JPET Fast Forward. Published on June 1, 2018 as DOI: 10.1124/jpet.118.249367
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JPET#249367

Enhancement of 5-Fluorouracil cytotoxicity by Pyridoxal 5'-phosphate and Folinic acid in tandem.

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Running title: Potentiation of 5-fluorouracil by vitamin B6 and leucovorin

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Number of pages including Title and Running title pages, Abstract, main text, and Footnote page: 12
Number of tables: 1
Number of figures: 4
Number of references: 31
Number of words in Abstract: 248
Number of words in Introduction: 690 including reference citations
Number of words in Discussion: 644 including reference citations

Abbreviations used in the text: CI, Combination index; F_a, fraction of cells affected; IC_{50}, fifty percent inhibitory concentration; FUra, 5-fluorouracil; FdUMP, fluorodeoxyuridine monophosphate; K_D, dissociation constant; PM, pyridoxamine; PN, pyridoxine; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; H_4PteGlu, tetrahydrofolate; CH_2-H_4PteGlu, 5,10-methylenetetrahydrofolate; CH_3-H_4PteGlu, 5-methyltetrahydrofolate; FA, 5-HCO-H_4PteGlu, folinic acid, leucovorin; SHMT, Serine hydroxymethyltransferase; TS, Thymidylate synthase.

Recommended section assignment: Chemotherapy, Antibiotics, and Gene Therapy

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Abstract

The present study originates from the assumption that, in tumors, levels of naturally occurring pyridoxal 5'-phosphate (PLP) are too small to allow conversion of tetra hydro pteroylglutamate (H_4PteGlu) into methylene tetra hydro pteroylglutamate (CH_2-H_4PteGlu) in amounts required to improve inhibition of thymidylate synthase by 5-fluorouracil (FUra) through ternary complex stabilization. The hypothesis relates to the low affinity for cofactor of the PLP-dependent serine hydroxymethyl transferase (SHMT), the enzyme that catalyses formation CH_2-H_4PteGlu by transfer of the Cβ of serine to H_4PteGlu. Intracellular concentrations of PLP are smaller than the dissociation constant (K_D) of SHMT for cofactor, which suggests that enzyme activity should be sensitive to PLP level changes. Three cancer cell lines were supplemented with PLP to investigate the influence of this cofactor on FUra cytotoxicity. Cells were exposed to FUra, FUra and folinic acid (FA), FUra and PLP, and FUra combined with both FA and PLP. The Median effect principle for concentration-effect analysis and combination indices were used to determine interactions on cytotoxicity. FUra cytotoxicity *in vitro* was enhanced by FA and PLP in tandem. Synergistic cytotoxic interaction of FUra with FA and PLP was demonstrated in HT29, and L1210 cells. Summation was found in HCT116 cells. Parenteral pyridoxamine was administered in mice to explore erythrocyte production of PLP *in vivo*. Cofactor attained levels in the range of the K_D for binding to SHMT and it was rapidly cleared from cells. Pharmacokinetics of pyridoxamine suggests that modulation of FUra by vitamin B6 could be achieved *in vivo*.

Introduction

Fluorodeoxyuridine monophosphate (FdUMP), the active metabolite of 5-fluorouracil (FUra), binds to thymidylate synthase (TS) and the cofactor N5-N10 methylene tetra hydro pteroylglutamate (CH_2-H_4PteGlu) to form a ternary complex [FdUMP-TS-CH_2-H_4PteGlu] with concomitant inactivation of the TS (Santi et al., 1974; Danenberg and Danenberg, 1978; Lockshin and Danenberg, 1981). Stability of the complex increases as CH_2-H_4PteGlu level is augmented over a wide concentration range up to levels greater than 450 μM. Supplementation of cancer cell lines exposed to FUra or fluorodeoxyuridine with high concentration N5-formyl tetra hydro pteroylglutamate (5-HCO-H_4PteGlu; folinic acid; leucovorin) *in vitro* results in greater formation of ternary complex than with fluoropyrimidines as single agents, leading to potentiation of the cytotoxic effect (Ullman et al., 1978).

These findings led to the design of regimens of FUra combined with folinic acid for patients with gastrointestinal carcinomas that possess greater antitumor efficacy than FUra as a single agent (Machover et al., 1982; Piedbois et al., 1992). Further changes in amounts of folates or use of the pure [6S]-folinic acid instead of the [6R,S] mixture of enantiomers (Machover et al., 1992) did not convincingly improve antitumor effect.

