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COMBINATION OF REDUCED FOLATES WITH METHOTREXATE OR 5-FLUOROURACIL

COMPARISON BETWEEN 5-FORMYLTETRAHYDROFOLATE (FOLINIC ACID) AND 5-METHYLTETRAHYDROFOLATE *IN VITRO* ACTIVITIES

MARIE-CHRISTINE ETIENNE,* JEAN-LOUIS FISCHEL,* PATRICIA FORMENTO,* MAURICE SCHNEIDER,* THIERRY GUILLOT,† MIA BARDON† and GÉRARD MILANO*‡

*Centre Antoine-Lacassagne, 36 Voie Romaine, 06054 Nice Cedex, and †Lederle Laboratories, 74 Rue d'Arceuil, Silic 275, 94578 Rungis, France

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Abstract—Folinic acid (*dl* FA) is increasingly used in clinical oncology. The active isomer *l* FA is intensively metabolized into *l* 5-methyltetrahydrofolate (*l* 5MTHF), the relative proportions of *l* FA, *d* FA and *l* 5MTHF in blood varying considerably between oral and i.v. FA administration. The purpose of the study was to compare the *in vitro* activities of pure *l* FA and pure *l* 5MTHF at equivalent drug exposure [area under curve (AUC)], taking into account their respective chemical stability in the culture medium. The *in vitro* growth inhibition [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) test] was evaluated on five human tumor cell lines after methotrexate (MTX)-folate or 5-fluorouracil (5FU)-folate exposures. Not only were the activities of *l* FA and *l* 5MTHF compared, but also clinically relevant mixtures of *l* FA + *d* FA + *l* 5MTHF corresponding to the proportions found at steady state during oral (PO mixture, 4, 39 and 57%, respectively) and i.v. administrations (i.v. mixture, 7, 81 and 12%, respectively). Measurement of folates demonstrated the marked lability of *l* 5MTHF (65.8% loss over 5 days in the culture medium) as compared to *l* FA (2.6% loss). Whatever the pharmacological model tested (MTX-folate or 5FU-folate), comparison of the folate effects at equivalent drug exposure taking into account their relative stability showed that *l* 5MTHF was never more potent than *l* FA. Moreover, a higher efficiency of *l* FA was demonstrated for the cell line most sensitive to 5FU; in this case, as expected, the i.v. mixture was more potent than the PO mixture. This study shows that depending on the tumor, *l* FA can be more potent than its main circulating metabolite *l* 5MTHF. Along with the limited capacity of oral absorption, the choice between oral and i.v. route for FA administration in patients should take into consideration the different pharmacological activities between *l* FA and *l* 5MTHF which suggest that the oral route is potentially detrimental to the optimal activity of the 5FU–FA combination as compared to i.v. administration.

Folinic acid (FA,§ 5-CHO-H_4PteGlu) is increasingly used in clinical oncology. Along with its classical prescription as a rescue of high-dose methotrexate (MTX) therapy [1], FA has been demonstrated to potentiate the cytotoxic effects of another major antimetabolite, 5-fluorouracil (5FU). The molecular basis of the synergistic interaction between 5FU and FA has been thoroughly researched. FA increases the intracellular pool of 5,10-methylenetetrahydrofolate which stabilizes the complex formation between 5-fluorodeoxyuridine monophosphate (5-FdUMP) and thymidylate synthase [2, 3]. The results of recent clinical trials using the 5FU–FA combination have confirmed this pharmacological rationale, demonstrating a significant improvement of response in advanced colorectal cancer [4, 5], breast cancer [6] and head and neck cancer [7].

Until now, FA has been available as a racemic mixture (*dl* FA) containing 50% of each diastereoisomer. The biological activity of *dl* FA is supported by its natural *l* form (6*S* isomer) whereas the *d* form (6*R* isomer) is considered as inactive [8]. FA diastereoisomers exhibit different pharmacokinetic behaviors [9, 10], the plasma half-life of *l* FA being significantly shorter than that of *d* FA [4, 10]. This results from the rapid metabolism of *l* FA into the active co-factor *l* 5-methyltetrahydrofolate (*l* 5MTHF, 5-CH_3-H_4PteGlu) [11, 12], which is the major physiological plasma reduced folate, whereas *d* FA does not undergo metabolism [10]. FA is given to patients either by i.v. or oral route [13–15], this choice being dictated principally by practical reasons such as the ambulatory ability of the patient. A close look at FA pharmacokinetics reveals that at steady state, during continuous FA administration, the relative proportions of *l* FA, *d* FA and *l* 5MTHF in blood vary considerably between oral and i.v. administration [9, 10, 16]. Considering plasma concentrations measured at steady state (Css), *l* 5MTHF is the preponderant folate during repeated oral intakes [9], while *d* FA is the major folate during continuous venous infusion [16]. The high concentrations of *d* FA in blood (at least 10 times that of *l* FA) have stimulated basic pharmacological studies and the results obtained up to now [17, 18]

‡ Corresponding author.

