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Original Paper

Relevance of Tumoral Folylpolyglutamate Synthetase and Reduced Folates for Optimal 5-Fluorouracil Efficacy: Experimental Data

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The purpose of this study was to investigate folate-related predictors of 5-fluorouracil (5-FU) cytotoxicity in the presence or absence of ℓ-folinic acid (ℓ-FA). Intracellular concentrations of the reduced folates (tetrahydrofolate + 5,10-methylenetetrahydrofolate) and folylpolyglutamate synthetase (FPGS) activity were determined in 14 human cancer cell lines expressing a spontaneous sensitivity to 5-FU. On these 14 cell lines grown without ℓ-FA supplementation, a significant positive correlation was demonstrated between basal intracellular folate concentration and FPGS activity. 5-FU sensitivity (IC_{50} range 0.6–25.4 μM) was not related to the basal intracellular folate concentration, whereas, significantly, it was linked to FPGS activity (range 2.5–11.1 pmol/min/mg protein): the higher the FPGS activity, the greater the 5-FU sensitivity. Under ℓ-FA supplementation (0.01–300 μM), intracellular reduced folates increased continuously without evidence of saturation in all cell lines; the pattern of accumulation was independent of the FPGS activity. ℓ-FA enhanced 5-FU cytoxicity by a factor of 1.9–6.4 in 12 of the 14 cell lines. In the 12 FA-sensitive cell lines, the ℓ-FA concentrations allowing 90% of maximum 5-FU potentiation [ℓ-FA]90 ranged between 0.7 and 107.9 μM (median 1.9); in contrast, the intracellular concentrations of reduced folates allowing 90% of maximum 5-FU potentiation were much less variable (range 7.6–38.3, median 24.8 pmol/mg protein). In the presence of [ℓ-FA]90, 5-FU sensitivity remained significantly correlated to the basal FPGS activity. In addition, reduced folates were measured in 96 tumoral samples (50 head and neck, 16 colon, 30 liver metastases from colorectal cancer) taken before treatment. Almost all investigated tumours had folate concentrations below the median concentration required for optimal 5-FU potentiation *in vitro*: median levels (range, pmol/mg protein) were 3.8 (0–17.7) for head and neck, 5.8 (2.3–12.0) for colon and 12.1 (1.7–118.5) for liver metastases. Above all, these data establish the relevance of FPGS activity for predicting the efficacy of 5-FU modulated by FA or not and point to the potential clinical interest of FPGS determination in human tumours. © **1997 Elsevier Science Ltd.**

Key words: 5-fluorouracil, folinic acid, reduced folates, folylpolyglutamate synthetase, chemosensitivity

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INTRODUCTION

DESPITE BEING one of the oldest anticancer drugs, 5-fluorouracil (5-FU) is increasingly used in cancer chemotherapy and occupies a major place not only in advanced colon cancer, but also in head and neck cancer [1] and breast cancer [2]. One explanation for this phenomenon is the wide possibility of 5-FU pharmacomodulation which markedly increases the antitumour efficacy of this antimetabolite [3]. Among the different approaches to 5-FU modulation, the

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association with folinic acid (FA) is a major one [3]. Numerous studies have established the beneficial effects of 5-FU–FA therapy in advanced colorectal cancer patients in terms of objective response [4–10] and survival [11]. Noteworthy, recent randomized trials comparing 5-FU–FA versus no treatment as adjuvant therapy following surgery in colorectal cancer patients have demonstrated an advantage for 5-FU–FA in terms of disease-free survival and overall survival [12, 13].

The mechanism of 5-FU cytotoxicity is complex since 5-FU is activated through different pathways leading to at least three cytotoxic compounds: fluorodeoxyuridine monophosphate (FdUMP) which inhibits thymidylate synthase (TS) and subsequent DNA synthesis; fluorouridine triphosphate (FUTP) which is directly incorporated into RNA; and fluorodeoxyuridine triphosphate (FdUTP) for which incorporation into DNA has been suggested [14]. The relative importance of these pathways seems to depend on the administered dose and schedule. Thus, experimental studies have demonstrated that short-term exposure to high 5-FU-concentrations kills the cells primarily through an RNA effect, whereas prolonged exposure to low concentrations is cytotoxic mainly via TS inhibition [15]. Interestingly, clinical studies showing the prognostic value of TS overexpression for predicting resistance to 5-FU-based therapy have been performed in digestive-tract cancer patients receiving 5-FU by continuous infusion for 3 or 4 weeks [16, 17]. Thus, together with the pharmacokinetic rationale, the above data clearly point to TS being the main 5-FU target when 5-FU is given by continuous infusion. The mechanism of cytotoxicity is not as clearly characterised regarding short i.v. administration. Enhancement of 5-FU cytotoxicity by FA is based on the optimal inhibition of TS resulting from an increase in the intracellular pool of 5,10-methylenetetrahydrofolate (CH_2FH_4) which, in turn, stabilises the inactive complex formed between TS and FdUMP [18]. Inside the cells, reduced folates are polyglutamated by a specific enzyme, FPGS, which can add up to seven glutamate residues to the monoglutamate form. Both cellular retention [19] and affinity to TS [20, 21] are increased with CH_2FH_4 polyglutamated forms. Recently, Wang and associates [22] reported an *in vitro* study showing that 5-FU resistance was related to a decrease in the FPGS activity in a tumoral cell line model.

