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Intracellular metabolism of 5-methyltetrahydrofolate and 5-formyltetrahydrofolate in a human breast-cancer cell line

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Abstract. This report describes the intracellular metabolism of 5-methyltetrahydrofolate (5-methyl-H_4PteGlu) and 5-formyltetrahydrofolate (5-formyl-H_4PteGlu) to the various folate forms and their respective polyglutamated states in the MCF-7 human breast-cancer cell line. The intracellular folate distribution observed in MCF-7 cells treated with 5-methyl-H_4PteGlu was similar to that seen in cells treated with 5-formyl-H_4PteGlu. In cells exposed to 5-formyl-H_4PteGlu for 24 h, the folate pool consisted of 103 ± 10 pmol/mg 10-formyl-H_4PteGlu, 120 ± 18 pmol/mg H_4PteGlu, and 71 ± 18 pmol/mg 5-methyl-H_4PteGlu versus 88 ± 5, 54 ± 20 and 87 ± 10 pmol/mg, respectively, for cells exposed to 5-methyl-H_4PteGlu. Only the difference seen in H_4PteGlu levels between cells exposed to either 5-methyl-H_4PteGlu or 5-formyl-H_4PteGlu reached statistical significance ($P < 0.05$). In the absence of vitamin B12, exposure to 5-methyl-H_4PteGlu resulted in 154 ± 17 pmol/mg 5-methyl-H_4PteGlu along with only 8 ± 5 pmol/mg 10-formyl-H_4PteGlu and 4 ± 2 pmol/mg H_4PteGlu, thus demonstrating the marked dependence on vitamin B12 for the metabolism of 5-methyl-H_4PteGlu to the other intracellular folates. 5-10-Methylene- H_4PteGlu (2 ± 1.3 pmol/mg) was detected only in cells exposed to 5-formyl-H_4PteGlu for 24 h, not in cells treated with 5-methyl-H_4PteGlu. The profile of polyglutamates detected in cells treated with either 5-formyl-H_4PteGlu or 5-methyl-H_4PteGlu for 24 h was not significantly different, although cells treated with 5-methyl-H_4PteGlu tended to have less conversion to the higher polyglutamates (Glu3 – Glu5) as compared with those treated with 5-formyl-H_4PteGlu. In 5-methyl-H_4PteGlu-treated cells grown in the absence of vitamin B12, the pentaglutamate was the only polyglutamate form detected, accounting for only 11% of the total folate pool. Since there does not appear to be a greater formation of the optimal reduced-folate forms necessary to achieve enhanced thymidylate synthase (TS) inhibition through ternary-complex formation in cells exposed to 5-methyl-H_4PteGlu versus 5-formyl-H_4PteGlu, these studies suggest that the use of 5-methyl-H_4PteGlu would not be advantageous over that of 5-formyl-H_4PteGlu in combination regimens with the fluoropyrimidines.

Key words: 5-Methyl-tetrahydrofolate – 5-Formyl-tetrahydrofolate – Active folate metabolites – Thymidylate synthase

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Introduction

The combination of 5-fluorouracil and the reduced folate 5-formyltetrahydrofolate (citrovorum factor, leucovorin, 5-formyl-H_4PteGlu) has become standard therapy for patients with advanced adenocarcinoma of the large bowel. The interaction of 5-formyl-H_4PteGlu with 5-fluorouracil has been extensively investigated in both in vitro and in vivo preclinical models and in patient tumor samples [11, 14, 19, 24–27]. These studies have clearly demonstrated the ability of 5-formyl-H_4PteGlu treatment to stabilize the ternary complex of thymidylate synthase, fluorodeoxyuridine monophosphate (FdUMP), and the reduced folate substrate 5-10-methylene-H_4PteGlu. A variety of schedules and doses of 5-formyl-H_4PteGlu have been employed clinically in attempts to optimize the serum level required for enzyme inhibition [12, 13, 20]. Previous laboratory studies have shown that metabolism of 5-formyl-H_4PteGlu to 5-10-methylene-H_4PteGlu and the polyglutamate forms in time-dependent and proportional to the dose/exposure concentration of 5-formyl-H_4PteGlu [5, 16, 17]. The folate polyglutamates have a prolonged intracellular retention and are approximately 100-fold more potent in stabilizing ternary-complex formation [1, 21]. Prolonged or repetitive dosing schedules of 5-formyl-H_4PteGlu appear to be optimal as compared with brief 5-formyl-H_4PteGlu exposures in that they result in higher levels of the more potent polyglutamate forms.