§ Abbreviations: FA, folinic acid; 5MTHF, 5-methyltetrahydrofolic acid; 5FU, 5-fluorouracil; MTX, methotrexate; 5-FdUMP, 5-fluorodeoxyuridine monophosphate, DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; AUC, area under curve; CF, correction factor.

including our group [19] have failed to demonstrate that the presence of *d* FA, up to 1000 times more concentrated than *l* FA, could interfere with the biological activity of *l* FA.

5MTHF has been demonstrated to exhibit pharmacological activities *in vitro* [12, 20] and *in vivo* [21]. However, published data appear contradictory regarding the comparative pharmacological activities of FA and 5MTHF [11, 12, 22, 23]. Thus, there is a lack of pharmacological basis for the choice of the route of administration of *dl* FA. Moreover, in some of these studies [12, 22] the marked instability of 5MTHF relative to FA in cell culture condition was not taken into account, and this could lead to an overestimation of the effects of FA as compared to 5MTHF. The purpose of the present study was to compare the effects of pure *l* FA and pure *l* 5MTHF at equivalent drug exposure, taking into account their computed concentration–time profile. Two pharmacological models were used to evaluate the activities of folates: the rescue of MTX growth inhibition and the potentiation of 5FU activity. Four human tumor cell lines representative of the spectrum of the clinical use of MTX and 5FU, and one non-malignant (lymphoblastoid) cell line were investigated. *l* FA and *l* 5MTHF were tested not only as single drugs but also in combination with *d* FA, the proportions of each folate corresponding to those found in plasma at steady state during repeated i.v. and oral administration of *dl* FA in cancer patients.

MATERIALS AND METHODS

Chemicals. An injectable preparation of 5FU (0.385 M in water) from Roche laboratories (Neuilly, France) was used. A working solution of MTX (5.5×10^{-3} M) was prepared in distilled water with MTX from Roger Bellon Laboratories (Neuilly, France), and stored at −20°. Pure *l* FA, *d* FA and *l* 5MTHF stereoisomers (purity of 97.9, 98.9 and 98.5%, respectively) were kindly provided by Lederle Laboratories (Dr D. Lecompte, Oullins, France); working solutions (10^{-3} M) were prepared in 0.9% NaCl and aliquots were stored at −20°. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, phosphate-buffered saline (PBS), *l* glutamine and fetal bovine serum were from Gibco (Paisley, U.K.). Penicillin and streptomycin were from Merieux (Lyon, France). Transferrin was from Flow Laboratories (Irvine, U.K.). 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), dimethylsulfoxide and ascorbic acid were from the Sigma Chemical Co. (La Verpilliere, France).