The clinical use of 5-FU–FA chemotherapy is still hampered by unsolved issues such as the choice of optimal schedule and optimal FA dose as well as the identification of 5-FU–FA responsive tumours. We have previously attempted to partially answer these questions by investigating the FA concentrations which allow optimal potentiation of 5-FU cytotoxicity on a large panel of human cancer cell lines [23]. In the cells responding to FA supplementation, the optimal ℓ-FA concentrations varied considerably between lines, from 0.05 to 200 μM (i.e. a 4000-fold range). The next step, which we present here, was to investigate more closely the profile of intracellular reduced folate concentrations on a wide panel of 14 cancer cell lines grown in culture medium containing increasing ℓ-FA concentrations. The panel of investigated cell lines covered the tumour localisations usually treated with 5-FU–FA chemotherapy. In order to understand fully the factors governing 5-FU potentiation by FA, the profile of intracellular reduced folates (CH_2FH_4 and its direct precursor tetrahydrofolate (FH_4)) was analysed with regard to the FPGS activity expressed in each cell line. In addition to these investigations, the intratumoral reduced folate concentrations required *in vitro* for optimal 5-FU efficacy were compared with those encountered in tumours taken from patients before treatment. To this end, tumoral reduced folates were measured in 96 tumoral biopsies obtained from cancer patients at the time of diagnosis.

MATERIALS AND METHODS

Chemicals

5-FU was provided by Roche Laboratories (Neuilly, France). Pure ℓ-FA was kindly provided by Wyeth-Lederle Laboratories (Rungis, France). ^{3}H-FdUMP labelled at position 6 (23 Ci/mmol) was obtained from Moravek Biochemicals (Brea, California, U.S.A.) and ^{14}C-glutamic acid tetralabelled (264 Ci/mol) was obtained from Amersham (Les Ulis, France). CH_2FH_4 was prepared from FH_4 supplied by Fluka Biochemika (Buchs, Switzerland), according to the procedure provided by Professor Priest (University of South Carolina, South Carolina. U.S.A.). Purified TS (3.7 unit/mg protein) from *Lactobacillus casei* was also supplied by Professor Priest. Sephadex G25 fine gel was obtained from Pharmacia Biotech (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM) folic acid-free, glutamine and fetal bovine serum (FBS) were obtained from Gibco (Paisley, U.K.). Penicillin and streptomycin were obtained from Merieux (Lyon, France). All other chemicals including aminopterin, MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and $d\ell$-5-methyltetrahydrofolate were obtained from Sigma Chemical Co. (St Quentin Fallavier, France). Cytosolic proteins were measured according to the Bradford assay using the Bio-Rad protein assay kit from Bio-Rad Laboratories (Munich, Germany) with human purified albumin as standard.

Cell lines

Culture conditions. Fourteen human cancer cell lines of different origin (4 breast, 4 colon, 1 duodenum, 1 pancreas, 4 head and neck) were investigated (Table 1). All expressed a spontaneous sensitivity to 5-FU (the cells had never been previously exposed to 5-FU). Cell doubling times were between 1.3 and 6.3 days (median 2.5). Cells were grown in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO_2. Cells were routinely cultured in DMEM medium supplemented with 10% FBS (concentration of active folates in the FBS was 10 nM accounting for 1 nM in the culture medium), 2 mM glutamine, 50 000 U/l penicillin and 80 μM streptomycin. In order to remain as close as possible to the physiological folate concentration in humans, cells were grown in a folate-controlled medium for 10 days before starting the experiments (i.e. folate-free DMEM medium supplemented with 40 nM of $d\ell$-5-methyltetrahydrofolate plus 0.1 mM of ℓ-ascorbic acid for folate stabilisation). All experiments were then performed in this folate-controlled medium which was renewed every 5–6 days. For cytotoxicity experiments, cells were grown in 96-well microtitration plates (0.32 cm^2/well). In addition, cells were cultured for 5 days in 175 cm^2 plates for FPGS measurement (basal condition) and for

