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Augmentation of 5-fluoro-2′-deoxyuridine cytotoxicity by 5-phenethyl-2′-deoxyuridine in human gastric cancer cells in culture

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5-Phenethyl-2′-deoxyuridine (PEUdR) augmented 5-fluoro-2′-deoxyuridine (FUdR) cytotoxicity up to 100-fold in several human gastric cancer cell lines. PEUdR also potentiated 5-fluorouracil (5-FU) cytotoxicity about 5-fold. In contrast, PEUdR reversed 5-fluorouridine (FUR) cytotoxicity in all cell lines studied. PEUdR was not cytotoxic up to 200 μM. PEUdR inhibited the incorporation of [^{3}H]thymidine and [^{14}C]uridine into acid-insoluble fractions, and also inhibited uptake of [^{3}H]thymidine into KATO III cells. Thus, PEUdR inhibits pyrimidine nucleoside transport and salvage enzymes, which potentiates the cytotoxicity of FUdR and reverses the effect of FUR in human gastric cancer cells. These results may contribute to more effective cancer chemotherapy with FUdR and 5-FU.

Key words: Potentiation, 5-fluoro-2′-deoxyuridine cytotoxicity, human gastric tumors, 5-phenethyl-2′-deoxyuridine.

5-Fluorouracil (FU)[1] is one of the most promising antitumor agents for several solid cancers, including gastric and colon cancers, but its side effects include leukopenia, vomiting and diarrhea.[2–4] There have been many attempts to synthesize derivatives, such as tegafur[5] and carmofur,[6] that are more active and less toxic than FU. There have also been attempts to increase the antitumor activity of FU[7] and reduce its toxicity by combining it with an FU antagonist.[8] FU has also been combined with leucovolin,[9] a key substrate of FU metabolism. Combinations of FU or FU derivatives with cytotoxic compounds have synergistic effects in cultures,[10] in experimental animals[11] and in patients.[12]

In this article we describe augmentation of the cytotoxicity of 5-fluoro-2′-deoxyuridine (FUdR), which is believed to be a proximate metabolite of FU, by the non-cytotoxic pyrimidine nucleoside 5-phenethyl-2′-deoxyuridine (PEUdR) (Figure 1), which is known as an antitumor active compound,[13] in human gastric cancer cells in culture. All data were obtained with 96-well microplates cultures at 37°C for 96 h in a humidified incubator with 5% CO_2, seeded with 2000 cells per well in RPMI 1640 medium containing HEPES buffer, sodium bicarbonate, L-glutamine and antimicrobial drugs, and supplemented with 10% fetal bovine serum. The cell growth was determined by the MTT assay method. The nucleoside PEUdR itself did not inhibit cell growth at concentrations up to 200 μM against any of the following four human gastric cancer cell lines: KATO III, MKN 28, MKN 45 and MKN 74 (Table 1). When the cells were cultured with both FUdR and PEUdR (25–200 μM), their growth was inhibited, and the degree of inhibition did not directly depend on the concentration of PEUdR (50–200 μM). A typical example of the effect of PEUdR on the cytotoxicity of FUdR is shown in Figure 2. Inhibition of cell growth depended on the concentration of FUdR but not on the concentration of PEUdR. Data from all cell lines are summarized in Table 2 with the maximum augmentation ratio (MAR), which was calculated as the IC_{50} without PEUdR divided by the minimum IC_{50} in the presence of PEUdR. PEUdR at 100 μM potentiated FUdR cytotoxicity in all human cell lines tested. In the KATO III, MKN 28, MKN 45 and MKN 74 cell lines, there were 38.6-, 100-, 2.5- and 6.2-fold increases in cytotoxicity, respectively, as measured by IC_{50}.

