Novel Derivatives of 5-Fluorouridine and 5-Fluorouracil Having Potent Antitumor and Lower Immunosuppressive Activities

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Received October 4, 1991 Accepted December 16, 1991

ABSTRACT—We studied the biological activities of several 5-fluorouridine (5-FUR) and 5-fluorouracil (5-FU) derivatives to find novel antitumor drugs with lower immunosuppressive effects. We examined 5-FUR and 5-FU derivatives acylated with (2-n-propyl-n-pentanoyl)glycine (KN-539). Among the examined compounds, we found satisfactory activities in a derivative of 5-FUR, 2',3',5'-tris-O-[N-(2-n-propyl-n-pentanoyl)glycyl]-5-fluorouridine (UK-21), and a derivative of 5-FU, 1-[N-(2-n-propyl-n-pentanoyl)glycyl]amino-n-hexylcarbamoyl]-5-fluorouracil (UK-25). UK-21 (0.05–0.2 mmole/kg, p.o., 10 days) and UK-25 (0.1–0.4 mmole/kg, p.o., 10 days) suppressed Meth A and E.L.4 tumor growths in the corresponding syngeneic hosts (BALB/c mice and C57BL/6 mice, respectively) without decreasing body weight and blood leukocyte count. UK-21 and UK-25 suppressed the proliferation of KB tumor cells in vitro (IC₅₀: 3.0 × 10⁻¹¹ M and 4.4 × 10⁻⁷ M, respectively) at concentrations almost equivalent to those of 5-FUR and 5-FU, respectively. These results suggest that UK-21 and UK-25 express their antitumor activity as 5-FUR and 5-FU, respectively. Neither UK-21 nor UK-25 suppressed thymus weight and humoral antibody production against sheep red blood cells (SRBC) in ddY mice, although 1-(2-tetrahydrofuryl)-5-fluorouracil (FT-207) and 5-FU suppressed them in their respective therapeutic dose ranges for tumors. Thus, UK-21 and UK-25 are expected to develop into anticancer drugs with lower immunotoxicological effects.

5-Fluorouracil (5-FU) is an anticancer agent that was first synthesized in 1957 (1). 5-FU is widely used for many types of cancer because it has a suppressive effect on a wide spectrum of cancers; and it is even effective against carcinomas of the stomach, intestine, breast and other adenocarcinomas, which are relatively resistant to chemotherapy.

It is well-known that a metabolite of 5-FU, 5-fluorodeoxyuridine monophosphate (5-FdUMP), exerts its antitumor activity mainly through competitive antimetabolic action against thymidylate synthetase in DNA synthesis (2), although another metabolite, 5-fluorouridine triphosphate (5-FUTP), exerts its cytotoxic action by being incorporated into RNA or inhibiting the synthesis of uridine (3–5). It is also known that tumor cells are most sensitive to 5-FU from the G₁ phase to the S phase of the cell proliferation cycle, because the main mode of action of 5-FU is as an antimetabolite as mentioned above. Thus, a better
therapeutic effect would be expected by main-
taining its effective plasma concentration as
long as possible. From this aspect, 1-(2-
tetrahydrofuryl)-5-fluorouracil (FT-207, ftor-
afur) was developed as a drug which could be
metabolized into 5-FU gradually in the liver to
give a long lasting effective plasma concentra-
tion level of 5-FU (6). Following these studies,
numerous attempts were made to develop new
derivatives of 5-FU. At present, 1-hexyl-
carbamoyl-5-fluorouracil (HCFU, carmofur)
has been clinically used (7, 8). It is said that
5'-deoxy-5-fluorouridine (5'-DFUR, doxi-
fluridine), a recently developed derivative of
5-FU, is metabolized into 5-FU by pyrimidine
nucleoside phosphorylase which shows higher
activity in tumors than in normal tissues, re-
sulting in a high concentration of 5-FU in
tumor cells and less side-effects (9–11). Up
till now, development of 5-FU derivatives has
focused on enhancing the anticancer potency
and lowering the side-effects. However, the
trials have not always been focused on de-
creasing its immunosuppressive action as a
side-effect, although there are reports examin-
ing the effect of anticancer drugs on immune
responses (12, 13).

Anticancer agents act on not only tumor
cells but also normal cells, bringing about vari-
ous side-effects such as immunosuppression,
injury of bone marrow and dysfunctions of the
digestive system. The immunosuppression
weakens the therapeutic effect of chemothera-
petics (14), because it causes not only dam-
age in the immunological defense function to-
ward infections but also a dysfunction of im-
munological resistance against the tumor.
Hence, the immunosuppressive activity of
anticancer agents is a serious and paradoxical
side-effect from the aspect of its therapeutic
efficacy.