Effectiveness of the biochemical modulation of the fluoropyrimidines by folates varies among cancer cells. Variation is thought to be due to differences in capacities for polyglutamation (Romanini et al., 1991), and for expansion of CH_2-H_4PteGlu pools. From results of prior reports, it is unlikely that supplementation of cancer cells with any amount of folate would result in rise of CH_2-H_4PteGlu to concentrations required to increase the tightness of FdUMP binding to TS for maximum stability of the ternary complex. In most of these studies when cancer cells were exposed to folates in great amounts, only limited increase of CH_2-H_4PteGlu concentration occurred, followed by rapid decline after discontinuation of folate exposure (Romanini et al., 1991; Houghton et al., 1990; Priest et al., 1993; Voeller and Allegra, 1994; Wright et al., 1989; Boarman and Allegra, 1992; Zhang and Rustum, 1991; Machover et al., 2001).

One possible explanation for these findings is the rapid turnover of folates in cancer cells resulting from interconversion of folate cofactors (Nixon et al., 1973), including the irreversible reduction of CH_2-H_4PteGlu to N5-methyl tetra hydro pteroylglutamate (CH_3-H_4PteGlu) (Figure 1). Poor expansion of CH_2-H_4PteGlu pools in cancer cells may also result from insufficient production of this folate. Synthesis of CH_2-H_4PteGlu from H_4PteGlu results from two pathways. One is the transfer of Cβ of serine to H_4PteGlu with formation of glycine and CH_2-H_4PteGlu catalysed by serine hydroxymethyl transferase (SHMT), a ubiquitous pyridoxal 5'-phosphate (PLP)-dependent enzyme which includes the cytoplasmic SHMT1, and the mitochondrial SHMT2 isoforms (Florio et al., 2011; Ueland et al., 2015). The second pathway is the Glycine cleavage system that catalyzes glycine cleavage up to formation of CH_2-H_4PteGlu in mitochondria (Ueland et al., 2015; Kikuchi et al., 2008).

The rationale for the present hypothesis lies in the low affinity for binding of SHMT apoenzyme to cofactor. PLP was found to bind purified bovine liver SHMT1 with dissociation constant (K_D) as high as 27 μM (Jones III and Priest, 1978), and it was reported to bind rabbit liver SHMT with a K_D of 700 nM (Schirch et al., 1973). Human recombinant SHMT1 bound to cofactor with a K_D of 850 nM in one study (Perry et al, 2007), and in another study human recombinant SHMT1 and SHMT2 bound to cofactor with K_Ds of 250 nM, and 440 nM, respectively (Giardina et al., 2015). By contrast, levels of naturally occurring PLP in human erythrocytes under basal conditions are as small as 30-100 nmol/L of 100% packed red cells (Zempleni and Kübler, 1994). Although mechanisms of intracellular cofactor supply to PLP-dependent enzymes are only partially known (Di Salvo et al., 2011), these features indicate that SHMT activity should be sensitive to PLP concentration changes.

We hypothesized that supplementation of cancer cells with high amounts of PLP would facilitate production of CH_2-H_4PteGlu and modulate FUra through formation and stabilization of the ternary complex resulting in augmentation of the cytotoxic effect. To test the hypothesis, experiments were conducted in three cancer cell line models *in vitro* to investigate for interactions between FUra, folinic acid and PLP on cell growth.

Materials and Methods

Cell lines and cytotoxicity studies *in vitro*

The human colorectal carcinoma cell lines HT29, and HCT116 and the murine L1210 lymphocytic leukemia were obtained from American Type Culture Collection (ATCC). These cell lines were chosen for their sensitivity to the cytotoxic action of the fluoropyrimidines and for their use as models for preclinical experiments *in vivo*. Cells were thawed from mycoplasma-free frozen stocks and were controlled for contamination. The three cell lines were grown in customized DMEM cell culture medium without any B6-vitamer (Gibco; Life Technologies) supplemented with 10% FBS and antibiotics (streptomycin, 50 μg/ml, and penicillin, 50 Unit/ml) at 37°C in an atmosphere with 5% CO_2. We analyzed HT29 cells growing in customized medium without vitamin B6 and did not observe any impact on growth after as much as five successive passages of 96-hour cultures. We also verified that neither folinic acid nor PLP as single agents and in combination carry cytotoxic properties *per se* in the concentrations used in the present study.

