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Irreversible cytotoxic effect of a novel lowly immunosuppressive antitumor fluorouridine derivative, UK-21

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Previously, we reported that the 5-fluorouridine derivative, 2′,3′,5′-tris-*O*-[*N*-(2-*n*-propyl-*n*-pentanoylglycyl]-5-fluorouridine (UK-21), is a newly synthesized lowly immunosuppressive and potent antitumor drug in comparison with other fluorouridine derivatives such as 5-fluorouracil (5-FU), 5-fluorouridine (5-FUR) and 5-fluorodeoxyuridine (5-FUDR). In order to elucidate the molecular mechanism of antitumor activity of UK-21, we compared the effect of the four drugs on cell proliferation, cell cycle progression and macromolecular syntheses. When KB cells were subjected to a colony-forming inhibition assay designed to expose the cells to the drugs for 4–96 h and wash out, UK-21 and 5-FUR inhibited the colony formation at concentrations ranging from 0.01 to 0.1 μM, whereas 1–100 μM was needed for the cytotoxicity of 5-FU and 5-FUDR. By exposure for 24–48 h, all these drugs inhibited cell growth and caused accumulation of the cells in S or G_2 phase at almost the same concentrations of 0.32–8 μM. These results suggest that the cytotoxic effects of UK-21 and 5-FUR are irreversible, while those of 5-FU and 5-FUDR are reversible. To confirm this, KB cells were treated with UK-21 and/or 5-FU for 1 h, and continued to be cultured for 1–7 days, resulting in the inhibition of the cell growth by UK-21 in a dose-dependent manner at concentrations of 10–100 μM, but not by 5-FU even at 100 μM. UK-21, 5-FUR and 5-FU showed a linear relationship between exposure time and IC_{50} in the colony formation assay with a slope of almost −1, but 5-FUDR did not, suggesting that UK-21, 5-FUR and 5-FU are cell cycle non-specific inhibitors, while 5-FUDR is a cell cycle-specific inhibitor. UK-21 and 5-FUR, but not 5-FU and 5-FUDR inhibited the incorporation of [^{3}H]uridine into the acid insoluble fraction, while UK-21 and 5-FUDR, but not 5-FUR and 5-FU inhibited the incorporation of [^{3}H]thymidine. These results suggest that irreversible cytotoxic effects of UK-21 like 5-FUR are exerted through inhibition of RNA synthesis.

Key words: Antitumor mechanism, 5-fluorouridine derivative, macromolecular synthesis.

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Introduction

Anticancer drugs which exert a direct cytotoxic or cytostatic effect on neoplastic cells have commonly such detrimental effects as immunosuppression, gastrointestinal and bone marrow disturbances.[1]

In previous studies, we reported that a newly synthesized 5-fluorouridine derivative, 2′,3′,5′-tris-*O*-[*N*-(2-*n*-propyl-*n*-pentanoylglycyl]-5-fluorouridine (UK-21), shows excellent antitumor activity in mice with a low level of immunological side effects such as suppression of humoral, cellular and tumor immune responses.[2–5] Recently we reported[5] that UK-21 given i.p. suppressed the growth of L1210, P388 and EL4 leukemias inoculated i.v. into the corresponding syngeneic mice, as well as the growth of Lewis lung carcinoma transplanted s.c. into C57BL/6 mice and its subsequent metastasis to the lung. UK-21 showed its suppressive activity at a dose almost 10 times lower than that of 5-fluorouracil (5-FU). The suppressive effect of UK-21 on IgM and IgG antibody formation in mice immunized with ovalbumin was clearly weaker than that of 5-fluorouridine (5-FUR), 5-FU and cyclophosphamide (CY) at doses producing comparable antitumor activity. These results suggested the potential of UK-21 as a novel anticancer drug with cytotoxic mechanisms different from those of 5-FU.