We reported previously that α-mercaptopro-
pionylglycine (α-MPG) and sodium dipropyl-
acetate (DPA) enhanced the humoral immune
response against sheep erythrocytes (15, 16),
and antagonized the immunosuppressive activi-
ties of prednisolone and antitumor agents
including cyclophosphamide, azathioprine,
 methotrexate, actinomycin D and mitomycin C
(17). α-MPG and DPA showed antitumor
effects through their immunostimulative activi-
ties (18). Thereafter, we (19) reported that a
novel compound related to α-MPG and DPA,
(2-n-propyl-n-pentanoyl)glycine (KN-539),
showed also a host-dependent antitumor activ-
ity.

5-Fluorouridene (5-FUR) has a very strong
antitumor activity, but is not used as an an-
cancer drug because of its serious side-effects.
In this series of studies on newly synthesized
derivatives of 5-FUR and 5-FU, we tried to
reduce their immunosuppressive effects while
keeping a high antitumor activity by conjugat-
ing them with KN-539.

MATERIALS AND METHODS

Compounds

5-Fluorouridine (5-FUR)- and 5-fluorouracil
(5-FU)-derivatives (UK-2, UK-2', UK-3, UK-
7, UK-10, UK-10', UK-20, UK-21, UK-22 and
UK-25) examined in this study are shown in
Fig. 1. These compounds as well as 5'-deoxy-
5-fluorouridine (5'-DFUR, doxifluridine) and
1-hexylcarbamoyl-5-fluorouracil (HCFU, car-
mofur) were synthesized at the Medicinal Re-
search Institute of Ube Industries, Ltd. (Ube,
Japan). 5-FU (Nacalai Tesque, Inc., Kyoto,
Japan), 5-FUR (Sigma Chemical Co., St.
Louis, MO) and 1-(2-tetrahydrofuryl)-5-
fluorouracil (FT-207, ftorafur, Taiho Phar-
maceutical Industry Co., Ltd., Tokushima,
Japan) were also used. The examined com-
pounds were either suspended or dissolved in
0.2% carboxymethylcellulose sodium salt
(CMC) before oral administration except for
UK-20, UK-21 and UK-22 which could not be
suspended in CMC solution. These 3 com-
pounds were either suspended or dissolved in
ethanol and then mixed with olive oil to give
5% ethanol solution. The reference drugs
underwent the same procedures.

Animals

Male BALB/c mice, C57BL/6 mice and ddY
mice (Japan SLC, Inc., Shizuoka, Japan) were
used at 7–8 weeks of age. They were maintained with free access to pellet food and water in filtered laminar air flow isolation cages at 21 ± 1°C temperature and 60% humidity.

**Antitumor effect in vivo**

Meth A tumor cells derived from BALB/c mice and E.L.4 tumor cells derived from C57BL/6 mice were maintained by weekly passage in the cavity of the respective syngeneic hosts as an ascites form. To examine the antitumor effect of the test compounds, 1 × 10⁶ cells of Meth A tumor or 5 × 10⁵ cells of E.L.4 tumor was inoculated subcutaneously into the flank of BALB/c mice or C57BL/6 mice, and the compounds were given orally for 10 consecutive days starting the day of transplantation. The tumor growth and blood leukocyte count were determined on day 10 after the transplantation. If necessary, the animals were killed under ether anesthesia on day 10 to weigh their thymus and spleen. The size of a tumor growing in the subcutis was measured with Vernier calipers in terms of 2 diameters at right angles and expressed as volume (mm³) calculated as follows:

\[ \frac{4}{3} \pi \times \left( \frac{\text{long diameter}}{2} \right) \times \left( \frac{\text{short diameter}}{2} \right)^2 \]

To determine the blood leukocyte count, 20 µl of blood collected from the tail vein of mice was mixed with 10 ml of Isoton II and 3 drops of Zapoglobin 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.

**Antitumor effect in vitro**

L-929 fibroblast cells of murine origin and
KB epidermal carcinoma cells of human origin were used. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% heat inactivated fetal bovine serum, 10 U/ml of penicillin G and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO2 in air. The subcultured cells were harvested from the culture flask by using 0.2% trypsin to examine the effect of the test compounds on their proliferation.

