Circumvention of drug resistance in cancer cells by a new fluoropyrimidine analogue

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Abstract

The potentiation of antitumor drugs by their analogues is one of strategies for overcoming cellular resistance to chemotherapy. In attempts to improve the efficacy of 5-fluorouracil (5-FU) and overcome 5-FU resistance, we synthesized new 5-FU derivatives and evaluated the potentiation effect on 5-FU cytotoxicity. 5-Fluorouracil-1-yl-3-butanone (5-FUBO), a newly synthesized 5-FU derivative, significantly potentiated the cytotoxic effect of 5-FU, and the potentiation of 5-FU by 5-FUBO in 5-FU resistant cells was higher than in their drug-sensitive parental cells. Surprisingly, it potentiated the cytotoxicities of vincristine and vinblastine. The potentiation of these drugs by 5-FUBO in mutidrug-resistant (MDR) cells was more than in their drug-sensitive parental cells. It was also shown that 5-FUBO increased the intracellular accumulation of [^{3}H]vincristine in P388/M cells, and this is related with increased cytotoxicity of vincristine. Northern blot analysis showed that the potentiation effect of MDR-related antitumor drugs by 5-FUBO was caused by inhibition of MDR1 gene expression. Also, 5-FUBO cytotoxicity in K562 cells was increased significantly upon transformation with c-Ha-ras.

Keywords: 5-fluorouracil, 5-fluorouracil-1-yl-butanone, multidrug-resistant cells, MDR1 gene, 5-FU resistance

Introduction

The fluororinated pyrimidine, 5-fluorouracil (5-FU) is an antimetabolite that is frequently used in the treatment of gastrointestinal, breast, head, neck and genitourinary cancers (Kubota et al., 1988). 5-FU has multiple biochemical actions that may be responsible for its cytotoxicity.

Three explanations have been given for the cytotoxic action of 5-FU. One widely accepted explanation is that 5-fluoro deoxyuridine-5'-monophosphate, an active metabolite of 5-FU, inhibits thymidylate synthetase irreversibly (Houghton et al., 1986). Another explanation is that 5-FU is incorporated into RNA (Cohen et al., 1985), and disturbs gene expression (Akazawa, 1986). A third explanation is that 5-FU is incorporated into DNA (Swayer et al., 1984) and interferes with DNA metabalism (Swayer et al., 1984). However, although 5-FU is effective in some cancer patents when given alone or in combination with other agents, most clinicians report that the response rate to 5-FU is only about 10 to 30% and remission, if achieved, is generally of short duration (Fujii et al., 1989a,b). In an effort to enhance the 5-FU activation, various antitumor agents and nucleosides have been used in combination with the fluoropyrimidine (Darnouski et al., 1985; Nishiyama et al., 1988; Fujii et al., 1989a,b). Multidrug resistance (MDR) is an important problem in cancer therapy. In human cells, the MDR phenotype is thought to be primarily a consequence of the increased expression of the MDR1 gene. This encodes for a transmembrane glycoprotein of 170 kDa (P-gp170) (Ueda et al., 1987). It was also reported that 5'-deoxy-5-fluorouridine (dFUrd) increased daunorubicin uptake in MDR cells with P-gp170 expression, and this is related with increased cytotoxicity (Van Der Heyden et al., 1994). Geng and colleagues reported that the NIH3T3 cells transformed by c-Ha-ras were more sensitive to dFUrd than their parental cells (Geng et al., 1991). In a previous study, we reported that a new 5-FU derivative, 5-fluorouracil-1-yl-3-butanone (5-FUBO) showed strong antitumor activity against various cancer cell lines (Kim et al., 1994).

In the present work, we demonstrate that 5-FUBO significantly potentiated the cytotoxic effect of 5-FU and partially overcome the 5-FU resistance. We also searched for altered sensitivity of MDR cells to 5-FUBO in combination with MDR-related antitumor drug, vinblastine or vincristine. The results demonstated that the potentiation of these MDR-related drugs by 5-FUBO in MDR cells was more than in drug-sensitive parental cells.

Materials and Methods

Chemicals
5-FUBO and its derivatives were synthesized by J. C. Kim (Pusan National University, Korea) as described elsewhere (Kim *et al.*, 1994). Acetyl-CoA was obtained from Sigma (St. Louis, MO). [^{14}C]Chloramphenicol and [G-^{3}H]vincristine sulphate were purchased from Amersham (Arlington Heights, IL).