5-FU, a widely used anticancer agent, is metabolically converted to various phosphorylated fluoroanalogs such as 5-fluorouridine monophosphate (5-FUMP), 5-fluorouridine triphosphate (5-FUTP), 5-fluorodeoxyuridine monophosphate (5-FdUMP) and 5-fluorodeoxyuridine triphosphate (5-FdUTP). 5-FdUMP exerts its anticancer activity mainly through competitive antimetabolic action against thymidylate synthetase in DNA synthesis.[6] The other metabolite, 5-FUTP, may be incorporated into RNA, followed by cytocidal activity.[7–9] Kanamura *et al.*[9] found that the

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amount of 5-FU incorporated into the RNA of L1210 cells showed a good correlation with its cytocidal action. Akizawa *et al.*[8] 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-FUR is more effective to cell killing than 5-FU. However, 5-FUR has not been clinically used as an anticancer drug because of its severe side effects.[5,10]

In the present paper, we compared UK-21 with 5-FUR, 5-FU and 5-fluorodeoxyuridine (5-FUDR) in the action profile on KB cells *in vitro*, in order to investigate the cytocidal mechanism of UK-21.

Materials and methods

Chemicals

UK-21 (Figure 1; molecular weight 812) was synthesized at Ube Laboratories of Ube Industries (Ube, Japan) as described elsewhere.[5] 5-FUR was purchased from Sigma (St Louis, MO). 5-FU and 5-FUDR were obtained from Nacalai Tesque (Kyoto, Japan). UK-21 was dissolved in ethanol or dimethylsulfoxide; other agents were administered in a culture medium.

Cell and cell culture

Human epidermal carcinoma cells, KB, were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 4 mM glutamine and non-essential amino acids [0.01 ml/ml of non-essential amino acid solution 10 mM, liquid (Life Technologies, NY)]. The cells were grown in a 5% CO_2 incubator at 37°C under a humidified atmosphere. The logarithmically growing KB cells were harvested by trypsinization and used for the experiments described below. Cell number was counted with a hemocytometer.

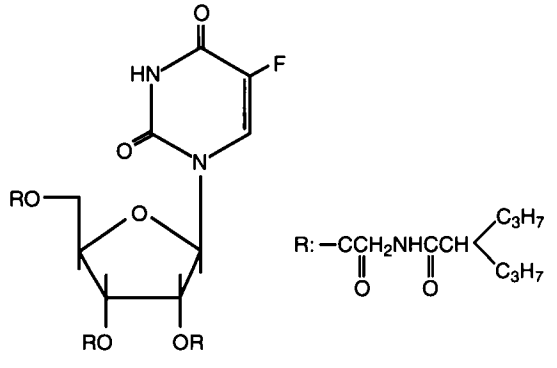

Figure 1. Chemical structure of UK-21.

Cytotoxicity assay

Cytotoxicity was determined by a colony-forming assay. Five hundred cells suspended in 2.7 ml of the medium were seeded into a 60 mm dish (Corning, NY) and cultured for 1 day. Then, the drugs were added to the dish in a volume of 0.3 ml (the final ethanol concentration was less than 0.5%). After 4–100 h incubation, the cells were washed twice with phosphate buffered saline and allowed to continue colony formation. At 8 days after the seeding, the developed colonies were washed, fixed with methanol and stained with 10% Giemsa solution for counting. IC_{50}, the drug concentration yielding 50% colony formation, was calculated at each exposure time by the probit method using a computer.

Analysis of cell cycle progression by flow cytometry

Two hundred thousand KB cells suspended in 3 ml of the medium were seeded into a 60 mm dish. The next day, the medium was replaced with the same amount of medium containing drugs at various concentrations. After incubation for 4, 24 or 48 h with the drugs, the cells were harvested with trypsinization, fixed with 70% ethanol, treated with 1 mg/ml of RNase (type I; Sigma, St Louis, MO) for 20 min at 37°C and then stained with 50 μg/ml of propidium iodide. The DNA distribution pattern of the KB cells was examined for 10^4 events by flow cytometry (FACScan; Becton Dickinson Immunocytometry System, San Jose, CA).