Twenty thousand L-929 cells were co-cultured with the test compounds per well of a 96-well flat microplate (Coming Laboratory Science Co., NY) for 48 hr. After the cultivation, the cells were washed twice with Hank's balanced salt solution (HBSS), followed by assaying the amount of DNA per well as described previously (20). Briefly, the cells in each well were lysed and transferred to a test tube using 2.0 ml of 0.1% sodium dodecyl sulfate (SDS) solution, and then 2.0 ml of 6 μg/ml ethidium bromide solution was added. The fluorescence intensity (Ex, 525 nm; Em, 600 nm) which depended on the DNA content of the solution was measured 15 min later, using commercial available ds-DNA (Type I, Sigma, MO) as a reference. The number of cells in a well was calculated using the fluorescence standard curve prepared with L-929 cells.

To study the effect of the test compounds on the proliferation of KB cells, 5 × 10^3 cells/well of KB cells were pre-cultured 24 hr in a 24-well flat plate (Corning Laboratory Science Co., NY). Then, the test compound was added to the wells and the cultivation was continued for 4 days. After the cultivation, the cells were washed twice with HBSS and dispersed with 0.2 ml/well of 0.2% trypsin. The enzymatic reaction was stopped by adding 0.2 ml of calf serum and the cell suspension was mixed with 9.6 ml of Isoton II. The number of cells was determined by a cell counter (Coulter Counter, Model Z; Coulter Electronics, Inc., Hialeah, FL).

The test compounds were dissolved in dimethylsulfoxide (DMSO) and sterilized by using a filter for organic solvents (0.45 μm, FHLP, Millipore, Bedford, MA). The final concentration of DMSO was set to be less than 0.5% by diluting with the culture medium to avoid the effect of DMSO on the cell proliferation.

Humoral antibody production
Sheep red blood cells (SRBC; Toyo Kessei Co., Ltd., Tokyo, Japan) were washed with ethylenediaminetetraacetic acid (EDTA)-gelatin veronal buffer (EDTA-GVB, pH 7.4), GVB (pH 7.4) containing Ca^{2+} and Mg^{2+}, and saline. The washed cells were resuspended in saline. These procedures were performed under sterile conditions. The ddY mice were immunized with 0.2 ml of 10^9 cells/ml SRBC suspension, i.v. The test compounds were administered orally to the mice for 10 consecutive days starting the day of immunization. Blood was collected from the retro-orbital venous plexus of the mice 5 and 10 days after the immunization. The animals were killed under ether anesthesia on day 10 to weigh their thymus and spleen. The separated serum was subjected to enzyme-linked immunosorbent assay (ELISA) (21) to measure the anti-SRBC antibody titer. Briefly, 0.1 ml of the serum diluted in phosphate-buffered saline (PBS, pH 7.4) was incubated at 37°C for 2 hr with SRBC attached directly to the bottom of a positively charged aminoplate well (96-well, flat bottomed plate, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The final dilutions of the serum collected on day 5 were 100-fold for assaying IgM and IgG antibody titers. For the serum collected on day 10, the dilutions were 100-fold for the IgM antibody titer and were 1000-fold for the IgG antibody titer. Either 0.1 ml/well of 500-fold diluted alkaline phosphatase-conjugated affinity purified goat anti-mouse IgM or IgG preparations (Cappel, West Chester, PA) were used as secondary antibodies. The alkaline phosphatase activity in a well was determined using 4-aminopyrine as a substrate (22). Optical density (OD; sample: 492 nm, reference: 690 nm;
Titertek Multiskan MCC/340, Flow Lab., VA) was considered as the antibody titer.

Statistics
Results were expressed as means ± S.E. Wilcoxon's rank sum test (U-test) was employed to analyze the statistical difference between two groups in the data of tumor size. Either Student's t-test or Welch's t-test after the F-test were used for the other data. P < 0.05 was considered to indicate a significant difference.

RESULTS

5-FUR related compounds
Antitumor activity of UK-2 and UK-3: We examined the antitumor activities of UK-3 (a derivative of 5-FUR acylated in the 5'-hydroxyl group with KN-539), UK-2′ (an α-anomer of 5-FUR) and UK-2 (an α-anomer of UK-3).

UK-2 (0.2–0.6 mmole/kg), UK-3 (0.01–0.1 mmole/kg), FT-207 (0.2 mmole/kg) and 5-FU (0.1 mmole/kg) were given to BALB/c mice transplanted with Meth A tumor according to the procedure described under Materials and Methods (Fig. 2). UK-2 showed an antitumor effect only in high doses, without dose-dependency. On the other hand, UK-3 showed a strong dose-dependent antitumor effect at lower doses than FT-207 and 5-FU. However, at a dose of 0.1 mmole/kg of UK-3, body weight and blood leukocyte count of the mice decreased markedly and one out of eight mice died by day 10.

