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EFFECTS OF 5-METHYLTETRAHYDROFOLATE ON THE ACTIVITY OF FLUOROPYRIMIDINES AGAINST HUMAN LEUKEMIA (CCRF-CEM) CELLS*

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Abstract—The growth inhibitory effects of 5-fluorouracil (FUra) or 5-fluoro-2′-deoxyuridine (FdUrd) combined with 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) were determined, as a function of time, dose, and sequence of exposure, on human T-lymphoblast leukemia cells, CCRF-CEM. Synergistic inhibitory effects on cell growth were obtained when exponentially growing CCRF-CEM cells were exposed to 5-CH_3-H_4PteGlu (1-100 μM) for 4 hr and to FUra (250 μM) or FdUrd (0.5 μM) during the last 2 hr. Synergism was dependent on 5-CH_3-H_4PteGlu dose (100 > 10 > 1 μM) and did not occur at 0.1 μM. No clear dependence of synergism on sequence was observed with FUra and 5-CH_3-H_4PteGlu combinations (5-CH_3-H_4PteGlu → FUra, 5-CH_3-H_4PteGlu + FUra, or FUra → 5-CH_3-H_4PteGlu). With 5-CH_3-H_4PteGlu and FdUrd combinations, synergism was dependent on sequence of exposure (5-CH_3-H_4PteGlu + FdUrd, 5-CH_3-H_4PteGlu → FdUrd were synergistic, but FdUrd → 5-CH_3-H_4PteGlu was not). Thymidine (0.1 μM), added after drug treatment, substantially rescued CCRF-CEM cells from 5-CH_3-H_4PteGlu → FUra cytotoxicity. L-methionine (1500 mg/l) completely protected CCRF-CEM cells from enhanced cytotoxicity of the combination, 5-CH_3-H_4PteGlu-FdUrd. The results are consistent with the hypothesis that the mechanism by which 5-CH_3-H_4PteGlu potentiates fluoropyrimidine cytotoxicity is the enhancement of complex formation between thymidylate synthase and 5-fluorodeoxyuridylate, as a consequence of an increase of intracellular levels of 5,10-methylenetetrahydrofolate generated from 5-CH_3-H_4PteGlu. Also, enhanced stability of the complex in the presence of high levels of this folate coenzyme may contribute to the synergism observed. These data provide a rationale basis for further trials of folate coenzymes and fluoropyrimidine combinations in the clinic.

Fluoropyrimidines, 5-fluorouracil (FUra)‖ and 5-fluoro-2′-deoxyuridine (FdUrd), are active agents against various types of solid tumors, including carcinomas of the breast, colon, ovary, head and neck [1]. In these tumors, the response rates vary from 10 to 40% and complete remissions are unusual, when these drugs are used as single agents. Early clinical trials have shown no significant activity of these compounds in acute leukemias and lymphomas. A more recent report of Costanzi *et al.* [2] suggests, however, that FUra may be effective in the treatment of adults with acute leukemia.

The cytotoxicity of fluoropyrimidines may result from different biochemical events involving nucleotide derivatives of these drugs. Fluoropyrimidine nucleotides are incorporated into RNA and interfere with the functioning of all species [3] and fluoropyrimidine deoxynucleotides may be incorporated into DNA [4]. In most tumor cells, however, the inhibition of thymidylate (dTMP) biosynthesis by fluorodeoxyuridylate (FdUMP), the active common metabolite of FUra and FdUrd, appears to be the most critical determinant of the action of these drugs [5–9]. FdUMP binds tightly to thymidylate (dTMP) synthase only in the presence of the folate coenzyme, 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu) [10, 11]. Although the ternary complex among dTMP synthase, FdUMP, and 5,10-CH_2-H_4PteGlu is a covalent one, nevertheless it does dissociate in the absence of excess of the folate coenzyme [12]. As this process occurs intracellularly, the released enzyme would be protected from further FdUMP binding by expanded deoxyuridylate pools [13] with consequent resistance to fluoropyrimidine kill.