Cells were exposed in 12 well-cell plates to FUra in various concentrations under four conditions; either as a single agent [FUra]; in combination with [6R,S]-folinic acid [FUra-FA]; in combination with PLP (Sigma-Aldrich) [FUra-PLP]; and with both [6R,S]-folinic acid, and PLP combined [FUra-FA-PLP]. Cells were exposed to PLP at the single concentration of 160 μM, and to folinic acid at 20 μM, a concentration reported to be required for maximum potentiation of the fluoropyrimidines *in vitro* (Ullman et al., 1978). Cells were harvested 72 hours from start of exposure. Cell viability was measured with the Trypan Blue dye exclusion

test in Malassez chambers and by flow cytometry. For the latter method, living cells defined by light double scatter were counted in a BD Accuri C6 flow cytometer (BD Biosciences). Experiments were done in duplicate. Control cells for each experimental condition were that grown in the absence of FUra, either with FA or PLP as single agents, with FA and PLP in combination, or with none of these.

Analysis of drug interaction on cytotoxicity. Statistical methods

Cell growth inhibition data obtained with [FUra], [FUra-FA], [FUra-PLP], and [FUra-FA-PLP] in the three cancer cell lines were analyzed according to the mass-action law-based Median-effect principle for concentration-effect analysis. The combination index (CI) proposed by Chou and Talalay was used for determination of synergism, additive effect and antagonism (Chou and Talalay, 1984). The Median-effect equation correlates the dose (*i.e.*, the concentration) of the drug and the effect produced in the form $f_a/f_u = (D/D_m)^m$, where D is the dose of drug; D_m is the median-effect dose signifying potency (the IC_{50}); f_a is the fraction of cells affected by the dose; f_u is the fraction of cells unaffected (f_u=1-f_a); and m is a coefficient for shape of the dose-effect curve (sigmoidicity). The conformity of experimental data to the Median-effect principle is represented by the linear correlation coefficient r of the median-effect plot. The Combination index equation $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$ was used for studies of cytotoxic interaction between drugs. The CI equation dictates that dose of Drug 1 $(D)_1$, and dose of Drug 2 $(D)_2$ in combination inhibit cell growth by x%. $(Dx)_1$ and $(Dx)_2$ are the doses of Drug 1, and of Drug 2 alone that also inhibit x%. $(Dx)_1$ and $(Dx)_2$ are calculated from the Median-effect equation, where D is designated for x% growth inhibition. Values for CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism between drugs, respectively.

The experimental schema for studies of drug interaction included [FUra-FA] as Drug1, [FUra-PLP] as Drug 2, and the combination [FUra-FA-PLP]. Combination indices were calculated with the CI equation from the effect on cell growth produced by drugs [FUra-FA], and [FUra-PLP] used alone, and by the combination [FUra-FA-PLP] at 1:1 constant concentration ratio assuming, for simplicity in data analysis, that the bio modulators (*i.e.*, folinic acid, and PLP) affect FUra cytotoxicity through mutually exclusive mechanisms (*i.e.*, that have similar modes of action). For representation of synergism, summation and antagonism, the combination indices for [FUra-FA-PLP] were plotted as CI with respect to percent of cells inhibited (f_a).

Median-effect parameters, median-effect and dose-effect plots, combination indices, CI-f_a plots, and calculations of error were obtained with the CalcuSyn v2 Software (Biosoft, Cambridge, UK). Dose-effect standard error was calculated assuming that D_m has lognormal distribution. Estimated 1.96 SD for combination indices was calculated using a Monte-Carlo simulation (Belen'kii and Schinazi, 1994).

Statistical analysis of cytotoxicity was done by two-way Fisher analysis of variance (ANOVA) for cell effect in regard to factors defined as (a) concentration of FUra, and (b) experimental condition (*i.e.*, [FUra], [FUra-FA], [FUra-PLP], and [FUra-FA-PLP]) in pooled data from the three cancer cell lines. Normal

distribution of data was determined with the Kolmogorov-Smirnov normality test. The effect on cell growth (f_a) between the four experimental conditions was tested according to the various concentrations of FUra. Concentration of FUra and experimental condition were tested for interaction. In absence of interaction found between factors (described below), one way ANOVA defined by experimental condition was performed, followed by post-hoc tests (Tukey) with multi-comparisons between experimental conditions two by two. Subsequent univariate analysis and post-hoc tests were performed for each cell line separately. Statistical analysis was done with the R software, v3.2.3.