The concentration of UK-2 inhibiting L-929 cell proliferation in vitro by 50% (IC50) was 6 × 10⁻⁴ M, which was higher than that of FT-207 (IC50: 3.5 × 10⁻⁵ M). The activity of UK-2′ (IC50: 2 × 10⁻⁵ M) was as potent as that of 5-FU (IC50: 1.5 × 10⁻⁵ M). On the other
hand, UK-3 (IC$_{50}$: $3 \times 10^{-7}$ M) showed the same activity at 200 times lower concentration than 5-FU.

**Antitumor activity of UK-10**: Next, the antitumor effect against Meth A tumor was examined for UK-10, 5'-aza derivative of UK-3.

UK-10, UK-3 and FT-207 were given p.o. in doses of 0.02 - 0.6, 0.05 and 0.2 mmole/kg, respectively. UK-3 and FT-207 clearly suppressed the tumor growth, comparably to the results shown in Fig. 2, while UK-10 showed no such suppression even at the highest dose of 0.6 mmole/kg (data not shown).

The effect of UK-10 and its mother compound UK-10' was examined on KB cell proliferation in vitro. The IC$_{50}$ values of UK-10 and UK-10' were $3 \times 10^{-4}$ M and $3 \times 10^{-5}$ M, respectively. Their cytotoxic activities were weak compared with that of FT-207 (IC$_{50}$: 1.2 $\times 10^{-5}$ M). Hence, the substitution of the 5'-hydroxyl group of 5-FUR with an amino group reduced the activity both in vivo and in vitro.

**Antitumor activity of UK-20, UK-21 and UK-22**: UK-20, UK-21 and UK-22 are 5-FUR derivatives esterified with KN-539 at the 2', 3' and/or 5' positions.

UK-20, UK-21 and UK-22 showed dose-dependent antitumor effects on Meth A tumor in BALB/c mice (Fig. 3). Their activities were stronger than those of FT-207 and 5-FU. UK-20 and UK-22 at a dose of 0.2 mmole/kg, however, decreased body weight and blood leukocyte count. Moreover, at this dose of UK-20 and UK-22, 5 out of 8 and 2 out of 8 mice died by day 10, respectively. On the other hand, in the mice administered UK-21, decreases in body weight and peripheral blood cell

![Fig. 3](image_url)
leukocyte count were not so severe and no deaths were observed.

UK-20, UK-21 and UK-22 also suppressed the growth of E.L.4 tumor in C57BL/6 mice dose-dependently in lower doses than FT-207 (Fig. 4). However, 5 out of 8 mice died at the dose of 0.1 mmole/kg of UK-20, and all of mice died at the dose of 0.2 mmole/kg of UK-20 or UK-22 by day 10. On the other hand, the decrease in body weight by UK-21 was less than those by UK-20 and UK-22, and no mice died.

IC50 value of UK-20, UK-21 and UK-22 on the proliferation of KB tumor cells in vitro were $2 \times 10^{-9}$ M, $3 \times 10^{-11}$ M and $5.6 \times 10^{-9}$ M, respectively. Their effects were clearly stronger than that of 5-FU (IC50: $1.8 \times 10^{-7}$ M). The potencies of UK-20 and UK-22 were almost the same as that of 5-FUR (IC50: $1.9 \times 10^{-9}$ M), and UK-21 showed a stronger activity than 5-FUR.

Effect on humoral antibody production: The effects of UK-20, UK-21, UK-22, FT-207, 5-FU and 5-FUR were examined on humoral antibody production in ddY mice immunized with SRBC (Table 1). 5-FUR at the dose of 0.1 mmole/kg tended to decrease the IgM antibody titer on day 5 and decreased it significantly on day 10. On the other hand, 0.1 mmole/kg of UK-22 increased IgM antibody titer on day 10. Administration of the other compounds had no obvious effect on IgM antibody production. For IgG antibody production, UK-20 at 0.1 mmole/kg, FT-207 at 0.8 mmole/kg and 5-FUR at 0.1 mmole/kg decreased the titer significantly on day 5, and FT-207 at 0.8 mmole/kg and 5-FUR at 0.1 mmole/kg decreased it on day 10.

![Fig. 4. Effect of UK-20, UK-21, UK-22, FT-207 and 5-FU on E.L.4 tumor growth in C57BL/6 female mice. Animals were transplanted with $10^5$ E.L.4 tumor cells s.c. into their flanks. Drugs were given p.o. for 10 consecutive days starting the day of transplantation. The measurements were carried out on the day after the final administration. Data represent means ± S.E. of 6 to 8 animals. The tumor size in the control mice was $116 ± 18$ mm$^3$. †: Statistically significant difference from the control mice at $P < 0.01$.](image-url)
In addition, 0.8 mmole/kg of FT-207 and 0.1 mmole/kg of 5-FUR decreased the thymus weight significantly (Table 3). None of the examined compounds affected the spleen weight except for 5-FUR at a dose of 0.1 mmole/kg, which showed a tendency to decrease it (data not shown).

