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Biochemical Pharmacology 61 (2001) 867–876

Biochemical Pharmacology

Cytotoxic synergism of methioninase in combination with 5-fluorouracil and folinic acid

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Received 17 March 2000; accepted 6 September 2000

Abstract

Potentiation of the cytotoxic activity of 5-fluorouracil (FUra) by folinic acid (5-HCO-H_4folate) is due to elevation of the methylene tetrahydrofolate (CH_2-H_4folate) level, which increases the stability of the ternary complex of thymidylate synthase (TS), fluorodeoxyuridine monophosphate, and CH_2-H_4folate that inactivates the TS. Methionine deprivation results in the production of tetrahydrofolate (H_4folate) and, subsequently, CH_2-H_4folate from methyl tetrahydrofolate, as a consequence of the induction of methionine synthesis. We hypothesized that the efficacy of FUra could be augmented by the combination of high-concentration 5-HCO-H_4folate and recombinant methioninase (rMETase), a methionine-cleaving enzyme. Studies *in vitro* were performed with the cell line CCRF-CEM. Cytotoxic synergism of FUra + rMETase and FUra + 5-HCO-H_4folate + rMETase was demonstrated with the combination index throughout a broad concentration range of FUra and rMETase. A subcytotoxic concentration of rMETase reduced the IC_{50} of FUra by a factor of 3.6, and by a factor of 7.5, in the absence and in the presence of 5-HCO-H_4folate, respectively. 5-HCO-H_4folate increased the intracellular concentrations of CH_2-H_4folate and H_4folate from their baseline levels. Concentrations of folates were not changed by exposure to rMETase. Levels of free TS in cells treated with FUra + 5-HCO-H_4folate and with FUra + rMETase were lower than those in cells exposed to FUra alone. The decrease of TS was still more pronounced in cells treated with FUra + 5-HCO-H_4folate + rMETase. The synergism described in this study will be a basis for further exploration of combinations of fluoropyrimidines, folates, and rMETase.

Keywords: Fluoropyrimidines; Folates; Methioninase; Synergism; Thymidylate synthase

1. Introduction

Potentiation of the cytotoxic activity of FUra and FdUrd by reduced folates was demonstrated *in vitro* in murine [1,2] and human [3,4,5] cell lines derived from hematopoietic neoplasms and solid tumors, and in murine colon tumors *in vivo* [6,7]. Synergism is due to the formation of a ternary complex of FdUMP, CH_2-H_4folate, and TS, with concomitant inactivation of the TS [1,2,4,8,9]. The stability of the ternary complex increases as the concentration of CH_2-H_4folate is increased. These findings led to the design of chemotherapy regimens of FUra and high-dose 5-HCO-H_4folate that are used with efficacy for treatment of patients with various types of carcinomas, mainly colorectal [10–12].

The most abundant folate in cells and in plasma resulting from the metabolism of the active levorotatory enantiomer of folinic acid ([6S]-5-HCO-H_4folate) is CH_3-H_4folate [12–14]. CH_3-H_4folate is mobilized to enter the active folate cofactor pool only through formation of H_4folate as a consequence of de novo methionine synthesis [15–18].

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Abbreviations: FUra, 5-fluorouracil; FdUrd, fluorodeoxyuridine; FdUMP, fluorodeoxyuridine monophosphate; 5-HCO-H_4folate, folinic acid, 5-formyl tetrahydrofolate; H_4folate, tetrahydrofolate; CH_2-H_4folate, 5,10-methylene tetrahydrofolate; CH_3-H_4folate, 5-methyl tetrahydrofolate; TS, thymidylate synthase; rMETase, recombinant methioninase; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00560-3

We hypothesized that the cytotoxic effect of FUra could be augmented by the combination of 5-HCO-H_4folate and a sustained depletion of methionine with recombinant METase (L-methionine-α-deamino-γ-mercaptoethane lyase), a purified enzyme that cleaves methionine and exerts antitumor activity [19–21].

Normal and tumor cells can synthesize methionine provided sufficient folate, cobalamin, and homocysteine are supplied [15,22]. However, most tumor cells require larger amounts of methionine than what they can synthesize, and, in the absence of an exogenous supply, they experience growth inhibition or die [23–25]. Methionine deprivation increases the activity of methionine synthase (5-methyl tetrahydrofolate:homocysteine methyltransferase) [15,22,26] and thereby utilizes CH_3-H_4folate for production of H_4folate and possible conversion to CH_2-H_4folate, which potentiates the effect of FUra at the level of TS [1,8,9].

To test the hypothesis, we depleted methionine in the extracellular compartment of the human leukemic cell line CCRF-CEM *in vitro* with rMETase to determine the cytotoxic interaction with FUra in the absence and in the presence of 5-HCO-H_4folate. Intracellular concentrations of CH_2-H_4folate and H_4folate were determined in cells exposed to rMETase alone, and rMETase together with 5-HCO-H_4folate. Levels of free TS were measured in cells treated with FUra, 5-HCO-H_4folate, and rMETase.

2. Materials and methods

2.1. Chemicals

FUra (Fluorouracile®) was purchased from Laboratories Roche. Folinic acid as calcium salt (Lederfoline®) was obtained from Lederle. It consists of a mixture of equal parts of the active levorotatory [6S]-5-HCO-H_4folate and the inactive [6R]-5-HCO-H_4folate [12].

Recombinant METase from *Pseudomonas putida* was produced at AntiCancer Inc. METase is a homotetramer of molecular mass of approximately 170 kDa. It catalyzes the α, γ-elimination of methionine to α-ketobutyrate, methanethiol, and ammonia in the presence of pyridoxal 5′-phosphate. One unit of enzyme is the amount that catalyzes the formation of 1 μmol of α-ketobutyrate per minute. The K_m for methionine is 0.8 mM. Production of rMETase was achieved with a gene vector cloned in *Escherichia coli*. Purification procedures yielded preparations of rMETase that were more than 98% pure, with low endotoxin levels [19,20]. The enzyme was obtained as a lyophilized powder containing sodium phosphate buffer and NaCl. Its specific activity was 20.2 U/mg of protein.