Short-term treatment with UK-21 and 5-FU

Two hundred thousand KB cells suspended in 3 ml of the medium were incubated in a 60 mm dish for 1 day. Then, the drugs were added to the cells at various concentrations. After 1 h incubation with the drugs, the cells were washed twice with phosphate buffered saline and supplemented with 3.0 ml of the culture medium. They were continued to be cultured for 1–7 days. The cells were harvested by

trypsinization and the number of viable cells was determined by the Trypan blue dye exclusion method.

Incorporation of [^{3}H]thymidine and [^{3}H]uridine into the acid insoluble fraction

Twenty thousand KB cells suspended in 2 ml of the medium were seeded into a 35 mm dish. After 1 day cultivation, drugs were added to the medium and the culture was continued for a further 1 h. One μCi/ml of either [^{3}H]thymidine (40–60 Ci/mmol; New England Nuclear, Boston, MA) or [^{3}H]uridine (40–60 Ci/mmol; New England Nuclear) was added to the medium 30 min before the end of the cultivation. Radioactivity incorporated into the acid-insoluble fraction of the cells was measured as described elsewhere.[11] Briefly, the culture medium was removed and the cells were lysed by incubation with 1 ml of 0.05 M Tris buffered solution (pH 7.5) containing 1 mM EDTA, 0.15 M NaCl and 0.6% sodium dodecylsulfate at 37°C for 10 min. Then, 1 ml of 10% trichloroacetic acid (TCA) was added to the lysate. After 10 min standing at room temperature, acid-insoluble material was collected on glass fiber filter (GF/C; Whatman, Maidstone, UK), washed with 5% TCA and ethanol, then dried. The radioactivity on the filter was counted in the toluene base scintillation fluid.

Statistics

Comparison of the two regression slopes in Figure 3 was performed by calculating *t*-values. A value of $p \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Time and concentration dependence of cytotoxic effect of UK-21, 5-FUR, 5-FU and 5-FUDR

In order to examine the effect of concentration and time, we measured the cytotoxicity of UK-21, 5-FUR, 5-FU and 5-FUDR to KB cells over a wide range of concentrations and exposure times. All four drugs exhibited a dose-dependent suppressive effect on colony formation (Figure 2). UK-21 and 5-FUR showed the cytotoxicity at concentrations ranging from 0.01 to 0.1 μM, whereas 5-FU and 5-FUDR showed it at higher concentrations of 1 to 100 μM. Although the cytotoxicities of UK-21, 5-FUR and 5-FU are dose dependent at any exposure time of