5-FU related compounds

Antitumor activity of UK-7 and UK-25: UK-7 and UK-25 are 5-FU derivatives possessing the KN-539 residue in the side-chain of the 1-position. The effect of oral administration of UK-7, UK-25, FT-207 and 5-FU was examined on Meth A tumor growth in BALB/c mice as well as on body weight and blood leukocyte count (Fig. 5). UK-7 and UK-25 showed a strong dose-dependent antitumor effect. Their antitumor activities at a dose of 0.4 mmole/kg were more potent than those of FT-207 at 0.4 mmole/kg and 5-FU at 0.1 mmole/kg. However, UK-7 at 0.4 mmole/kg decreased body weight and blood leukocyte count, while UK-25 did not decrease them at any dose, but rather increased blood leukocyte count at 0.1 mmole/kg. A significant decrease

### Table 1. Effect of UK-20, UK-21, UK-22, FT-207, 5-FU and 5-FUR on production of humoral antibody in ddY female mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>mmole/kg</th>
<th>Day 5</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM (1/100)</td>
<td>IgG (1/100)</td>
</tr>
<tr>
<td>Control</td>
<td>0.025</td>
<td>0.66 ± 0.14(100)</td>
<td>0.68 ± 0.08(100)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.93 ± 0.11(142)</td>
<td>0.82 ± 0.07(119)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.68 ± 0.05(103)</td>
<td>0.58 ± 0.06(85)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.57 ± 0.07(87)</td>
<td>0.41 ± 0.05(60) *</td>
</tr>
<tr>
<td>UK-21</td>
<td>0.05</td>
<td>0.81 ± 0.11(123)</td>
<td>0.65 ± 0.09(95)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.78 ± 0.10(118)</td>
<td>0.84 ± 0.08(123)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.51 ± 0.06(78)</td>
<td>0.43 ± 0.11(63)</td>
</tr>
<tr>
<td>UK-22</td>
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<td>0.74 ± 0.08(112)</td>
<td>0.85 ± 0.13(124)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.66 ± 0.09(101)</td>
<td>0.52 ± 0.10(76)</td>
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<tr>
<td></td>
<td>0.1</td>
<td>0.69 ± 0.05(104)</td>
<td>0.49 ± 0.05(72)</td>
</tr>
<tr>
<td>FT-207</td>
<td>0.2</td>
<td>0.78 ± 0.10(118)</td>
<td>0.62 ± 0.07(90)</td>
</tr>
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<td>0.4</td>
<td>0.70 ± 0.10(106)</td>
<td>0.63 ± 0.08(93)</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.56 ± 0.05(85)</td>
<td>0.39 ± 0.05(58) *</td>
</tr>
<tr>
<td>5-FU</td>
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<td>0.74 ± 0.10(112)</td>
<td>0.74 ± 0.09(109)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.66 ± 0.08(101)</td>
<td>0.57 ± 0.08(83)</td>
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<td>0.2</td>
<td>0.59 ± 0.05(90)</td>
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<tr>
<td>5-FUR</td>
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<td>0.74 ± 0.10(108)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.69 ± 0.10(105)</td>
<td>0.45 ± 0.06(65)*</td>
</tr>
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<td></td>
<td>0.1</td>
<td>0.31 ± 0.03(46)</td>
<td>0.20 ± 0.03(28)*</td>
</tr>
</tbody>
</table>

Animals were immunized i.v. with 2 × 10⁶ SRBC. Drugs were given p.o. for 10 consecutive days starting the day of immunization. The measurement was carried out 5 and 10 days after the immunization. *: Dilution of assayed serum for titration by ELISA. Data represent means ± S.E. of 6 animals. †: Statistically significant difference from the control mice at P < 0.05 and P < 0.01, respectively.
Fig. 5. Effect of UK-7, UK-25, FT-207 and 5-FU on body weight, Meth A tumor growth and blood leukocyte count in BALB/c female mice. Animals were transplanted with $10^6$ Meth A tumor cells s.c. into their flanks. Drugs were given p.o. for 10 consecutive days starting the day of transplantation. The measurements were carried out on the day after the final administration. Data represent means ± S.E. of 8 animals. The tumor size in the control mice was $498 ± 38$ mm$^3$. *$\dagger$: Statistically significant difference from the control mice at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 6. Effect of UK-25, HCFU, 5'-DFUR, FT-207 and 5-FU on E.L.4 tumor growth in C57BL/6 female mice. Animals were transplanted with $10^5$ E.L.4 tumor cells s.c. into their flanks. Drugs were given p.o. for 10 consecutive days starting the day of transplantation. The measurements were carried out on the day after the final administration. Data represent means ± S.E. of 7 to 8 animals. The tumor size in the control mice was $172 ± 29$ mm$^3$. *$\dagger$: Statistically significant difference from the control mice at $P < 0.05$ and $P < 0.01$, respectively.
in blood leukocyte count was also observed at 0.1 mmole/kg of 5-FU.

