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ORIGINAL ARTICLE

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Synergistic cytotoxic effect between serine-threonine phosphatase inhibitors and 5-fluorouracil: a novel concept for modulation of cytotoxic effect

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Abstract *Purpose:* The present study was undertaken to look for an agent or agents able to modulate the cytotoxic effect of 5-fluorouracil (FUra) and to investigate the role of serine-threonine phosphatase inhibitors on the cytotoxic effect of FUra. *Methods:* The cytotoxicities of FUra and protein phosphatase inhibitors (PPIs) were evaluated by two different methods: a clonogenic assay and a proliferation assay. In the clonogenic assay, cancer cells were treated with various concentration of FUra with or without PPIs for 72 h. The drug-containing medium was replaced by fresh medium, the cultures incubated for an additional 10 days, and the colonies enumerated. In the proliferation assay the cells were treated with FUra alone or in combination with PPIs for 96 h and cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the uptake of the tetrazolium dye. Thymidine kinase (TK) activity was determined based on the catalytic phosphorylation of [^{3}H]d-thymidine to [^{3}H]dTMP. Incorporation of FUra into DNA and RNA was determined by treating the cells with [2-^{14}C]fluorouracil for 72 h and measuring the radioactivity in the isolated DNA and RNA fractions. *Results*: The serine-threonine phosphatase inhibitors caliculin A (CAL), okadaic acid (OA) and microcystin-LR (MCLR) dose-dependently inhibited the growth of Clone 20 and Clone 5 cells of Colon 26 murine colon adenocarcinoma cells, human cervical cancer HeLa cells, human gastric cancer MKN 7 cells, and murine sarcoma S-180 cells in vitro. Among the compounds tested, MCLR at non-toxic concentrations was found to increase FUra incorporation into RNA and DNA in Clone 20 cells by 60% and 127%, respectively, to increase TK activity alone (two-fold) as well as in combination with FUra (threefold), and to potentiate the cytotoxicity of FUra synergistically and cytospecifically in vitro. The cytotoxicity of FUra alone or in combination with MCLR, but not that of PPIs alone, was abrogated almost completely by exogenous thymidine (dThd), suggesting that inhibition of thymidylate synthetase (TS) is the growth-limiting event in the cytotoxic action of FUra even in combination with MCLR. *Conclusions:* The findings presented here suggest that MCLR synergistically and cytospecifically potentiates the antitumor activity of FUra with substantial improvement in the therapeutic index of FUra via enhancement of both DNA- and RNA-directed cytotoxicity.

Keywords Synergism · Cytotoxicity · 5-Fluorouracil
Phosphatase inhibitors · Microcystin-LR · Thymidine kinase

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Introduction

Since its discovery as an antineoplastic agent by Heidelberger in 1957, 5-fluorouracil (FUra) has been used for the treatment of a broad spectrum of solid tumors, although the response rates are moderate and cures are rarely achieved [33, 40]. It is also extensively used as a palliative

treatment for disseminated visceral cancers as well as curative therapy for many kinds of epithelial neoplasms [1, 6]. Among the anticancer drugs in use, FUra is the single most active chemotherapeutic agent, particularly in the treatment of colorectal adenocarcinoma [21, 28].

To date, for the curative treatment of colorectal carcinoma, neither a single drug nor a combination is available. Due to severe hematological toxicity, FUra is regarded as a highly toxic drug with a narrow margin of safety. Many approaches have been made to increase the therapeutic index of FUra, such as combination therapy with other antineoplastic drugs, modulation of FUra metabolism by biochemical means [33] and synthesis of newer more effective analogues [17]. However, combinations of other anticancer drugs with FUra do not usually produce a better clinical response and the higher doses of FUra used frequently result in severe adverse reactions. FUra-based cancer chemotherapy has recently undergone a dramatic change with the combination of noncytotoxic agents to modulate the cytotoxic effect of FUra resulting in a marked increase in the therapeutic index. However, an agent able effectively to potentiate the antitumor effect of FUra with marginal toxicity is still not clinically available.

Potentiation of the cytotoxic effect of FUra by the alkaline phosphatase inhibitors levamisole and orthovanadate has been reported [19, 20]. These studies have shown that inhibition of alkaline phosphatase is responsible for the increased cytotoxicity of FUra in vitro when administered in combination. An additive interaction between FUra and levamisole has also been reported in human colorectal carcinoma cell lines [12]. Levamisole has also been used clinically in combination with FUra for the treatment of Dukes' C colon carcinoma [24, 29] and advanced gastrointestinal malignancy [2, 36]. The increased cytotoxicity of FUra in combination with the phosphatase inhibitors levamisole and sodium orthovanadate led us to investigate the effects of other phosphatase inhibitors on FUra cytotoxicity.