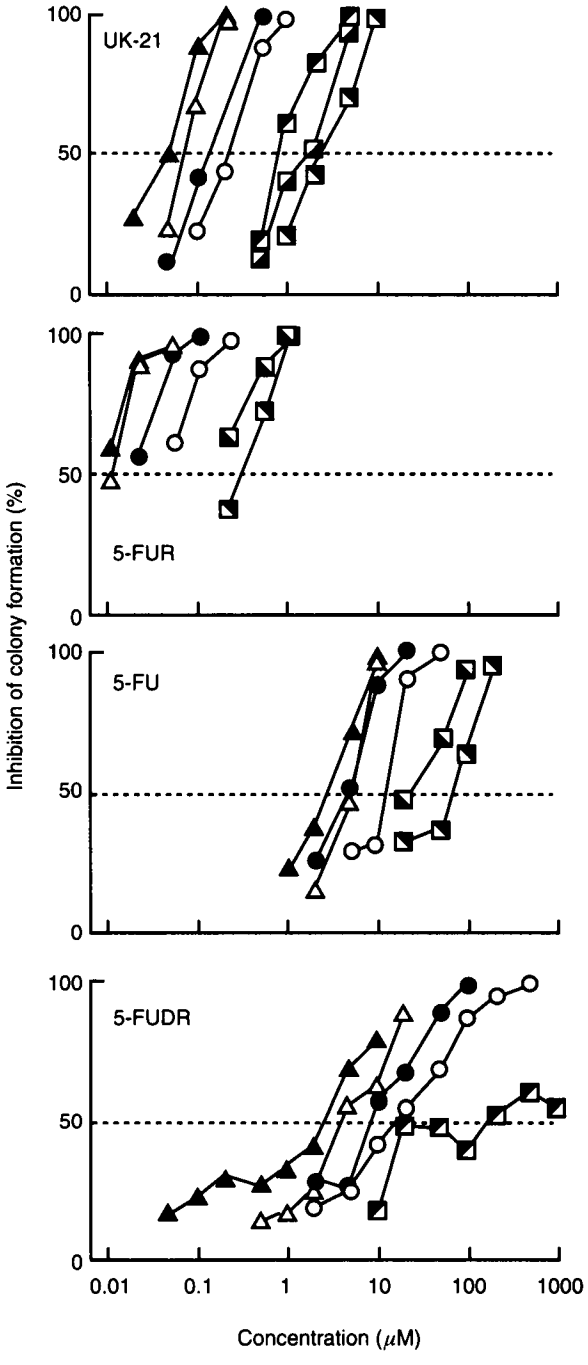

Figure 2. The concentration and time dependence of the cytotoxicity of UK-21, 5-FUR, 5-FU and 5-FUDR to KB cells. The cytotoxicity of the drugs was determined by the colony-forming inhibition assay as described in Materials and methods. The cells were exposed to the drugs for 4 (◥), 6 (◢), 8 (◣), 9 (◤), 24(○) 48 (●), 72 (△) or 96 (▲) h.

4–96 h, that of 5-FUDR showed a time dependency only when cells were exposed to the drug for more than 24 h. The data in Figure 2 were then plotted using IC_{50} as ordinate and exposure time as abscissa, respectively (Figure 3). The relationship between the exposure time and IC_{50} was linear with UK-21, 5-FUR and 5-FU, but not with 5-FUDR. The slopes of UK-21 and 5-FUR were −1.26 and −1.18, respectively (no statistically significant difference). The slope of 5-FU was −0.9 (statistically different from that of UK-21). The inhibitive potency of UK-21 on colony formation was clearly stronger than that of 5-FU and 5-FUDR, and was weaker than that of 5-FUR.

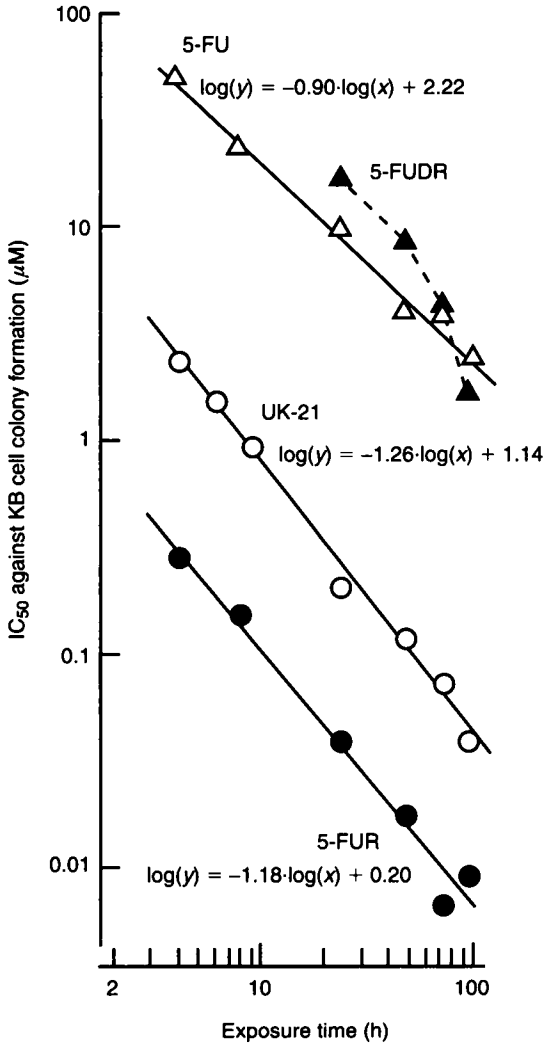

Figure 3. Relationship between the exposure time and IC_{50} of UK-21, 5-FUR, 5-FU and 5-FUDR in KB cells. IC_{50} values for the drugs, estimated from the dose–response curves in Figure 2, were plotted against exposure times. IC_{50} of 5-FUDR for 5 h exposure time could not be plotted.