Following 5-formyl-H_4PteGlu administration, 5-methyl-H_4PteGlu accounts for a large proportion of the biologically active folate detected in the plasma [18, 23]. It has been assumed that 5-methyl-H_4PteGlu has equivalent biologic activity as compared with 5-formyl-H_4PteGlu with respect to the synergistic interaction between the folates and the fluoropyrimidines. However, relatively few studies have focused on its intracellular metabolic fate. One of the principal reasons for investigating the metabolic fate of 5-methyl-H_4PteGlu is that this folate is metabolized to 5-10-methylene-H_4PteGlu by enzymatic pathways that are different from those used by 5-formyl-H_4PteGlu. Furthermore, since 5-methyl-H_4PteGlu is the principal plasma folate, mammalian cells have developed transport and metabolic mechanisms specifically designed for its handling. These features suggest that 5-methyl-H_4PteGlu may have advantages over 5-formyl-H_4PteGlu in its ability to be transported and metabolized to 5-10-methylene-H_4PteGlu and the respective polyglutamate forms.

We also attempted to investigate the role of vitamin B12 (cobalamin) in the metabolism of 5-methyl-H_4PteGlu, since this vitamin is required by the methyl transfer reaction that regenerates methionine from homocysteine via methionine synthetase. This transfer of a methyl group represents the initial step in the cellular metabolism of 5-methyl-H_4PteGlu. In patients with gastrointestinal malignancies, the malignant process and/or surgical resection may result in B12 deficiency due to decreased dietary intake or to decreased absorption resulting from either a lack of intrinsic factor produced by the gastric parietal cells or a loss of the absorbing mucosal surfaces in the ileum.

A study by Houghton and colleagues [15] compared the metabolism of 5-formyl-H_4PteGlu and 5-methyl-H_4PteGlu to the combined 5-10-methylene-H_4PteGlu and H_4PteGlu pools in a human colon-cancer xenograft model. Following 4-h infusions of leucovorin, they noted a 6.6- and 2.5-fold increase in the intratumoral levels of the combined pool of 5-10-methylene-H_4PteGlu and H_4PteGlu in HxELC2 and HxGC3 xenografts, respectively. With 5-methyl-H_4PteGlu infusion, they found an approximately 1.5-fold expansion of the combined folate pool in each of the four xenografts studied. These investigators suggested that 5-methyl-H_4PteGlu was not as efficient as 5-formyl-H_4PteGlu in its metabolism to 5-10-methylene-H_4PteGlu and H_4PteGlu pools. These studies did not address specific changes in the 5-10-methylene-H_4PteGlu or other intracellular reduced-folate pools.

Since 5-methyl-H_4PteGlu is one of the major folate metabolites found in plasma following 5-formyl-H_4PteGlu administration and may represent a potential alternative folate for clinical use, we compared the metabolic fate of this folate with that of 5-formyl-H_4PteGlu in the MCF-7 human breast-cancer cell line.

Materials and methods

Materials. (6S)-[3′,5′,7-^{3}H]-5-formyl-H_4PteGlu (specific activity, 40 Ci/mmol) and (6S)-[3′,5′,7-^{3}H]-5-methyl-H_4PteGlu (specific activity, 28 Ci/mmol) were purchased from Moravek Biochemicals (Brea, Calif.). The radiopurity of the compounds as determined by high-performance liquid chromatography (HPLC) was ≥98% and they were used without further purification. Minimum essential medium without folic acid was purchased from Gibco (Gaithersburg, Md.). RPMI 1640 media, glutamine, phosphate-buffered saline (PBS), and fetal calf serum were purchased from Biofluids (Rockville, Md.). Sep-pak C18 cartridges and Pic Reagent A were purchased from Waters Associates (Milford, Mass.). (6R, S)-5-Formyl-H_4PteGlu was obtained from Burroughs Wellcome Co. (Research Triangle Park, N.C.), and (6S)-5-formyl-H_4PteGlu was kindly provided by Lederle Laboratories (Pearl River, N.Y.). Beta-mercaptoethanol (2-mercaptoethanol), bovine serum albumin (fraction V), vitamin B12, and reduced folate standards, including (6R, S)-5-methyl H_4PteGlu and (6R, S)-H_4PteGlu, were purchased from Sigma Chemical Co. (St. Louis, Mo.). 5-10-Methylene-H_4PteGlu was prepared as previously described [19]. Methanol was purchased from J.T. Baker Inc. (Phillipsburg, N.J.). Acetonitrile was purchased from Fisher Scientific (Fair Lawn, N.J.). Pico Aqua scintillation cocktail was purchased from Packard Instrument Co. (Meriden, Conn.).