Intracellular conversion of B6 vitamers *in vivo*

Vitamin B6 is the generic name that encompasses six inter convertible compounds (*i.e.*, B6 vitamers), namely pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), and their respective 5'-phosphorylated forms, PNP, PMP and the cofactor PLP. Erythrocyte pharmacokinetics of B6 vitamers was studied in mice after parenteral PM in high doses to explore the physiologic limits of cells to synthesize and accumulate PLP *in vivo* in search for attaining intracellular levels of PLP in the range of K_D values for binding of cofactor to apo SHMT or greater. We measured mouse erythrocyte levels of PMP, PL, and PLP resulting from conversion of parenteral PM. Female Balb/C mice aged 6 weeks, caged and fed under standard conditions were given intraperitoneal PM (Sigma-Aldrich) either at 150 mg/kg or at 450 mg/kg at time 0 only (*t*0), or twice at time 0 and after 12 hours from start (*i.e.*, at times *t*0 and at *t*12h). For each PM dose explored, groups of two mice each subjected to one injection of PM at *t*0 were sacrificed and sampled after 1, 3, 6, and 12 hours from start, and two animals that received 2 injections of PM were sampled 12 hours after the 2nd injection (*i.e.*, 24 hours after start of the experiment). Blood was collected in heparin. Measurements of B6 vitamers were done by HPLC (Bisp et al., 2002).

Results

Cytotoxicity of FUra was increased by folinic acid and PLP

Dose-effect data (Figure 2) including 50% inhibitory concentrations (IC_{50}s) changes in each experimental condition (Table 1) indicate that, in the three cell lines studied, FUra cytotoxicity was enhanced by FA, and by PLP, with the strongest effect resulting from the combination of FUra with both PLP and FA. IC_{50} values obtained in each of the three cell lines with FUra combined with each bio modulator individually were of same magnitude, approximately half of that with FUra as a single agent. IC_{50} values of FUra in combination with PLP and FA in tandem were approximately 8-fold smaller than that of FUra as a single agent in HT29 and L1210 cells, and was approximately 4-fold smaller than that with FUra as a single agent in HCT116 cells.

Two-way Fisher ANOVA identified a significant difference in cell effect between the four experimental conditions ($p = 3.73.10^{-11}$). The test also found that the concentration of FUra affect significantly cell growth

($p < 2.2.10^{-16}$). Lastly, no significant interaction was found between the two factors tested (*i.e.*, concentration of FUra, and experimental condition) throughout the entire FUra concentration range from 0.01 μM to 3 μM ($p = 0.2929$). Post-hoc tests (Tukey) comparing experimental conditions two by two in pooled data from the three cancer cell lines, found a statistically significant difference ($p < 0.001$) in comparisons of [FUra-FA-PLP] *vs* [FUra]. Tests did not reach significant differences in other comparisons between experimental conditions. Univariate Fisher ANOVA and Tukey post-hoc tests performed in each cancer cell line separately found significant differences in cell effect in comparisons of [FUra-FA-PLP] *vs* [FUra] in HT29 ($p = 0.002$), HCT116 ($p = 0.05$), and L1210 ($p <0.001$) cancer cells. As for tests performed with pooled data, no significant differences were found in other comparisons two by two between experimental conditions.

Combination Indices for [FUra-FA-PLP] were of synergistic significance (<1) for fractions of affected cells (F_a) up to 0.70 in HT29 cells, and up to 0.80 in L1210 cells (Table 1 and Figure 3). Below these fractional effect limits, CI simulations followed a continuous trend for synergy of the effect of combined FA and PLP in tandem on FUra cytotoxicity. For HCT116 cells, CI values were scattered. Only a small number of experimental values were of synergistic significance, while the majority was above 1. In HCT116 cells, CI simulations followed a trend for summation of the effect of FA and PLP combined on FUra cytotoxicity within a fractional effect range of approximately 0.40 to 0.60 with values above 1 outside these limits.

Parenteral pyridoxamine resulted in high erythrocyte PLP levels

Basal PLP concentration in erythrocytes was 31±7 nmol/L of 100% packed red cells (Figure 4). Parenteral PM resulted in rapid increase of intracellular PLP attaining a maximum concentration between 1 hour and 3 hours after injection. Mean peak erythrocyte PLP concentrations were 382 nmol/L of 100% packed red cells in mice that received PM at 150 mg/kg, and 2,326 nmol/L of 100% packed red cells in mice that received PM at 450 mg/kg. PLP levels rapidly decreased after the 3rd hour, approaching baseline concentrations by 12 hours after injection. This observation was similar in animals that received two injections of PM (*i.e.*, at times *t*0, and *t*12h), indicating that cellular clearance of newly synthesized PLP is rapid and that cofactor is not appreciably accumulated in cells when injections are repeated at 12-hour interval. Metabolic conversion of PM also resulted in production of large amounts of PL, and PMP. No signs of acute toxicity were observed during the time of the study in mice receiving either one or two injections of PM at any of the two doses administered.