In the past few decades much effort has been expended on exploring the underlying mechanism responsible for the antitumor effect of FUra. FdUMP, an intermediate product of FUra metabolism, binds covalently to thymidylate synthetase (TS) with the formation of a ternary complex with 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4 folate) resulting in inhibition of DNA synthesis due to decreased availability of thymidylate which is indispensable for synthesis of DNA [15, 16, 30]. Incorporation of FUra into cellular RNA resulting in RNA dysfunction [9, 10, 11, 22, 38, 41] is considered to play a vital role in the cytotoxicity of FUra. In the 1980s, several investigations revealed that FUra is also incorporated into DNA but to a lesser extent [22, 23, 27], but the role of this effect in the cytotoxicity of FUra has not yet been elucidated [32].

For the regulation of gene expression and cell growth, phosphorylation is the main regulatory mechanism of signal transduction pathways. The phosphatase inhibitor microcystin-LR (MCLR), a metabolite of cyanobacteria of the genus *Microcystis* which grows worldwide in fresh and brackish water [3], is a potent inhibitor of protein phosphatase-1 and -2A as well as a powerful tumor promoter [18, 26, 42]. Inhibition of these enzymes in the liver is apparently associated with hepatocyte deformation due to reorganization of microfilaments [7].

In the study reported here we investigated the effects of some protein phosphatase inhibitors (PPIs) of marine origin on cancer cell growth, FUra cytotoxicity, thymidine kinase (TK) activity, and incorporation of FUra into DNA and RNA.

Materials and methods

Radiochemicals and drugs

[methyl-^{3}H]d-Thymidine (48 Ci/mmol), [5-^{3}H]dUMP (16 Ci/mmol), and [2-^{14}C]-fluorouracil (56 mCi/mmol) were purchased from Moravek Biochemicals (Brea, Calif.). 5-FUra, MCLR, caliculin A (CAL), okadaic acid (OA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Dojindo, Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of reagent or analytical grade.

Cell lines

Two subclones derived from the murine Colon 26 adenocarcinoma cell line, Clone 20 and Clone 5, were collected from Dr. Yutaka Tanaka of Nippon Roche Research Center, Japan. Human cervical cancer HeLa cells, murine sarcoma S-180 cells, and human gastric cancer MKN 7 cells were kindly donated by the National Cancer Center, Japan. Clone 20, Clone 5, S-180 and MKN 7 cells were maintained in RPMI-1640 medium (GIBCO BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO BRL), penicillin G (100 U/ml) and streptomycin (100 μg/ml) in a humid atmosphere (100% relative humidity) containing 5% v/v CO_2. HeLa cells were maintained in DMEM (GIBCO BRL) supplemented with 10% FBS. All cell lines were passaged every 3 days and maintained in exponential growth.

Animals and tumor inoculation

Male ICR mice weighing 18–20 g were obtained at 6 to 8 weeks of age from Japan Shizuoka Laboratory Animal Center (Hamamatsu). The experiments with mice were conducted in accordance with the "Guide for Animal Experimentation", an official publication of Chiba University, Japan. A single-cell suspension of S-180 sarcoma cells (1×10^6 cells/mouse) was inoculated subcutaneously into the right inguinal flank.

Cytotoxicity assay

Proliferation assay. Cells from routine, subconfluent culture were counted by trypan blue exclusion, and dispensed to 96-well culture plates (Sumitomo, Japan) at a density of 1000 cells/well. The cultures were incubated at 37°C and after a lag-phase of 24 h, the medium from the wells was replaced by drug-containing medium with corresponding controls. For thymidine rescue experiments 10 μ*M* (final concentration) deoxythymidine (dThd) was added simultaneously with FUra and/or PPIs. The drugs were tested in quadruplicate for each concentration. After 96 h of drug exposure, the growth-inhibitory effects were evaluated with the MTT assay. Details of the assay procedure have been described extensively elsewhere [35].

Clonogenic assay. Cells were plated at a density of 150 cells/well in 24-well plates (Sumitomo, Japan) in medium containing 10% dialyzed FBS and after 24 h for cell attachment, various concentrations of FUra alone and in combination with 1 μ*M* MCLR were added. After 72 h of drug exposure, the drug-containing medium was replaced with fresh medium containing 10% FBS. After incubation of the plates for an additional 10 days, the wells were rinsed once in normal saline, dried, stained with 1% crystal violet, and the colonies were counted.