Table 1. In vitro *investigations*

Cell lines											
					Basal folates (pmol/mg protein)		Maximal folates* (pmol/mg protein)		Folate concentrations at 90% of F_{max} Extracellular (μM)	Intracellular (pmol/mg protein)	
Origin	Name	5-FU IC_{50} (μM)	F_{max}	FPGS activity (pmol/min/mg protein)	CH_2FH_4	CH_2FH_4 + FH_4	CH_2FH_4	CH_2FH_4 + FH_4	[ℓ-FA]90	$[CH_2FH_4]90$	$[CH_2FH_4 + FH_4]90$
Breast	ZR 75	1.0 ± 0.3	5.3 ± 1.3	10.6 ± 0.6	0.5 ± 0.1	1.20 ± 0.3	27.6 ± 2.7	68.4 ± 12.4	1.9 ± 0.5	11.0 ± 1.4	32.5 ± 4.9
	CAL 51	4.1 ± 0	1.0 ± 0	2.5 ± 0.1	ND	ND	34.1 + 1.8	106.3 ± 4.6	–	–	–
	MCF 7	6.3 ± 2.0	5.6 ± 0.7	5.2 ± 0.8	0.9 ± 0.3	1.3 ± 0.5	32.8 ± 15.3	56.6 ± 15.0	1.4 ± 0.4	15.3 ± 3.1	32.5 ± 3.8
	T 47 D	10.2 ± 1.1	6.4 ± 0.8	3.7 ± 0.2	ND	ND	6.6 ± 1.9	18.5 ± 5.7	50.0 + 5.8	6.0 + 0.1	18.1 + 2.5
Colon	WIDR	3.6 ± 0.2	4.3 ± 0.3	11.1 ± 1.7	0.4 ± 0	1.4 ± 0.1	12.7 ± 1.2	66.8 ± 8.9	60.2 ± 14.8	9.8 ± 4.9	29.8 ± 10.9
	SW 620	13.8 ± 2.7	2.1 ± 0.6	4.0 ± 0.7	0.4 ± 0.1	0.9 + 0.1	42.4 ± 8.4	90.3 ± 13.6	1.4 ± 0.2	8.1 ± 1.4	19.3 ± 7.3
	CAL 14	2.9 ± 0.6	1.9 ± 0.1	7.0 ± 3.0	ND	0.7 ± 0.4	13.4 ± 1.8	58.6 ± 10.4	NA	NA	NA
	COLO 205	0.8 ± 0.1	4.9 ± 0.1	8.3 ± 1.5	3.3 ± 2.1	5.7 ± 3.2	16.1 ± 3.9	43.9 ± 12.0	1.1 ± 0.5	3.3 ± 0.1	7.6 ± 0.8
Duodenum	HUTU 80	9.7 ± 0.9	1.0 ± 0	2.8 ± 0.3	ND	ND	16.1 ± 0.2	45.8 ± 3.3	–	–	–
Pancreas	HS 766T	16.5 ± 2.4	2.2 ± 0.6	3.1 ± 0.6	ND	ND	15.1 ± 1.8	53.7 ± 4.5	107.9 ± 62.6	6.7 ± 3.4	19.3 ± 9.7
Head and neck	CAL 27	1.8 ± 0.2	5.3 ± 1.7	6.4 ± 0.6	0.8 ± 0.3	2.4 ± 0.9	18.8 ± 5.8	52.6 ± 11.9	1.7 ± 0.6	11.2 ± 2.3	38.3 ± 3.8
	KB	7.3 ± 1.1	2.8 ± 0.6	5.8 ± 1.6	1.3 ± 0.1	3.2 ± 0.7	26.8 ± 2.3	66.9 ± 7.3	0.7 ± 0.2	13.8 ± 3.0	36.8 ± 7.1
	HEP 2	25.4 ± 3.5	3.3 ± 0.1	5.3 ± 0.3	ND	ND	20.2 ± 1.3	61.3 ± 1.0	6.2 ± 3.4	12.4 ± 4.8	24.8 ± 8.8
	CAL 33	0.6 ± 0.1	3.3 ± 0.1	7.6 ± 0.2	ND	ND	21.1 ± 9.2	43.6 ± 16.9	4.7 ± 2.0	8.7 ± 3.4	19.7 ± 5.5
All cell lines	Median	5.2	3.3	5.6	ND	0.8	19.5	57.6	1.9	9.8	24.8
	Mean	7.4	3.5	6.0	0.5	1.2	21.7	59.5	21.6	9.7	25.3
	S.D.	7.2	1.8	2.7	0.9	1.6	9.9	21.1	35.7	3.6	9.5

Results are given as mean ± SE from three separate experiments.

F_{max}, see definition in Materials and Methods.

ND, not detectable i.e. < 0.3 pmol/mg protein; NA, not assessable (lack of precision in the fitting for an accurate determination of optimal concentrations).

* With 300 μM of ℓ-FA in the culture medium (5-day incubation).

intracellular reduced folate determination (in the absence or presence of increasing ℓ-FA concentrations). After 5 days of growth (70–80% of confluence), cells were harvested, washed three times in phosphate-buffered saline at 4°C and cell pellets containing approximately 50×10^6 cells were stored in liquid nitrogen. All investigations (cytotoxicity and biochemical determinations, including FPGS measurement) were performed during three independent experiments.

Evaluation of cytotoxicity. Cells were plated in 96-well microtitration plates in order to obtain exponential growth for the whole duration of the experiment (initial cell density 5000–7000 cells/well depending on the cell line). Twenty-four hours later, cells were exposed for 5 days to various 5-FU, ℓ-FA or 5-FU + ℓ-FA concentrations. 5-FU concentrations ranged between 0.01 and 500 μM (14 concentrations) and ℓ-FA concentrations ranged between 0.01 and 300 μM (6 concentrations). Experimental conditions were tested in sextuplicate. The growth of cells in the presence of ℓ-FA was similar to that of cells without ℓ-FA. Growth inhibition was assessed by the MTT test at the end of the 5-FU ± ℓ-FA exposure [24]. Results were expressed as the percentage of absorbance compared to controls without drug.