Discussion

Concentration-effect data studied according to the Median effect principle indicate enhancement of the cytotoxic action of FUra by high-concentration folinic acid. Similarly, data suggest increase of FUra cytotoxicity by PLP. The strongest growth inhibiting effect resulted from the combination of FUra, FA and PLP in the three cancer cell lines studied. Statistical analysis of cell effect data confirms that the combination

of FUra, folinic acid and pyridoxal 5'-phosphate in tandem produces significantly greater cytotoxicity than FUra as a single agent, which supports enhancement of the action of FUra by the two modulators in combination. In contrast with the concentration-effect data obtained according to the Median effect principle, statistical analysis could not significantly differentiate FUra combined with one single modulator (*i.e.*, FA or PLP) from FUra as a single agent. Combination indices and CI-f_a plots indicate that FA and PLP interact synergistically on FUra cytotoxicity in HT29 and L1210 cells within a large cell effect range. In HCT116 cells the two bio modulators produce summation of their effects on FUra cytotoxicity over a narrow fractional effect range.

Effects of B6 vitamers on FUra cytotoxicity have not been reported previously. The present data fit with the assumption of cellular changes produced by the bio modulators that contribute synergistically for improvement of FUra cytotoxic effect in two of the three cancer cell lines studied. From our results and the rationale presented we hypothesize that folinic acid in combination with vitamin B6 facilitate expansion of CH_2-H_4PteGlu pool in cancer cells leading, in the presence of FdUMP, to increased ternary complex formation and stabilization with concomitant inhibition of the TS. However, the mechanisms of the interaction remain to be determined.

Evidence for folate-mediated one-carbon metabolism changes through SHMT related to vitamin B6 availability was previously reported. One study has shown that vitamin B6 deficiency in rat reduced PLP levels and SHMT activity in liver, together with decreased remethylation of homocysteine in methionine with serine-derived methyl groups, *i.e.*, generated through SHMT-catalyzed synthesis of CH_2-H_4PteGlu (Martínez et al., 2000). In another study, investigators found that vitamin B6 restriction decreased cellular PLP levels and SHMT activity in MCF-7 human mammary carcinoma cells *in vitro*, and was accompanied by decrease in levels of S-adenosyl-methionine. Addition of PLP increased SHMT activity up to 4.2-fold in extracts of MCF-7 cells grown in vitamin B6 deficient medium, which reflects the fraction of the SHMT pool that was present as inactive apoenzyme (Perry et al., 2007). These findings strongly support the present hypothesis of expansion of methylenetetrahydrofolate pools due to increase in SHMT activity by supplying PLP in cancer cells.

The pharmacokinetic study presented herein demonstrates that erythrocytes rapidly metabolize pyridoxamine *in vivo*, resulting in high intracellular levels of PLP. Erythrocyte PLP attains levels close to or greater than most K_D values reported for binding of cofactor to SHMT, which suggests that activity of the enzyme may be increased by intracellular PLP expansion within cancer cells *in vivo*. However, decline of intracellular PLP to basal levels occurs in less than 12 hours from injection. The mechanisms underlying the absence of PLP accumulation within cells have not been elucidated. Our findings in mice are in accordance with that reported on intracellular pharmacokinetics of PMP, PL, and PLP after parenteral administration of PN in humans (Ueland et al., 2015; Zempleni and Kübler, 1994).

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Effective uptake of PLP by erythrocytes *in vitro* was previously demonstrated (Suzue and Tashibana, 1970; Maeda et al., 1976). However, intracellular cofactor levels in cancer cells under exposure to high concentration PLP have not yet been determined. Correlating levels of PLP required for enhancement of FUra-dependent cytotoxicity in cancer cells *in vitro* to that attained in erythrocytes after parenteral pyridoxamine may help finding schemas for preclinical studies.

Further exploration of the bio modulation of fluoropyrimidines by folinic acid and vitamin B6 in tandem should be considered for improvement of the cytotoxic effect in cancer cells.

Acknowledgments: The authors thank Philippe Machover for statistical support.