Incorporation of [^{14}C]FUra into DNA and RNA

To the cultures of Clone 20 cells, [2-^{14}C]FUra (56 mCi/mmol) alone and in combination with MCLR, both at a final concentration of 5 μ*M* were added at subconfluency. Incorporation of labeled FUra into DNA and RNA was determined as described previously [34] with minor modifications. In brief, following 72 h of drug treatment, the cells were washed twice with phosphate-buffered saline (PBS) after trypsinization and counted in a hemocytometer. Cells were treated with 0.4 *M* $HClO_4$ for 15 min and centrifuged to precipitate DNA and RNA. Following two washes with PBS, the precipitate was incubated with DNase-free RNase (15 μg in 100 ml Tris-HCl containing 1 m*M* EDTA at pH 7.4) for 15 min at room temperature, and the ribonucleotides were extracted with 5 *M* $HClO_4$. On centrifugation, the DNA settled as a pellet leaving the RNA in the supernatant. The pellet was washed three times prior to solubilization of the DNA in a solubilizer containing 0.1% sodium dodecyl sulfate in 0.1 *M* sodium hydroxide. Radioactivity in both the fractions was measured as described previously [34]. FUra incorporation was expressed as picomoles per 10^6 cells per 24 h.

TK assay

TK activity was determined based on the catalytic phosphorylation of [^{3}H]dThd to [^{3}H]dTMP as described previously [43]. In brief, the cell extract was incubated with 0.5 μCi [methyl-^{3}H]thymidine in a final volume of 50 μl containing 50 m*M* Tris-HCl buffer (pH 8.0), 1 m*M* ATP, 5 m*M* magnesium chloride, 0.2% bovine serum albumin (BSA), at 37°C for 15 min followed by incubation at 100°C for 5 min to stop the reaction. An aliquot was applied to an ion-exchange paper (Whatman DE-81) which was washed sequentially with 1 m*M* ammonium formate, methanol, and ether, and the radioactivity retained on the paper was measured using a liquid scintillation spectrophotometer (Beckman LS 6500). Enzyme activities were normalized to total protein concentration, determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, Calif.) with BSA as a standard. TK activity was expressed as picomoles per milligram protein per hour.

TS assay

The tumors were frozen in liquid nitrogen immediately after removal. The frozen tissues were homogenized with ice-cold homogenizing buffer containing 200 m*M* Tris-HCl, 20 m*M* β-mercaptoethanol, 100 m*M* NaF, and 15 m*M* cytidine 5′-monophosphate (CMP) using a Polytron PT homogenizer. TS activity was determined based on the conversion of [5-^{3}H]dUMP to dTMP, releasing tritiated water [39]. The reaction mixture consisted of 25 μl 7000 *g* supernatant, 5 μl 6.5 m*M* 5,10-CH_2-H_4 folate, 10 μl [5-^{3}H]dUMP (10 μ*M* final concentration) and was incubated for 30 min at 37°C. The reaction was stopped by the addition of 50 μl ice-cold 35% trichloroacetic acid and 250 μl 10% neutral activated charcoal. The suspension was centrifuged and 150 μl of the supernatant was used for counting of radioactivity by liquid scintillation.

HPLC analysis

The enzyme preparations from the tumors were analyzed using a DEAE-5PW ion-exchange column (7.5×75 mm, Tosch Company, Japan). The column was equilibrated with buffer 'A' containing 5 m*M* Tris-HCl (pH 8.5) and 10% v/v glycerol prior to each application of sample. An aliquot of enzyme extract of about 0.2 ml was injected onto the column and was eluted with a NaCl gradient system (buffer 'A' and buffer 'A' containing 0.7 *M* NaCl). The elution velocity was maintained at 0.25 ml/min and the NaCl gradient was controlled with the help of a gradient pump modulator (PX-8010, Tosch Company) to create a concave NaCl curve in the elution pattern.

Statistical analysis

The level of significance of differences between treatment groups at each drug concentration and differences between means were analyzed using one-way ANOVA and Newman-Keuls' range test, respectively. Student's *t*-test was employed to evaluate the differences between two groups.

Results

In vitro growth-inhibitory effect of PPIs

The PPIs CAL, OA, and MCLR showed inhibition of cell growth in all cell lines tested at nanomolar to micromolar concentrations with a wide variation in sensitivity. Among the cell lines examined, HeLa and Clone 20 cells appeared to be relatively resistant to FUra (IC_{50} 6.13 and 4.08 μ*M*, respectively). Clone 20 cells also exhibited a higher tolerance to OA and MCLR, but HeLa cells showed the highest tolerance to CAL of the cell lines tested (Table 1).