Recently, it has been shown that modulation by 5-formyltetrahydrofolate (leucovorin, LV) may

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‖ Abbreviations used: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FUTP, 5-fluorouridine 5′-triphosphate; FdUTP, 5-fluoro-2′-deoxyuridine 5′-triphosphate; FdUMP, 5-fluoro-2′-deoxyuridine; 5,10-CH_2-H_4PteGlu, 5,10-methylenetetrahydrofolate; LV, leucovorin, 5-formyltetrahydrofolate; dThd, thymidine; Hyp, hypoxanthine; 5-CH_3-H_4PteGlu, 5-methyltetrahydrofolate; PRPP, phosphoribosylpyrophosphate.

enhance the potency of fluoropyrimidines, FUra and FdUrd, against tumor cells of various origin, including murine leukemia cells only marginally sensitive to these drugs [14–16]. This effect is presumably the consequence of an increase in the intracellular pools of folate cofactors including 5,10-CH_2-H_4PteGlu, leading to an enhanced formation of the ternary complex [5, 15, 17] and to a stabilization of it [14, 18].

Following *in vivo* administration, however, LV is rapidly converted to 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) [19–21]. The blood level of this compound exceeds that of the physiological (*l*)-diastereoisomer of LV within two hours of drug administration [21].

Based on these observations, we analyzed the interactions of the latter folate coenzyme and FUra or FdUrd in human leukemic lymphoblasts, CCRF-CEM, during simultaneous or sequential exposure.

Our results demonstrate that 5-CH_3-H_4PteGlu enhances the cytotoxicity of both fluoropyrimidines against these cells and that the observed synergism can be reversed by thymidine (dThd) rescue and L-methionine protection. This indicates that inhibition of dTMP synthase by fluoropyrimidines is potentiated by 5-CH_3-H_4PteGlu pretreatment or coadministration and that potentiation occurs via the methionine synthetase-dependent conversion of 5-CH_3-H_4PteGlu to H_4PteGlu and subsequent conversion to 5,10-CH_2-H_4PteGlu.

MATERIALS AND METHODS

Chemicals. (*dl*)-5-CH_3-H_4PteGlu calcium salt was a generous gift of Bioresearch S.p.A. (Milan, Italy); FUra and FdUrd were offered by Hoffman-LaRoche, S.p.A. (Milan); hypoxanthine (Hyp) and thymidine (dThd) were purchased from Sigma Chemical Co., (St. Louis, MO); L-methionine from Merck (Darmstadt, West Germany). Media and sera for cell culture were obtained from Gibco Lab. (Grand Island, NY); antibiotics were offered by Farmitalia-Carlo Erba, S.p.A. (Milan). Plasticware for cell culture was purchased from Costar (Cambridge, MA).

Cell line. The CCRF-CEM human leukemic T-lymphoblast cells were maintained as suspension cultures in RPMI 1640 medium supplemented with 10% horse serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37° in a 5% CO_2 atmosphere and subcultured twice a week [22]. Under these conditions the doubling time of exponentially-growing cells was 21 hr.

Cell growth inhibition studies. Exponentially-growing cells were exposed to drugs at an initial density of about 2×10^5 cells/ml according to experimental design. All drugs were dissolved in water and freshly prepared before each experiment. After drug treatment cells were harvested by centrifugation, washed twice, and resuspended in drug-free medium supplemented with 10% horse serum at a concentration of about 2×10^4 cells/ml. Subsequent changes in cell number were followed over 5–7 days using a Coulter counter (Model D, Counter Electronics, Ltd., Luton, Bedfordshire, England). To compare the effects of drug treatment on growth of cells resuspended at a slightly different initial density, the cell numbers were converted to total cell division (TCD) according to the formula:

$$\mathrm{TCD} = \frac{\log_{10}(N_t/N_o)}{\log_{10}2}$$

in which N_o is the initial cell number following resuspension, and N_t is the cell number at time, t. Growth curves were generated plotting TCDs as a function of time [23]. For quantitating the effects of 5-CH_3-H_4PteGlu and fluoropyrimidine (FUra or FdUrd) combined treatment on cell growth, the difference between the product of the percent growth inhibition of each individual agent and the percent growth inhibition of the 5-CH_3-H_4PteGlu-fluoropyrimidine combination at 72 hr after the end of the treatment and resuspension of cells in drug-free medium was calculated. No difference indicates an additive effect, while positive or negative values (±10%) indicate a synergistic or an antagonistic effect on cell growth, respectively.

Since 5-CH_3-H_4PteGlu treatment was not inhibitory of cell growth at concentrations up to 100 μM, expected percent cell growth inhibition of combined 5-CH_3-H_4PteGlu-fluoropyrimidines under conditions of additivity is equal to percent growth of single fluoropyrimidine-treated cells. Any significant positive difference (>10%) with observed percent growth of cells treated with this combination indicates synergism.