PEUdR also enhanced the cytotoxicity of FU in these cell lines, but the magnitude of the effect was much smaller: KATO III, 1.5-fold; MKN 28, 2.3-fold; MKN 45, 5.2-fold; and MKN 74, 1.1-fold. It is not

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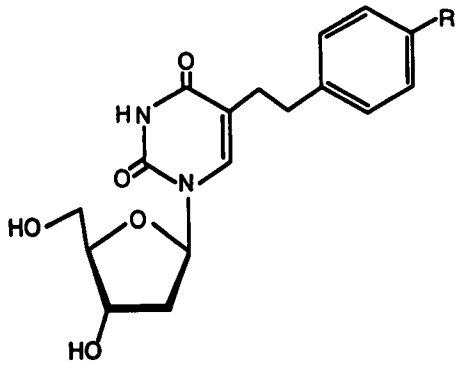

R=H, PEUdR; R=F, FPEUdR; R=Me, MePEUdR.

Figure 1. Chemical structure of PEUdR and its derivatives.

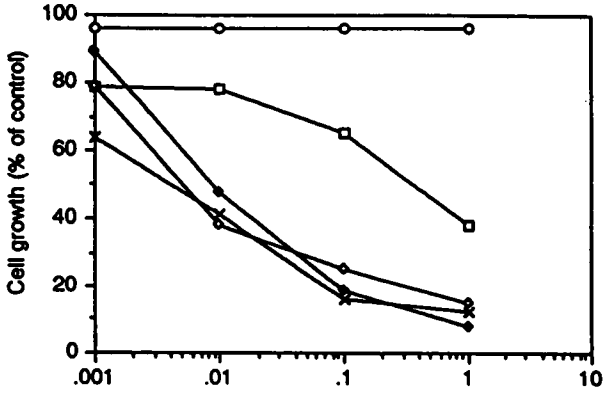

Figure 2. Augmentation of FUdR cytotoxicity by PEUdR in KATO III cells: (○) 200 μM PEUdR alone, (□) FUdR alone, (◆) FUdR plus 200 μM PEUdR, (×) FUdR plus 100 μM PEUdR and (◇) FUdR plus 50 μM PEUdR.

Table 1. Effect of PEUdR on growth of human cancer cells and murine leukemia cells in culture[a]

Concentration (μM)[b]	Cell growth (% of control)[c]				
	KATO III	MKN 28	MKN 45	MKN 74	L1210
200	100	100	100	100	50
100	100	100	100	100	98
50	100	100	100	100	100
25	100	—	—	—	100
12.5	—	—	—	—	100

[a] Human gastric cancer cells (KATO III, MKN 28, MKN 45 and MKN 74) were seeded at 2×10^3 cells per well (1×10^4 cells for the L1210 cell line) and cultivated for 96 h (48 h for the L1210 cell line) at 37°C in a humidified incubator with 5% CO_2.
[b] PEUdR was added to the medium solution until it accounted for 10% of the total volume.
[c] Cell growth was measured by the MTT assay method.

Table 2. IC_{50} values of FUdR with various concentrations of PEUdR against several types of tumor cells[a]

Concentraion of PEUdR (mM)[b]	IC_{50} (μg/ml)[a]				
	KATO III	MKN 28	MKN 45	MKN 74	L1210
0	0.27	1.0	0.015	0.80	1.6
25	0.0055	—	—	—	0.01
50	0.0058	0.038	0.004	0.11	0.0032
100	0.007	0.01	0.006	0.13	0.0052
200	0.01	0.01	0.003	0.079	—[b]
MAR[c]	49.1	100.0	5.0	10.1	500.0

[a] The concentration that resulted in 50% inhibition of growth (IC_{50}) was calculated from dose–response curves.
[b] At this concentration, PEUdR alone inhibited growth.
[c] MAR was calculated as the IC_{50} without PEUdR divided by the minimum IC_{50} in the presence of PEUdR.

clear why PEUdR had only a small effect on FU cytotoxicity, but one possibility is polymorphic metabolic activation of FU in the cells.