To determine whether the cytotoxic effects of these PPIs were mediated by TS, the cells were coincubated

Table 1 Growth inhibitory effects of PPIs and FUra in various cancer cell lines in vitro. Cells from subconfluent cultures were plated in the wells of a 96-well plate at a cell density of 1000–2000 cells/well. After attachment for 24–48 h, cells were treated with either FUra or each of the PPIs as indicated below for 72–96 h depending on the cell line used. Growth inhibitory effects were evaluated by the MTT assay as described in Material and methods. The IC_{50} value for each agent was determined from a dose response curve constructed for each agent. Values are the means ± SE from three or four independent determinations

Cell line	IC_{50} values			
	CAL (n*M*)	OA (n*M*)	MCLR (μ*M*)	FUra (μ*M*)
Clone 20 Colon 26 murine adenocarcinoma	0.82 ± 0.05	13.50 ± 2.10	9.00 ± 1.51	4.08 ± 0.65
Clone 5 Colon 26 murine adenocarcinoma	0.51 ± 0.06	11.95 ± 0.94	6.88 ± 1.38	1.63 ± 0.08
HeLa human cervical cancer	1.85 ± 0.21	10.65 ± 0.35	4.57 ± 0.62	6.13 ± 1.05
S-180 murine sarcoma	0.46 ± 0.02	12.70 ± 1.52	6.61 ± 0.93	1.25 ± 0.10
MKN 7 human gastric cancer	0.75 ± 0.10	9.50 ± 0.67	5.35 ± 0.21	3.90 ± 0.43

with various concentrations of dThd and PPIs or FUra for different lengths of time. In no case abrogation of the cytotoxic effect was observed except for FUra with 15 μ*M* dThd which abrogated the cytotoxic effect almost completely (data not shown) suggesting that PPIs exert cytotoxic effect through different pathway other than TS.

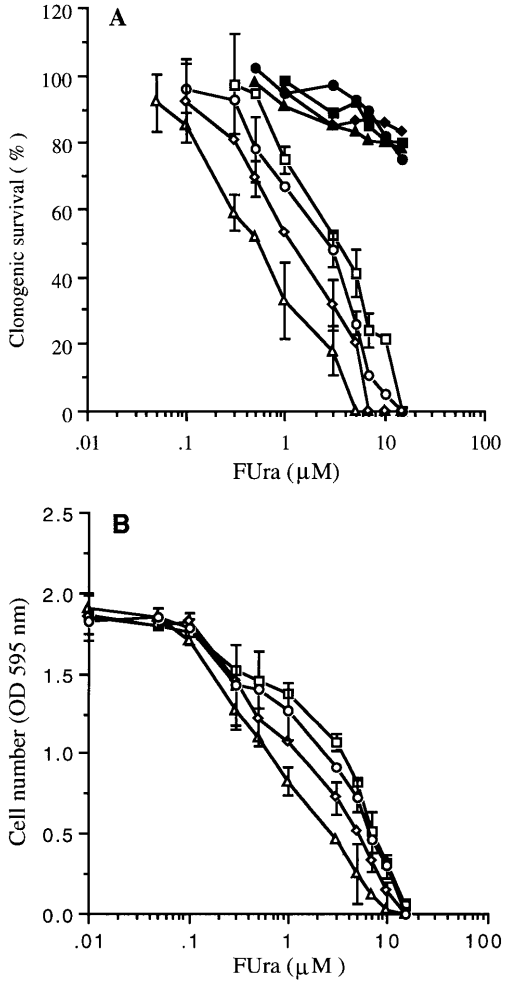

Fig. 1A, B Synergistic growth inhibitory effect of PPIs in combination with FUra in Clone 20 cells in vitro. **A** Clonogenic assay: Clone 20 cells were seeded at a density of 150 cells/well in 24-well plates. The cells were treated for 72 h with various concentration of FUra with or without PPIs, as follows: FUra *open squares*, FUra + CAL (0.1 n*M*) *open diamonds*, FUra + OA (2 n*M*) *open circles*, FUra + MCLR (1 μ*M*) *open triangles*. The *solid symbols* indicate the additional presence of 10 μ*M* dThd. The drug-containing medium was replaced by fresh medium followed by incubation for an additional 10 days. The colonies were counted. The mean cloning efficiency was about 63% in the absence of drug. The results are representative of three separate experiments and are presented as means ± SE. **B** Proliferation assay: cells were plated at a density of 1×10^3 cells/well in 96-well plates and after 48 h of incubation the cells were treated with the indicated concentrations of FUra alone or in combination with various PPIs for 96 h. The symbols indicate the same drug treatments as above (**A**). Inhibition of growth was evaluated by the MTT assay as described in the Materials and methods. Each point represents the mean ± SE from three or four individual experiments