Cell lines. The characterization of the MCF-7 human breast-cancer cell line used for these experiments has previously been described [22]. The cells were grown as a continuous monolayer in 75-cm^2 plastic tissue-culture flasks (Falcon Labware, Oxnard, Calif.) in two different types of media: minimum essential media (MEM) without folic acid (with and without the addition of vitamin B12 at a concentration of 5 μg/l) and RPMI 1640. The concentration of B12 in the MEM is approximately 10-fold that found in plasma (0.205–0.876 μg/l). Both media were supplemented with 10% fetal calf serum, 2 m*M* glutamine, 1 m*M* 2-mercaptoethanol, and 50 n*M* (6S)-5-formyl-H_4PteGlu. All cells were grown in the experimental media for at least two passages before their use in the experiments. For each of the experimental points, 1×10^6 cells were plated onto 75-cm^2 plastic tissue-culture flasks, and after 96 h of growth (60% confluency) they were used in the various experiments.

Stability of 5-methyl-H_4PteGlu. The stability of 5-methyl-H_4PteGlu was determined by incubation in media at 37° C for various intervals of up to 24 h. 5-methyl-H_4PteGlu at a concentration of 10 μ*M* was incubated in the presence or absence of 2-mercaptoethanol (0.1–10 m*M*). The amount of 5-methyl-H_4PteGlu remaining after incubation for intervals of 0, 2, 6, and 24 h was quantitated by HPLC. Since 2-mercaptoethanol is potentially toxic to cells, its growth-inhibitory effects on MCF-7 cells was determined. Approximately 10^5 cells were plated in 25-cm^2 tissue-culture flasks with folate-free media supplemented with 10% fetal calf serum, 2 m*M* glutamine, and 50 n*M* (6S)-5-formyl-H_4PteGlu. After 24 h, various concentrations of 2-mercaptoethanol ranging from 0.5 to 10 m*M* were added to the flask. A flask containing no 2-mercaptoethanol was used as a control. After 48 h an aliquot was removed from each flask and the number of cells were determined using a ZBI Coulter counter (Hialeah, Fla.).

Intracellular folate-pool measurements. MCF-7 cells were exposed to either (6S)-[^{3}H]-5-formyl-H_4PteGlu or (6S)-[^{3}H]-5-methyl-H_4PteGlu for 2, 6, and 24 h. The radiolabeled folates were diluted to the final desired concentration by the addition of unlabeled (6R, S)-5-formyl-H_4PteGlu (10 μ*M*) or (6R, S)-5-methyl-H_4PteGlu (10 μ*M*). Therefore, the concentrations listed in the text and the figures represent the final concentration of only the biologically active folate, i.e., 5 μ*M* for 5-formyl-H_4PteGlu and 5-methyl-H_4PteGlu. We have previously reported that the intracellular metabolism of (6S)-5-formyl-H_4PteGlu was not altered in MCF-7 breast-cancer cells by the addition of up to a 20-fold excess of (6R)-5-formyl-H_4PteGlu [5]. At the end of the specified exposure period, the cells were washed two times with ice-cold PBS and then harvested in 1 ml saline with the aid of a rubber cell scraper. A 100-μl aliquot was removed for subsequent protein analysis. The folates were extracte from the remainder of the cell suspension according to previously published techniques [2, 3]. The labeled folates were separated by HPLC using a Waters Model 510 pump and a Waters Model 440 ultraviolet absorption detector with a fixed wavelength of 256 nm according to methods described elsewhere [2, 3]. The

recovery rate for the various folates (except for 5-10-methylene-H_4PteGlu) ranged from 60% to 70%, and no correction for recovery was applied as all comparisons were made between cells processed under identical conditions and at the same time. The retention times for the folates were as follows: paraaminobenzoate, 4–5 min; para-aminobenzoyl glutamate, 7.5–8 min; 10-formyl-H_4PteGlu, 11 min; H_4PteGlu, 13.5 min; 5-formyl-H_4PteGlu, 15 min; H_2PteGlu, 18.5 min; and 5-methyl-H_4PteGlu, 28 min.