RESULTS

The inhibitory effects of 5-CH_3H_4PteGlu, fluoropyrimidines, and their combinations on cell growth were determined by measurement of cell regrowth after washing and resuspension in drug-free medium following drug treatment, as described in "Materials and Methods". 5-CH_3-H_4PteGlu used in this investigation was the commercially-available form produced by chemical reduction of folic acid, thus containing equal amounts of the (*l*) and the (*d*) diastereoisomers. Reported 5-CH_3-H_4PteGlu concentrations refer to total racemic mixture (*dl*) of the folate.

Exposure of various durations (4 hr to continuous) to 5-CH_3-H_4PteGlu at concentrations between 0.01 and 100 μM did not produce any significant change in the growth of CCRF-CEM cells.

In Fig. 1 the results of exposing cells to 5-CH_3-H_4PteGlu (1–100 μM) for 4 hr and to FUra (250 μM) during the last 2 hr of exposure to the reduced folate are shown. The inhibitory effects of sequential 5-CH_3-H_4PteGlu and FUra on cell growth were greater than those observed with FUra alone. Observed synergy was dose-dependent on 5-CH_3-H_4PteGlu concentration (100 > 10 > 1 μM). Synergism did not occur at 5-CH_3-H_4PteGlu concentration of 0.1 μM (data not shown).

Marked synergism was noted when FdUrd was substituted for FUra at a concentration of 0.5 μM (approximately as toxic as 250 μM FUra) in the previously described sequential schedule (Fig. 2). Dose-dependent synergism occurred at a 5-CH_3-H_4PteGlu concentration of 1 μM. Degree of synergism was

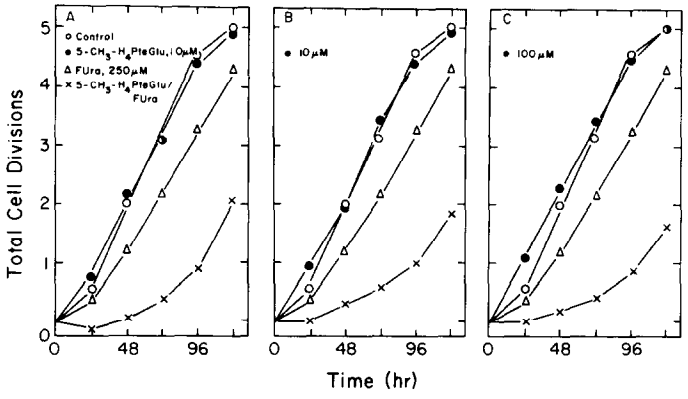

Fig. 1. Inhibitory effects of sequential 5-CH_3-H_4PteGlu-FUra treatments on growth of CCRF-CEM cells. Cells at a density of about 2×10^5/ml were exposed to 5-CH_3-H_4PteGlu (●) (panel A. 1 μM; B. 10 μM; C. 100 μM) for 4 hr and to FUra (250 μM) alone (△) or in combination for the last 2 hr of folate exposure (×). After drug exposure, cells were washed twice, resuspended in drug-free medium at a density of about 2×10^4/ml, and their growth followed. Growth curves of control (○) and treated cells were generated by plotting total cell divisions (TCDs) versus time. TCDs were calculated as described in "Materials and Methods." The data represent the mean of duplicate samples from a single representative experiment.

greater for 5-CH_3-H_4PteGlu-FdUrd, as compared to 5-CH_3-H_4PteGlu-FUra sequential treatment.

When cells were exposed to 10 μM 5-CH_3-H_4PteGlu and to 100 μM FUra for 4 hr in sequence (that is, 5-CH_3-H_2PteGlu → FUra or FUra → 5-CH_3-H_4PteGlu) or simultaneously (5-CH_3-H_4PteGlu + FUra), synergistic inhibitory effects on cell growth were observed (Fig. 3A). No clear dependence of synergism on sequence of exposure was observed. However, while sequential administration of 5-CH_3-H_4PteGlu and FUra (5-CH_3-H_4PteGlu → FUra) and the simultaneous exposure of cells to the reduced folate and the fluoropyrimidine (5-CH_3-H_4PteGlu + FUra) obtained maximum inhibitory effects on cell growth, the reverse sequence, FUra → 5-CH_3-H_4PteGlu, resulted in slightly less synergism.