The effect of UK-21, 5-FUR, 5-FU and 5-FUDR on cell growth and cell cycle progression

We examined the effect of drugs on cell proliferation by estimating the increase and decrease of cell number (Table 1) as well as cell cycle progression. UK-21 at 0.32 μM concentration inhibited cell growth slightly. At 1.6 μM and more, the drug inhibited the growth completely, although a slight increase in cell number was observed when cells were incubated at 24 h in the presence of 1.6 or 8 μM of UK-21. The other drugs, 5-FUR, 5-FU and 5-FUDR, also inhibited cell growth even at the lowest concentration. UK-21, 5-FUR and 5-FUDR at 40 and 200 μM concentrations caused a reduction in cell number, suggesting that the three drugs are cytocidal to cells at these concentrations.

Since UK-21 and three fluoro-compounds inhibited cell growth at the concentrations examined, we then examined by flow cytometry in which phase the cell cycle progression is affected (Figure 4). Cells with 2C and 4C DNA content correspond to cells in G_1

Table 1. Number of recovered KB cells at 4, 24 and 48 h after addition of UK-21, 5-FUR, 5-FU and 5-FUDR

	μM	Number of recovered KB cells ($\times 10^5$)		
		4 h	24 h	48 h
Control		4.6	10.2	21.2
UK-21	0.32	ND	8.2	13.8
	1.6	4.8	6.8	4.4
	8	4.6	5.8	3.7
	40	3.4	4.2	1.9
	200	2.9	0.75	0.08
5-FUR	0.32	ND	4.3	4.9
	1.6	4.5	5.6	5.9
	8	4.8	4.8	0.23
	40	3.9	3.7	0.08
	200	4.7	ND	ND
5-FU	0.32	ND	7.0	6.8
	1.6	4.6	6.0	5.0
	8	4.1	4.7	3.4
	40	4.6	5.0	3.2
	200	4.8	5.2	3.2
5-FUDR	0.32	ND	5.2	4.8
	1.6	4.5	5.4	3.8
	8	4.3	2.3	3.7
	40	3.8	4.4	0.15
	200	6.6	ND	ND

Agents at various concentrations were added to the culture medium 1 day after seeding of 2×10^5 KB cells. The cells were harvested by trypsinization at 4, 24 and 48 h after the addition of the agent. ND: Not done

and G_2/M phase, respectively, and cells with DNA between the two are in S phase. At 1.6 μM, where the four drugs showed cell growth inhibition, cell cycle progression was affected. In the presence of UK-21, S phase cells increased slightly at 24 h, then the cells in $S + G_2/M$ phase accumulated at 48 h. 5-FUR and 5-FUDR caused cell accumulation in G_1 phase and reduction in G_2 phase. 5-FU rendered the cells arrested in S phase at 48 h. At 8 μM, these drugs caused cell accumulation in G_1 phase. These results indicate that the four drugs inhibited cell growth and cell cycle progression at nearly identical concentrations if the drugs are present continuously.

Effect of short-term treatment with UK-21 and 5-FU on KB cell proliferation

UK-21 and 5-FUR were cytotoxically effective at concentrations ranging from 0.01 to 0.1 μM. By contrast, higher concentrations were needed for the cytotoxicity of 5-FU and 5-FUDR when the toxicity was examined by ability of colony formation (Figures 2 and 3). However, the four drugs inhibited cell growth at nearly the same concentrations (Table 1), suggesting that the toxic effects of UK-21 and 5-FUR are irreversible, while those of 5-FU and 5-FUDR are reversible. To confirm this, KB cells were treated with 10–100 μM of UK-21 and 100 μM of 5-FU for 1 h, and continued to be cultured for 1–7 days (Figure 5). UK-21 inhibited the cell growth in a dose-dependent manner. On the other hand, 5-FU, even at 100 μM, only inhibited slightly the increase in cell number.