TS from *Lactobacillus casei* was provided by D.G. Priest and M. Bunni (Medical University of South Carolina, Charleston). It was obtained from an *E. coli* strain that overproduces the enzyme. *L. casei* TS was purified to a specific activity of 4.1 U/mg protein. [6-^{3}H]FdUMP (22.0 Ci/mmol) was purchased from Moravek Biochemicals. L-[4,5-^{3}H]leucine (50 Ci/mmol) was from NEN Life Sciences Products, Inc., and Sephadex G-25 and dextran (T-70) were from Pharmacia. [6R,S]-5,10-CH_2-H_4folate was purchased from Schircks Laboratories.

Medium (RPMI 1640) and fetal bovine serum (FBS) were purchased from Gibco Laboratories. Tetrazolium-formazan reagents (MTT), DMSO, activated charcoal, BSA, DL-homocysteine, and all other reagents were obtained from Sigma Chemical Co.

2.2. Cell line

The CCRF-CEM human T-lymphoblastic leukemia cell line was thawed from mycoplasma-free frozen stocks and was controlled for contamination. Cells were maintained in suspension culture in RPMI 1640 supplemented with 10% FBS and antibiotics (streptomycin, 50 μg/mL, and penicillin, 50 U/mL) at 37° in an atmosphere containing 5% CO_2 and were subcultured twice a week. The doubling time in the exponential phase of growth was 22 hr. One part of the studies for measurement of intracellular folates was carried out on CCRF-CEM cells adapted to grow under low-folate conditions in cell culture medium which differed from RPMI 1640 only by a concentration of folic acid that was 100-fold lower (i.e. 0.023 μM). This condition did not alter cell viability.

2.3. Cytotoxicity studies

Exponentially growing cells at an initial density of approximately 2×10^5 cells were exposed to the drugs under study. Cells were exposed to 5-HCO-H_4folate two hr prior the addition of FUra. After 4 hr of incubation with FUra, cells were exposed to rMETase and dispensed into 96-well cell culture plates (Nunc) at a density of 2×10^4 cells in 100 μL per well. Each plate contained eight different cell culture assays consisting of (a) control without drugs, (b) 5-HCO-H_4folate, (c) FUra, (d) FUra + 5-HCO-H_4folate, (e) rMETase, (f) 5-HCO-H_4folate + rMETase, (g) FUra + rMETase, and (h) FUra + 5-HCO-H_4folate + rMETase, and one blank sector without cells. Plates were incubated at 37° in 5% CO_2. Twenty-four hours and 48 hr after the start of the experiment, rMETase was added again into wells of assays e, f, g, and h. Cells were exposed to rMETase every 24 hr three times to ensure a sustained depletion of methionine.

The study of the interaction of rMETase with FUra and 5-HCO-H_4folate and its effect on cytotoxicity was performed over concentration ranges of 0.02 U/mL to 0.225 U/mL, and of 1 μM to 500 μM, for rMETase and FUra, respectively. The 5-HCO-H_4folate was used at a single concentration of 100 μM in all experiments (i.e. 50 μM of [6S]-5-HCO-H_4folate) to produce a high degree of expansion of intracellular folates, as described previously in CCRF-CEM cells [3].

Cell viability was measured at 72 hr with the MTT colorimetric assay [27]. In this assay, the number of viable

cells is directly proportional to the cellular production of formazan, which is measured spectrophotometrically. A solution of MTT at 5 mg/mL was added to each well and was maintained during 4 hr at 37°. Formazan was solubilized with DMSO and measured at 570 nm in a microplate reader (Multiskan MCC/340 Mk II, Labsystems).

2.4. Analysis of the effect of interaction of FUra with rMETase and 5-HCO-H_4folate upon cytotoxicity

Data obtained with both subcytotoxic and cytotoxic concentrations of rMETase combined with FUra and FUra + 5-HCO-H_4folate were studied according to the median-effect principle for concentration-effect analysis. The combination index (CI) proposed by Chou and Talalay was used for determination of synergism, additivity, and antagonism [28].

The CI was calculated for the combination of FUra + rMETase and FUra + 5-HCO-H_4folate + rMETase. To calculate the CI, we used a mutually exclusive assumption. The calculation procedure involved three steps:

(1) The median-effect equation was fitted to each single-drug efficacy value with unweighted least-squares regression. The median-effect equation states that

$$\text{Log}\,(f_a/f_u) = m \log (D) - m \log (D_m),$$

where D is the concentration of a drug; f_a is the fraction inhibited by the concentration D (a given fraction corresponds to the percent of the cell population inhibited); f_u is the fraction not inhibited by the concentration D, defined as $f_u = 1 - f_a$ (a given fraction not inhibited corresponds to the percent of control); D_m is the concentration required to produce the median effect (defined as IC_{50}); and m is a coefficient signifying the sigmoidicity of the concentration-effect curve. Values for the slopes of median-effect curves (m), the x-intercepts, and D_m with their standard error were obtained for FUra, FUra + 5-HCO-H_4folate, and rMETase.

(2) For a given observed effect obtained with each combination of drugs (i.e. a given percent inhibited), we calculated, with the median-effect equation, the concentration $(D_x)_1$, $(D_x)_{1'}$, and $(D_x)_2$ that result in the same degree of effect for FUra, FUra together with 5-HCO-H_4folate, and rMETase, respectively.

(3) For each combination of drugs, the CI is expressed as

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

for the combination FUra + rMETase, and

$$CI = (D)_{1'}/(D_x)_{1'} + (D)_2/(D_x)_2$$

for the combination FUra + 5-HCO-H4folate + rMETase,

where $(D)_1$, $(D)_{1'}$, and $(D)_2$ are the concentrations, in each combination, of FUra, FUra together with 5-HCO-H_4folate, and rMETase, respectively. Combination experiments were performed with non-constant drug concentration ratios of FUra and rMETase. Synergism and antagonism were determined from the combination indices calculated for each combination of FUra and rMETase at a given concentration of each agent. Synergism ($CI < 1$) and antagonism ($CI > 1$) for combinations of FUra + rMETase and FUra + 5-HCO-H_4folate + rMETase were represented graphically (e.g., in Fig. 1) as plots of the CI with respect to the percent inhibited.