Tumour biopsies

Tumour biopsies from 96 patients were obtained prior to treatment at the time of diagnosis. Biopsies from 50 head and neck tumours, 16 colon tumours and 30 liver metastases from colorectal carcinoma were investigated. In addition, for 17 head and neck cancer patients, a biopsy was also available from normal tissue in the symmetrical position. At time of sampling, one part of the biopsy was examined for histological diagnosis. The other part was pulverised in liquid nitrogen into a fine powder and stored in liquid nitrogen until assayed for biochemical investigations.

Biochemical investigations

Reduced folate assay. On the day of the assay, frozen powders from biopsies (10–30 mg) were homogenised with a Polytron in 300 μl of buffer A (50 mM Tris–HCl buffer pH 7.4 containing 1 mM EDTA and 50 mM ℓ-ascorbic acid). For the cell lines, cell suspensions (50×10^6 cells/ml) were performed in buffer A. The homogenates were then sonicated on an ice-bed three times (5 sec each time) at 10 sec intervals. After centrifugation of the homogenates for 5 min at 15 000*g* (4°C), the supernatants were boiled for 3 min in order to denature the enzymes responsible for the cycling of folates.

Reduced folates (FH_4 and CH_2FH_4) were measured according to the entrapment assay described by Bunni and associates [25]. This assay is based on the stoichiometric formation of a stable ternary complex between CH_2FH_4, TS, and FdUMP, allowing CH_2FH_4 to be quantified whatever its polyglutamylation level. Each cytosol was assayed at three different volumes (between 10 and 70 μl) in the presence or absence of formaldehyde which allows the chemical conversion of FH_4 into CH_2FH_4. The measurement of FH_4 was obtained from the difference between formaldehyde-containing tubes and those without formaldehyde. The cytosol was incubated in the presence of an excess of purified TS (0.225 μM final concentration) and an excess of ^{3}H-FdUMP (0.35 μM final concentration) in a total volume of 100 μl (in buffer A) containing 6.66 mM final concentration of formaldehyde or not. Controls containing increasing known concentrations of CH_2FH_4 (0.5–50 nM final concentrations) were performed in each series, as well as blanks without cytosol for quantitation of the binary complex formed between TS and ^{3}H-FdUMP. After 30 min incubation at room temperature, the reaction was stopped by the addition of 10 μl of sodium dodecylsulphate (11%), followed by 10 min boiling to stabilise the ternary complexes. The excess of ^{3}H-FdUMP was then retained on 400 μl microcolumns (ref 72700 from Sarstedt, Nümbrecht, Germany) extemporaneously filled with Sephadex G25 gel. Twenty-five microliters of the reagent mixture were applied on to the microcolumn (duplicates). After 10 min centrifugation at room temperature (1300*g*), the eluting fractions containing the ternary complexes were counted. Results were expressed as pmol of folates per mg of cytosolic proteins. The recovery calculated from the controls was 90% on average (coefficient of variation 8%). The limit of sensitivity was 0.3 pmol/mg protein. The coefficient of variation for the intra- and interassay reproducibility, determined on cell aliquots obtained from a pool of cell pellets, were 9.4% and 25.0%, respectively.

FPGS assay. Due to the small size of head and neck biopsies, FPGS was only assayed in colon and liver biopsies. On the day of the assay, frozen powders from biopsies (50–500 mg) were homogenised with a Polytron in ten volumes of buffer B (10 mM Tris–HCl buffer pH 7.5 containing 1.5 mM EDTA, 0.5 mM dithiothreitol and 10 mM sodium molybdate). For *in vitro* investigations, basal FPGS activity was measured on cells grown in the folate-controlled medium, at 70–80% confluence. Cell pellets were homogenised (50×10^6 cells/ml) in buffer B supplemented with 0.2 mg/ml trypsin soybean inhibitor, freeze-thawed three times and sonicated on an ice-bed four times (10 sec each time) at 10 sec intervals. Cytosols were obtained after centrifugation of the homogenates for 30 min at 105 000*g* (+4°C).

FPGS activity was measured according to a method derived from that of Montero and Llorente [26] based on the incorporation of an additional ^{14}C-glutamic acid residue into the glutamate chain of aminopterin. Each cytosol was assayed in duplicate. The assay consisted of incubating 100 μl of cytosol with ^{14}C-glutamic acid (isotopic dilution, 250 μM final concentration) and aminopterin (250 μM final concentration) in a total volume of 250 μl (final concentrations of the buffer at pH 8.9 are 100 mM Tris–HCl, 20 mM $MgCl_2$, 20 mM KCl, 10 mM ATP and 100 mM β-mercaptoethanol). After 2 h incubation at 37°C, the reaction was stopped by the addition of 50 μl of 40% trichloracetic acid (the reaction was linear according to duration of incubation from 1 h to 4 h). Tubes were then centrifuged for 10 min at 3000*g*. The supernatant (80 μl injected) was analysed for the presence of ^{14}C-aminopterin diglutamates by high performance liquid chromatography using an RP18 5 μm Lichrospher 100 column (250 × 4 mm ID) from Merck (Darmstadt, Germany). Mobile phase A contained 100 mM ammonium acetate/acetonitrile (99/1, pH 5.5) and phase B, 100 mM ammonium acetate/acetonitrile (90/10, pH 5.5). The elution (flow rate 1.5 ml/min) was as follows: 0–15 min, 100% phase A; 15–40 min, 100% phase B; 40–60 min, 100% phase A for equilibration. Typical retention time was 2 min for ^{14}C-glutamic acid and 21 min for