5-10-Methylene-tetrahydrofolate quantitation. The 5-10-methylene-H_4PteGlu pool was quantitated in a separate set of experiments using MCF-7 cells labeled with either (6S)-[^{3}H]-5-formyl-H_4PteGlu or (6S)-[^{3}H]-5-methyl-H_4PteGlu according to previously published methods [3]. For these experiments, the cells were exposed to either 5 μ*M* (6S)-[^{3}H]-5-formyl-H_4PteGlu or 5 μ*M* (6S)-[^{3}H]-5-methyl-H_4PteGlu for 24 h, after which they were washed two times with ice-cold PBS and harvested. The intracellular folates were extracted as described above and separated by HPLC using a modified mobile phase consisting of 76% Pic A (adjusted to pH 4.0 with 1 *N* HCl) and 24% methanol. The folate pool was quantitated using an in-line scintillation counter. The retention time for 5-10-methylene-H_4PteGlu was 26 min. Authentication of the 5-10-methylene-H_4PteGlu was accomplished by coelution with a standard compound and by specific metabolism of the putative 5-10-methylene-H_4PteGlu peak to H_2PteGlu in the presence of thymidylate synthase and deoxyuridylate [8].

Intracellular folate-polyglutamate measurements. MCF-7 cells were exposed to either 5 μ*M* (6S)-[^{3}H]-formyl-H_4PteGlu or 5 μ*M* (6S)-[^{3}H]-5-methyl-H_4PteGlu for intervals of 2, 6, and 24 h. At the end of each period, the cells were washed two times with ice-cold PBS and then harvested in 1 ml saline with the aid of a rubber cell scraper. A 100-μl aliquot was removed for protein quantitation. The folate polyglutamates were extracted from the remainder of the cell suspension by boiling for 90 s in 2 ml of a 2% ascorbate/2% 2-mercaptoethanol solution (pH 6.0). The denatured protein was removed by centrifugation at 10,000 *g* for 5 min. The polyglutamated folates were then concentrated using a C-18 Sep-pak cartridge and separated by HPLC using a 30-min linear gradient from 20% to 35% acetonitrile in Pic A (pH 5.5) according to previously published methods [5].

Protein measurement. A 100-μl aliquot of cell suspension was sonicated with five 3-s bursts using a Branson Model 350 sonicator equipped with a microtip. The cell debris was pelleted by centrifugation at 10,000 *g* for 10 min, and the protein in the supernatant was quantitated using the method of Bradford [7].

Calculations. The disintegrations per minute obtained by counting an aliquot of the extracted labeled folates were converted to total intracellular folate content (expressed in picomoles per milligram of protein) by dividing the total disintegrations per minute per flask of cells by the total amount protein per flask of cells and then dividing the result by the specific activity of the labeled compound; abbreviated as follows: (dpm/flask)/(mg protein/flask)/(dpm/pmol folate) = pmol folate/mg protein.

Results

As a result of the susceptibility of 5-methyl-H_4PteGlu to oxidation, we measured the half-life of this reduced folate in complete growth media at 37° C. We found that less than half of the parent compound remained as 5-methyl-H_4PteGlu after 6 h. Previous experience using dihydrofolate for tissue-culture experiments suggested that low concentrations of 2-mercaptoethanol could be used to stabilize folates while having little effect on the viability of the MCF-7 breast-cancer cells [4]. We first determined that the maximal amount of 2-mercaptoethanol that did not produce growth inhibition of the cells over a 24-h incubation period was a concentration of 1 m*M*. An examination of the half-life of 5-methyl-H_4PteGlu along with 2-mercaptoethanol revealed that a 1 m*M* concentration resulted in a prolongation of the half-life of the folate in the media to