Cells and plasmids
Human colorectal carcinoma cell line HT29 was kindly provided by Dr. Y. S. Kim (UCSF, U.S.A.). Golden hamster embryo lung cell line GHE-L was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Mouse leukemia cell line P388, mouse mammary carcinoma cell line FM3A and human leukemia cell line K562 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The MDR sublines of P388 and FM3A cells (P388/M and FM3A/M, respectively) were obtained by stepwise selection in colchicine (Kim *et al.*, 1993). pMMTV *ras* H-transfected K562 cells which overexpressed the Ha-*ras* was kindly donated by G. M. Cooper (Harvard Medical School, U.S.A.). The plasmid p432-CAT, which contains promoter region (970 bp *Pst*I fragment) of human *MDR1* gene and bacterial chloramphenicol acetyltransferase (CAT) gene, was obtained from Y. Sugimoto (the Japanese Foundation of Cancer Research, Japan).

Growth inhibition assay
Cells were seeded (2 X 10^4) in 96-well flat-bottom microtitration plates. After 24 h, cell were treated for 96 h by antitumor agent with indicated concentration of 5-FUBO. The effect of antitumor drugs on cellular growth in the absence or the presence of 5-FUBO was determined using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay (Mosman, 1983).

DNA transfection and CAT assay
Subconfluent cultures of GHE-L cells were transfected with 10 μg of p432-CAT according to the calcium phosphate coprecipitation method (Graham *et al.*, 1973). In order to test the effect of 5-FUBO on p432-CAT expression, 5-FUBO was added to the culture medium for the transfected cells, and CAT assay was carried out 24 h later. CAT assays were performed according to the method of Gorman *et al.* (1982).

Drug accumulation assay
The cells were plated into 24-well tissue culture dishes and allowed to grow to 90% confluency. The cells were washed with PBS and then incubated in 0.5 ml of RPMI 1640 containing 24 nM [^{3}H]vincristine (8.45 Ci/mmol)

for 30, 60 and 120 min at 37°C. The cultures were rapidly washed three times with ice-cold PBS. Intracellular [^{3}H]vincristine was solubilized wtih 0.3 ml of 1% SDS in PBS and quantified by liquid scintillation counting.

Northern blot analysis
Total RNA was isolated from P388/M cells using Ultraspec™-II RNA isolation kit (Biotecx). Denatured RNA samples (20 μg/well) were fractionated by electrophoresis in 1% agarose gel containing 7% formaldehyde and transferred to Hybond nylon membrane (Amersham), and hybridized for 20 h at 42°C in the same buffer containing the ^{32}P-labeled *MDR1* probe. After washing with 0.1% SDS and 0.1x SSC, the membranes were subjected to autoradiography.

Results and Discussion

The antitumor activity of 5-FUBO against drug-sensitive parental cells and their MDR sublines was compared in Table 1. 5-FUBO showed 2.8-fold higher antitumor activity in P388/M cells than in parental P388/S cells. Similarly, the antitumor activity of 5-FUBO in FM3A/M cells was 3.3-fold higher in comparison with the parental FM3A/S cells. This result suggested that 5-FUBO was more efficient against MDR cells than their drug-sensitive parental cells.

Moreover, we investigated the combination effect of antitumor drug and 5-FUBO on drug sensitivity against HT-29 cells. The IC$_{50}$ value of 5-FUBO for the cells was about 0.5 μg/ml, and this concentration of 5-FUBO potentiated the antitumor activities of 5-FU, vincristine and vinblastine by 5.1-, 4.0- and 3.2-fold, respectively (Table 2). It is interesting that 5-FUBO was rather effective against MDR sublines of cancer cells than their parental cells and potentiated the cytotoxicities of antitumor drugs in HT29 cells.

To investigate the combination effect of 5-FUBO and

Table 1. Effect of 5-fluorouracil-1-yl-3-butanone(5-FUBO) on growth of parental and drug resistant cancer cells. The IC$_{50}$ value is defined as the concentration of the drug required to produce 50% inhibition of cell growth. Data obtained from triplicate experiments.

Cells	IC$_{50}$ (μg/ml) of 5-FUBO
P388	0.42
P388/M	0.15
FM3A	0.54
FM3A/M	0.16

Table 2. Effect of 5-FUBO on sensitivities to antitumor drugs of HT29 cells. Data were obtained from triplicate experiments. VCR, vincristine; VBL, vinblastine.

Cells	Drugs	IC$_{50}$ (ng/ml)		Fold enhancement
		None	+5-FUBO (0.5 μg/ml)	
HT29	5-FU	360	70	5.1
	VCR	1.2	0.3	4.0
	VBL	3.2	1.0	3.2

Table 3. Effect of 5-FUBO on 5-FU cytotoxicity of parental and various 5-FU resistant FM3A cells. Data were obtained from triplicate experiments.

Cells	IC$_{50}$ of 5-FU (ng/ml)		Fold enhancement
	None	5-FUBO (0.1 μg/ml)	
FM3A	20	20	1.0
FM3A/R-1	35	15	2.3
FM3A/R-2	100	38	2.6
FM3A/R-3	130	31	4.2
FM3A/R-4	250	48	5.2

Table 4. Effect of 5-FUBO on sensitivities to antitumor drugs of parental and multidrug-resistant cancer cells.