When FdUrd at a dose of 0.5 μM was substituted for FUra in these sequential combinations, synergism was noted to be dependent on sequence of administration (Fig. 3B). Degree of synergism was greater with the sequence 5-CH_3-H_4PteGlu + FdUrd, as compared to that of the sequence 5-CH_3-H_4PteGlu → FdUrd; no synergism was observed using FdUrd → 5-CH_3-H_4PteGlu.

Thymidine (dThd) (0.1 μM), added to medium after drug treatment, substantially rescued CCRF-CEM cells from cytotoxicity of sequential 5-CH_3-H_4PteGlu-FUra (5-CH_3-H_4PteGlu, 10 μM, 4 hr, followed by FUra, 100 μM, 4 hr), but did not rescue cells from the cytotoxic effects of FUra alone (Fig. 4). Higher concentrations of dThd (1 μM), however, were able to rescue cells from the cytotoxicity of FUra alone (data not shown).

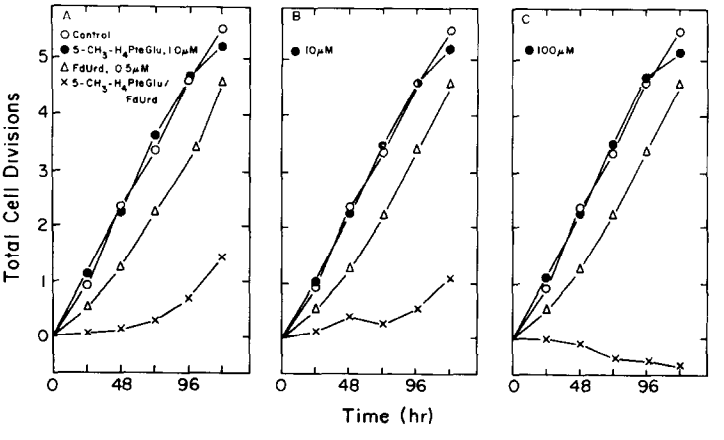

Fig. 2. Inhibitory effects of sequential 5-CH_3-H_4PteGlu-FdUrd treatment on growth of CCRF-CEM cells. Cells at a density of about 2×10^5/ml were exposed to a 5-CH_3-H_4PteGlu (●) (panel A. 1 μM; B. 10 μM; C. 100 μM) for 4 hr and to FdUrd (0.5 μM) alone (△) or in combination for the last 2 hr of folate exposure (×). (○) represent control growth. After drug exposure, cells were treated as described in the legend to Fig. 1. The data points represent the mean duplicate samples from a single representative experiment.