Experimental conditions. Four human tumor cell lines were used, including: 2 colorectum, WIDR (ref. CCL 218 from ATCC, Rockville, MD, U.S.A.) and CAL 14 (from our institute); 1 osteosarcoma, HOSTE (ref. CRL 1543 from ATCC); 1 squamous cell carcinoma of the head and neck, CAL 27 (from our institute). In addition, we tested a non-malignant (lymphoblastoid) cell line obtained from the blood of a disease-free individual, RPMI (ref. CCL 156 from ATCC). Doubling times were 38, 74, 57, 45 and 27 hr for WIDR, CAL 14, HOSTE, CAL 27 and RPMI, respectively. Cells were routinely cultured in a humidified incubator (Sanyo) at 37° containing 8% CO_2 in air. Cells were grown in RPMI 1640 (lymphoblastoid cell line growing in suspension) or DMEM (all other cell lines growing as monolayer) media supplemented with 10% fetal bovine serum, penicillin (50,000 IU/L), streptomycin (86 μM) and *l* glutamine (2 mM). Cells were exposed to the drugs 24 hr after plating (96-well flat-bottom plates, initial density 3500–20,000 cells per 0.38 cm^2 well). For the rescue of MTX effects, cells were incubated for 24 hr in the presence of MTX, and reduced folates were added 12 hr after the start of MTX exposure and maintained for 5 days (24 hr after the onset of MTX exposure, cells were rinsed and a new medium supplemented with reduced folates was added). Growth inhibition measurement was performed at the end of the folate exposure. For the potentiation of 5FU activity, cells were incubated for 5 days in the presence of both 5FU and reduced folates, then they were rinsed and grown in a drug-free medium until growth inhibition evaluation (2 days after). MTX concentrations ranged from 5×10^{-8} to 5×10^{-4} M (14 points) and 5FU concentrations from 77 to 7680 nM (14 points). The effects of pure *l* FA, pure *l* 5MTHF and *l* FA + *d* FA + *l* 5MTHF were tested at equimolarity of active folates (i.e. *l* FA + *l* 5MTHF). Folate concentrations were monitored over 6 days in the culture medium; the effects of folates were then compared at equivalent folate exposure [area under curve $(AUC)_{0-120hr}$]. Two mixtures were tested (i) the p.o. mixture (4% *l* FA, 39% *d* FA, 57% *l* 5MTHF) representative of the proportions of reduced folates found in plasma at steady state during repeated oral administration of 100 mg *dl* FA every 4 hr for 5 days [9]; (ii) the i.v. mixture (7% *l* FA, 81% *d* FA, 12% *l* 5MTHF) representative of the proportions of reduced folates found in plasma at steady state during continuous i.v. administration of a widely used dose of 500 mg/m^2/day *dl* FA for 5.5 days [16]. The relative proportions *l* FA/*l* 5MTHF in p.o. and i.v. mixtures were thus 1/14.3 and 1/1.7, respectively. The initial total concentrations of active reduced folates tested were 5×10^{-8}, 5×10^{-7}, 5×10^{-6} and 5×10^{-5} M.

Evaluation of growth inhibition. The effects of the different drug combinations were assessed by the MTT semi-automated test [24]. The day of evaluation (control cells at approximately 80% of confluence), cells were rinsed once with PBS. Then DMEM medium (100 μL/well) and MTT (2 mg/mL, 50 μL/well) were added. The duration of MTT incubation was 4 hr at 37°. Wells were then cleaned out and dimethylsulfoxide was added (100 μL/well). After mixing, the absorbance was measured on a Titertek Twinreader ($\lambda = 540$ nm). Results were expressed as the relative percentage of absorbance compared to controls without drugs. Each experimental condition was performed in sextuplicate and each experiment was repeated once. Within a sextuplicate, the variability (coefficient of variation) was less than 6%.

Evaluation of the stability of reduced folates. This experiment was conducted apart from the *in vitro* growth inhibition studies. The stability of folates in the culture medium (supplemented DMEM in 75-

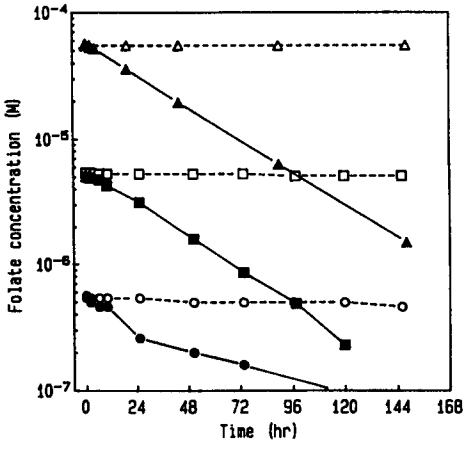

Fig. 1. Stability analysis of pure *l* FA (open symbols) and pure *l* 5MTHF (filled symbols) in the culture medium as a function of time. Initial concentrations were 5×10^{-5} M (triangle), 5×10^{-6} M (square) and 5×10^{-7} M (circle). See Materials and Methods.