Synergistic cytotoxic effect of PPIs with FUra

The effects of PPIs in combination with various concentration of FUra on growth inhibition were evaluated by a proliferation assay and a clonogenic assay. In Clone 20 cells, under the experimental condition of the clonogenic assay, 3.40 μ*M* FUra alone caused a 50% reduction in the number of colonies (Fig. 1A). Surprisingly, treatment with 1 μ*M* MCLR reduced the IC_{50} of FUra to 0.54 μ*M* (6.30-fold). MCLR inhibited cell growth maximally followed by CAL, but OA caused little inhibition (Table 2). The results of the proliferation assay (Fig. 1B) were comparable to those of the clonogenic assay (Fig. 1A). To examine the pathways involved in this synergistic effect, a dThd rescue experiment was carried out. The cytotoxicity of FUra alone or in combination with PPIs was markedly abrogated by 10 μ*M* dThd, indicating that the sole locus of action, even in combination, lies on TS (Fig. 1A). However, at higher concentrations of FUra, thymidine rescue was not possible (data not shown). Potentiation of FUra cytotoxicity by MCLR was also observed in HeLa and MKN 7 cells, but not in Clone 5 or S-180 cells (data not shown).

The nature of the interaction between FUra and MCLR was assessed by calculating the combination index (CI) using the equation $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where $(D)_1$ and $(D)_2$ are the concentrations of the two drugs $(Dx)_1$ and $(Dx)_2$. Data obtained from the MTT assay were subjected to median effect analysis for calculation of the CI using the equation $fa/fu = (D/Dm)^m$, where D is the dose, fa and fu are the fractions of the system affected and unaffected, respectively, by the dose D, Dm is the dose required to produce the median effect, and m is a coefficient signifying the sigmoidicity of the dose-effect curve [4, 5]. The combination of MCLR and FUra at a molar ratio of 3:1 showed the maximum CI, indicating significant synergism between the two, followed by FUra and CAL (Fig. 2). The FUra and OA combination exhibited a slight synergistic effect.

Effect of MCLR on TK activity

TK activity in Clone 20 tumor cells in vitro was found to increase moderately after treatment with 5 μ*M* FUra and about twofold with 5 μ*M* MCLR for 48 h at 37°C (Fig. 3). When the cells were treated with the same concentration of FUra in combination with 5 μ*M* MCLR for same length of time and under the same experimental condition, TK was increased by more than threefold (Fig. 3) indicating interruption in DNA synthesis due to depletion of thymidylate.

TS inhibition and TK increase by FUra

Administration of FUra orally to mice bearing S-180 sarcoma markedly increased TK activity and dramati-

Table 2 Potentiation of cytotoxicity of FUra by PPIs. Proliferation assay: Clone 20 cells were treated with FUra alone or in combination with various PPIs. Clonogenic assay: Clone 20 cells were seeded at a density of 150 cells/well in a 24-well plate using medium containing dialyzed serum. After 24–48 h, the cells were treated with FUra and/or each of the PPIs for 72 h. For the thymidine rescue experiment 10 μ*M* dThd was also added. After removal of the drugs, the cells were allowed to form colonies for an additional 10 days, after which the colonies were stained and counted. The IC_{50} values for the proliferation assay were calculated from the data presented in Fig. 1A, and those for the clonogenic assay were calculated from the data presented in Fig. 1B

Relative to FUra

	Clonogenic assay		Proliferation assay	
Additions	IC_{50} (μ*M*)	Fold change	IC_{50} (μ*M*)	Fold change
None	3.40	1.0	4.08	1.0
OA (2 n*M*)	2.74	1.24	3.10	1.32
CAL (0.1 n*M*)	1.21	2.81	1.63	2.50
MCLR (1 μ*M*)	0.54	6.30	0.75	5.44
dThd (10 μ*M*)	>15.0			
dThd + OA	>15.0			
dThd + CAL	>15.0			
dThd + MCLR	>15.0			