Effect of UK-21, 5-FUR, 5-FU and 5-FUDR on incorporation of [^{3}H]thymidine and [^{3}H]uridine into acid insoluble fraction

To investigate the early events which lead to cell cycle arrest, the effect of fluorouridine derivatives was examined on the incorporation of [^{3}H]thymidine and [^{3}H]uridine into acid insoluble fraction of KB cells after exposure of the cells to the drugs for 1 h (Figure 6). UK-21 inhibited both [^{3}H]thymidine and [^{3}H]uridine incorporation in a concentration-dependent fashion, and the inhibition of [^{3}H]uridine incorporation was greater than that of [^{3}H]thymidine incorporation. 5-FUR enhanced [^{3}H]thymidine incorporation and inhibited

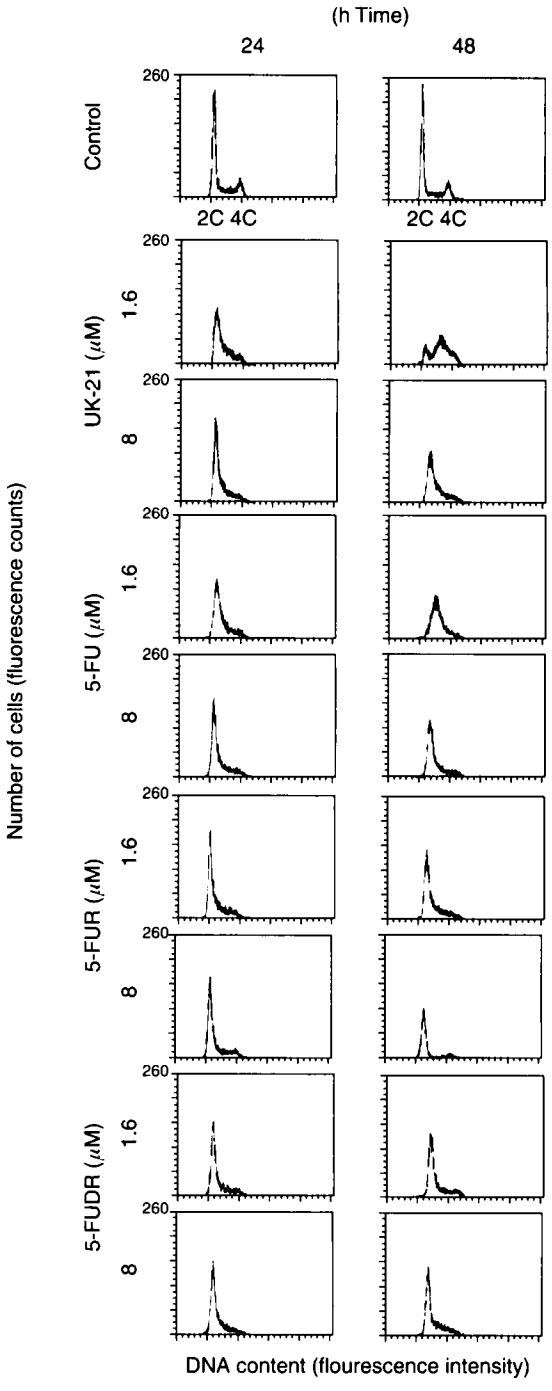

Figure 4. Effect of UK-21, 5-FUR, 5-FU and 5-FUDR on cell cycle progression in KB cells. Cells were exposed to UK-21, 5-FUR, 5-FU and 5-FUDR at various concentrations, and the distribution of the cells in the cell cycle was examined by flow cytometry.