^{14}C-aminopterin diglutamate. Results were expressed as pmol/min/mg protein. The limit of sensitivity was 0.4 pmol/min/mg protein. The intra- and interassay reproducibility, determined on cell aliquots obtained from a pool of cell pellets, gave coefficients of variation of 7.2% and 9.4%, respectively.

Analysis of data

For *in vitro* investigations, curve fittings were done on GraphPad software (ISI, Philadelphia, Pennsylvania, U.S.A.). For each studied parameter, the mean values of three separate experiments were calculated. For reduced folates, analyses were performed on the concentration of CH_2FH_4 alone and on the concentration of $CH_2FH_4 + FH_4$ (sum). The analysed parameters were:

— the 5-FU concentrations causing a 50% growth inhibition (5-FU IC_{50}) compared to controls without drug.

— the 5-FU cytotoxicity enhancement factor (F) defined as 5-FU IC_{50} without ℓ-FA divided by 5-FU IC_{50} in the presence of ℓ-FA (F was calculated for each ℓ-FA concentration tested).

— the maximal 5-FU cytotoxicity enhancement factor (F_{max}) defined as the plateau of the sigmoid curve fitting the evolution of F as a function of ℓ-FA concentrations. The F_{max} values allowed FA-sensitive cell lines ($F_{max} > 1$) to be distinguished from FA-resistant cell lines ($F_{max} = 1$).

— [ℓ-FA]90 defined as the ℓ-FA concentration allowing 90% of F_{max} to be reached.

— the 5-FU IC_{50} in the presence of [ℓFA]90, obtained by dividing the 5-FU IC_{50} value by F_{max}.

—[CH_2FH_4]90 and [$CH_2FH_4 + FH_4$]90 defined as the reduced folate concentrations allowing 90% of F_{max} to be reached, based on the fitting (rectangular hyperboles) of F as a function of intracellular reduced folate concentrations.

— the maximal reduced folate concentrations (maximal folates) observed in cells grown in the presence of the highest ℓ-FA concentration tested (300 μM).

— the accumulation factor of reduced folates, defined as the ratio of maximal folate concentration divided by the basal concentration (basal concentrations below the detection limit were thus considered equal to 0.3 pmol/mg protein).

For statistics, non-parametric tests were used. Correlations were analysed using the Spearman rank test. For clinical investigations, the distribution of reduced folates was compared between the three localisations by means of the Kruskal–Wallis test. The comparison of folate concentrations between tumoral and normal tissues was made using the Wilcoxon paired test. The limit of significance was $P = 0.05$. Statistical analyses were done on Statgraphics Plus software (Uniware, Paris, France).

RESULTS

Investigations on cell lines

Investigations without ℓ-FA supplementation. There was a wide variability in spontaneous sensitivity to 5-FU between cell lines, with 5-FU IC_{50} ranging between 0.6 and 25.4 μM (mean 7.4, median 5.2, 1st–3rd quartile 1.8–10.5). Concentrations of intracellular reduced folates measured in cells grown in the folate-controlled medium are shown in Table 1: basal CH_2FH_4 concentrations ranged between not detectable (7 cell lines) and 3.3 pmol/mg protein (1st–3rd

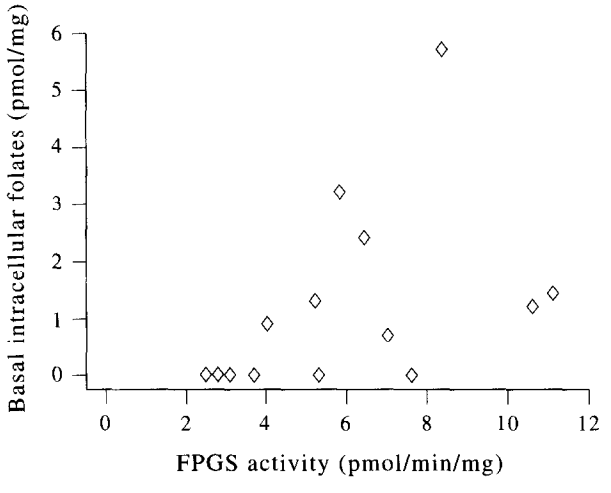

Figure 1. Plot of basal intracellular reduced folate concentration ($CH_2FH_4 + FH_4$) versus FPGS activity for the 14 cell lines grown in the folate-controlled medium (Spearman rank correlation: $r = 0.60$, $P = 0.030$). For each parameter, the mean values of three independent experiments are plotted (see Table 1 for standard errors).