Next, the effects of UK-25, HCFU, 5'-DFUR, FT-207 and 5-FU were examined on E.L.4 tumor growth in C57BL/6 mice (Fig. 6). UK-25 suppressed the tumor growth significantly in doses of 0.1–0.4 mmole/kg. The antitumor activity of UK-25 at the a dose of 0.4 mmole/kg was less than that of HCFU at the dose of 0.4 mmole/kg, but was almost the same as those of 5'-DFUR at 0.8 mmole/kg, FT-207 at 0.4 mmole/kg and 5-FU at 0.2 mmole/kg.

The IC50 value of UK-25 for the inhibition of KB cell proliferation in vitro was $4.4 \times 10^{-7}$ M, while those of FT-207 and 5-FU were $1.6 \times 10^{-5}$ M and $6.6 \times 10^{-7}$ M, respectively.

Therefore, the cytotoxic activity of UK-25 against KB cells was similar to that of 5-FU but stronger than that of FT-207.

**Effect of humoral antibody production:** We examined the effects of UK-7, UK-25, HCFU, FT-207 and 5-FU on humoral antibody production in ddY mice immunized with SRBC (Table 2). For IgM antibody production, UK-7 at a dose of 0.2 mmole/kg and FT-207 at 0.2 mmole/kg increased the titer on day 5, while FT-207 at a dose of 0.8 mmole/kg and 5-FU at 0.2 mmole/kg decreased the titer. On day 10, UK-7 at 0.1 mmole/kg, HCFU at 0.4 mmole/kg and FT-207 at 0.8 mmole/kg decreased the titer. For IgG antibody production, the low or middle dose of all the examined compounds increased or showed a

<table>
<thead>
<tr>
<th>Antibody titer (OD at 492 nm and percentage to control)</th>
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<tr>
<td>Day 5</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>mmole/kg</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>UK-7</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>UK-25</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>HCFU</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>FT-207</td>
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<tr>
<td>0.4</td>
</tr>
<tr>
<td>0.8</td>
</tr>
<tr>
<td>5-FU</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>0.2</td>
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</tbody>
</table>

Animals were immunized i.v. with $2 \times 10^8$ SRBC. Drugs were given p.o. for 10 consecutive days starting the day of immunization. The measurement was carried out 5 and 10 days after the immunization. a; Dilution of assayed serum for titration by ELISA. Data represent the means ± S.E. of 6 animals. *; Statistically significant difference from the control mice at $P < 0.05$ and $P < 0.01$, respectively.
tendency to increase the titers on days 5 and 10. Even at the high dose, UK-7 and UK-25 did not decrease the IgG antibody titers on days 5 and 10, while HCFU and FT-207 decreased the titer on day 10 at the high dose, and 5-FU also showed a tendency to decrease the IgG antibody titer on days 5 and 10. In this experiment, UK-25 did not affect the thymus weight at any dose used (Table 3). However, significant decreases in thymus weight were observed at doses of 0.4 mmole/kg of UK-7, 0.2–0.4 mmole/kg of HCFU, 0.4–0.8 mmole/kg FT-207 and 0.2 mmole/kg of 5-FU. All of the examined compounds had no effect on spleen weight (data not shown).

Table 4 summarizes the results obtained in this study for the effect of UK-21, UK-25 and the reference drugs on the growth of Meth A tumor in BALB/c mice, humoral immune response and thymus weight in ddY mice. The antitumor activity was statistically assessed by the probit-method using a computer. It was disclosed that UK-21 showed an antitumor activity at a little higher dose than HCFU and 5-FU, while UK-25 showed it at a dose lower than that of FT-207 and almost twice those for HCFU and 5-FU. It was also clear that the suppressive effects of UK-21 and UK-25 on the humoral immune response and thymus weight were less than that of the reference compounds.