The effect of a subcytotoxic concentration of rMETase (0.02 U/mL) was also studied by direct comparison of the cytotoxicity measured with FUra as a single drug and FUra combined with 5-HCO-H_4folate, with that achieved with the combination of these agents and rMETase. Potentiation of the cytotoxic effect of FUra was considered present when the percent of the cell population inhibited with FUra plus 5-HCO-H_4folate, rMETase, or 5-HCO-H_4folate plus

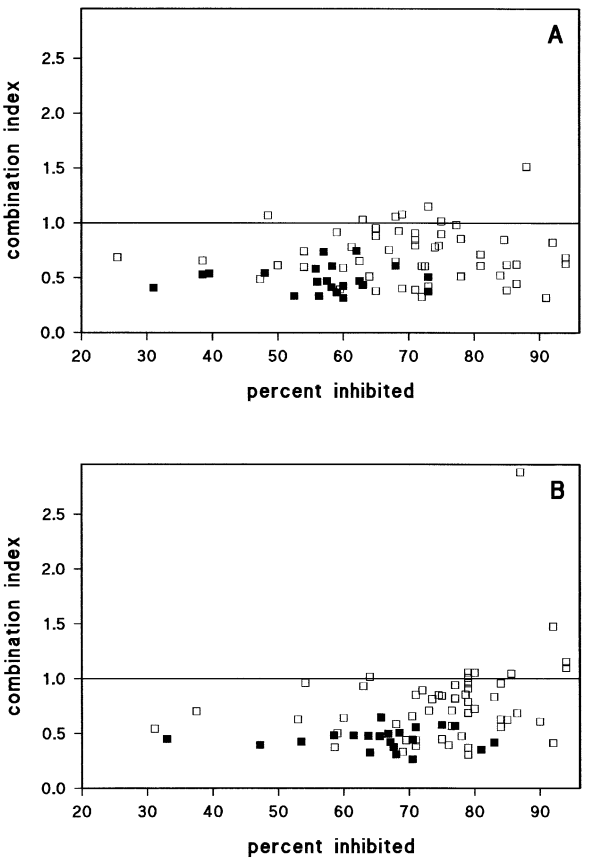

Fig. 1. Plots of combination index (CI) with respect to percent of cell population inhibited in CCRF-CEM cells. Squares represent the CI calculated for combinations of FUra + rMETase (A) and FUra + 5-HCO-H_4folate + rMETase (B) producing a given effect. A and B each comprise results from 134 assays, including 51 assays with rMETase at the subcytotoxic concentration of 0.02 U/mL. Each square represents a combination data point defined by the concentration of FUra and rMETase, and corresponds to a single assay or to an average of two to seven replicates. The combination data points for assays at 0.02 U/mL of rMETase are represented by black squares. Open squares represent combination data points in which rMETase was present at concentrations greater than 0.02 U/mL. The line CI = 1 indicates the boundary between synergism ($CI < 1$) and antagonism ($CI > 1$).

rMETase was greater than that observed with FUra as a single drug. The ICs of the drugs (i.e., the concentrations of a drug that produce a given percent inhibited) were calculated with the median-effect equation, and their standard errors were obtained with the δ-method [29].

2.5. Measurement of methionine concentration in cell culture

The concentration of methionine was measured in the supernatant of CCRF-CEM cell cultures growing exponentially in RPMI 1640 supplemented with 10% FBS exposed to various concentrations of rMETase for 3 hr, 6 hr, and 24 hr. Measurements were done by ion-exchange chromatography, as previously described [30].

2.6. Measurement of intracellular folates

Intracellular concentrations of CH_2-H_4folate and H_4folate were measured in two separate experiments in exponentially growing CCRF-CEM cells exposed to rMETase under culture conditions that did not produce cytotoxicity.

In Experiment 1, cells grown in RPMI 1640 (which contains 2.3 μM folic acid), and cells adapted to grow in medium with low-concentration folic acid (0.023 μM), were exposed for 72 hr to 100 μM of 5-HCO-H_4folate, to rMETase at 0.02 U/mL, and to 5-HCO-H_4folate + rMETase with the same schedules as those for cytotoxicity studies.

In Experiment 2, we tested the effect on folate pools of a subcytotoxic schedule of short-term exposure to a high concentration rMETase. Cells growing in RPMI 1640 were exposed to rMETase at 0.2 U/mL for 6 hr, to 100 μM of 5-HCO-H_4folate for 24 hr, and to rMETase + 5-HCO-H_4folate combined. In experiments with rMETase + 5-HCO-H_4folate, the enzyme was added for 6 hr of the last part of the 24-hr exposure period to 5-HCO-H_4folate. To test the possible effect on intracellular folate metabolism due to the homocysteine-cleaving activity of *P. putida* METase [31], we carried out folate measurements with the same experimental protocol, but with additional exposure of cells to DL-homocysteine at 148 μM for 24 hr.

Intracellular CH_2-H_4folate and H_4folate were measured with the Ternary Complex Assay, as described by Priest *et al*. [14]. For extraction of folates, cells were suspended in cold buffer [50 mM Tris-HCl (pH 7.4), 50 mM sodium ascorbate, and 1 mM EDTA] to a cell density of 10 to 20 × 10^6 cells/mL. Cells were lysed in a boiling water bath for 3 min and centrifuged at 14.000 × *g* for 5 min at 4°, and supernatant was collected. Cell-free extracts of 10 to 20 × 10^6 cells were assayed in each experiment. Reaction mixtures for analysis of CH_2-H_4folate contained 20 mUnits of *L. casei* TS and 12.5 pmol [^{3}H]FdUMP in 100 μL of buffer. Quantitation of the sum of CH_2-H_4folate + H_4folate was done in the presence of 6.5 mM formaldehyde. The mixture was incubated at 25° for 30 min to allow ternary-complex formation. The reactions were stopped by addition of 1% SDS and boiling for 10 min. Aliquots (25 μL) were eluted over 400-μL minicolumns (Sarstedt AG) of Sephadex G-25 by centrifugation. The eluant containing ternary complex-bound [^{3}H]FdUMP was diluted in scintillation cocktail (Pico-Fluor 40, Packard Instruments), and radioactivity was measured in a scintillation counter (Rackbeta, LKB Wallac). Under these conditions with TS and [^{3}H]FdUMP in excess, all of the CH_2-H_4folate present in a sample becomes bound, and is equal to bound [^{3}H]FdUMP. The concentration of folates was expressed in pmol/10^6 cells.