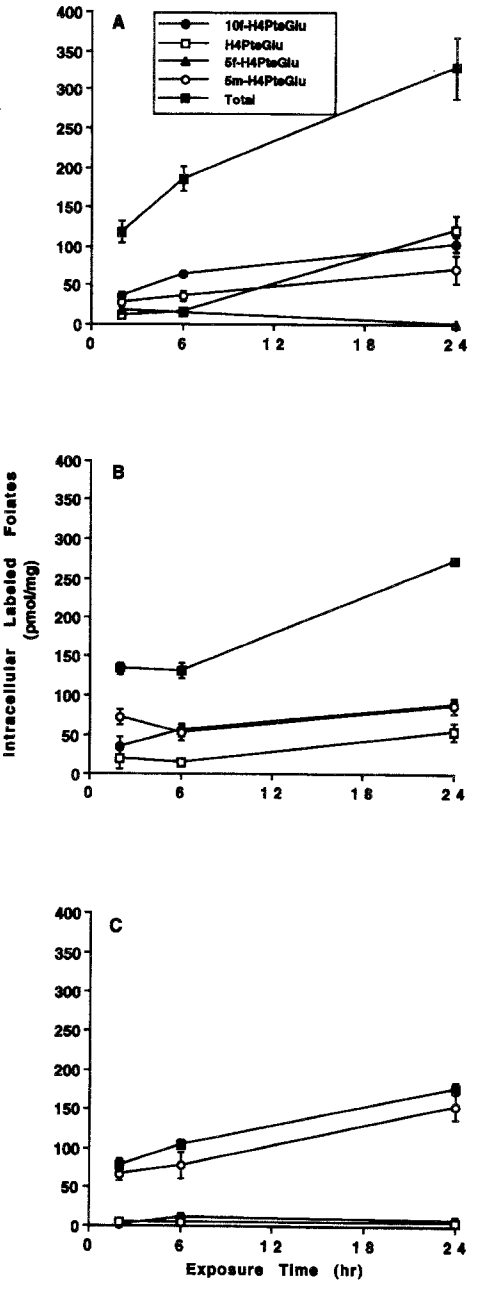

Fig. 1A–C. Intracellular folate metabolism during exposure to either 5-methyl-H_4PteGlu or 5-formyl-H_4PteGlu in MCF-7 human breast-cancer cells. MCF-7 breast-cancer cells were grown in MEM folate-deplete media containing 50 n*M* (6S)-5-formyl-H_4PteGlu, 10% fetal calf serum, 1 m*M* 2-mercaptoethanol, and 2 m*M* glutamine at an initial plating density of 1×10^6 cells/plate. The cells were allowed to grow until they reached 60% confluency. The cells were then exposed to radiolabeled 5 μ*M* 5-formyl-H_4PteGlu (**A**), 5-methyl-H_4PteGlu (**B**), or 5-methyl-H_4PteGlu in the absence of vitamin B12 (**C**) for 2, 6, and 24 h in 1 m*M* 2-mercaptoethanol. After each exposure period, the cells were washed twice in ice-cold PBS and harvested. The labeled intracellular folate pools represented in the figure were extracted from the cells, separated, and quantitated by HPLC. Each point represents the mean value ± SE for 3–5 independent experiments

approximately 24 h. To ensure that 1 m*M* 2-mercaptoethanol would not interfere with intracellular folate metabolism, we measured the metabolism of 5-formyl-H_4PteGlu in the presence or absence of 1 m*M* 2-mercaptoethanol and found no significant difference in the folate pools detected in the breast-cancer cell line. Folate-pool profiles obtained after exposure of cells to 5-formyl-H_4PteGlu in the absence of 2-mercaptoethanol have been published elsewhere [5].

Using MCF-7 human breast-cancer cells as our model system, we examined the metabolism of both 5-formyl-H_4PteGlu and 5-methyl-H_4PteGlu to the various folate pools over time and at a fixed exposure concentration. Experiments were performed using 5 μ*M* radiolabeled 5-formyl-H_4PteGlu or 5 μ*M* radiolabeled 5-methyl-H_4PteGlu to treat cells grown in the absence or presence of vitamin B12 at a supraphysiologic concentration of 5 μg/l (normal human-serum vitamin B12, 0.205–0.876 μg/l) for intervals of 2, 6, and 24 h. As illustrated in Figs. 1A and 1B), cells exposed to 5-formyl-H_4PteGlu and to 5-methyl-H_4PteGlu had a 2- to 3-fold increase in the total labeled intracellular folate pool between 2 and 24 h of exposure. The relative proportion of each folate form was stable during the 24-h period of exposure. The metabolism of 5-formyl-H_4PteGlu and 5-methyl-H_4PteGlu to the various reduced folates was similar (Fig. 1), differing only in a decreased amount of H_4PteGlu in the 5-methyl-H_4PteGlu-exposed cells (20%, 54 pmol/mg) as compared with those exposed to 5-formyl-H_4PteGlu (32%, 120 pmol/mg). This was the only significant ($P < 0.05$) difference noted between a 24-h exposure to either 5-formyl-H_4PteGlu or 5-methyl-H_4PteGlu in the presence of vitamin B12. Exposure of cells to 5-methyl-H_4PteGlu in the absence of vitamin B12 (Fig. 1C) resulted in approximately one-half as much total folate expansion at each time point as compared with cells treated with equal concentrations of 5-methyl-H_4PteGlu in the presence of vitamin B12. The folate pool profiles obtained in these cells were significantly different, with relatively little conversion of 5-methyl-H_4PteGlu being noted, which represented approximately 90% of the total folate pool.