Table 3. Effect of 5-FUR related compounds (UK-20, UK-21, UK-22), 5-FU related compounds (UK-7, UK-25) and reference drugs on thymus weight in ddY female mice

<table>
<thead>
<tr>
<th>5-FUR related compounds and reference drugs</th>
<th>5-FU related compounds and reference drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmole/kg thymus weight (mg)</td>
<td>mmole/kg thymus weight (mg)</td>
</tr>
<tr>
<td>Control</td>
<td>68.0 ± 6.6</td>
</tr>
<tr>
<td>UK-20 0.025</td>
<td>64.1 ± 3.8</td>
</tr>
<tr>
<td>0.05</td>
<td>78.8 ± 4.7</td>
</tr>
<tr>
<td>0.1</td>
<td>51.0 ± 5.1</td>
</tr>
<tr>
<td>UK-21 0.05</td>
<td>67.6 ± 4.0</td>
</tr>
<tr>
<td>0.1</td>
<td>67.0 ± 4.0</td>
</tr>
<tr>
<td>0.2</td>
<td>70.6 ± 2.4</td>
</tr>
<tr>
<td>UK-22 0.025</td>
<td>69.0 ± 8.5</td>
</tr>
<tr>
<td>0.05</td>
<td>69.6 ± 6.1</td>
</tr>
<tr>
<td>0.1</td>
<td>64.3 ± 4.0</td>
</tr>
<tr>
<td>FT-207 0.2</td>
<td>66.1 ± 5.3</td>
</tr>
<tr>
<td>0.4</td>
<td>45.8 ± 8.8</td>
</tr>
<tr>
<td>0.8</td>
<td>14.6 ± 1.0</td>
</tr>
<tr>
<td>5-FU 0.05</td>
<td>67.8 ± 5.8</td>
</tr>
<tr>
<td>0.1</td>
<td>66.5 ± 3.3</td>
</tr>
<tr>
<td>0.2</td>
<td>50.5 ± 6.6</td>
</tr>
<tr>
<td>5-FUR 0.025</td>
<td>55.1 ± 4.2</td>
</tr>
<tr>
<td>0.05</td>
<td>64.3 ± 4.2</td>
</tr>
<tr>
<td>0.1</td>
<td>34.5 ± 6.4</td>
</tr>
</tbody>
</table>

*): See legend to Table 1 for details. **): See legend to Table 2 for details. The measurements were carried out 10 days after the immunization.
DISCUSSION

First, in the present paper, 5-FUR was acylated with KN-539 to decrease the side-effects. UK-3, an 5'-ester of 5-FUR with KN-539, showed antitumor activity to Meth A tumor transplanted into BALB/c mice in quite a low dose range, 0.02–0.1 mmole/kg. However, UK-3 decreased body weight and blood leukocyte count dose-dependently, suggesting that UK-3 still had marked side-effects including bone marrow injury associated with a strong antitumor activity. UK-2, an a-isomer of UK-3, showed antitumor activity only at the high dose of 0.4 mmole/kg. Moreover, high concentrations of UK-2 and UK-2', a-isomer of 5-FUR and the mother compound of UK-2, were required to show cytotoxicity to L-929 cells in vitro (IC_{50}; 6 × 10^{-4} M and 2 × 10^{-5} M, respectively) compared with UK-3 (IC_{50}; 3 × 10^{-7} M), suggesting that the natural form /3-isomer was necessary for 5-FUR to show the unique antitumor activity.

Next, we examined the antitumor activity of UK-20', a 5'-aza analogue of 5-FUR, and UK-21, a 5'-aza analogue of UK-3. As described in the Results, their antitumor activities were markedly decreased. These results suggested that the hydroxyl group at the 5'-position was essential for UK-3 to show the strong antitumor activity. UK-3 might release KN-539 easily and exert its activity in the form of 5-FUR, without being converted into 5-FU. This was also supported by the fact that UK-3 showed cytotoxicity to L-929 cells in vitro in a similar concentration range as 5-FUR did, and UK-2', an unnatural a-isomer of 5-FUR, needed much higher concentrations than 5-FUR to exhibit cytotoxicity.

Therefore, UK-20, UK-21 and UK-22, derivations of the natural /3-isomer 5-FUR, were examined for their antitumor activities and side effects. UK-20 is a derivative of 5-FUR esterified with KN-539 at the 2'- and/or 3'- and 5'-positions. UK-21 is a KN-539 triester of 5-FUR. UK-22 is also a KN-539 diester of 5-FUR at the 2'- and 3'-positions. UK-20, UK-21 and UK-22 at doses of 0.05–0.2 mmole/kg suppressed the growths of Meth A and E.L.4 tumor to the same extent or greater than FT-207 at doses of 0.2–0.6 mmole/kg and 5-FU at doses of 0.1–0.2 mmole/kg. Among the derivatives, the order of potencies was: UK-20 > UK-22 > UK-21. However, UK-20 at doses of 0.1 and 0.2 mmole/kg and UK-22 at a dose of 0.2 mmole/kg decreased body weight gain and blood leukocyte count. Furthermore, some of mice treated with UK-