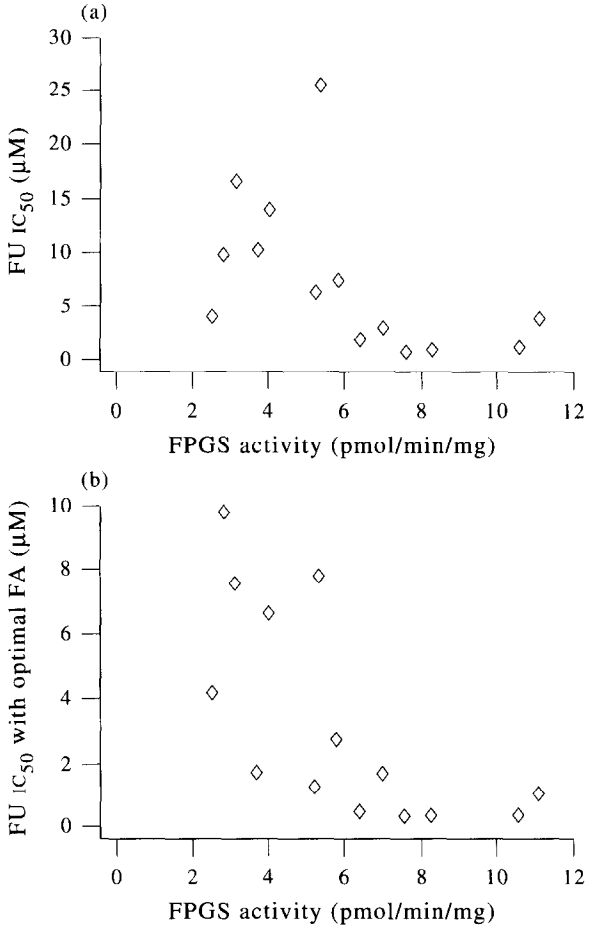

Figure 2. Plot of 5-FU IC_{50} in the absence of ℓ-FA (a) or in the presence of [ℓ-FA]90 (b) as a function of FPGS activity for the 14 cell lines investigated (Spearman rank correlation: $r = -0.68$, $P = 0.014$ (a); $r = -0.78$, $P = 0.004$ (b). For each parameter, the mean values of three independent experiments are plotted (see Table 1 for standard errors).