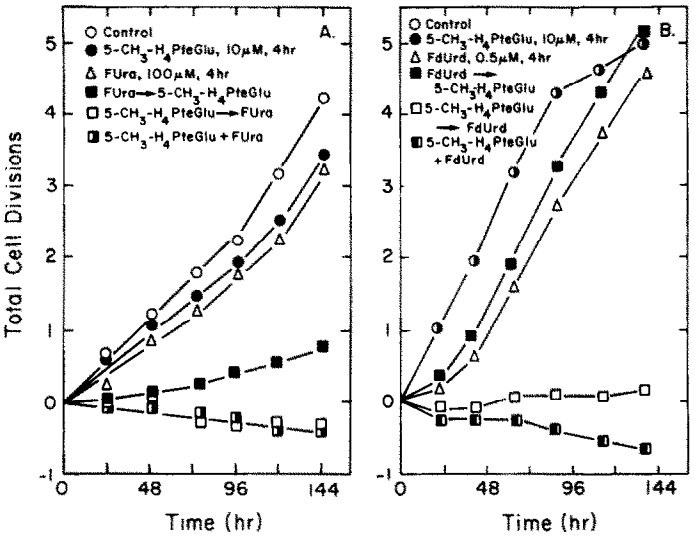

Fig. 3. Sequence-dependent effects of 5-CH_3-H_4PteGlu and FUra or FdUrd on CCRF-CEM cell growth. Cells at a density of about 2×10^5/ml were exposed to either 5-CH_3-H_4PteGlu (10 μM) or to a fluoropyrimidine (FUra, 100 μM; or FdUrd, 0.5 μM). Each drug exposure either alone or in simultaneous or sequential combination, was for 4 hr. After 4 hr exposure to the first drug, cells were washed twice and re-exposed to the second drug at the same density. After the final wash, the cells were resuspended in drug-free medium and treated as described in the legend to Fig. 1. Results represent mean values of duplicate determinations from a single representative experiment. Symbols used are: ○, control; ●, 5-CH_3-H_4PteGlu (10 μM); △, fluoropyrimidine (Panel A. FUra, 100 μM; B. FdUrd, 0 5 μM); ■, fluoropyrimidine (A. FUra; B. FdUrd) → 5-CH_3-H_4PteGlu; □, 5-CH_3-H_4PteGlu → fluoropyrimidine (A. FUra; B. FdUrd); ◨, 5-CH_3-H_4PteGlu + fluoropyrimidine (A. FUra; B. FdUrd).

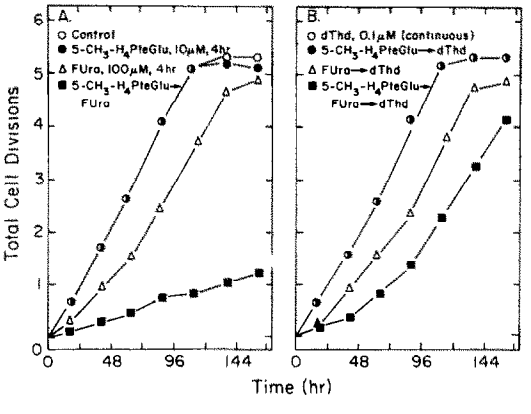

Fig. 4. Effects of dThd rescue on cytotoxicity of the sequential combination 5-CH_3-H_4PteGlu → FUra. CCRF-CEM cells at a density of about 2×10^5/ml were first exposed to 5-CH_3-H_4PteGlu (10 μM) (●) or no drug (○) for 4 hr. After washing in drug-free medium, cells were then exposed at the same density to FUra (100 μM) for 4 hr, followed either by no treatment (△) or by 5-CH_3-H_4PteGlu (■). After the final wash, cells were resuspended in drug-free medium without or with dThd (0.1 μM) (Panel A. and B., respectively), at a density of about 2×10^4/ml and their growth followed. Growth curves of control and drug-treated cells were generated as described in the legend to Fig. 1. Results represent mean values of duplicate determinations from a single representative experiment.

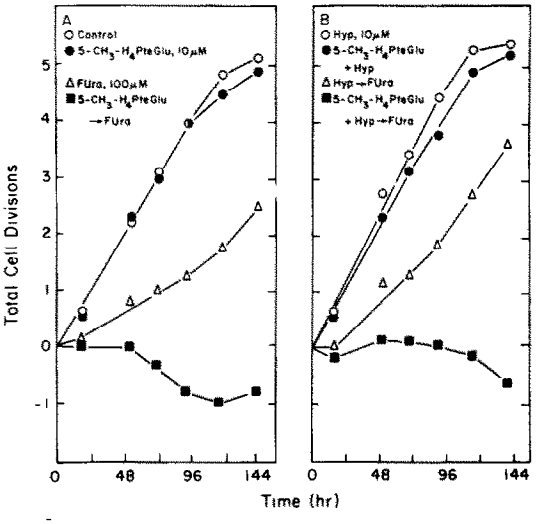

Fig. 5. Effects of Hyp on cytotoxicity of the sequential combination 5-CH_3-H_4PteGlu → FUra. CCRF-CEM cells at a density of about 2×10^5/ml were first exposed to 5-CH_3-H_4PteGlu (10 μM) (●) or no drug (○) for 4 hr, in the absence or in the presence of Hyp (10 μM) (Panel A. and B., respectively). After washing in drug-free medium, cells were exposed at the same density to FUra (100 μM) for 4 hr, following either no treatment (△) or 5-CH_3-H_4PteGlu (■). After the final wash, cells were treated as described in the legend to Fig. 1. Results represent mean values of duplicate determinations in a single representative experiment.

