effect of the agents on the thymus function remains unclear. We examined the thymus weight but not the thymus function for supporting T-cell maturation. The reduction in WBC counts induced by UK-21 was also lower than that produced by any other agent.

Therefore, it is clear that UK-21 has adequately effective antitumor activity and produces low-level immunological side effects, although the mechanism of the side-effect reduction of UK-21 remains unclear. In a previous study [14], we compared UK-21, tegafur, and 5-FU for their effects on cellular immune responses, including sheep red-blood-cell-induced delayed-type hypersensitivity (SRBC-DTH) in ddY mice and Meth A tumor-induced

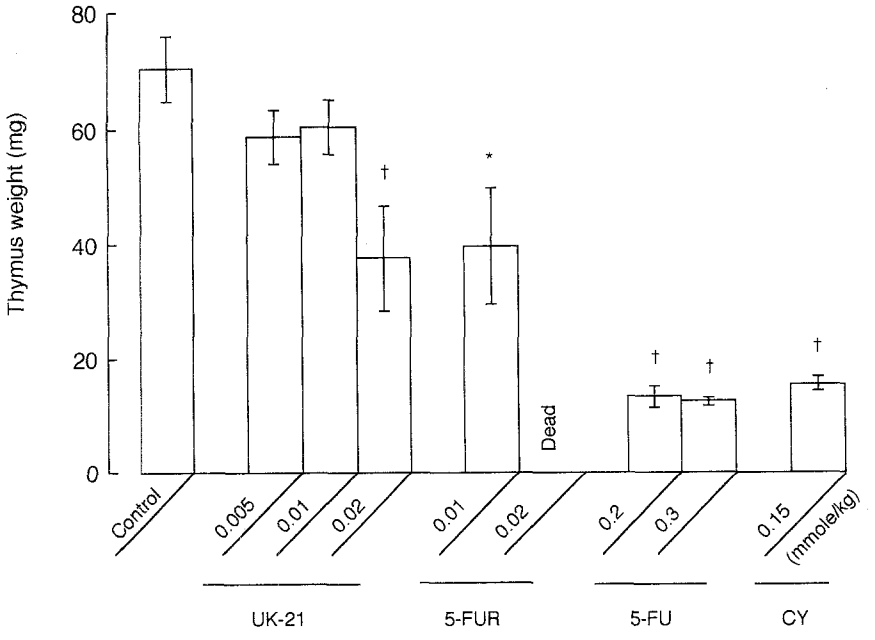

Fig. 6. Effect of UK-21, 5-FUR, 5-FU, and CY on the thymus weight in ddY mice immunized with ovalbumin. Each column represents the mean value ± SE for 6–7 mice. * $P \leq 0.05$; † $P \leq 0.01$ vs control values (two-tailed t-test). See the legend to Fig. 2 for the experimental protocol

delayed-type hypersensitivity (Meth A-DTH) in BALB/c mice. Either an oral therapeutic dose (UK-21, 0.1–0.4 mmol/kg; tegafur, 0.2–0.4 mmol/kg; 5-FU, 0.1–0.2 mmol/kg) or an oral overdose (UK-21, 0.4–0.8 mmol/kg; tegafur, 0.8–1.2 mmol/kg; 5-FU, 0.4–0.6 mmol/kg) was given to mice sensitized s.c. with 5×10^5 or 5×10^7 SRBC for 5 days from the day of sensitization. The results indicated that the suppressive effects of UK-21 on SRBC-DTH were weaker than those of tegafur and 5-FU. In these experiments, the thymus weights were also measured. The therapeutic index (50% suppressive dose on thymus weight/50% suppressive dose on Meth A tumor growth in BALB/c mice) was 4.6 for UK-21, 1.5 for tegafur and 2.0 for 5-FU. Furthermore, UK-21 enhanced Meth A-DTH but tegafur and 5-FU did not at the respective oral therapeutic doses.

These results suggested that the suppressive effect of oral UK-21 on the cellular immune response was weak, as was its inhibitory effect on the thymus. It is quite likely that UK-21 is easily incorporated into tumor cells and then converted to 5-FUR to exert its cytotoxicity, because UK-21 exhibits cytotoxicity at concentrations comparable with those of 5-FUR in vitro [12] as described in the introduction to this paper. As a mechanism of the low-level immunological side effects of UK-21, it may be that UK-21 is less subject to conversion to 5-FUR in normal tissues/cells including thymus, lymphoid and myeloid cells. This possibility requires further investigation.

Heidelberger and Dushinsky [2] examined the antitumor activity of many fluorinated analogs of pyrimidine, including 5-FUR, and could not find any analog superior to 5-FU. 5-FUR shows excellent tumoricidal activity but has not been used as an antitumor drug because of its serious side effects. In spite of many subsequent attempts to find antitumor agents among 5-FUR analogs, none of the analogs has come into clinical use. These unsuccessful results indicate the difficulty involved in reducing the side effects of 5-FUR. However, the results reported in this paper suggest that the chemical modification of 5-FUR is a possible way of developing a novel anticancer drug.

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