We also examined the expansion of 5-10-methylene-H_4PteGlu in breast-cancer cells that had been exposed to either 5-methyl-H_4PteGlu or 5-formyl-H_4PteGlu. In these independent experiments, in MCF-7 cells grown in low-folate media containing 50 n*M* 5-formyl-H_4PteGlu as the only folate source other than serum, we found no detectable level of 5-10-methylene-H_4PteGlu after a 24-h period of exposure to 5 μ*M* concentrations of either of the labeled folates. Presumably, the expansion of the 5-10-methylene-H_4PteGlu pool under these low-folate conditions fell below the detection threshold of the assay (0.5 pmol/mg). In contrast, examination of the 5-10-methylene-H_4PteGlu pool in breast-cancer cells grown in folate-replete RPMI 1640 media (2.2 μ*M* folic acid) following a 24-h period of exposure to 5 μ*M* 5-formyl-H_4PteGlu resulted in a detectable 5-10-methylene H_4PteGlu pool of 2.0±1 pmol/mg. However, when cells grown in folate-replete media were exposed to 5 μ*M* radiolabeled 5-methyl-H_4PteGlu for 24 h, there was no detectable level of 5-10-methylene-H_4PteGlu.

In addition to intracellular metabolism to the various folate pools, we also examined the metabolism of 5-formyl-H_4PteGlu and 5-methyl-H_4PteGlu to the various polyglutamated states in an independent set of experiments. There was little difference in the total folate pool detected in cells exposed to either 5-formyl-H_4PteGlu or 5-methyl-H_4PteGlu