2.7. Measurement of thymidylate synthase

Determination of free TS was performed in cells growing under the same culture conditions as those for cytotoxicity studies. Measurements were performed 48 hr and 72 hr after the start of the experiment in 8 different cell culture assays, as described above. The 5-HCO-H_4folate was used at 100 μM. The concentrations of FUra were 1 μM and 5 μM. Cells were exposed to rMETase at a single subcytotoxic concentration of 0.02 U/mL.

Titration of free TS was performed with the TS radioligand binding assay, as described by Moran et al. [32]. Formation of the covalent ternary [TS-FdUMP-CH_2-H_4folate] complex allows quantitation of TS active sites by incubation of cell extracts with excess CH_2-H_4folate and [^{3}H]FdUMP and subsequent separation of free and enzyme-bound radioactivity with charcoal.

Cells were disrupted by sonication in 50 mM phosphate, pH 7.4, containing 0.25 M sucrose. The sonicates were centrifuged, and acid-soluble extracts were prepared. A standard or unknown quantity of TS was mixed with 6.25 nmol of CH_2-H_4folate in 25 μL of buffer [1 mL of 1 M phosphate (pH 7.2), 14 μL of 2-mercaptoethanol, 2 mL of a solution containing 10 mg of BSA per mL, and 17 mL of water] and 6 pmol of [^{3}H]FdUMP in a total volume of 125 μL. After 20 min of incubation at 30°, 1 mL of ice-cold suspension of dextran T-70- and BSA-treated charcoal was added, and the suspensions were mixed and centrifuged for 20 min at 4400 × *g*. A 0.9-mL sample of the supernatant, which retains TS-bound [^{3}H]FdUMP, was assayed for radioactivity in 8.1 mL of scintillation cocktail. Protein was measured with the Lowry method. Free TS was expressed in cpm per mg of supernatant protein.

Free TS levels in cells treated with FUra, FUra + 5-HCO-H_4folate, FUra + rMETase, and FUra + 5-HCO-H_4folate + rMETase for 48 hr and 72 hr were represented graphically (e.g., in Fig. 6) as percent of the preexisting free TS levels measured at each assay time point in control cells grown in the absence of FUra. Cells exposed to 5-HCO-H_4folate, rMETase, and 5-HCO-H_4folate + rMETase were used as controls for cells treated with FUra + 5-HCO-H_4folate, FUra + rMETase, and FUra + 5-HCO-H_4folate + rMETase, respectively. Cells grown in the absence of drugs were used as control for cells treated with FUra as a single agent.

2.8. Measurement of protein synthesis

The interaction of rMETase at the subcytotoxic concentration of 0.02 U/mL on protein synthesis was estimated by measurement of cellular incorporation of L-[^{3}H]leucine. Exponentially growing cells were exposed to rMETase for 24 hr, 48 hr, and 72 hr. Fresh enzyme was added to the culture every 24 hr. Approximately 10^5 cells were incubated for 1 hr in cell culture medium containing 1 μCi/mL of L-[^{3}H]leucine. Cells were washed in PBS, filtered, and dried at 37°. Macromolecules were precipitated with trichloroacetic acid. The precipitate was washed with methanol and dried at 37°. The preparation was diluted in scintillation cocktail and assayed for radioactivity.

3. Results

3.1. Cytotoxicity studies

3.1.1. Studies of rMETase

At a concentration of 0.02 U/mL, rMETase did not produce any measurable cytotoxic effect, either when it was used as a single drug or when it was combined with 5-HCO-H_4folate. Cytotoxicity appeared at 0.04 U/mL of rMETase (mean percent of control, 86%) and was augmented with increasing concentrations of the enzyme. The IC_{50} ± SE for rMETase was 0.157 ± 0.01 U/mL. Therefore, rMETase at 0.02 U/mL corresponds to a subcytotoxic level on a concentration-dependent cytotoxicity curve for CCRF-CEM cells.

3.1.2. Studies of the combinations FUra + 5-HCO-H_4 folate, FUra + rMETase, and FUra + 5-HCO-H_4 folate + rMETase

A total of 134 combination assays with the entire concentration ranges of FUra and rMETase were performed, and seventy-three different combination data points defined by the concentrations of FUra and rMETase were calculated (Fig. 1). Synergism was found in 90% and in 89% of the combinations of FUra + rMETase and FUra + 5-HCO-H_4folate + rMETase, respectively. The median value of the CI was 0.61 for FUra + rMETase and 0.60 for FUra + 5-HCO-H_4folate + rMETase. Synergism was found throughout a broad range of concentrations of FUra and rMETase studied, including all of the combinations comprising rMETase at the subcytotoxic concentration of 0.02 U/mL. Antagonism was observed only in a small number of assays producing cell population inhibition above 70–80%, either with rMETase at 0.225 U/mL or with FUra above 100 μM. The patterns of the CI plots were similar for FUra + rMETase and for FUra + 5-HCO-H_4folate + rMETase. It was not possible to compare these plots to assess the effect of 5-HCO-H_4folate on the synergistic interaction of FUra and rMETase.

Recombinant METase at the subcytotoxic concentration of 0.02 U/mL greatly augmented the cytotoxicity of FUra both as a single drug and in combination with 5-HCO-H_4folate (Fig. 2). The increased effect was maintained over the entire range of concentrations of FUra studied. The effect of FUra + rMETase was greater than that achieved with FUra as a single drug and with FUra + 5-HCO-H_4folate. The cytotoxicity was highest with FUra + 5-HCO-H_4folate + rMETase.

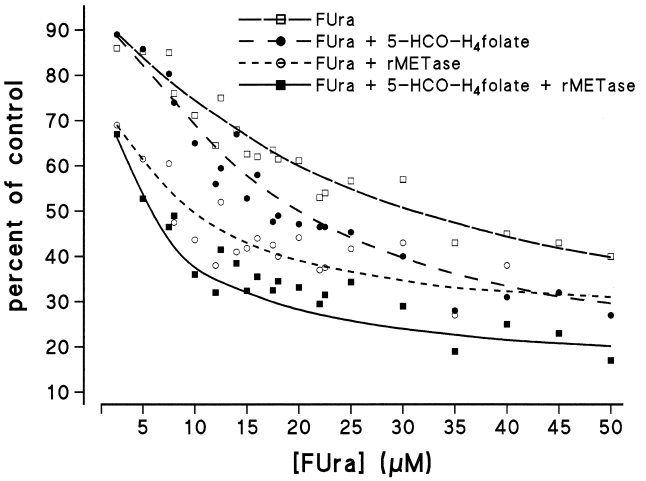

Fig. 2. Cytotoxic effect of FUra, FUra + 5-HCO-H_4folate, FUra + rMETase, and FUra + 5-HCO-H_4folate + rMETase, measured at increasing concentrations of FUra in CCRF-CEM cells. Experiments were done with rMETase at the subcytotoxic concentration of 0.02 U/mL. 5-HCO-H_4folate alone did not produce any measurable cytotoxicity (mean percent of control, 99%). At 0.02 U/mL, METase did not produce any cytotoxic effect, either when it was used as a single drug (mean percent of control, 97%) or in combination with 5-HCO-H_4folate (mean percent of control, 97%).

