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

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Abstract

Potentiation of the cytotoxic activity of 5-fluorouracil (FUra) by folinic acid (5-HCO-Hfolate) is due to elevation of the methylene tetrahydrofolate (CH2-Hfolate) level, which increases the stability of the ternary complex of thymidylate synthase (TS), fluorodeoxyuridine monophosphate, and CH2-Hfolate that inactivates the TS. Methionine deprivation results in the production of tetrahydrofolate (Hfolate) and, subsequently, CH2-Hfolate 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-Hfolate 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-Hfolate + rMETase was demonstrated with the combination index throughout a broad concentration range of FUra and rMETase. A subcytotoxic concentration of rMETase reduced the IC50 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-Hfolate, respectively. 5-HCO-Hfolate increased the intracellular concentrations of CH2-Hfolate and Hfolate 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-Hfolate 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-Hfolate + rMETase. The synergism described in this study will be a basis for further exploration of combinations of fluoropyrimidines, folates, and rMETase. © 2001 Elsevier Science Inc. All rights reserved.

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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 in vivo [6,7]. Synergism is due to the formation of a ternary complex of FdUMP, CH2-Hfolate, and TS, with concomitant inactivation of the TS [1,2,4,8,9]. The stability of the ternary complex increases as the concentration of CH2-Hfolate is increased. These findings led to the design of chemotherapy regimens of FUra and high-dose 5-HCO-Hfolate 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-Hfolate) is CH2-Hfolate [12-14]. CH2-Hfolate is mobilized to enter the active folate cofactor pool only through formation of Hfolate as a consequence of de novo methionine synthesis [15-18].
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°C. 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$_4$folate upon cytotoxicity

Data obtained with both subcytotoxic and cytotoxic concentrations of rMETase combined with FUra and FUra + 5-HCO-H$_4$folate 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$_4$folate + 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

$$
\log\left( \frac{f_s}{f_a} \right) = m \log D - m \log D_m,
$$

where D is the concentration of a drug; $f_s$ is the fraction inhibited by the concentration D (a given fraction corresponds to the percent of the cell population inhibited); $f_a$ is the fraction not inhibited by the concentration D, defined as $f_a = 1 - f_s$ (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$_4$folate, 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$_1$), (D$_2$)$_1$, and (D$_2$)$_2$ that result in the same degree of effect for FUra, FUra together with 5-HCO-H$_4$folate, and rMETase, respectively.

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

$$
CI = \frac{(D_1)(D_2)}{(D_1)} + \frac{(D_2)}{(D_2)}
$$

for the combination FUra + rMETase, and

$$
CI = \frac{(D_1)(D_2)}{(D_1)} + \frac{(D_2)}{(D_2)}
$$

for the combination FUra + 5-HCO-H$_4$folate + rMETase.

where (D$_1$), (D$_1$)$_1$, and (D$_1$)$_2$ are the concentrations, in each combination, of FUra, FUra together with 5-HCO-H$_4$folate, 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$_4$folate + 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$_4$folate, 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$_4$folate, rMETase, or 5-HCO-H$_4$folate plus...
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-[3H]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-[3H]leucine. Cells were washed in PBS, filtered, and dried at 37°C. Macromolecules were precipitated with trichloroacetic acid. The precipitate was washed with methanol and dried at 37°C. 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-H₄folate. Cytotoxicity appeared at 0.04 U/mL of rMETase (mean percent of control, 86%) and was augmented with increasing concentrations of the enzyme. The IC₅₀ ± 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 FUr + 5-HCO-H₄folate, FUr + rMETase, and FUr + 5-HCO-H₄folate + rMETase

A total of 134 combination assays with the entire concentration ranges of FUr and rMETase were performed, and seventy-three different combination data points defined by the concentrations of FUr and rMETase were calculated (Fig. 1). Synergism was found in 90% and in 89% of the combinations of FUr + rMETase and FUr + 5-HCO-H₄folate + rMETase, respectively. The median value of the CI was 0.61 for FUr + rMETase and 0.50 for FUr + 5-HCO-H₄folate + rMETase. Synergism was found throughout a broad range of concentrations of FUr 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 FUr above 100 μM. The patterns of the CI plots were similar for FUr + rMETase and for FUr + 5-HCO-H₄folate + rMETase. It was not possible to compare these plots to assess the effect of 5-HCO-H₄folate on the synergistic interaction of FUr and rMETase.