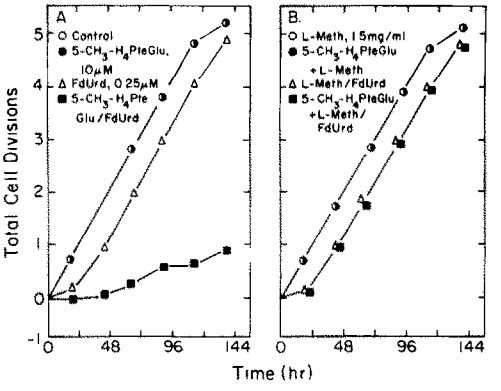

Fig. 6. Effects of L-methionine protection on cytotoxicity of the sequential combination 5-CH_3-H_4PteGlu-FdUrd. CCRF-CEM cells at a density of about 2×10^5/ml were exposed to 5-CH_3-H_4PteGlu (10 μM) (●) or no drug (○) for 4 hr, in the absence or in the presence of L-methionine (1500 mg/l) (Panel A. and B., respectively) which was administered 4 hr earlier. During the last 2 hr FdUrd (0.25 μM) was added to culture medium following either no treatment (△) or 5-CH_3-H_4PteGlu (■). After treatment, cells were washed and treated as described in the legend to Fig. 1. Results represent mean values of duplicate determinations in a single representative experiment.

Hypoxanthine (Hyp), a preformed purine base which requires phosphoribosylpyrophosphate (PRPP) as the phosphoribose donor for nucleotide formation, given for 4 hr at concentrations varying from 1 to 100 μM concomitantly with 5-CH_3-H_4PteGlu (10 μM) did not protect CCRF-CEM cells from the cytotoxicity of subsequently-administered FUra (4 hr, 100 μM) (Fig. 5).

L-Methionine, added to the culture medium at a concentration of 1500 mg/l at 4 hr before, and then during, drug exposure, substantially protected cells from the cytotoxicity of sequential 5-CH_3-H_4PteGlu (4 hr, 10 μM) and FdUrd (0.25 μM, last 2 hr), without affecting cytotoxicity of FdUrd alone (Fig. 6).

DISCUSSION

Previous laboratory studies have demonstrated that responsiveness of human tumors to fluoropyrimidines, FUra and FdUrd, is limited by the availability of the intracellular folate cofactor, 5,10-CH_2-H_4PteGlu necessary for covalent binding between dTMP synthase and FdUMP and that inhibition of dTMP synthesis can be maximized by the use of an exogenous source of folate cofactors, such as LV [5, 14–18]. Initial clinical studies using high doses of LV and FUra, initiated on the basis of these laboratory data, obtained encouraging response rates in advanced gastrointestinal tumors, about double those expected from FUra treatment alone (50 and 37% response rates in previously untreated gastric and colorectal cancer patients, respectively) [24–29].

When LV is administered either orally or parenterally, rapid conversion to 5-CH_3-H_4PteGlu occurs [19–21] and the blood level of this compound exceeds that of physiological (*l*)-diastereoisomer of LV within 2 hr of drug administration [19]. Because of these pharmacokinetic patterns, the interactions of 5-CH_3-H_4PteGlu and fluoropyrimidines at the cellular level become of critical importance in view of the clinical application of folate–fluoropyrimidine combinations.

Intracellularly, 5-CH_3-H_4PteGlu undergoes conversion to tetrahydrofolate via methionine synthetase, in the presence of homocysteine and vitamin B_{12}, and then to 5,10-CH_2-H_4PteGlu [30]. Thus the possibility exists that this folate coenzyme might modulate the action of fluoropyrimidines at the dTMP synthase level or at levels of other folate-dependent enzymes.

Pretreatment of short duration (2 hr) with 5-CH_3-H_4PteGlu over a large range of concentrations (1–100 μM), enhanced the inhibitory effects of both FUra and FdUrd administered for the following 2 hr at approximately equitoxic concentrations (250 and 0.5 μM, respectively) on CCRF-CEM cell growth (Figs 1 and 2). Our data clearly demonstrate the direct dependence of the synergism of the dose of the folate used, in that the maximum effect occurred at 100 μM 5-CH_3-H_4PteGlu and decreased with decreasing 5-CH_3-H_4PteGlu concentrations (100–1 μM), until disappearance at 0.1 μM.

Also, the degree of synergism was higher for 5-CH_3-H_4PteGlu-FdUrd combinations than for 5-CH_3-H_4PteGlu-FUra combinations.