Recombinant METase at 0.02 U/mL reduced the IC_{50} of FUra by a factor of 3.6, and by a factor of 7.5, in the absence and in the presence of 5-HCO-H_4folate, respectively (Table 1). In the absence of rMETase, 5-HCO-H_4folate reduced the IC_{50} of FUra by a factor of only 1.5.

From the studies at the subcytotoxic concentration of rMETase, we could establish a ranking for cytotoxic potency as follows: FUra + 5-HCO-H_4folate + rMETase > FUra + rMETase > FUra + 5-HCO-H_4folate > FUra.

Table 1
IC_{50} of FUra used as a single drug and in combination with rMETase and 5-HCO-H_4folate

Drug and combination of drugs	IC_{50} ± SE (μM)
FUra	33.8 ± 2.8
FUra + 5-HCO-H_4folate	22.4 ± 1.5
FUra + rMETase	9.5 ± 1.0
FUra + 5-HCO-H_4folate + rMETase	4.5 ± 1.0

Experiments were done with rMETase at the subcytotoxic concentration of 0.02 U/mL. rMETase did not produce any cytotoxic effect, either when it was used as a single drug or in combination with 5-HCO-H_4folate. The IC_{50} values were determined with the median-effect equation, and their standard error were calculated with the δ-method [28,29].

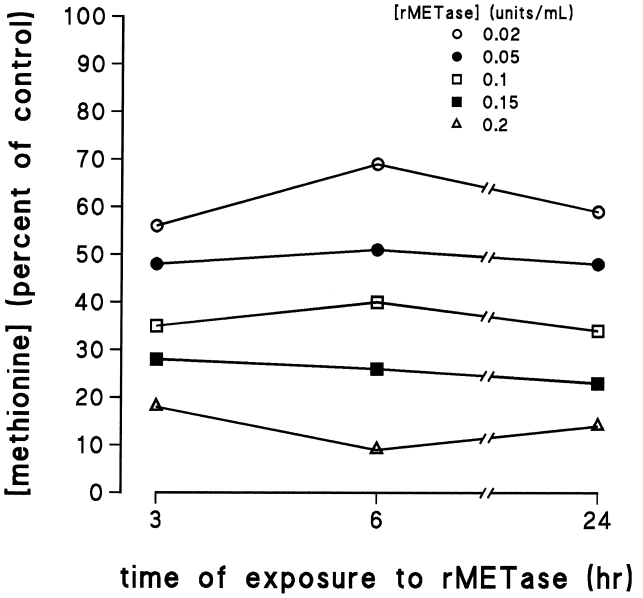

Fig. 3. Methionine levels in the supernatant of CCRF-CEM cell cultures growing exponentially exposed to various concentrations of rMETase. In the absence of rMETase, the mean concentration of methionine was 80 μM.

3.2. Methionine concentration in cell culture

The mean concentration of methionine in the supernatant of CCRF-CEM cell cultures was 80 μM. Exposure to rMETase decreased the levels of methionine, and the effect was augmented with increasing concentrations of the enzyme (Fig. 3). The degree of methionine depletion achieved with a given concentration of rMETase was maintained during the 24-hr duration of the experiment.

3.3. Intracellular pools of methylene tetrahydrofolate and tetrahydrofolate

In Experiment 1, in untreated cells grown for 72 hr in RPMI 1640, the mean baseline concentration of CH_2-H_4folate was 0.54 ± 0.12 pmol/10^6 cells, and that of H_4folate + CH_2-H_4folate was 1.75 ± 0.54 pmol/10^6 cells (Fig. 4A). Exposure to 100 μM of 5-HCO-H_4folate for 72 hr expanded the intracellular folates over the baseline by a factor of 2 and by a factor of 1.4 for CH_2-H_4folate and H_4folate + CH_2-H_4folate, respectively. In untreated cells grown for 72 hr in low-folate medium, the mean baseline concentration of CH_2-H_4folate was 0.22 ± 0.02 pmol/10^6 cells, and that of H_4folate + CH_2-H_4folate was 0.39 ± 0.03 pmol/10^6 cells (Fig. 4B). Exposure to 100 μM of 5-HCO-H_4folate for 72 hr increased the folate pools over the baseline by a factor of 6.75 and by a factor of 5 for CH_2-H_4folate and H_4folate + CH_2-H_4folate, respectively. The maximum intracellular concentration of CH_2-H_4folate and H_4folate attained after exposure to high-concentration 5-HCO-H_4folate in cells grown in standard medium was similar to that in cells adapted to grow in medium with a low folate concentration. Exposure to 0.02 U/mL rMETase for 72 hr had no influence on folate pools of CCRF-CEM cells grown either in standard medium and in low-folate medium.