Cells	Drugs	IC$_{50}$ (ng/ml)		Fold enhancement
		None	5-FUBO (0.5 μg/ml)	
FM3A	VCR	90	44	2.0
	VBL	51	34	1.5
FM3A/M	VCR	390	160[a]	2.4
	VBL	83	41[a]	2.
P388	VCR	24	24	1.0
	VBL	38	38	1.0
P388/M	VCR	190	90[a]	2.1
	VBL	88	38[a]	2.3

[a] Cells were treated with antitumor drugs in the presence of 0.1 μg/ml 5-FUBO for 96 h. Data were obtained from triplicate experiments.

5-FU and the possibility of overcoming 5-FU resistance by 5-FUBO, we have isolated a series of sublines resistant to 5-FU, and the effect of 5-FUBO on sensitivity to 5-FU of parental and 5-FU-resistant FM3A cells was examined (Table 3). The results showed that 5-FUBO enhanced the antitumor activity of 5-FU and the potentiation ratio was increased as the higher degree of 5-FU resistance in 5-FU-resistant cells.

Also, the potentiation of antitumor agents by 5-FUBO was compared in drug-sensitive and their MDR cells (Table 4). 5-FUBO (0.5 μg/ml) potentiated the cytotoxicities of vincristine and vinblastine by 2- and 1.5-fold in parental FM3A/S cells, but there was no effect in P388/S cells. By contrast, low concentration (0.1 μg/ml) of 5-FUBO potentiated vincristine or vinblastine cytotoxicity more than 2-fold in FM3A/M and P388/M cells. These results indicate that the potentiation of these MDR-related drugs by 5-FUBO in MDR cells was more than in drug-sensitive parental cells, and 5-FUBO can partially overcome the drug resistance.

The cellular accumulation of vincristine is often reduced in MDR cells (Matsuo et al., 1990). To assess the effect of 5-FUBO on the intracellular accumulation of [^{3}H]vincristine, P388/M cells were pretreated with 5-FUBO (1 μg/ml) for 24 h, and accumulation of vincristine was measured at 30, 60 and 120 min (Figure
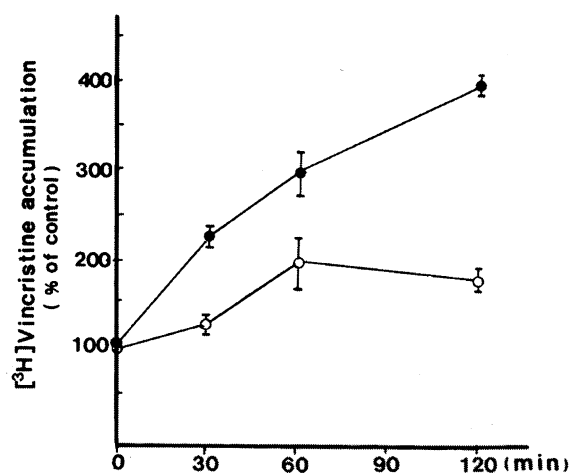

Figure 1. Effect of 5-FUBO on [^{3}H]vincristine accumulation in P388/M cells. The cells were incubated in the absence (○) or presence (●) of 1 μg/ml 5-FUBO for 24 h. [^{3}H]Vincristine was then added and its intracellular accumulation after 30, 60 or 120 min was detemined. Values represent the mean ± S.D. accumulation of [^{3}H]vincristine (solvent control = 100%) in one of two similar experiments.

1). Pretreatment of 5-FUBO (1 μg/ml) caused 2-fold increase in intracellular [^{3}H]]vincristine accumulation, when the cells were incubated for 120 min with the drug. The result shows that 5-FUBO improves the [^{3}H]vincristine accumulation in MDR cells, and this is associated with the potentiation of VCR cytotoxicity by 5-FUBO. Thus, we examined whether the synergistic effect of 5-FUBO with antitumor drugs was caused by inhibition of *MDR1* gene expression. Northern blot analysis using *MDR1* probe showed that the expression of *MDR1* gene was reduced in P388/M cells by 5-FUBO
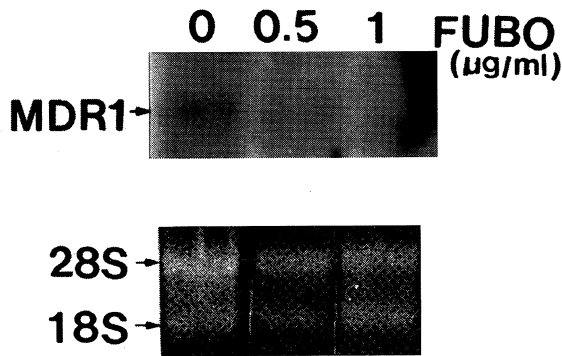
0   0.5   1   FUBO
                    (μg/ml)

MDR1

28S
18S

Figure 2. Effect of 5-FUBO on the expression of *MDR1* gene. Total cellular RNA (20 μg/lane) isolated from P388/M cells was resolved by electrophoresis, transfered to Hybond nylon membrane, and sequentially hybridized with ^{32}P-labeled *MDR1* probe.