cm^2 culture flasks without cells at 37°) was evaluated as a function of time over 6 days (7–10 sampling points). Stability of *l* FA and *l* 5MTHF was tested separately at 5×10^{-5}, 5×10^{-6} and 5×10^{-7} M (the sensitivity of the assay did not allow an accurate monitoring of the folate concentration to be performed at 5×10^{-8} M). At each sampling time, 0.5 mL of the culture medium was taken in a tube containing 0.75 mg of ascorbic acid in order to avoid folate oxidation. Samples were immediately stored at −20° until analysis. Folates were assayed using a modified HPLC method developed previously in our laboratory [25]. Briefly, 25–50 μL of the culture medium were directly injected onto an RP18, 5 μm, 250 × 4 mm Hibar-Lichrocart column (Merck, France). Mobile phase consisted in methanol/Pic A low UV (Waters) in H_2O (30/70), pH 7 (1.2 mL/min). UV absorption was measured at 313 nm. Retention times were 5.4 and 8.9 min for FA and 5MTHF, respectively. Intra- and inter-assay reproductibilities were 5 and 8%, respectively for FA, and 10 and 12%, respectively for 5MTHF. The limit of sensitivity was 3×10^{-8} M for both FA and 5MTHF. Folate exposure during 5 days ($AUC_{0-120hr}$) was computed from the concentration–time curves according to trapezoidal rule. This allowed a correction factor (CF) to be calculated for *l* FA and *l* 5MTHF. CF corresponded to the ratio between measured $AUC_{0-120hr}$ and theoretical $AUC_{0-120hr}$ (initial concentration × 120).

Analysis of the effects of folates. MTX and 5FU IC_{50} were defined as the drug concentration causing a 50% inhibition in growth as compared to control. IC_{50} values were automatically computed from the sigmoid concentration–response curves generated from GraphPAD software (San Diego, U.S.A.). For each folate considered (pure *l* FA, pure *l* 5MTHF, i.v. mixture, p.o. mixture), folate exposure over 5 days ($AUC_{0-120hr}$) was computed as CF × initial concentration × 120. Exposure to active folates in the i.v. and p.o. mixtures was calculated according to their relative concentrations of *l* FA and *l* 5MTHF. In order to compare the activities of each folate considered at equivalent exposure, the ratio of MTX or 5FU IC_{50} without folate/MTX or 5FU IC_{50} with folate was plotted as a function of the computed $AUC_{0-120hr}$. Curves were plotted according to the cubic spline function of GraphPAD software. Correlations between the degree of reversal of MTX effects or the degree of potentiation of 5FU effects, and the folate concentration (5×10^{-8}–5×10^{-5} M, 4 points) were tested using the non-parametric Spearman rank correlation test (Statgraphics software, Uniware, Paris, France).

RESULTS

Measurement of *l* FA and *l* 5MTHF concentrations in the medium under cell culture conditions confirms the physico-chemical lability of *l* 5MTHF as compared to *l* FA (Fig. 1). The ratios between computed $AUC_{0-120hr}$ and theoretical $AUC_{0-120hr}$ (initial concentration × 120) were 95.2, 97.1 and 100% for *l* FA at 5×10^{-7}, 5×10^{-6} and 5×10^{-5} M, respectively; they were 32.9, 35.8 and 33.9%, respectively for *l* 5MTHF. The *l* 5MTHF degradation appeared to be independent of the initial concentration over this 100-fold range. We therefore used the means of the above percentages in order to evaluate the CF (97.43 and 34.15% for *l* FA and *l* 5MTHF, respectively) and thus compute the $AUC_{0-120hr}$ for each folate considered (Table 1).

Regarding the reversal of MTX effects, the 3 cell lines tested (CAL 27, HOSTE, RPMI) exhibited

Table 1. Calculation of the active folate $AUC_{0-120hr}$ according to the initial concentration in the medium

Initial concentration of active folates* (M)	$AUC_{0-120hr}$ (μM × hr)			
	Pure *l* FA	Pure *l* 5MTHF	i.v. mixture	p.o. mixture
5×10^{-8}	5.8	2.0	3.4	2.3
5×10^{-7}	58.5	20.5	34.5	23.0
5×10^{-6}	584.6	204.9	344.6	230.0
5×10^{-5}	5846.0	2049.0	3446.0	2300.0

* Total concentration of active reduced folates i.e. *l* FA + *l* 5MTHF.