In contrast, the cytotoxicity of 5-fluorouridine (FUR) was reduced by PEUdR in relatively low concentrations of FUR in all cell lines tested. A typical result of KATO III cells is shown in Figure 3, the degree of reverse depended on the concentration of PEUdR. It seems that the mode of the reverse of FUR cytotoxicity is similar to that of the uridine reverse effect (Tokuzen *et al.*, unpublished results). The mechanism of this reduction is not clear.

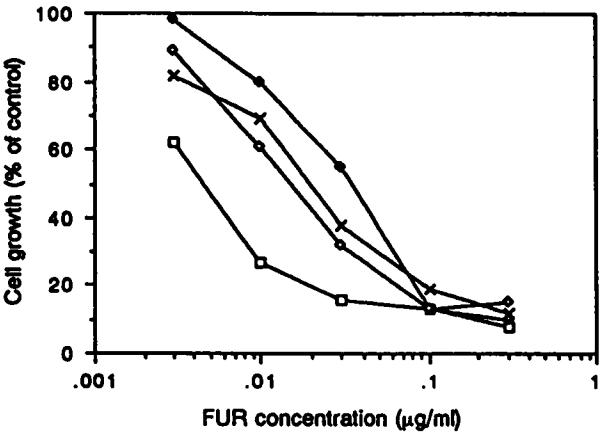

Figure 3. Reverse of FUR cytotoxicity by PEUdR in KATO III cells: (□) FUR alone, (◆) FUR plus 200 μM PEUdR, (×) FUR plus 100 μM PEUdR and (◇) FUR plus 50 μM PEUdR.

To find a more potent potentiator of FUdR cytotoxicity *in vitro*, two more derivatives, 5[2-(*p*-fluorophenyl)ethyl]-2′-deoxyuridine (FPEUdR) and 5[2-(*p*-methylphenyl)ethyl]-2′-deoxyuridine (MePEUdR) (Figure 1), were newly synthesized in our laboratory and their effect on FUdR cytotoxicity was tested with KATO III cells. FPEUdR was more potent than PEUdR (IC_{50} value of FUdR with 100 μM FPEUdR was 0.0048 μg/ml and the AR was 41.7-fold) and MePEUdR was less potent than PEUdR (IC_{50} value of FUdR with 100 μM MePEUdR was 0.009 μg/ml and the AR was 22.2-fold). It is difficult to draw conclusions about structure–activity relationships because there are so few derivatives, but it seems that compounds with a bulky and hydrophobic substituent (in this case methyl) at the *p*-position on the phenyl group are less effective than those with a less bulky fluoro substituent, which causes polarization of the phenyl group.

To understand how PEUdR affected metabolism of nucleosides and nucleotides, the incorporation of two radioisotope-labeled precursors of DNA and RNA, [^{3}H]thymidine ([^{3}H]TdR) and [^{14}C]uridine ([^{14}C]UR), into the acid-insoluble fraction was studied. Cultured KATO III cells were studied with three concentrations of PEUdR, i.e. 50, 100 and 200 μM. Incorporation of both thymidine and uridine was inhibited, and the degree of inhibition depended on concentration (Figure 4) and on time. When the cells were incubated for 4 h with 200 μM of PEUdR, the incorporation of [^{3}H]TdR was 78% less than control and the incorporation of [^{14}C]UR was 84% less than control. To find out if PEUdR inhibits transport of pyrimidine nucleosides, incorporation of the precursor into an acid-soluble fraction was measured. PEUdR inhibited [^{3}H]TdR