Synergistic inhibitory effects on cell growth were observed when 5-CH_3-H_4PteGlu (10 μM, 4 hr) preceded FUra (100 μM, 4 hr) or FdUrd (0.5 μM, 4 hr) administration (5-CH_3-H_4PteGlu → FUra, 5-CH_3-H_4PteGlu → FdUrd), or when the folate and one of the fluoropyrimidines were given simultaneously (5 - CH_3 - H_4PteGlu + FUra, 5 - CH_3 - H_4PteGlu + FdUrd, 4 hr exposure) (Fig. 3A and B). A slightly lower degree of synergism was noted when FUra was given prior to 5-CH_3-H_4PteGlu (FUra → 5-CH_3-H_4PteGlu, Fig. 3A), while no synergism occurred when FdUrd was substituted for FUra in this reverse sequence schedule (FdUrd → 5-CH_3-H_4PteGlu, Fig. 3B).

These differences in the degree and sequence-dependence of synergism might be explained by the different rate of conversion of the two fluoropyrimidines to the common active metabolite, FdUMP. In CCRF-CEM cells, in fact, FdUrd will presumably reach higher FdUMP levels in a shorter time as compared to FUra. As described by Piper and Fox [31] these cells lack uridine and thymidine phosphorylases. Thus, FdUrd is entirely activated to FdUMP via thymidine kinase, while FUra is only partially converted to the fluorodeoxynucleotide via a multienzymatic pathway including orotate phosphoribosyl transferase and ribonucleotide reductase, which presumably will require longer time.

Exposure after drug treatment to dThd, the pyrimidine nucleoside that circumvents the block of dTMP synthesis induced by FdUMP, subtantially rescued CCRF-CEM cells from cytotoxic effects of the combination of 5-CH_3-H_4PteGlu-FUra (Fig. 4), at a concentration (0.1 μM) that did not rescue those of FUra alone. These data suggest that the prolonged inhibition of dTMP synthase by FdUMP might be

the primary event responsible for the enhancement of FUra cytotoxicity by 5-CH_3-H_4PteGlu.

L-methionine is the amino acid derived from the B_{12}-dependent methylation of homocysteine by methionine synthetase. In this reaction, 5-CH_3-H_4PteGlu acts as the methyl group donor. This enzymatic step is limiting for the conversion of 5-CH_3-H_4PteGlu into the intracellular folate coenzyme pools. L-methionine (1500 mg/l) protected CCRF-CEM cells from the cytotoxicity of the sequential combination 5-CH_3-H_4PteGlu-FdUrd (Fig. 6), indicating that conversion of this folate to tetrahydrofolate via methionine synthetase is critical to synergism.

It is generally accepted that (*d*)-5-CH_3-H_4PteGlu is devoid of activity as a folate coenzyme. The biochemical and biological effects on this unnatural isomer, however, are still unknown. It has previously been shown that (*d*)-5-CH_3-H_4PteGlu is able to utilize the specific membrane carrier system for reduced folates and 2,4-diamino analogs as effectively as the natural (*l*) isomer [32]. Thus it was possible that (*d*)-5-CH_3-H_4PteGlu might have also efficiently competed for uptake with (*l*)-5-CH_3-H_4PteGlu in the human leukemia cell line studied. As a consequence of this event a potential effect of the unnatural derivative (*d*) as an inhibitor of folate-dependent enzymes would have been of importance, also in regard to the interactions with fluoropyrimidines. (*d*)-5-CH_3-H_4PteGlu might, for instance, enhance FUra metabolism via orotate phosphoribosyl transferase by increasing PRPP intracellular levels as a consequence of inhibition of folate-dependent enzymes in the purine pathway. To verify this possible interaction, we exposed cells, concomitantly with 5-CH_3-H_4PteGlu, to Hyp, a preformed purine base which competes with FUra for the common co-substrate for nucleotide formation, the phosphoribose donor, PRPP [33–35]. This would have prevented potentiation of subsequently-administered FUra. We were unable to show protection of 5-CH_3-H_4PteGlu-FUra cytotoxicity by Hyp at concentrations up to 100 μM (Fig. 5).

These data support the hypothesis that 5-CH_3-H_4PteGlu-fluoropyrimidine synergism is a consequence of enhanced covalent binding between dTMP synthase and FdUMP [6, 16, 18] and of decreased dissociation of this complex [15–19] in the presence of increased levels of the folate coenzyme, 5,10-CH_2-H_4PteGlu generated intracellularly from 5-CH_3-H_4PteGlu. They also encourage clinical investigations of combinations of folate (LV or 5-CH_3-H_4PteGlu) and fluoropyrimidines in leukemia and lymphoma patients.

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