Table 2. Reversal of MTX growth inhibition in the presence of reduced folates

Cell line	Initial folate concentration* (M)		MTX IC_{50} (μM)			
		Control†	/FA	/5MTHF	i.v. mixture	p.o. mixture
HOSTE‡	5×10^{-8}	(1) 2	6.6	1	2.9	2
		(2) 0.8	1.4	0.7	1.2	0.8
	5×10^{-7}	(1) 2	>100	5.8	37	9.4
		(2) 0.8	32	4	21	4.7
	5×10^{-6}	(1) 2	>100	>100	>100	>100
		(2) 0.8	>100	40	>100	83
CAL 27	5×10^{-8}	(1) 2.8	3.5	2.2	2.6	2.1
		(2) 1.2	1.7	0.9	1.3	1.1
	5×10^{-7}	(1) 2.8	36	3.5	9.8	3.6
		(2) 1.2	7.3	1.3	2.8	1.9
	5×10^{-6}	(1) 2.8	>150	23	>150	35
		(2) 1.2	53	5	26	8.1
	5×10^{-5}	(1) 2.8	>150	150	>150	>150
		(2) 1.2	255	13.8	153	55
RPMI	5×10^{-8}	(1) 2.3	10.2	2.8	3.8	3.7
		(2) 3.8	7.9	3.6	7	4.8
	5×10^{-7}	(1) 2.3	27.5	16.3	25.2	17.6
		(2) 3.8	45.9	7.4	26.9	13.3
	5×10^{-6}	(1) 2.3	69.6	58	71	50.5
		(2) 3.8	140.3	36.2	133.6	125.5
	5×10^{-5}	(1) 2.3	738	69	289	109.3
		(2) 3.8	402	70	342	149.5

Results of two separate experiments (1, 2) are reported for each experimental condition.
* Total concentration of active reduced folates, i.e. /FA + /5MTHF.
† Control with no folate added to the medium.
‡ MTX IC_{50} in the presence of reduced folate at 5×10^{-5} M is not given since it was always > 100 μM.

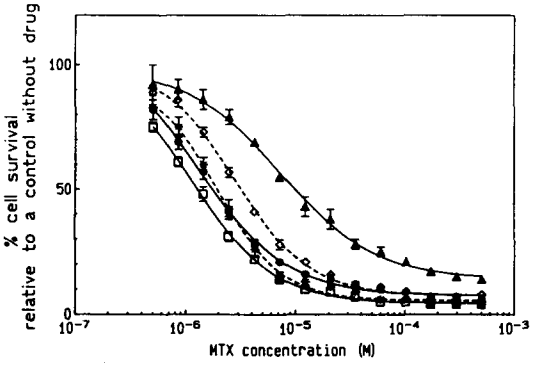

Fig. 2. Dose–response curves of MTX growth inhibition (mean survival ± SD for sextuplicates) in the presence of folates on CAL 27 cell line (□ = control without folates). Pure /FA (▲), pure /5MTHF (●), i.v. mixture (◇) and p.o. mixture (*) were tested at 5×10^{-7} M (total concentration of active folates).

comparable sensitivity to MTX (Table 2, controls). Under folate exposure, a more or less marked reversal of MTX growth inhibition was observed in all cell lines (Table 2). Figure 2 shows a representative example of the reversal of MTX effects on CAL 27 cell line (5×10^{-7} M total active folate concentration). Whatever the cell line and the folate considered, the degree of reversal of MTX growth inhibition was significantly related to the initial folate concentration (Spearman rank correlation, $P < 0.001$). Taking into account folate exposure, plots of the ratio between MTX IC_{50} without folate and MTX IC_{50} with folate confirmed this observation (Fig. 3); the greater the folate exposure, the smaller the IC_{50} ratio, i.e. the greater the reversal of the effects. Globally, these curves (log–log plot) show that a one log increment in folate exposure corresponds approximately to a one log decrease in IC_{50} ratio. This suggests a stoichiometric molecular antagonism between MTX and folates on cell viability. Comparison of MTX IC_{50} without folates/MTX IC_{50} with folate ratios between CAL 27 and RPMI cells within each folate condition indicated that for the same folate exposure the degree of growth inhibition reversal was significantly greater in RPMI cells than in CAL 27 cells (Wilcoxon paired test, $P = 2 \times 10^{-3}$). More interestingly, Fig. 3 shows the relative effects of the different folates considered. For CAL 27 cell line and whatever the intensity of folate exposure over 5 days, the reversal of MTX effects was less efficient with pure /5MTHF than with the parent drug /FA; accordingly, the i.v. mixture which exhibits a /FA/ /5MTHF ratio of 1/1.7 was more efficient than the p.o. mixture which exhibits a /FA/5MTHF ratio of 1/14.3. In the RPMI cell line, no marked differences in MTX growth inhibition reversal appeared between the different folates considered.