Authorship Contributions

Participated in research design: Machover, Goldschmidt, and Gaston-Mathé

Conducted experiments: Machover, Mollicone, Haghighi-Rad, and Dairou

Contributed new reagents or analytic tools: Machover, Mollicone, Haghighi-Rad, Gaston-Mathé, and Dairou

Performed data analysis: Machover, Goldschmidt, Desterke and Dairou

Wrote or contributed to the writing of the manuscript: Machover, Goldschmidt, Desterke, Saffroy, and Boucheix.

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Footnotes

Support: The present work was supported by the Institut du Cancer et d'Immunogénétique (ICIG), Hospital Paul-Brousse, 94800 Villejuif, France.

Authorizations: Murine experiments were performed under authorization No DR2015-09 delivered by CEEE-55 from the Ethics Committee for animal experimentation at University Claude-Bernard Lyons 1.

Legends for figures

Figure 1. Selected biochemical pathways of folates. H_2PteGlu: 7,8-dihydrofolate; H_4PteGlu: tetrahydrofolate; CH_2-H_4PteGlu: 5,10-methylenetetrahydrofolate; CH_3-H_4PteGlu: 5-methyltetrahydrofolate; CH^+-H_4PteGlu: 5,10-methenyltetrahydrofolate; 10-HCO-H_4PteGlu: 10-formyltetrahydrofolate; CHNH-H_4PteGlu: 5-formiminotetrahydrofolate; 5-HCO-H_4PteGlu: [6S]-5-formyltetrahydrofolate (folinic acid; leucovorin). **1**, Thymidylate synthase; **2**, Dihydrofolate reductase; **3**, PLP-dependent Serine hydroxymethyltransferase (including the cytoplasmic SHMT1 and the mitochondrial SHMT2 isoforms); **4**, Glycine cleavage system (including the carrier H-Protein bound to the mitochondrial inner membrane, and the enzymes P-protein, a PLP-dependent glycine dehydrogenase, T-protein, an amino methyltransferase, and L-protein, a dihydro lipoamide dehydrogenase); **5**, Methionine synthase; **6**, Methylenetetrahydrofolate reductase; **7**, Methylenetetrahydrofolate dehydrogenase; **8**, Methenyltetrahydrofolate cyclohydrolase; **9**, Phosphoribosylglycinamide formyltransferase; **10**, Aminoimidazole carboxamide ribonucleotide formyltransferase; **11**, Formiminotransferase cyclodeaminase; **12**, Formiminotetrahydrofolate cyclodeaminase; **13**, Formate tetrahydrofolate ligase;**14**, 5-Formyltetrahydrofolate cycloligase; **15**, activated methyl transfer enzymes; **16**, Betaine-homocysteine methyltransferase; **17**, Adenosylmethionine decarboxylase; PLP-dependent **18**, Cystathionine β-synthase, and **19**, Cystathionine γ-lyase. Met: L-methionine; HCy: L-homocysteine; AdoMet: S-adenosyl-L-methionine; AdoHCy: S-adenosyl-L-homocysteine.

Figure 2. Dose-effect plots of FUra as a single agent [FUra], FUra with folinic acid [FUra-FA], FUra with PLP [FUra-PLP], and FUra with both folinic acid and PLP combined [FUra-FA-PLP] in the human colorectal carcinoma cell lines HT29 and HCT116, and in the murine lymphocytic leukemia L1210. Symbols represent experimental points obtained for each condition. Plots were obtained with the CalcuSyn v2 software (Biosoft).

Figure 3. Combination index (CI) with respect to fraction of cells affected (f_a) plots in the human colorectal carcinoma cell lines HT29, and HCT116, and in the murine lymphocytic leukemia L1210. Solid squares represent experimental values of CI calculated for the combination of FUra, folinic acid and pyridoxal 5'-phosphate in tandem producing a given effect. Continuous line represents simulation of the CI with respect to f_a, and vertical bars represent ± 1.96 SD. Values for CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism between drugs, respectively. The experimental schema is detailed under Materials and Methods section. Plots and calculations of error were obtained with the CalcuSyn v2 software (Biosoft).

Figure 4. Mouse erythrocyte levels of PMP, PL, and PLP after intra peritoneal pyridoxamine (PM). Balb/C mice were given PM at 150 mg/kg or at 450 mg/kg at time 0 only, or twice at time 0 and after 12 hours from start. For each PM dose explored, measurements were done after 1, 3, 6, 12, and 24 hours from start of the experiment. Vertical bars indicate SE.

TABLE 1. Median-effect parameters and Combination indices of FUra as a single agent and in combination with folinic acid and pyridoxal 5'-phosphate in HT29, HCT116, and L1210 cancer cell lines.