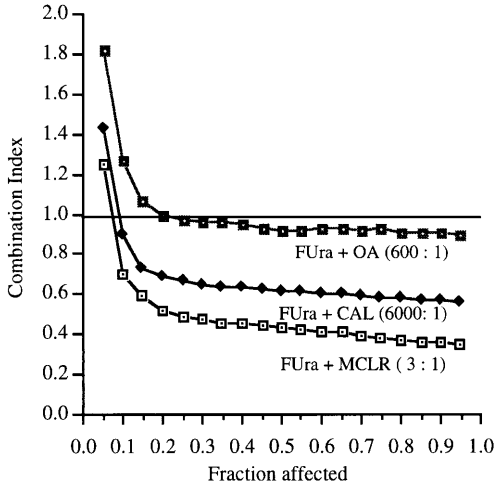

Fig. 2 Analysis of the cytotoxicity of combinations of FUra and PPIs against Clone 20 cells. To assess the nature of the interaction between FUra and PPIs, they were tested alone and in combination at various concentrations but at fixed molar ratios. Cell numbers were determined by the MTT assay. CI values less than 1.0 indicate synergism, equal to 1.0 indicate additivity, and greater than 1.0 indicate antagonism

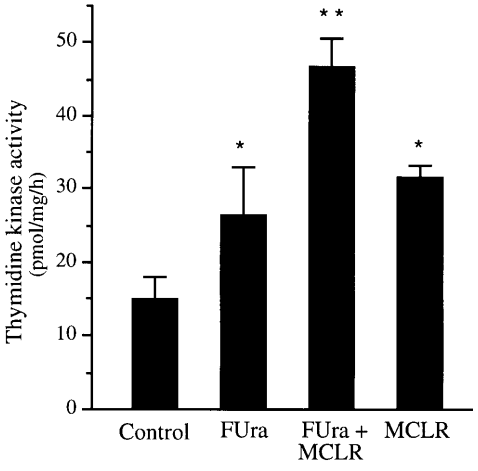

Fig. 3 TK activity in Clone 20 cells after treatment with FUra and MCLR in vitro. Exponentially growing Clone 20 cells were treated with equimolar concentrations (5 μM) of FUra and/or MCLR for 48 h and TK activity in cell extracts was determined as described in Materials and methods. Data are representative of four separate experiments. $*P<0.05$ vs control, $**P<0.01$ vs FUra-treated samples

cally decreased TS activity (Fig. 4). TK activity was increased 3.5-fold and TS activity was decreased nearly 20-fold 3 h after administration of 80 mg/kg FUra. The maximum increase in TK activity was found 3 h after FUra administration.

Phosphorylation of TK by FUra

In order to gain an insight into the increase in TK, we analyzed the S-180 sarcoma enzyme preparation by HPLC. Two distinct cytosolic TK peaks were resolved, designated as TK I and TK II (Fig. 5A). These two well-defined peaks, representing two isoforms of TK, were eluted with two different concentrations of NaCl in elution buffer, 0.03 *M* and 0.05 *M*, respectively. A third chromatographic peak, eluting with 0.2 *M* NaCl, represented TK of mitochondrial origin as revealed by its preferential utilization of cytidine 5-triphosphate (CTP) as a phosphate donor. Surprisingly, 3 h after administration of 80 mg/kg FUra to mice bearing S-180 tumor, the first peak disappeared completely and only one peak was eluted with 0.05 *M* NaCl (Fig. 5B), the concentration with which the second peak of the control sample eluted, indicating that the first peak had merged with the second peak (TK II), possibly due to phosphorylation of TK protein.

Increased FUra incorporation into DNA and RNA

As incorporation of FUra into DNA and RNA is thought to be responsible for its cytotoxic effect [40], we sought to determine whether MCLR could increase

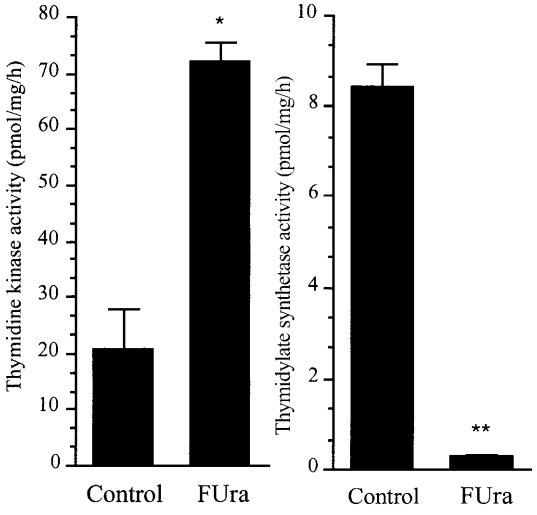

Fig. 4 Effect of FUra on TK and TS activity. S-180 sarcoma-bearing male ICR mice were given with 80 mg/kg FUra and the animals were killed after 3 h. Tumor extracts were prepared and analyzed for TK and TS activity as described in Materials and methods. The results are represented as means ± SE from three independent experiments. $*P < 0.05$, $**P < 0.01$ vs corresponding control

FUra incorporation into DNA and RNA. MCLR caused a substantial increase in FUra incorporation into RNA as well as DNA in Clone 20 cells. Surprisingly, MCLR increased incorporation of FUra into DNA by about 60% and into RNA by as much as 127% (Fig. 6), suggesting that RNA-directed cytotoxicity is also responsible for the synergistic antitumor effect of FUra and MCLR.