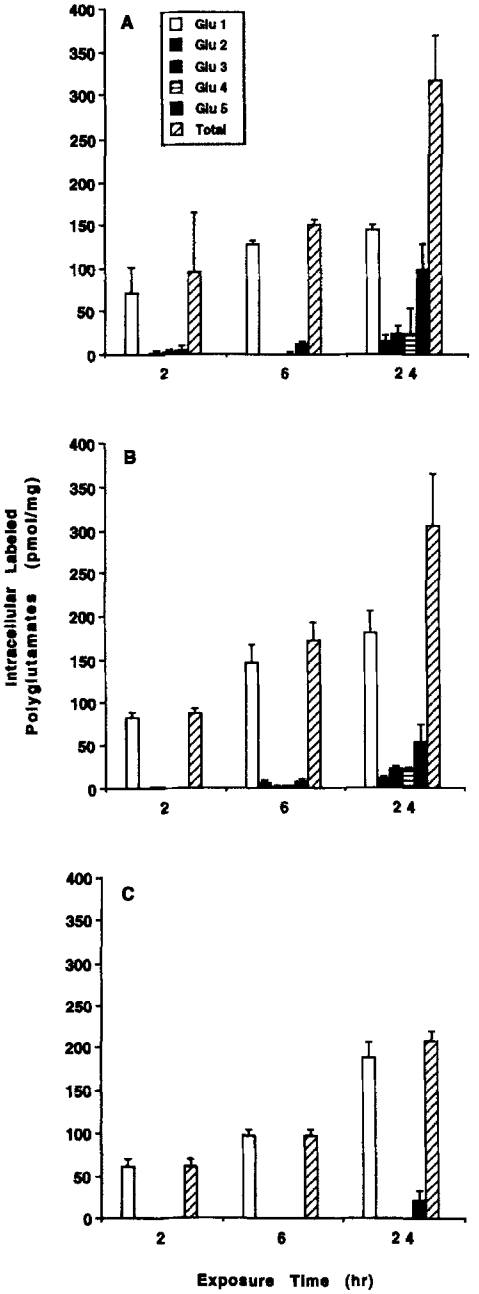

Fig. 2 A–C. Polyglutamate formation during exposure to either 5-methyl-H_4PteGlu or 5-formyl-H_4PteGlu in MCF-7 human breast-cancer cells. MCF-7 breast-cancer cells were grown in MEM folate-deplete media containing 50 n*M* (6S)-5-formyl-H_4PteGlu, 10% fetal calf serum, 1 m*M* 2-mercaptoethanol, and 2 m*M* glutamine at an initial plating density of 1×10^6 cells/plate. The cells were allowed to grow until they reached 60% confluency. The cells were then exposed to radiolabeled 5 μ*M* 5-formyl-H_4PteGlu (**A**), 5-methyl-H_4PteGlu (**B**), or 5-methyl-H_4PteGlu in the absence of vitamin B12 (**C**) for 2, 6, and 24 h in 1 m*M* 2-mercaptoethanol. After each exposure period, the cells were washed twice in ice-cold PBS and harvested. The folate polyglutamates (Glu1–Glu5) were extracted from the cells and then separated and quantitated by HPLC. Each point represents the mean value ± SE for 3–5 independent experiments

Table 1. Intracellular polyglutamation of folates during exposure to labeled 5-methyl-H_4PteGlu or 5-formyl-H_4PteGlu in human MCF-7 breast-cancer cells. Data are given as mean values ± SE

Folate used for exposure	Time of exposure (h)		
	2	6	24
	Glu 1-2 polyglutamates (pmol/mg)		
5-formyl-H_4PteGlu[a]	77±15	124±4	160±8
5-methyl-H_4PteGlu[a]	82±5	152±14	190±20
5-methyl-H_4PteGlu[b]	62±6	97±6*	189±4
	Glu 3-5 polyglutamates (pmol/mg)		
5-formyl-H_4PteGlu[a]	15±3	17±4	147±28
5-methyl-H_4PteGlu[a]	1.4±0.8*	12±1	99±15
5-methyl-H_4PteGlu[b]	0*	0*	23±9*

* $P < 0.05$ as compared with cells exposed to 5-formyl-H_4PteGlu
[a] Minimum essential media with vitamin B12 (5.0 μg/l)
[b] Minimum essential media alone

as illustrated in Fig. 2A and 2B. The polyglutamate levels (Glu >1) were low during the early time points, with a significant amount of polyglutamates being detected only after 24 h. Table 1 illustrates the sum of the formation of the lower polyglutamates (Glu1 and Glu2) and higher polyglutamates (Glu ≥3) at each time point after exposure to the labeled compounds under the various conditions tested. Cells exposed to 5-methyl-H_4PteGlu tended to have greater amounts of the lower polyglutamates at the expense of metabolism to the higher forms as compared with those treated with 5-formyl-H_4PteGlu, but this difference did not reach statistical significance. By 24 h, 46% ± 9% of the intracellular folates were present in the form of higher polyglutamates in 5-formyl-H_4PteGlu-exposed cells versus 33% ± 5% in those treated with 5-methyl-H_4PteGlu.

Although quantitative differences were observed in the total folate pool between exposure to 5-methyl-H_4PteGlu in the presence and absence of vitamin B12, there was a more profound qualitative difference in their polyglutamate metabolism as illustrated in Figs. 2B and 2C. In contrast to polyglutamation of the folates following exposure to either 5 μ*M* 5-formyl-H_4PteGlu or 5-methyl-H_4PteGlu with media containing vitamin B12, wherein all polyglutamate forms were apparent, the pentaglutamate was the only polyglutamate detected in cells treated with 5-methyl-H_4PteGlu in the absence of vitamin B12. Under these conditions, the pentaglutamate represented only 11% ± 5% of the total pool, the remainder consisting of the monoglutamate form. We detected no intermediate polyglutamate form at any of the three intervals examined in the breast-cancer cells treated with 5-methyl-H_4PteGlu in the absence of vitamin B12.