Cell Line[1]	Drug[2]	Parameter[3]			Combination Index value (1.96 SD) at fractional effect:		
		m	r	D_m (IC_{50}) and 95% CI in μM	IC_{25}	IC_{50}	IC_{75}
HT29	FUra	1.236	0.949	1.18 (0.76-1.82)	-	-	-
	FUra-FA	1.082	0.994	0.64 (0.55-0.74)	-	-	-
	FUra-PLP	0.883	0.960	0.66 (0.44-0.99)	-	-	-
	FUra-FA-PLP	0.539	0.989	0.14 (0.10-0.19)	0.18 (0.03)	0.43 (0.06)	1.08 (0.16)
HCT116	FUra	1.23	0.921	1.31 (0.74-2.32)	-	-	-
	FUra-FA	1.21	0.965	0.76 (0.53-1.10)	-	-	-
	FUra-PLP	0.40	0.965	0.46 (0.30-0.71)	-	-	-
	FUra-FA-PLP	0.53	0.941	0.31 (0.16-0.58)	1.43 (0.6)	1.07 (0.24)	1.61 (0.56)
L1210	FUra	1.75	0.969	0.65 (0.40-1.06)	-	-	-
	FUra-FA	1.89	0.990	0.30 (0.20-0.44)	-	-	-
	FUra-PLP	1.635	0.991	0.28 (0.19-0.41)	-	-	-
	FUra-FA-PLP	1.15	0.995	0.08 (0.05-0.11)	-	0.56 (0.06)	0.77 (0.06)

[1]Cancer cells were the human colorectal carcinoma cell lines HT29 and HCT116, and the L1210 murine lymphocytic leukemia.

[2]Cells were grown in customized DMEM cell culture medium without any B6-vitamer supplemented with 10% FBS and were exposed for 72 hours to FUra as a single agent, FUra and folinic acid (FA, 20 μM), FUra and pyridoxal 5'-phosphate (PLP, 160 μM), and FUra with both FA (20 μM), and PLP (160 μM).

[3]Median-effect parameters are the median-effect dose (D_m); and a coefficient (m) for shape (sigmoidicity) of the dose-effect curve. The linear correlation coefficient of the median-effect plot (r) represents conformity of experimental data to the Median-effect principle. Median-effect parameters, combination indices and calculations of error were obtained with the CalcuSyn v2 Software (Biosoft).