**Table 4. Antitumor activity and immunosuppressive activities of UK-21, UK-25 and reference drugs**

<table>
<thead>
<tr>
<th></th>
<th>Statistical analysis of antitumor activity(^{a1})</th>
<th>Suppression(^{b1}) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED(_{50}) mmole/kg</td>
<td>(95% confidence)</td>
</tr>
<tr>
<td>UK-21</td>
<td>0.19</td>
<td>0.16 – 0.22</td>
</tr>
<tr>
<td>UK-25</td>
<td>0.30</td>
<td>0.26 – 0.34</td>
</tr>
<tr>
<td>HCFU</td>
<td>0.15</td>
<td>0.11 – 0.18</td>
</tr>
<tr>
<td>FT-207</td>
<td>0.51</td>
<td>0.45 – 0.59</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.15</td>
<td>0.12 – 0.19</td>
</tr>
</tbody>
</table>

\(^{a1}\): BALB/c mice were transplanted with 1 × 10^6 Meth A cells, s.c. and given drugs p.o. for 10 consecutive days starting the day of transplantation. Antitumor activity was evaluated by the tumor size on day 10. Data were processed by the probit-method. \(^{b1}\): ddY mice immunized i.v. with 2 × 10^6 SRBC. Drugs were given p.o. for 10 days starting the day of immunization. –: No suppression, +: Slight suppression, ++: Obvious suppression, +++: Severe suppression.
20 or UK-22 at a dose of 0.2 mmole/kg died by day 10. On the other hand, UK-21 did not show such severe side-effects. All these compounds demonstrated their cytotoxicity to KB tumor cells in vitro at almost the same or lower concentration than 5-FUR, suggesting that these compounds were hydrolyzed easily to release KN-539 and the resulting 5-FUR caused the antitumor effect. However, it is not known why UK-21 showed stronger cytotoxicity to KB cells than its mother compound 5-FUR.

UK-20 at the highest dose (0.1 mmole/kg) suppressed IgG antibody production and decreased thymus weight on day 5, but did not suppress IgM and IgG antibody production on day 10. UK-21 and UK-22 did not affect any of these parameters at any dose. On the other hand, 5-FUR at the highest dose (0.1 mmole/kg) decreased IgM and IgG antibody production as well as thymus and spleen weights. FT-207 at the highest dose of 0.8 mmole/kg also showed marked decreases in thymus weight and IgG antibody production. These results for UK-20, UK-21 and UK-22 suggested that the conjugation of KN-539 to 5-FUR decreased immunotoxicological actions such as suppression of humoral immune response and injurious actions to the thymus and bone marrow as well as general toxicity. We do not yet know the mechanism for the decreased side-effects of UK-20, UK-21 and UK-22. Their pharmacokinetics including cellular incorporation, mode of cytocidal action, oral absorption and metabolism must be investigated to elucidate the mechanism of their strong antitumor activity and lower side-effects.

FT-207 is a masked compound of 5-FU and metabolized into 5-FU mainly in the liver to show its antitumor activity. The cytotoxicity curve of FT-207 against KB cells was shifted to higher concentrations than that of 5-FU, while UK-7 and UK-25 showed the same activity at almost the same concentrations as 5-FU. It is likely that UK-7 and UK-25 are easily converted into 5-FU and the resulting 5-FU exhibits the antitumor effect.

UK-7 decreased IgG antibody production on day 5 only at the lowest dose, but did not in the other doses. On the other hand, UK-25 did not decrease IgM and IgG antibody production as well as thymus weight at any dose. HCFU and FT-207 at the highest dose decreased IgM and IgG antibody production, especially the IgG antibody production on day 10. A marked decrease of thymus weight was observed by the treatment with HCFU, FT-207 and 5-FU at the highest dose. Hence, it was clear that either UK-7 or UK-25, especially UK-25, showed less immunotoxicological activity than the reference antitumor drugs in a dose range showing an antitumor effect as was in the case of UK-21.

In conclusion, we have developed novel derivatives of UK-21 and UK-25, which were conjugates of 5-FUR and 5-FU with KN-539. They showed strong antitumor activity but lower immunotoxicological effects. It was thought that UK-21 expressed its antitumor activity as 5-FUR, and UK-25 expressed it as 5-FU. The immunostimulative activity of KN-539 might reduce the immunosuppressive actions of 5-FUR and 5-FU.

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