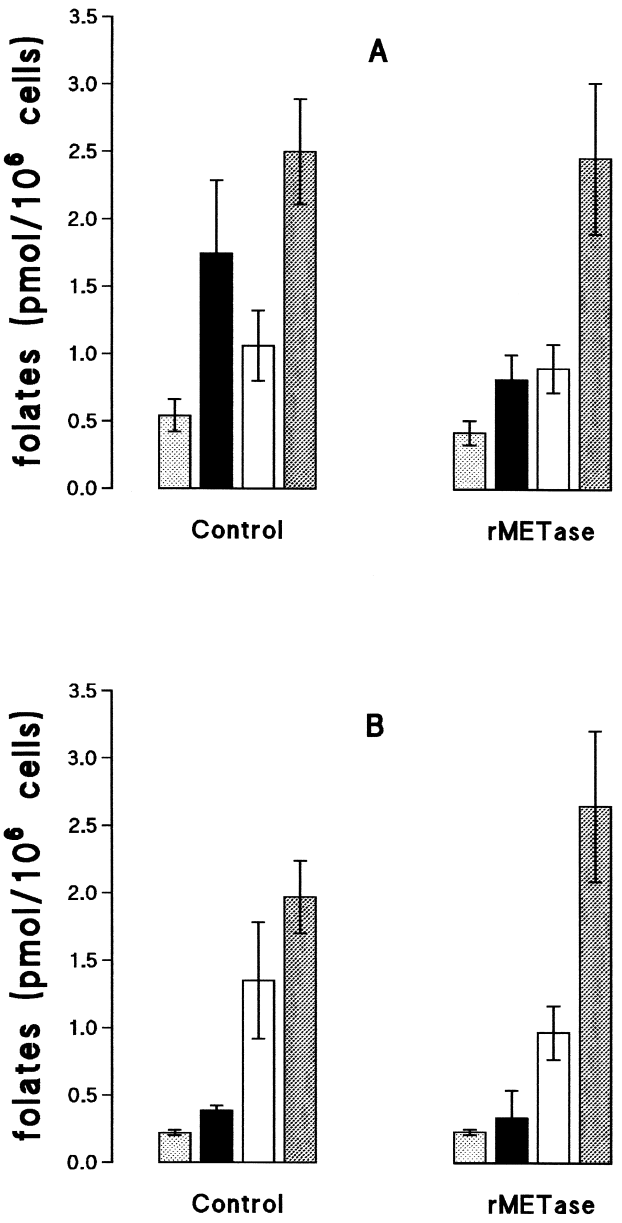

Fig. 4. Effect of exposure of CCRF-CEM cells grown in RPMI 1640 (A) and in medium with low-concentration folic acid (B) to 100 μM of 5-HCO-H_4folate for 72 hr, to rMETase at 0.02 U/mL for 72 hr, and to 5-HCO-H_4folate + rMETase combined, on intracellular CH_2-H_4folate and on CH_2-H_4folate + H_4folate concentrations. Histograms represent baseline concentrations of CH_2-H_4folate (light gray columns) and of CH_2-H_4folate + H_4folate (black columns) in cells that were not exposed to 5-HCO-H_4folate, and the concentrations of CH_2-H_4folate (white columns) and of CH_2-H_4folate + H_4folate (dark gray columns) in cells exposed to 5-HCO-H_4folate. Columns represent the mean of 3 separate determinations, each done in duplicate; bars, SEM.

In Experiment 2, in untreated cells, the mean base line concentration of CH_2-H_4folate was 0.8 ± 0.03 pmol/10^6 cells, and that of H_4folate + CH_2-H_4folate was 1.05 ± 0.05