As concerns 5FU activity, the 3 cell lines tested exhibited a mean 5FU IC_{50} of 1960, 5280 and 6470 nM for CAL 27, WIDR and CAL 14, respectively (Table

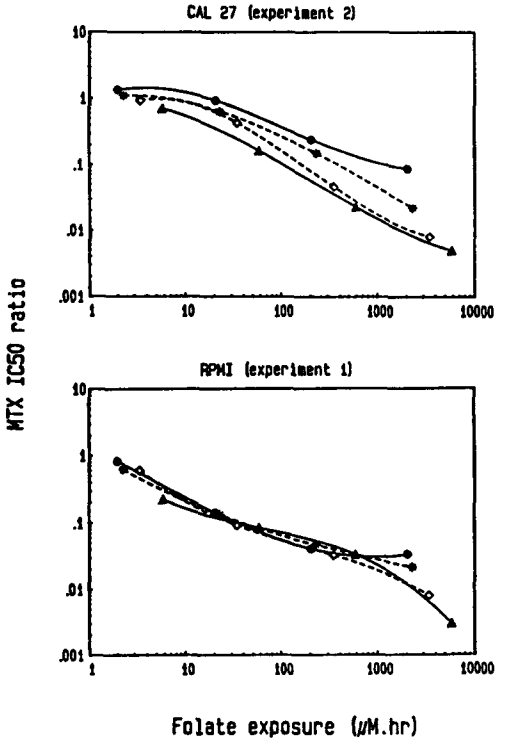

Fig. 3. Reversal of the MTX growth inhibition by folates considered at equivalent computed exposure. MTX IC_{50} without folates/MTX IC_{50} with folates (MTX IC_{50} ratio) was plotted as a function of the computed active folate $AUC_{0-120hr}$. The effects of pure *l* FA (▲), pure *l* 5MTHF (●), i.v. mixture (◇) and p.o. mixture (*) were compared.

3). The enhancement of 5FU effects by reduced folates was only demonstrated for the most sensitive cell lines, CAL 27 and WIDR. Growth inhibition of the CAL 14 cell line, the most resistant to 5FU, was not significantly modified by reduced folates (Table 3, Fig. 4). For CAL 27 and WIDR, regardless of the folate considered, a progressive increase in growth inhibition was observed as the folate concentration increases; plots of the ratio between 5FU IC_{50} without folate and 5FU IC_{50} with folate as a function of the folate exposure during 5 days illustrate this relationship (Fig. 4). For CAL 27 and WIDR the relative effects of the different folates considered show that *l* FA was more potent than *l* 5MTHF; accordingly the growth inhibition observed with the i.v. mixture was greater than that observed with the p.o. mixture (Fig. 4). The pattern of efficacy (*l* FA > i.v. mixture > p.o. mixture > *l* 5MTHF) was more marked in the CAL 27 cell line.

DISCUSSION

The experimental conditions used in the present study were as close as possible to clinical conditions encountered in cancer patients. This was achieved not only through the choice of the cell lines investigated, but also through the chosen time-schedules of the tested drugs. It has been demonstrated that continuous i.v. infusion of 5FU (usually 5 days) enhanced response rates as compared to i.v. bolus [26]. Moran and Scanlon [27] showed that increased duration of exposure to FA continuously enhances the *in vitro* effects of 5FU. Boarman and Allegra [28] recently provided a pharmacological rationale for the above observation, demonstrating that prolonged folate exposure results in a greater accumulation of intracellular polyglutamated anabolites of *l* FA. 5FU–folate combinations were thus tested over a 5 day co-incubation. In cancer patients MTX is usually administered as a 24 hr continuous infusion [1, 29, 30]. The schedule retained for folate exposure was based upon optimal conditions obtained in our previous investigations [19], showing that 18 hr after the onset of MTX incubation, growth inhibition could not efficiently be reversed by FA (we thus added reduced folates 12 hr after the start of MTX).

Standard media containing folic acid were used in the present study. The use of a folate-free medium could have theoretically allowed the activity of reduced folates to be more accurately evaluated; however, as noticed by Park *et al.* [31] who have studied 5FU–FA combinations, such a condition (folate-free medium) could have resulted in an overestimation of the effects of folates as compared to the physiological situation in which low concentrations of *l* 5MTHF are present. It must be stressed that in the majority of our experiments, the addition of pure *l* FA at concentrations as low as 5×10^{-8} M resulted in significant modifications of the growth inhibition as compared to controls (no folate added).