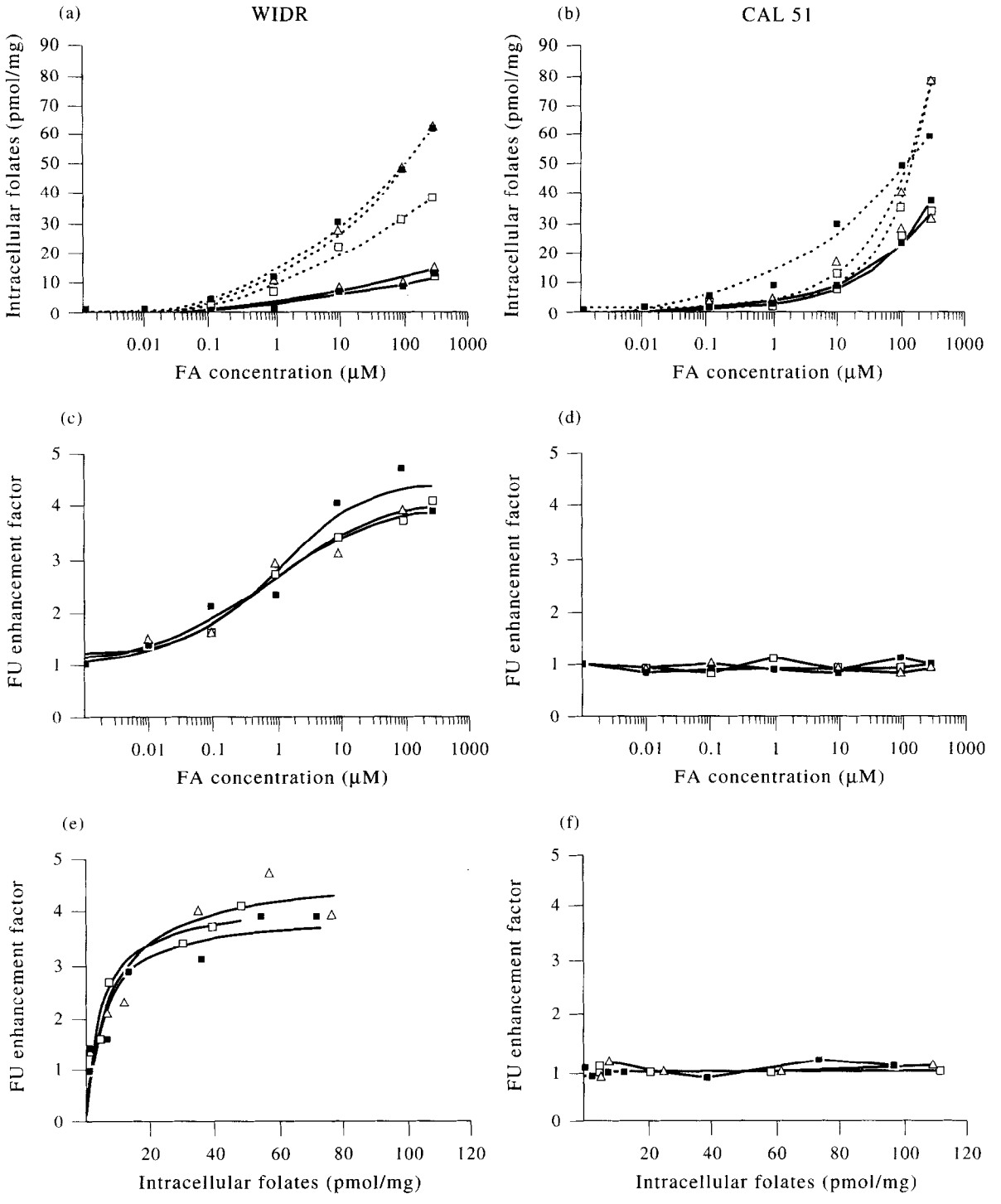

Figure 3. (a,b) Plot of intracellular FH_4 (broken lines) and CH_2FH_4 (solid lines) concentrations as a function of the ℓ-FA concentration applied in the culture medium. The fitting was calculated according to exponential curves. (c,d) Plot of the 5-FU enhancement factor (F = 5-FU IC_{50} divided by 5-FU IC_{50} in the presence ℓ-FA) as a function of the ℓ-FA concentration applied in the culture medium. The fitting was calculated according to sigmoid curves. (e,f) Plot of the 5-FU enhancement factor as a function of the intracellular reduced folate concentration ($CH_2FH_4 + FH_4$). The fitting was calculated according to rectangular hyperboles. Each figure shows three independent experiments, each symbol representing the mean value of one experiment (□, first experiment; ■, second experiment; △, third experiment).

quartile 0–0.8) and the sum ranged between not detectable (6 cell lines) and 5.7 pmol/mg protein (1st–3rd quartile 0–1.4). FPGS activity measured in the cells grown in the folate-controlled medium ranged between 2.5 and 11.1 pmol/min/mg protein (mean 6.0, median 5.6, 1st–3rd quartile 3.7–7.6). A significant positive correlation was demonstrated between the sum of basal reduced folates and the FPGS activity ($r = 0.60$, $P = 0.030$, Figure 1). The link between basal FPGS activity and 5-FU sensitivity was examined. Interestingly, the higher the FPGS activity, the

lower the 5-FU IC_{50} ($r = -0.68$, $P = 0.014$, Figure 2a) and thus the better the sensitivity to 5-FU. No significant relationship was found between intrinsic 5-FU sensitivity and basal CH_2FH_4 concentrations, nor the sum. 5-FU sensitivity was not related to the cell doubling time. Also, the FPGS activity was independent of the cell doubling time.

Investigations with ℓ-FA supplementation. Among the 14 cell lines investigated, 12 were sensitive to ℓ-FA supplementation ($F_{max} > 1$, meaning that 5-FU cytotoxicity was enhanced) and two cell lines were resistant to ℓ-FA supplementation ($F_{max} = 1$, meaning that 5-FU cytotoxicity was not increased) (Table 1). The evolution of intracellular reduced folates as a function of ℓ-FA concentrations followed an exponential law, both in FA-sensitive and FA-resistant cell lines. This observation is illustrated in Figure 3a for one FA-sensitive (WIDR) and Figure 3b for one FA-resistant cell line (CAL 51). At the maximal ℓ-FA concentration tested, no saturation was observed in the pattern of intracellular folate accumulation for all investigated cell lines. The maximal sum of folates ranged between 18.5 and 106.3 pmol/mg protein (median 57.6) and was independent of the FPGS activity. The accumulation factor of reduced folates was between 8 and 355 (median 73).

Figure 3(c, d) and (e, f) illustrate evolution of the 5-FU enhancement factor (F) as a function of ℓ-FA concentrations and intracellular reduced folate concentrations, respectively. In FA-sensitive cell lines, the 5-FU enhancement factor reached a plateau despite the fact that ℓ-FA concentrations or intracellular reduced folates increased continuously. In the 12 FA-sensitive cell lines, F_{max} values ranged between 1.9 and 6.4 (Table 1). The [ℓ-FA]90 for 5-FU modulation showed marked variability between cell lines, from 0.7 to 107.9 μM (mean 21.6, median 1.9, 1st–3rd quartile 1.4–50). In contrast, the $[CH_2FH_4]90$ value was much less variable between cell lines, ranging between 7.6 and 38.3 pmol/mg protein; the median value was 24.8 pmol/mg protein (mean 25.3, 1st–3rd quartile 19.3–32.5). No correlation was demonstrated between [ℓ-FA]90 and $[CH_2FH_4 + FH_4]90$.