0   0.1   0.5   FUBO
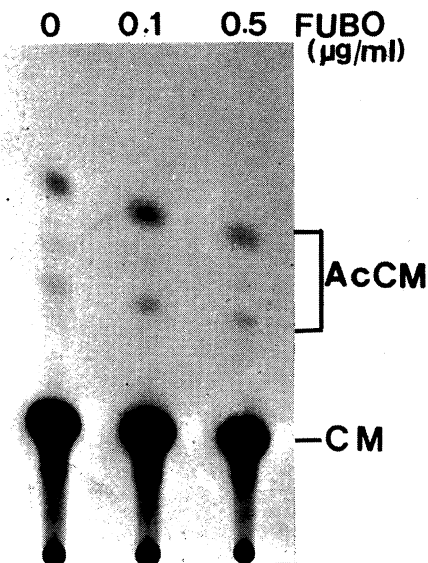   **(μg/ml)**

AcCM

—CM

Figure 3. Effect of 5-FUBO on MDRCAT activity in GHE-L cells. The cells were transiently transfected with 10 μg of p432 CAT. After 24 h of transfection, the cells were incubated in the presence of the indicated dose of 5-FUBO for 24 h prior harvesting.

Table 5. Combination effect of 5-FUBO on the cytotoxicity of antitumor drugs to parenal and ras-overexpressed K562 cells. Data were obtained from triplicate experiments.

Antitumor drugs	Added 5-FUBO (μg/ml)	Combination effect	
		K562	K562/*ras*
5-FU	0.2	1.0	2.0
	0.5	1.4	8.7
VCR	0.5	1.4	3.3
VBL	0.5	1.0	2.0
ADR	0.5	1.0	2.0

treatment (Figure 2). We also examined the effect of 5-FUBO on the activity of *MDR1* promotor. When p432-CAT was expressed in GHE-L cells, CAT activity was reduced in GHE-L cells by 5-FUBO treatment, although the same concentrations of 5-FUBO did not inhibit cell growth (Figure 3). Two independent assays demonstrate that 5-FUBO may regulate *MDR1* gene expression. Chin and colleagues reported that the promoter of the human *MDR1* gene is a target for the c-Ha-*ras* oncogene product, which implies that the *MDR1* gene could be activated during tumor progression associated with mutation in *ras* (Chin *et al.*, 1992). It is

of interest to explore the *ras* involvement in 5-FUBO activity against cancer cells. Thus, we investigated the possibility of *ras* involvement in 5-FUBO activation in *ras*-overexpressed K562 (K562/*ras*) cells. The 5-FUBO demonstrated the IC$_{50}$ value of 1.7 μg/ml for K562 cells and of 0.6 μg/ml for K562/*ras* cells. We compared the effect 5-FUBO on drug sensitivities to 5-FU, vincristine, vinblastine and adriamycin of K562/*ras* cells with those of K562 cells. As shown in Table 5, K562/*ras* cells showed that the 5-FU potentiation ratio of 5-FUBO was 2- and 8.7-fold at combination dose of 0.2 and 0.5 μg/ml 5-FUBO, respectively, but only 1.4-fold by combination of 0.5 μg/ml 5-FUBO in K562 cells. 5-FUBO (0.5 μg/ml) potentiated the cytotoxicities of vincristine, vinblastine and adriamycin by 3.3- to 2-fold in K562/*ras* cells, but only a slight effect in K562 cells. These findings suggested that the presence of activated *ras* genes might help to predict response to 5-FUBO.

Most antitumor drugs fail to induce clear response in the treatment of cancer. This can be explained by involvement of overexpression of the membrane glycoprotein P-gp170, which is associated with MDR and/or with involvement of *ras* (Chin *et al.*, 1992).

Previous studies reported that dFUrd exerted a positive effect on daunorubicin resistance and exhibited marked cytotoxic activity in MDR cells (Van Der Heyden *et al.*, 1994). It was also reported that dFUrd activation in NIH3T3 cells could be increased significantly upon transformation with c-Ha-*ras* (Geng *et al.*, 1991). 5-FUBO itself has marked cytotoxic activity in MDR cells and *ras*-overexpressed K562 cells. These finding suggest that 5-FUBO seems to be useful a candidate for the treatment of MDR cells. Futher studies are necessary to explore metabolism of the drug 5-FUBO.

Acknowledgement

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