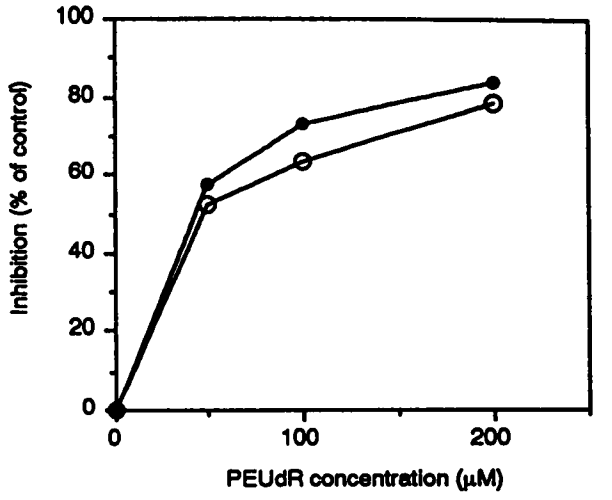

Figure 4. Inhibition of [^{3}H]TdR and [^{14}C]UR into acid-insoluble fractions in KATO III cells: (○) [^{3}H]TdR and (●) [^{14}C]UR.

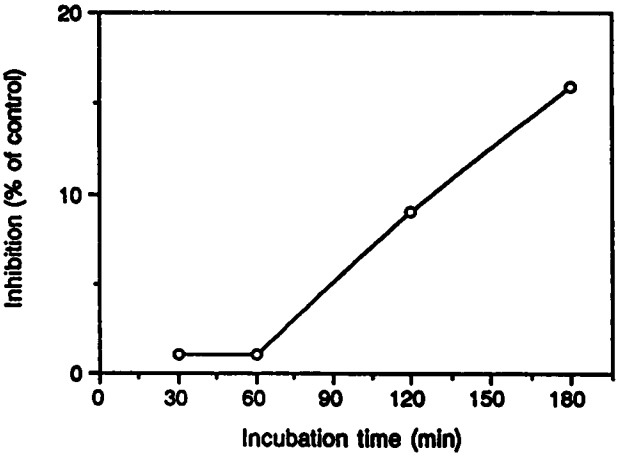

Figure 5. Inhibition of [^{3}H]TdR from the acid-soluble to the acid-insoluble fraction in KATO III cells in the presence of 100 μM of PEUdR compared with the absence of PEUdR.

uptake into KATO III cells. Furthermore, to find out if this nucleoside inhibits incorporation of [^{3}H]TdR from the acid-soluble to the acid-insoluble fraction (DNA synthesis), cells were incubated with [^{3}H]TdR at 19°C to label the nucleoside pool.[14] Then the cells were incubated at 37°C with a fresh medium in the presence or absence of PEUdR. PEUdR inhibited incorporation of [^{3}H]TdR from the acid-soluble to the acid-insoluble fraction as shown in Figure 5. The results suggest that PEUdR inhibits at least one thymidine salvage enzyme and the inhibition causes an imbalance between triphosphates in cooperation with the inhibition of thymidylate synthase (TSase) by FUdR.[15]

Next, [^{3}H]TdR was used to determine the ratios of thymidine (TdR), thymidine 5′-monophosphate

(TMP), thymidine 5'-diphosphate (TDP) and thymidine 5'-triphosphate (TTP) in the acid-soluble fraction (7×10^7 cells/ml, treated for about 6 h), by HPLC with DEAE-2SW (Tosoh) eluted along a linear gradient from 30 to 210 mM of phosphate buffer (pH 6.95) containing 20% acetonitrile, according to a previously reported method.[16] The per cent ratio of TdR, TMP, TDP and TTP was 40.85 : 20.95 : 20.60 : 17.61 for the normal control cells and 30.26 : 24.81 : 26.13 : 18.80 for cells treated with PEUdR (150 μM) for 4 h. These results are due to the inhibition of TdR transport by PEUdR and may include the inhibition of a thymidine salvage enzyme. Thus, the deoxynucleoside inhibits incorporation of [^{3}H]TdR from the acid-soluble to the acid-insoluble fraction. Furthermore, the counts per cell of [^{3}H]TdR in the acid- soluble fractions were higher than those in the control fraction for several different durations of incubation. This also indicates that PEUdR acts before DNA polymerase and after the active transport of pyrimidine nucleoside in KATO III cells.