In clinical practice, depending on the administration route of *dl* FA, different blood profiles of *l* FA and *l* 5MTHF are obtained at steady state [9, 10, 16]. Even if the blood profiles vary according to the dose and the schedule [10, 32, 33], the marked difference in the proportions of *l* 5MTHF plasma concentrations observed during i.v. and oral administration is unquestionable [9, 10, 16]. Thus, a better knowledge of the pharmacological activity of *l* 5MTHF relative to *l* FA could provide a more rational basis for the choice of the FA administration route.

So far, few studies have dealt with such a comparison. Recently, Houghton *et al.* [11] have compared the degree of expansion of 5,10 methylenetetrahydrofolate and tetrahydrofolate intracellular pools in human adenocarcinoma xenografts in mice after *dl* FA and *dl* 5MTHF i.v. infusion. They found an expansion of these pools by 253–661% of control during *dl* FA infusion whereas a limited pool size expansion (148–163% of controls) was observed during *dl* 5MTHF infusion. Without consideration of the different pharmacokinetic behavior between *dl* FA and *dl* 5MTHF in mice, the authors concluded that *dl* 5MTHF was less effective than *dl* FA at increasing the active folate intracellular pool. Evans *et al.* [23] have compared the effects of *dl* FA and *dl* 5MTHF on the potentiation of 5FU effects: incubation with folates was limited to 27 hr in a medium supplemented with 100 μM sodium ascorbate. Under these conditions the authors concluded to the stability of *dl* 5MTHF and found

Table 3. Potentiation of 5FU growth inhibition in the presence of reduced folates

Cell line	Initial folate concentration* (M)	5FU IC_{50} (nM) Control†	*l* FA	*l* 5MTHF	i.v. mixture	p.o. mixture
WIDR	5×10^{-8}	(1) 5760	3487	5207	4300	5500
		(2) 4800	2350	3180	2819	3080
	5×10^{-7}	(1) 5760	3180	2865	3425	4577
		(2) 4800	2112	2365	2127	2742
	5×10^{-6}	(1) 5760	3072	2964	2995	2742
		(2) 4800	1897	2243	1912	2104
	5×10^{-5}	(1) 5760	2557	2872	2895	2373
		(2) 4800	1252	2135	1889	2166
CAL 14	5×10^{-8}	(1) 6550	7661	7441	7854	8308
		(2) 6388	5211	4637	6044	4935
	5×10^{-7}	(1) 6550	6459	8497	7793	7045
		(2) 6388	4560	5139	6012	6754
	5×10^{-6}	(1) 6550	7048	9148	6699	7498
		(2) 6388	5175	4701	5140	5188
	5×10^{-5}	(1) 6550	8560	8950	7185	7871
		(2) 6388	4937	6884	5987	5386
CAL 27	5×10^{-8}	(1) 1460	1234	1414	1245	1339
		(2) 2456	1881	2294	1952	2266
	5×10^{-7}	(1) 1460	901	1445	978	1147
		(2) 2456	1399	2026	1573	1993
	5×10^{-6}	(1) 1400	824	1087	743	876
		(2) 2456	1286	1777	1411	1500
	5×10^{-5}	(1) 1460	640	880	750	835
		(2) 2456	1345	1400	1232	1391

Results of two separate experiments (1, 2) are reported for each experimental condition.
* Total concentration of active folate, i.e. *l* FA + *l* 5MTHF.
† Control with no folate added to the medium.

an equivalent potency for *dl* FA and *dl* 5MTHF activities at equimolarity. Balinska *et al.* [12] reported a greater MTX growth inhibition reversal with *dl* FA than with *dl* 5MTHF. These effects were associated with a marked reduction in intracellular MTX polyglutamate content and increased dihydrofolate reductase activity. For both mechanisms, *dl* FA was found to be more potent than *dl* 5MTHF. The stability of the metabolite was not taken into account whereas durations of incubation with folates were 24–72 hr. Nor was it in the study of Halpern *et al.* [22] comparing the reversal of MTX growth inhibition by reduced folates on a panel of normal adult cells and malignant cells; *dl* 5MTHF was shown to be as potent as *dl* FA for rescuing normal cells whereas, contrary to *dl* FA, *dl* 5MTHF was unable to rescue malignant cells. As compared to normal cells, the authors argued that malignant cells exhibited a relative deficiency in the vitamin B12-dependent enzyme 5MTHF:homocysteine methyltransferase (EC 2.1.1.13), which converts *l* 5MTHF into tetrahydrofolate, and concluded that a potential selective rescue of normal tissues occurred when 5MTHF was given.