Discussion

The present studies suggest that the intracellular metabolism of 5-formyl-H_4PteGlu versus 5-methyl-H_4PteGlu in the MCF-7 human breast-cancer cell line is similar. Although there tended to be greater conversion to the higher polyglutamate forms with 5-formyl-H_4PteGlu exposure as compared with 5-methyl-H_4PteGlu treatment, these differences did not reach statistical significance. Expansion of the 5-10-methylene-H_4PteGlu pool by either 5-formyl-H_4PteGlu or 5-methyl-H_4PteGlu fell below the detection threshold in cells grown in low-folate conditions; however, growth of cells in folate-replete conditions permitted quantitation of the 5-10-methylene-H_4PteGlu pool expansion in cells treated with 5 μ*M* 5-formyl-H_4PteGlu. In contrast, we could not detect 5-10-methylene-H_4PteGlu after exposure to 5 μ*M* 5-methyl-H_4PteGlu, even in cells grown in folate-replete media. This observation is consistent with the significant difference observed in H_4PteGlu levels after 24 h of exposure to 5-formyl-H_4PteGlu as compared with 5-methyl-H_4PteGlu. However, the low levels of 5-10-methylene-H_4PteGlu detected in the MCF-7 cell line preclude a definite statement of the superiority of metabolism to 5-10-methylene-H_4PteGlu in 5-formyl-H_4PteGlu-treated cells. Houghton and colleagues [15] determined that 5-methyl-H_4PteGlu exposure resulted in less expansion of the combined 5-10-methylene-H_4PteGlu and H_4PteGlu pool as compared with equivalent 5-formyl-H_4PteGlu exposure in the human colon-cancer xenograft model. These investigators noted a 2- to 3-fold greater increase in the combined intratumoral folate pool with 5-formyl-H_4PteGlu versus 5-methyl-H_4PteGlu exposure.

These observations suggest that 5-methyl-H_4PteGlu transport and/or metabolic pathways are not significantly more effective than those used by 5-formyl-H_4PteGlu and that 5-methyl-H_4PteGlu may not be as efficient as 5-formyl-H_4PteGlu in increasing the intracellular 5-10-methylene H_4PteGlu pool or in the formation of the higher polyglutamates. The differences in folate species observed between 5-methyl-H_4PteGlu and 5-formyl-H_4PteGlu may reflect differences in the manner in which the cell handles the intracellular fate of each of these folates rather than differences in their uptake or extracellular half-lives, since the total intracellular folate content was identical at each of the three exposure times investigated. Furthermore, evidence that the differences noted in 5-methyl-H_4PteGlu-exposed cells (in vitamin B12-containing media) were not due to incomplete vitamin B12 repletion derives from several observations, including 10-formyl-H_4PteGlu levels identical to those found in 5-formyl-H_4PteGlu-exposed cells, an increase in H_4PteGlu levels over time, a decrease in the percentage of 5-methyl-H_4PteGlu (rather than an accumulation) over time and identical total intracellular folate pools. The experiments performed with vitamin B12-depleted cells serve as support for this interpretation.

Interestingly, very little 5-methyl-H_4PteGlu is metabolized to either 10-formyl-H_4PteGlu or H_4PteGlu when no vitamin B12 is present in the media. These data suggest that the presence of vitamin B12 is a critical determinant in the metabolism of 5-methyl-H_4PteGlu to the various reduced-folate pools, as would be predicted given the requirement for vitamin B12 in the first metabolic step in the conversion of 5-methyl-H_4PteGlu to H_4PteGlu via methionine synthetase. It also appears that the presence of vitamin B12 may have an effect on the formation of higher polyglutamates. Cichowicz and Shane [6, 9 ,10] have found that 5-methyl-H_4PteGlu, which constitutes the greatest intracellular pool in cells exposed to 5-methyl-H_4PteGlu in the absence of vitamin B12, was 36% as efficient as a substrate for

purified mammalian folylpolyglutamyl snythetase as compared with H_4PteGlu. This issue may have clinical importance in those patients with malignancy who may be relatively vitamin B12-deficient due to either decreased intake or diminished absorption resulting from the presence of tumor or surgical resections.

As suggested in previous investigations, the present data also support the concept that longer periods of exposure to either 5-methyl-H_4PteGlu or 5-formyl-H_4PteGlu result in the formation of greater quantities of the various intracellular metabolites, including 5-10-methylene-H_4PteGlu (detectable only in the case of 5-formyl-H_4PteGlu exposure) and the higher polyglutamates. The higher polyglutamates, such as pentaglutamate, have up to a 100-fold enhanced ability to form ternary complexes with FdUMP and thymidylate synthase as compared with the monoglutamate form of 5-10-methylene-H_4PteGlu [1, 21]. Furthermore, folate pentaglutamates have a 20-fold longer intracellular retention (20 h) as compared with folate monoglutamates (1 h) [5].

In summary, these data indicate that the use of the more physiologic folate 5-methyl-H_4PteGlu does not appear to have an advantage over that of 5-formyl-H_4PteGlu in the formation of the active folate metabolites that are necessary for enhanced thymidylate synthase inhibition by the fluoropyrimidines. In addition, the metabolism of 5-methyl-H_4PteGlu to biologically active reduced folates is highly dependent on the presence of vitamin B12.

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