Figure 1

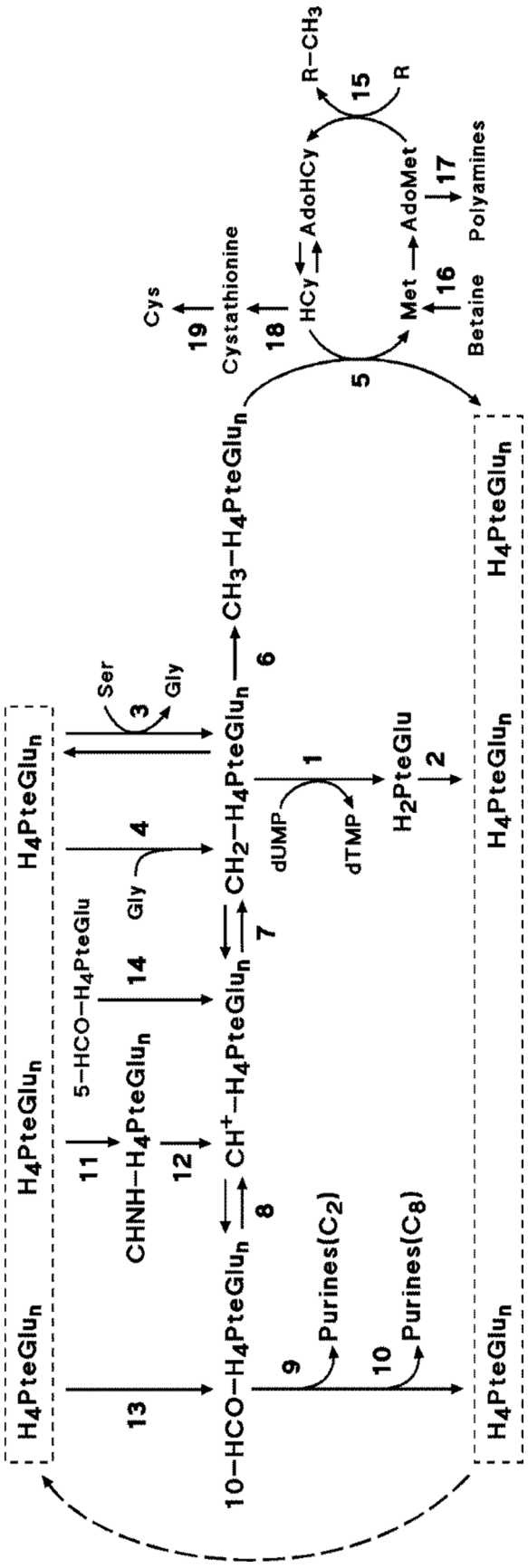

Figure 2

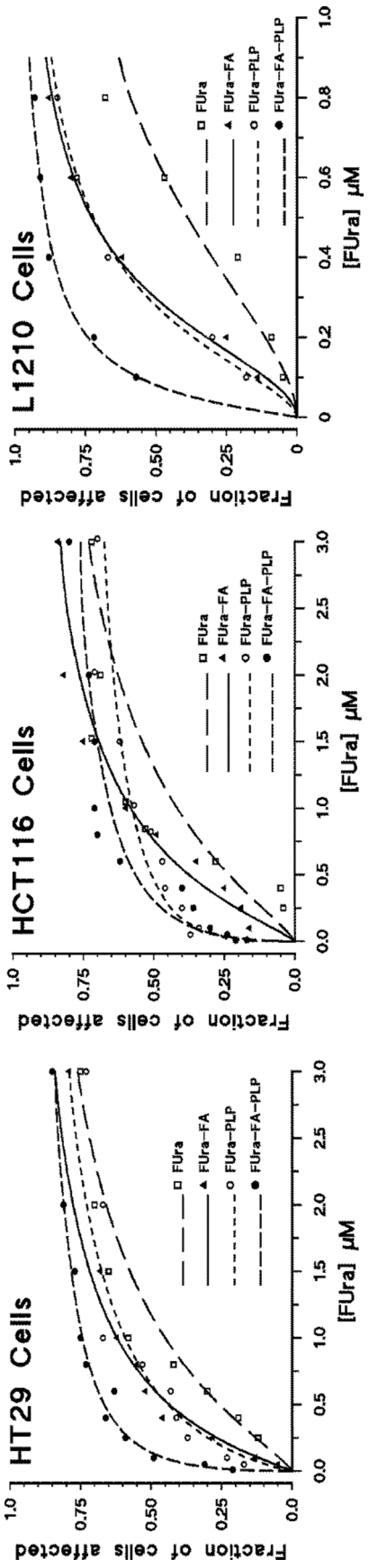

Figure 3

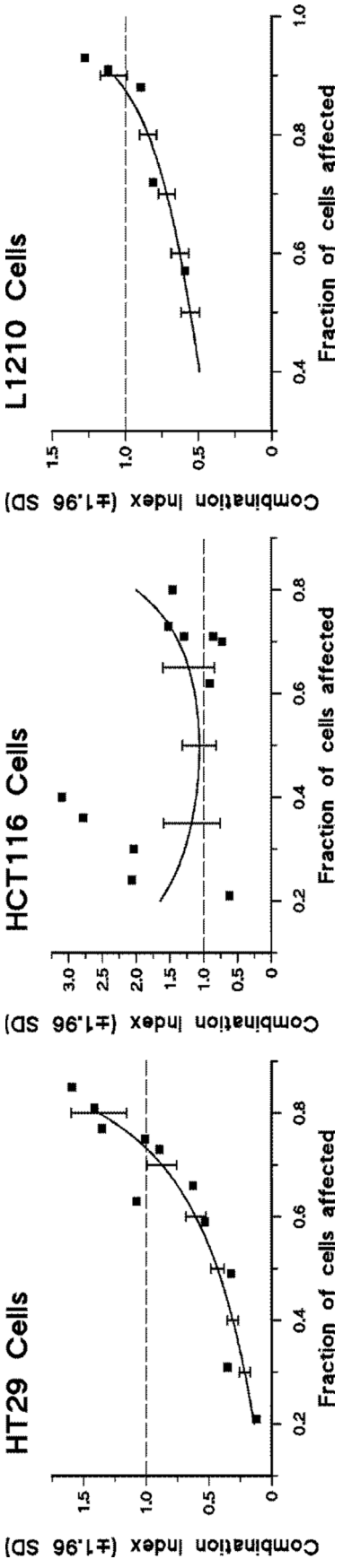

Figure 4