The aim of the present study was to compare the effects of pure *l* FA and pure *l* 5MTHF taking into account their relative stability under cell culture conditions. The pharmacological activities were thus compared at equivalent computed concentration × time.

As opposed to the results of Halpern *et al.* [22], *l* 5MTHF was shown to reverse the MTX growth inhibition for the two malignant models investigated. For CAL 27 cells, *l* FA was more potent than *l* 5MTHF; consequently the i.v. mixture was more potent than the p.o. mixture (Fig. 3). In the lymphoblastoid cell line, reversal of MTX growth inhibition was also observed but no marked differences appeared between the different folates tested. Regardless of the folate considered, globally, for a given folate exposure, the lymphoblastoid cells were more sensitive to the rescue than were CAL 27 cells. The mechanism by which reduced folates selectively protect normal tissues from MTX is not clearly established but the degree of MTX polyglutamation, which influences MTX retention, could be involved [34].

Potentiation of 5FU effects under folate exposure was observed in CAL 27 and WIDR cells only; the present results on CAL 27 confirmed our previous investigations [35]. The variability in the sensitivity to 5FU–folate combination could reflect differences in intracellular factors which have been demonstrated to be linked to 5FU–FA sensitivity: 5,10 methylene-tetrahydrofolate content, thymidylate synthase activity, FdUMP/dUMP ratio [36, 37]. One can also suggest differences in the importance of the intracellular activation routes of 5FU [38]. In WIDR cells, differences between the different folates tested were not strongly marked as opposed to CAL 27 cells in which *l* FA was always more potent than *l* 5MTHF and, as a result, the i.v. mixture was more effective than the p.o. mixture.

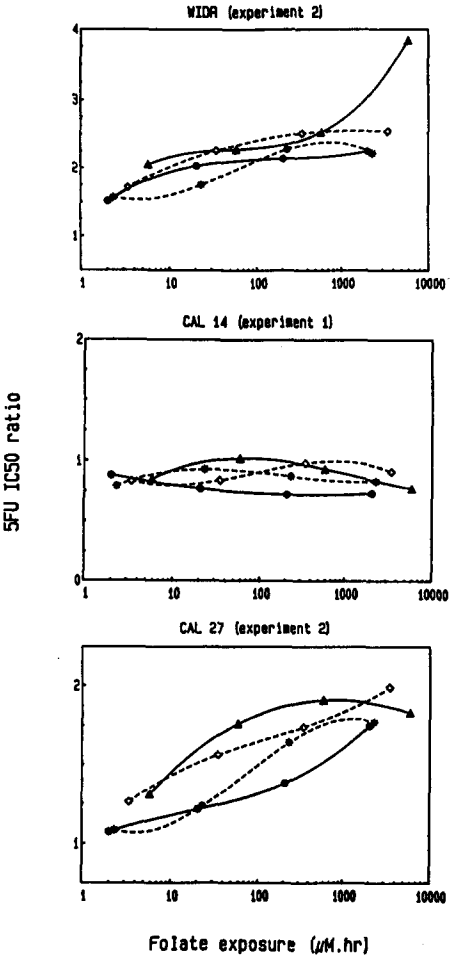

Fig. 4. Potentiation of the 5FU effects by folates considered at equivalent computed exposure. 5FU IC_{50} without folates/5FU IC_{50} with folates (5FU IC_{50} ratio) was plotted as a function of the computed active folate $AUC_{0-120hr}$. The effects of pure *l* FA (▲), pure *l* 5MTHF (●), i.v. mixture (◇) and p.o. mixture (*) were compared.

Finally, in the two pharmacological models tested and taking into account the folate stability, the most marked differences between *l* FA and *l* 5MTHF were observed for CAL 27 cells. Not considering the stability of *l* FA and *l* 5MTHF would have led to the erroneous conclusion that, whatever the cell line, *l* FA was always more potent than *l* 5MTHF. In contrast we conclude that, depending upon the cell line, *l* 5MTHF is only as potent as its parent drug, or less so. One can suggest that the variability in 5MTHF:homocysteine methyltransferase activity can partly explain these differences.

Along with the limited capacity of oral absorption [32], the choice between oral and i.v. route for FA administration should take into consideration the different pharmacological activities between *l* FA and *l* 5MTHF which suggests that the oral route is potentially detrimental to an optimal activity of 5FU–FA combination as compared to i.v. administration.

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