[^{3}H]uridine incorporation. 5-FU did not affect [^{3}H]uridine incorporation and enhanced [^{3}H]thymidine incorporation at the higher concentrations. 5-FUDR inhibited [^{3}H]thymidine incorporation.

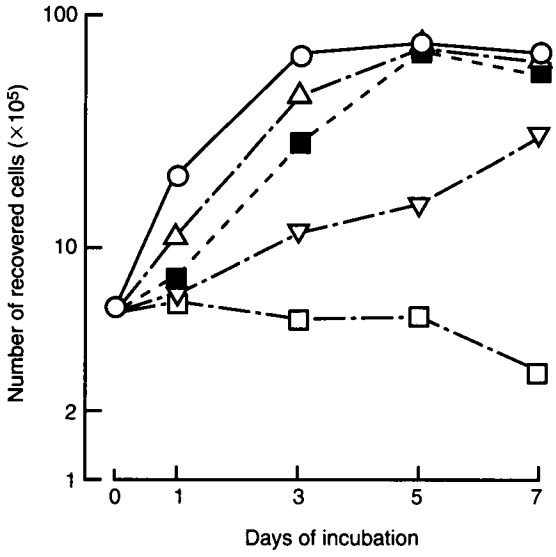

Figure 5. Effect of short-term exposure of KB cells to UK-21 and 5-FU. The cells were exposed to the drugs for 1 h, then cultured for 1–7 days without the drug. The numbers of viable cells were determined by the Trypan blue dye exclusion method. ○; Control; △; 10 μM of UK-21; ▽; 30 μM of UK-21; □; 100 μM of UK-21; ■; 100 μM of 5-FU.

Discussion

The present study shows that the mode of growth inhibition by UK-21 is similar to that of 5-FUR, and not to that of 5-FU or 5-FUDR; this is indicated from the data of the colony-forming inhibition assay, growth inhibition test, cell cycle analysis and macromolecular syntheses inhibition. We found that a 50 to 100 times higher concentration was required of 5-FU and 5-FUDR than of UK-21 and 5-FUR to inhibit the colony formation developed by at least 4 days culture after washing out the drugs (Figures 2 and 3). In contrast, all these drugs suppressed cell proliferation and cell cycle progression at almost identical concentrations when the cells were exposed to them continuously (Table 1 and Figure 4). These results suggest that the cytotoxic effects of UK-21 and 5-FUR are irreversible, whereas those of 5-FU and 5-FUDR are reversible. The results shown in Figure 5 support the irreversible cytotoxic effect of UK-21.

UK-21, 5-FUR and 5-FU showed a linear relationship between exposure time and IC_{50} in the colony formation assay with a slope of almost −1. On the other hand, 5-FUDR, an inhibitor of thymidylate synthetase, did not exhibit such a linear relationship. These results suggest that UK-21, 5-FUR and 5-FU are cell cycle non-specific inhibitors, and 5-FUDR is a