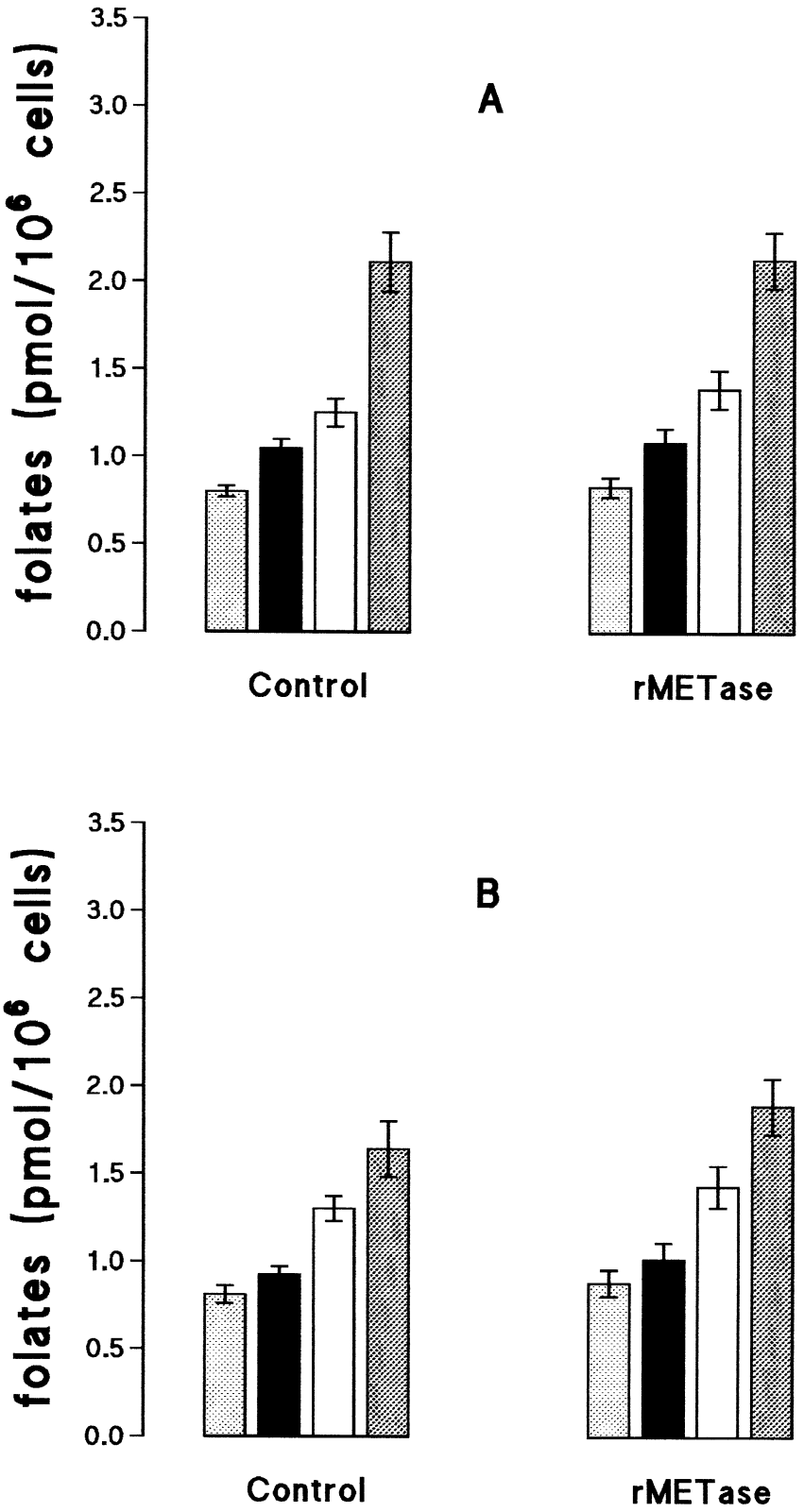

Fig. 5. Effect of exposure of CCRF-CEM cells to 100 μM of 5-HCO-H_4folate for 24 hr, to rMETase at 0.2 U/mL for 6 hr, and to 5-HCO-H_4folate + rMETase combined, on intracellular CH_2-H_4folate and on CH_2-H_4folate + H_4folate concentrations. Experiments were performed in the absence (A) and in the presence of 148 μM DL-homocysteine for 24 hr (B). Histograms represent baseline concentrations of CH_2-H_4folate (light gray columns) and of CH_2-H_4folate + H_4folate (black columns) in cells that were not exposed to 5-HCO-H_4folate, and the concentrations of CH_2-H_4folate (white columns) and of CH_2-H_4folate + H_4folate (dark gray columns) in cells exposed to 5-HCO-H_4folate. Columns represent the mean of 5 to 16 separate determinations, each done in duplicate; bars, SEM.

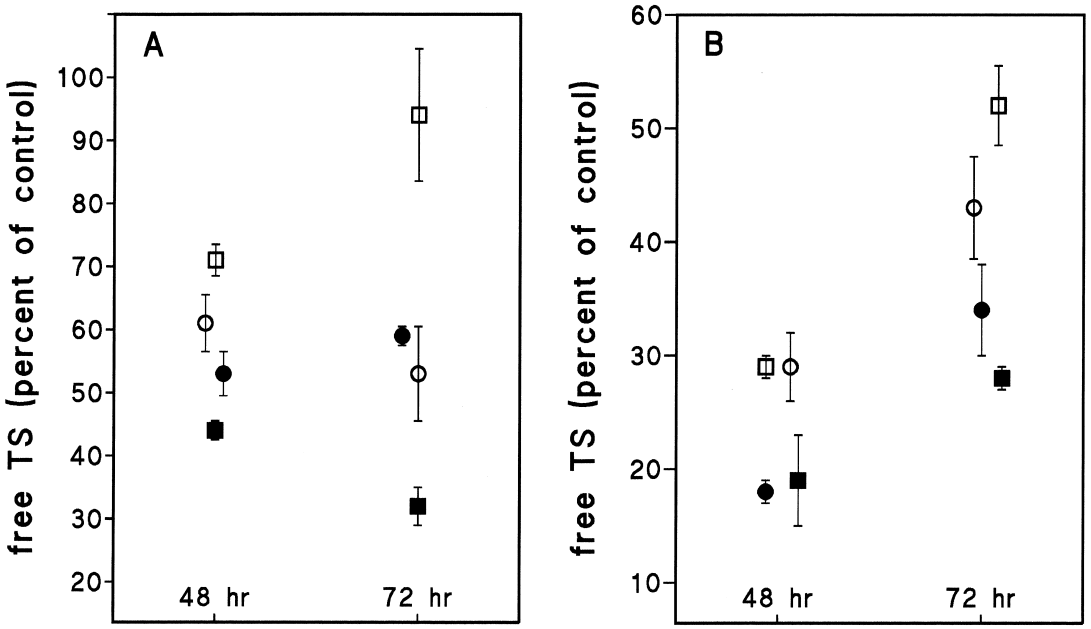

Fig. 6. Effect of exposure of CCRF-CEM cells to FUra, FUra + 5-HCO-H_4folate, FUra + rMETase, and FUra + 5-HCO-H_4folate + rMETase on free TS levels. Cells were exposed for 48 hr and 72 hr to FUra at 1 μM (A) and 5 μM (B), 5-HCO-H_4folate at 100 μM, and rMETase at the subcytotoxic concentration of 0.02 U/mL. Free TS levels in cells treated with FUra (open squares), FUra + 5-HCO-H_4folate (black circles), FUra + rMETase (open circles), and FUra + 5-HCO-H_4folate + rMETase (black squares) are represented graphically as percent of free TS levels measured in control cells grown in the absence of FUra. Each symbol represents the mean of 3 separate determinations, each done in duplicate; bars, SEM.

pmol/10^6 cells (Fig. 5A). After incubation for 24 hr with 100 μM of 5-HCO-H_4folate, the intracellular folate contents increased over the baseline by a factor of 1.6 and by a factor of 2 for CH_2-H_4folate and H_4folate + CH_2-H_4folate, respectively. Exposure to 0.2 U/mL of rMETase for 6 hr did not change the baseline concentration of folates from that in control cells, nor their expansion rates upon exposure to high-concentration 5-HCO-H_4folate for 24 hr. Incubation of the cells with homocysteine did not affect the pools of folates measured in the presence of rMETase, and 5-HCO-H_4folate + rMETase (Fig. 5B).

3.4. Free thymidylate synthase levels

Mean free TS levels in control cells grown in the absence of FUra after 48 hr of culture were 2.77 ± 0.26, 3.61 ± 0.15, 2.66 ± 0.16, and 3.59 ± 0.1 pmol/mg of protein in cells grown without drugs, and in cells exposed to 5-HCO-H_4folate, rMETase, and 5-HCO-H_4folate + rMETase, respectively. After 72 hr of culture, mean free TS levels in control cells were 1.63 ± 0.15, 2.47 ± 0.25, 2.26 ± 0.26, and 2.94 ± 0.13 pmol/mg of protein in cells grown without drugs, and in cells exposed to 5-HCO-H_4folate, rMETase, and 5-HCO-H_4folate + rMETase, respectively.