Discussion

In a recent study we demonstrated that subcutaneous inoculation of Clone 20 cells, a cachexigenic subclone of adenocarcinoma Colon 26, into mice resulted in marked body weight loss with an increase in hepatic nicotinamide *N*-methyltransferase activity. Neither carcass weight loss nor tumor burden could be prevented by administration of FUra alone [31]. Relative resistance of Clone 20 cells to FUra and the development of cachexia in the host animals led us to choose this tumor cell line to investigate the mechanism of the synergistic effect of PPIs on FUra cytotoxicity. In the present investigation the cytotoxicity of FUra was modulated by non-chemotherapeutic agents and the underlying mechanism was explored.

In the study reported here we demonstrated that the PPIs alone inhibited proliferation of various cancer cell lines cytospecifically (Table 1), and synergistically potentiated the cytotoxic effect of FUra (Fig. 1, Table 2) in vitro with an increase in TK activity (Fig. 3), and incorporation of FUra into RNA and DNA (Fig. 6). The cytospecificity of the PPIs was presumably due, at least in part, to differences in their permeability to the target

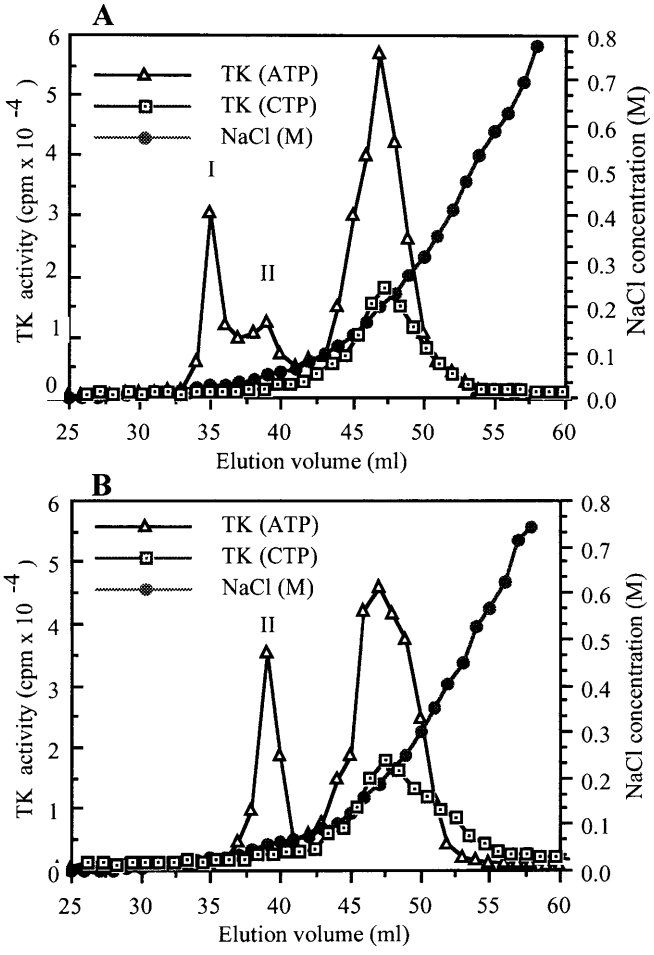

Fig. 5A, B HPLC analysis of TK enzyme in S-180 sarcoma-bearing male ICR mice. S-180 cells were inoculated s.c. into the mice and after 7 days when the tumors had grown to a reasonable size, the mice were treated orally with 80 mg/kg FUra or vehicle. The mice were killed 3 h after administration and the tumors were immediately frozen in liquid nitrogen. Tumors were homogenized with four volumes of homogenizing buffer containing 200 m*M* Tris-HCl (pH 7.4), 20 m*M* β-mercaptoethanol, 100 m*M* NaF, 15 m*M* CMP, and 1% CHAP, using a Polytron PT 1200 homogenizer and centrifuged at 105,000 *g* for 1 h. The enzyme preparations were put onto the HPLC column and eluted as described in Materials and methods. **A** TK activity pattern in tumor from a vehicle-treated mouse. **B** TK activity pattern in tumor from a FUra-treated mouse

site of the cells. OA showed a marginal growth-inhibitory effect in vitro that was slightly synergistic (Fig. 1) as shown by median effect analysis and CI (Fig. 2). Significant synergism was shown by MCLR and CAL in combination with FUra as also shown by the median effect analysis and CI (Fig. 2). CI values less than 1 indicate synergism, values equal to 1 indicate additivity, and values more than 1 indicate antagonism [4, 5]. The OA and FUra combination showed marginal synergism as shown by the CI and by its minimal growth-inhibitory effect (Fig. 1).