Recombinant METase at the subcytotoxic concentration of 0.02 U/mL greatly augmented the cytotoxicity of FUr, both as a single drug and in combination with 5-HCO-H₄folate (Fig. 2). The increased effect was maintained over the entire range of concentrations of FUr studied. The effect of FUr + rMETase was greater than that achieved with FUr as a single drug and with FUr + 5-HCO-H₄folate. The cytotoxicity was highest with FUr + 5-HCO-H₄folate + rMETase.

Recombinant METase at 0.02 U/mL reduced the IC₅₀ of FUr by a factor of 3.6, and by a factor of 7.5, in the absence and in the presence of 5-HCO-H₄folate, respectively (Table 1). In the absence of rMETase, 5-HCO-H₄folate reduced the IC₅₀ of FUr 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: FUr + 5-HCO-H₄folate + rMETase > FUr + rMETase > FUr + 5-HCO-H₄folate > FUr.

Table 1

<table>
<thead>
<tr>
<th>Drug and combination of drugs</th>
<th>IC₅₀ ± SE (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUr</td>
<td>33.8 ± 2.8</td>
</tr>
<tr>
<td>FUr + 5-HCO-H₄folate</td>
<td>22.4 ± 1.5</td>
</tr>
<tr>
<td>FUr + rMETase</td>
<td>9.5 ± 1.0</td>
</tr>
<tr>
<td>FUr + 5-HCO-H₄folate + rMETase</td>
<td>4.5 ± 1.0</td>
</tr>
</tbody>
</table>

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-H₄folate. The IC₅₀ values were determined with the median-effect equation, and their standard error were calculated with the delta method [28,29].
Fig. 5. Effect of exposure of CCRF-CEM cells to 100 μM of 5-HCO-CH$_3$folate for 24 hr, to rMETase at 0.2 U/mL for 6 hr, and to 5-HCO-CH$_2$folate + rMETase combined, on intracellular CH$_3$-H$_4$folate and CH$_2$-H$_4$folate 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$_3$-H$_4$folate (light gray columns) and of CH$_2$-H$_4$folate + H$_4$folate (black columns) in cells that were not exposed to 5-HCO-CH$_2$folate, and the concentrations of CH$_3$-H$_4$folate (white columns) and of CH$_2$-H$_4$folate + H$_4$folate (dark gray columns) in cells exposed to 5-HCO-CH$_2$folate. Columns represent the mean of 5 to 16 separate determinations, each done in duplicate; bars, SEM.
tion with 5-HCO-H$_2$folate was greatly augmented by rMETase at the subcytotoxic concentration of 0.02 U/mL. Cytotoxicity was highest with FUra + 5-HCO-H$_2$folate + rMETase.

The relationship of methionine metabolism to the cytotoxic 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$_2$folate [5]. Cisplatin increased the intracellular concentration of CH$_2$H$_2$folate and H$_2$folate in the cell line A2780 and caused enhancement of [TS-FdUMP-CH$_2$H$_2$folate] 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$_2$folate 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$_2$folate, the intracellular concentration of H$_2$folate and CH$_2$H$_2$folate expands to varying degrees in the different cell lines as the concentration of 5-HCO-H$_2$folate is increased. In most cells, the expansion is small, and the levels of CH$_2$H$_2$folate rapidly decrease after discontinuation of 5-HCO-H$_2$folate administration.

Expansion rates of intracellular CH$_2$H$_2$folate and H$_2$folate pools after exposure to 5-HCO-H$_2$folate 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$_2$folate and H$_2$folate attained in cells exposed to high-concentration 5-HCO-H$_2$folate were independent of the amount of folic acid contained in cell culture medium prior exposure to the 5-HCO-H$_2$folate.

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$_2$folate 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$_2$folate, 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$_2$folate + rMETase combined, do not result from inhibition of protein synthesis due to methionine depletion. Incorporation of L-[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$_2$folate, which is presumably due to stabilization of the ternary [TS-FdUMP-CH$_2$H$_2$folate] complex, supports the potentiation of FUra due to increased production of CH$_2$H$_2$folate resulting from the interaction of methionine depletion with CH$_2$H$_2$folate as methyl donor for methionine synthesis. However, we did not observe the increase in CH$_2$H$_2$folate and H$_2$folate 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$_2$folate, to result in a measurable increase of CH$_2$H$_2$folate + H$_2$folate pools [22,41].

Potentiation of FUra by rMETase, and by rMETase + 5-HCO-H$_2$folate, 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$_2$folate 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.

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