Results of experiments on the uptake of [^{3}H]TdR and [^{14}C]UR indicate that PEUdR inhibits transport of pyrimidine nucleosides (both thymidine and uridine) in KATO III cells, and also inhibits incorporation of [^{3}H]TdR into DNA. If DNA synthesis itself is inhibited by PEUdR, then PEUdR alone would inhibit cell growth. Studies of incorporation of the precursor into the acid-insoluble fraction indicate that the nucleoside PEUdR has its inhibitory effect in a salvage pathway of pyrimidine nucleoside biosynthesis. A typical nucleoside transport inhibitor blocks FUdR cytotoxicity as well as deoxyadenosine cytotoxicity.[17] At higher concentrations of PEUdR (200 μM), potentiation of FUdR cytotoxicity was not as high as with lower concentrations (50 and 100 μM), probably due to increased inhibition of FUdR transport. A part of the reverse effect of FUR cytotoxicity by PEUdR may be due to the inhibition of FUR transport.

PEUdR may act only on the pyrimidine biosynthetic pathway, including related catabolic pathways, such as those with UDP-sugar enzymes. PEUdR did not modulate the cytotoxicity of four other antitumor agents, thioguanosine, cisplatin, adriamycin and 5-trifluoromethyl-2'-deoxyuridine, in KATO III cells under similar conditions for FUdR. In contrast, at 200 μM PEUdR had inhibited the growth of murine L1210 cells by about 50% after 48 h of incubation, and it did not inhibit cell growth at lower concentrations. Addition of 100 μM PEUdR potentiated the cytotoxicity of FUdR more than 308-fold, as measured by IC_{50}.

Each human gastric cancer cell line and murine leukemia cell line has a different susceptibility to PEUdR. Murine L1210 cells are very susceptible to PEUdR. This high susceptibility may be due to the growth rate of the cell.

The nucleoside PEUdR and its 5'-monophosphate have been synthesized[18] and their biological activities have been tested. The nucleoside inhibits TSase of *Lactobacillus casei in vitro*.[13] DeClercq *et al.* concluded that PEUdR does not have antitumor activity such as we observed in these experiments on human gastric cancer cells. The inhibition of TSase may also be related to the potentiation of FUdR cytotoxicity seen in the present results, concomitant with inhibition of the pyrimidine salvage pathway. Details of these mechanisms are not clear yet. PEUdR modulates nucleoside metabolism but it is not lethal, possibly because it affects only enzymes in salvage pathways. This lack of cytotoxicity is important for any compound that is to be used *in vivo*. Indeed, treatment with the combination of FUdR (200 mg/kg/day) and PEUdR (200 mg/kg/day) resulted in a significantly longer life span (T/C, 173%) than did treatment with FUdR alone (T/C, 147%) in CDF1 mice with intraperitoneal L1210 cells (10^5 cells, intraperitoneal treatment on days 1 and 5) without any apparent toxicity (Maeda *et al.*, unpublished results).

The mechanism by which PEUdR augments FUdR cytotoxicity in the human gastric cancer cell line KATO III includes inhibition of pyrimidine nucleoside transport and inhibition of thymidylate synthase, as previously proposed. Also, the cytotoxicity of FUdR can be modulated by concomitant inhibition of pyrimidine biosynthesis enzymes in the salvage pathway. The PEUdR-induced inhibition of nucleoside transport may not be strongly influenced by the augmentation of FUdR cytotoxicity in these cells. However, the mechanism of augmentation is more complicated and it includes inhibition or enhancement of the effects of related enzymes. Knowledge of the mechanism of this augmentation can be helpful in understanding the mechanism of FU cytotoxicity.

The present observations can be applied to FU chemotherapy of some human solid cancers. They may contribute to more effective therapy with fewer side effects.

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