In experiments with FUra at 1 μM, a concentration that produces minimal inhibition of cell growth, mean free TS levels in cells exposed for 48 hr to FUra alone, FUra + 5-HCO-H_4folate, FUra + rMETase, and FUra + 5-HCO-H_4folate + rMETase were decreased to 71%, 53%, 61%, and 44% of control, respectively; in cells exposed for 72 hr, mean free TS levels were 94%, 59%, 53%, and 32% of control, respectively (Fig. 6A). In experiments with FUra at 5 μM, the free TS levels were smaller, and their variation rates from 48 hr to 72 hr were of lesser amplitude than those measured with 1 μM FUra (Fig. 6B). In these experiments, mean free TS levels in cells exposed for 48 hr to FUra, FUra + 5-HCO-H_4folate, FUra + rMETase, and FUra + 5-HCO-H_4folate + rMETase were decreased to 29%, 18%, 29%, and 19% of control, respectively; in cells exposed for 72 hr, mean free TS levels were 52%, 34%, 43%, and 28% of control, respectively.

3.5. Estimation of the interaction of rMETase on protein synthesis

Protein synthesis, as estimated by the cellular incorporation of L-[^{3}H]leucine, was not affected by exposure to rMETase at 0.02 U/mL. Incorporation of L-[^{3}H]leucine was 1.13, 1.01, and 1.02 of that in control at 24 hr, 48 hr, and 72 hr of exposure, respectively.

4. Discussion

The present study demonstrates cytotoxic synergism between FUra and rMETase, a methionine-depleting enzyme. A strong synergistic effect was found over a broad range of drug concentrations in most combinations of FUra + rMETase and FUra + 5-HCO-H_4folate + rMETase. The cytotoxicity of FUra both as a single drug and in combina-

tion with 5-HCO-H_4folate was greatly augmented by rMETase at the subcytotoxic concentration of 0.02 U/mL. Cytotoxicity was highest with FUra + 5-HCO-H_4folate + rMETase.

The relationship of methionine metabolism to the cytostatic activity of the fluoropyrimidines was suggested previously in a number of experiments *in vitro*. In CCRF-CEM cells, the addition of a high concentration of methionine prevented the potentiation of the cytotoxicity of FdUrd by CH_3-H_4folate [5]. Cisplatin increased the intracellular concentration of CH_2-H_4folate and H_4folate in the cell line A2780 and caused enhancement of [TS-FdUMP-CH_2-H_4folate] ternary-complex formation upon exposure to FdUrd [33]. The investigators hypothesized that their finding might be the consequence of impairment of methionine uptake by cells treated with cisplatin [34].

Interaction of methionine deprivation *in vivo* with the antitumor activity of FUra has been reported. Chemotherapy with FUra in Yoshida sarcoma-bearing rats fed with methionine-deprived total parenteral nutrition led to greater antitumor activity than did FUra in animals fed a normal diet [35]. In nude mice in which the human gastric cell line SC-1-NU had been transplanted, FUra, in animals fed a methionine-free diet, was more potent in inhibiting tumor growth than was FUra given to animals on a normal diet [36]. Mice transplanted with Lewis lung carcinoma were treated with FUra or rMETase, or with rMETase in combination with FUra [21]. The antitumor activity, measured both by the duration of survival from implantation and by the growth rate of the tumor, was greater in animals treated with FUra and rMETase in combination.

The pools of intracellular folates found in the present study and their expansion rates upon exposure to high-concentration 5-HCO-H_4folate are only slightly different from those previously reported in CCRF-CEM cells [3]. They are in the range of those found in most studies performed in various cell lines [2,3,13,37–40]. Data from these studies indicate that, upon exposure to 5-HCO-H_4folate, the intracellular concentration of H_4folate and CH_2-H_4folate expands to varying degrees in the different cell lines as the concentration of 5-HCO-H_4folate is increased. In most cells, the expansion is small, and the levels of CH_2-H_4folate rapidly decrease after discontinuation of 5-HCO-H_4folate administration.

Expansion rates of intracellular CH_2-H_4folate and H_4folate pools after exposure to 5-HCO-H_4folate were greater in cells adapted to growth in medium containing low-concentration folic acid than in those grown in standard medium. However, the highest levels of CH_2-H_4folate and H_4folate attained in cells exposed to high-concentration 5-HCO-H_4folate were independent of the amount of folic acid contained in cell culture medium prior exposure to the 5-HCO-H_4folate.

The present results demonstrate that cytotoxic synergism is accompanied by a decrease in free TS from preexisting levels. The decrease in the free TS level was of great magnitude with FUra combined with both 5-HCO-H_4folate and rMETase, and of intermediate extent with FUra together with each agent separately. These results are in agreement with data from cytotoxicity studies with FUra, 5-HCO-H_4folate, and rMETase at the subcytotoxic concentration of 0.02 U/mL, for which we established a ranking for cytotoxic potency among the cell culture assays comprising FUra. The findings suggest a cause-and-effect relationship between decrease of TS activity and cytotoxicity.

The diminished free TS levels in cells exposed to FUra + rMETase, and to 5-HCO-H_4folate + rMETase combined, do not result from inhibition of protein synthesis due to methionine depletion. Incorporation of L-[^{3}H]leucine and levels of free TS were not affected by exposure to rMETase alone at the subcytotoxic concentration of 0.02 U/mL.

The decrease of free TS in cells exposed to FUra + rMETase, and to FUra + rMETase + 5-HCO-H_4folate, which is presumably due to stabilization of the ternary [TS-FdUMP-CH_2-H_4folate] complex, supports the potentiation of FUra due to increased production of CH_2-H_4folate resulting from the interaction of methionine depletion with CH_3-H_4folate as methyl donor for methionine synthesis. However, we did not observe the increase in CH_2-H_4folate and H_4folate pools that was expected to occur when depletion of methionine increases the activity of methionine synthase [15,22,26]. This finding could be the consequence of rapid intracellular reduced folate cofactor turnover that prevents the actual overproduction of H_4folate, to result in a measurable increase of CH_2-H_4folate + H_4folate pools [22,41].

Potentiation of FUra by rMETase, and by rMETase + 5-HCO-H_4folate, is accompanied, and is likely to be caused, at least in part, by decreased levels of free TS. However, in the absence of a demonstrated increase of the production of CH_2-H_4folate induced by rMETase, the mechanism for decrease of free TS due to the combined action of FUra and rMETase is still unclear.

The strong synergistic cytotoxic effects described in the present study will be used as a basis for further exploration of combinations of fluoropyrimidines, folates, and rMETase.

Acknowledgments

The authors thank E.F. Lanzl for editorial assistance, and M. Bunni, D.G. Priest, and D. Rabier for their help. The research was supported in part by the Institut du Cancer et d'Immunogénétique (ICIG), Villejuif, France.

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