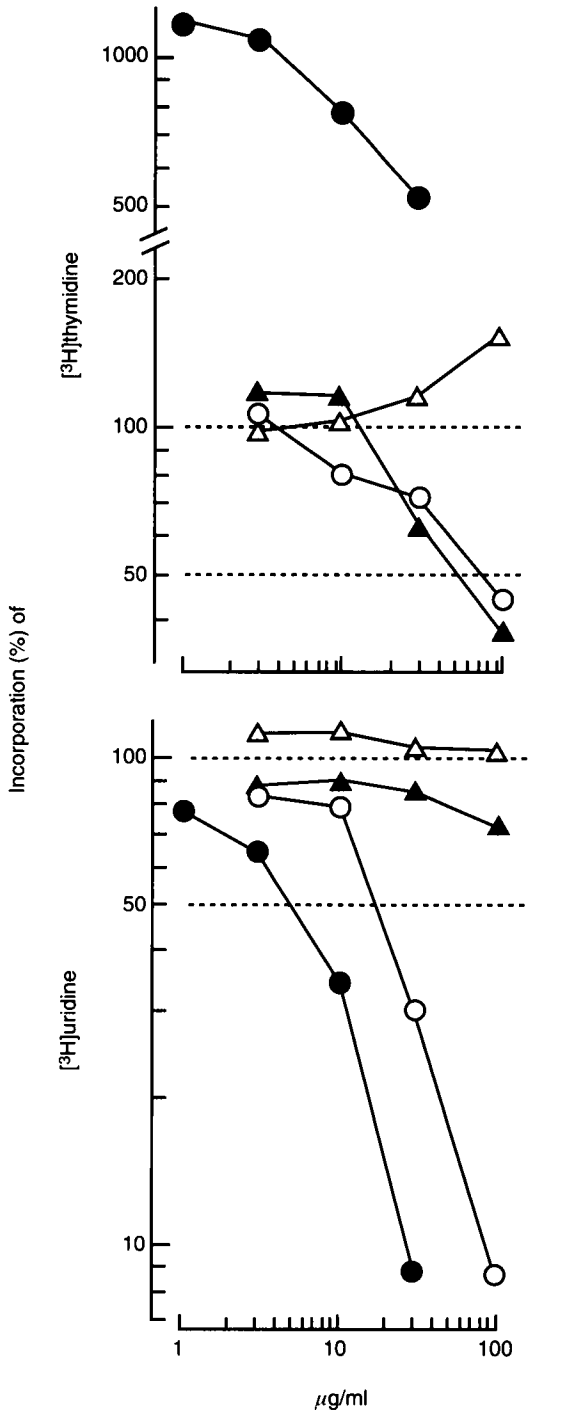

Figure 6. Effect of UK-21, 5-FUR, 5-FU and 5-FUDR on [^{3}H]thymidine and [^{3}H]uridine incorporations into acid soluble fraction of KB cells. The cells were incubated with the drugs for 1 h, and [^{3}H]thymidine and [^{3}H]uridine were added to the cells for 30 min before the end of the incubation. The [^{3}H]thymidine and [^{3}H]uridine incorporated were estimated with the radioactivity in the acid insoluble fraction. The radioactivity of the control cells was in a range of 2000–5000 c.p.m. for [^{3}H]thymidine and 15 000–16 000 c.p.m. for [^{3}H]uridine. ○, UK-21; ●, 5-FUR; △, 5-FU; ▲, 5-FUDR.

cell cycle (S phase)-specific inhibitor, according to the classification of Ozawa *et al.*[12,13].

For the mechanism of cytotoxicity of 5-FU and its analog, three possibilities are proposed: (i) incorporation of the drugs into DNA,[14] (ii) incorporation into RNA[7–9] or (iii) inhibition of thymidylate synthetase.[6] Both UK-21 and 5-FUR inhibited [^{3}H]uridine incorporation, although UK-21, unlike 5-FUR, also inhibited [^{3}H]thymidine incorporation. 5-FUDR inhibited [^{3}H]thymidine incorporation, but not [^{3}H]uridine incorporation. These results together with the data shown in Figures 2 and 3 suggest that the irreversible cytotoxic effect of UK-21 and 5-FUR is correlated with their inhibition of RNA synthesis, not of DNA synthesis. The mechanism of inhibition of RNA synthesis by UK-21 is not clear yet, but it seems likely that incorporation of UK-21 into RNA results in the inhibition of RNA chain elongation. It also remains to be resolved how UK-21 inhibits DNA synthesis. One possibility is that UK-21 inhibits thymidilate synthetase as 5-FUDR does.

UK-21 was drug-designed for antitumor agents with lower immunosuppressive effects.[2] UK-21 suppressed the *in vivo* growth of various experimental tumors such as L1210 and P388 at almost equal efficacy as 5-FUR and more than 5-FU and 5-FUDR in terms of the dose used.[5] Since UK-21 has a lower immunosuppressive effect and retains the antitumor activity of 5-FUR, the drug is of interest clinically.

The present study was designed to elucidate the cytocidal mechanism of UK-21, but not to examine the mechanism of the low level immunological side effects. Further studies will be required to clarify the latter point.

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