In combination with MCLR, FUra incorporation into RNA in Clone 20 cells seemed to be responsible for the synergistic potentiation of FUra cytotoxicity

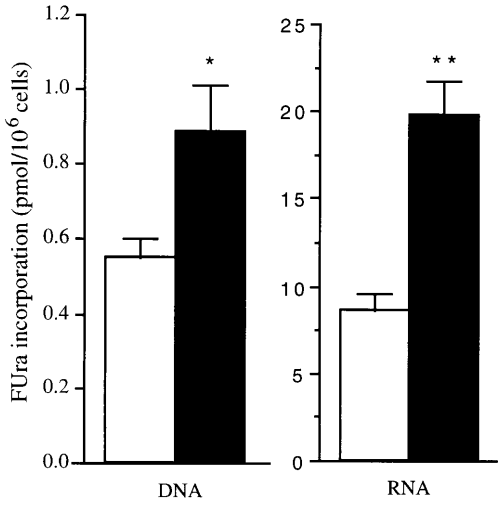

Fig. 6 Effect of MCLR on FUra incorporation into DNA and RNA in vitro. Subconfluent cultures of Clone 20 cells were exposed to 5 μ*M* [2-^{14}C]FUra with (*filled bars*) or without MCLR (*open bars*) for 24 h, DNA and RNA were extracted, and the radioactivity in the both fractions was determined as described in the Materials and methods. The *bars* represent the means ± SE from three separate determinations. *$P < 0.05$, **$P < 0.01$ vs corresponding controls

since it was observed that administration of dThd following FUra effectively reversed the de novo block to the synthesis dTMP, but failed to circumvent the cytotoxic effect completely. The remaining cytotoxicity after dThd treatment was probably due to incorporation of FUra into RNA in the form of FUTP. Thymidine rescue of Clone 20 cells from FUra cytotoxicity is consistent with the results of a previous study [37].

Increases in TK activity seem to have been due to inhibition of phosphatase(s) by a FUra intermediate, probably the fluoride ion itself and/or other fluorine-containing metabolite(s) that dephosphorylate the TK protein. The marked increase in TK activity following FUra treatment in response to MCLR (Fig. 3) presumably increased the formation of fluoride-containing pyrimidine nucleotides, reflecting more inhibition of TS activity and also potentiation of FUra incorporation into RNA resulting in potentiation of the cytotoxic effect. TK converts dFUrd and FUrd into dFUMP and FUMP, respectively. Increases in TK activity would enhance production of FdUMP, which would in turn cause more depletion of thymidylate required for DNA synthesis thereby resulting in an increased cytotoxic effect. The role of phosphatase inhibitors in TK activity has been studied previously [25], and it has been shown that the phosphatase inhibitor NaF increases and alkaline phosphatase decreases TK activity. This is in agreement with our finding that MCLR increased TK activity (Fig. 3).

On the other hand, OA at a dose that is much higher than the IC_{50} for protein phosphatases (1.2 n*M*) [8] failed to potentiate the cytotoxic effect of FUra significantly (Fig. 1), indicating that suppression of protein phosphatase enzymes is probably not the cause of the synergistic effects of PPIs with FUra.

The two cytosolic TK peaks (TK I and TK II) resolved from S-180 tumor cell extracts by HPLC are in agreement with previous work [13, 25]. These two isoforms of TK are the result of different degrees of phosphorylation and are not the products of different genes [13, 14]. The disappearance of the first peak was probably due to phosphorylation of TK protein by FUra, as reported by Lin et al. who showed by Western blotting that the first and the second peaks are mainly dephosphorylated and phosphorylated TK protein, respectively [25].

Furthermore, it seems that these PPIs phosphorylate various cellular proteins and the phosphorylation of many proteins is involved in the regulation of cell growth and function, and therefore might play a critical role in the process of synergism in cytotoxicity between FUra and PPIs. The mechanism of this synergism at the molecular level remains to be elucidated and these effects require testing in an animal model for the improvement of the therapeutic index of FUra. On the basis of the molecular mechanism it might be possible to design novel PPI derivatives with less toxicity and high target-specific activity, that in turn might give rise to safer and more effective cancer chemotherapy.

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