Considering all cell lines, the 5-FU IC_{50} values in the presence of [ℓ-FA]90 were inversely correlated to the FPGS activity ($r = -0.78$, $P = 0.004$, Figure 2b). No relationship was demonstrated between the 5-FU IC_{50} values in the presence of [ℓ-FA]90 and the levels of $[CH_2FH_4 + FH_4]90$. No relationship was demonstrated between F_{max} and either FPGS activity, basal reduced folate concentration or maximal sum of folates. In addition, we observed no relationship between F_{max} and intrinsic 5-FU sensitivity (5-FU IC_{50}). The [ℓ-FA]90 values required for 5-FU modulation were not correlated to the FPGS activity in investigated cell lines. However, the lower the basal reduced folate concentrations, the higher the ℓ-FA concentrations required for optimal 5-FU modulation ($r = -0.69$, $P = 0.030$).

Investigations on tumoral biopsies from patients

Intratumoral concentrations of reduced folates were measured in biopsies taken from patients before treatment. Table 2 summarises the results obtained from 50 head and neck tumours, 16 colorectal tumours and 30 liver metastases from colorectal cancers. The pattern of distribution of reduced folates was significantly different between the three tumour sites ($P = 2 \times 10^{-7}$). The median value of the $CH_2FH_4 + FH_4$ concentration was 3.8, 5.8 and 12.1 pmol/mg protein for head and neck carcinoma, colorectal carcinoma and liver metastases, respectively. Of 17 head and neck cancer patients, reduced folates showed a similar pattern of distribution in the tumour and in the normal tissues; in the normal tissue, $CH_2FH_4 + FH_4$ concentrations ranged between 1.8 and 16.8 pmol/mg protein (mean 4.4, median 3.7, 1st–3rd quartile 2.3–4.4).

In head and neck tumours and colon tumours, $CH_2FH_4 + FH_4$ concentrations were always below the median $[CH_2FH_4 + FH_4]90$ value found *in vitro* in the present study for 5-FU modulation (25 pmol/mg protein). Even though higher folate concentrations were present in liver metastases, only 19% of liver biopsies exhibited $CH_2FH_4 + FH_4$ concentrations greater than 25 pmol/mg protein. FPGS activity measured in the 16 colon tumours ranged between not detectable and 215.5 pmol/min/mg protein (mean 91.9, median 93, 1st–3rd quartile 39–139). In the 30 liver biopsies, FPGS activity ranged between not detectable and 223.7 pmol/min/mg protein (mean 89.9, median 80.2, 1st–3rd quartile 68–110). FPGS activity was not correlated with $CH_2FH_4 + FH_4$ concentrations, either in the colon or the liver biopsies.

DISCUSSION

Experimental conditions were designed to be as close as possible to the situation encountered in patients. In this regard, the chosen schedule for drug exposure reflected current clinical protocols [1, 8, 27]. Also, prolonged exposure favours the polyglutamylation of reduced folates [19]. Since the biological activity of racemic FA is supported by the natural ℓ-FA [28], all experiments were conducted with pure ℓ-FA. The efficient 5-FU and ℓ-FA concentrations found here were located within the range of plasma concentrations observed in patients treated with a similar administration schedule [27] and this validates, to some extent, the present *in vitro* model. Particular attention was given to the physiologically compatible folate concentration in the cul-

Table 2. Clinical investigations (pmol/mg protein)

	Head and neck carcinoma ($n = 50$)		Colorectal carcinoma ($n = 16$)		Liver metastases from colorectal carcinoma ($n = 30$)	
	CH_2FH_4	$CH_2FH_4 + FH_4$	CH_2FH_4	$CH_2FH_4 + FH_4$	CH_2FH_4	$CH_2FH_4 + FH_4$
Mean	2.0	4.8	3.2	6.1	9.1	18.0
Median	1.5	3.8	3.3	5.8	6.6	12.1
Min–max	ND–8.2	ND–17.7	1.4–5.1	2.3–12.0	1.1–47.9	1.7–118.5
1st–3rd quartile	0.6–2.7	2.4–5.9	2.3–4.1	4.3–7.3	3.7–9.7	6.8–18.7

ND, not detectable, i.e. <0.3 pmol/mg protein.

ture medium (20 nM) [29]. Overall, the aim of the present experimental investigations was to understand the role of folates and FPGS enzyme activity with regard to 5-FU sensitivity. In cell lines grown at low folate concentration (folate-controlled medium without ℓ-FA supplementation), present data show that the higher the FPGS activity, the greater the basal concentration of reduced folates (Figure 1). This observation confirms previous investigations reporting that intracellular retention of reduced folates is increased when folates show more polyglutamation [19]. Alternatively, when cells were exposed to high folate concentrations (folate-controlled medium with ℓ-FA supplementation), the total concentrations of intracellular $CH_2FH_4 + FH_4$ were no longer dependent on the FPGS activity. Although the polyglutamate forms of CH_2FH_4 and FH_4 were not measured in the present study, one could suggest a saturation of FPGS activity under high ℓ-FA concentrations in the culture medium. Such a saturation could lead to a decreased formation of long polyglutamate chains and consequently to lower cellular retention of intracellular folates as suggested by previous experimental data from Houghton and associates [30] and Zhang and Rustum [31]. In addition, the long polyglutamate chains of CH_2FH_4 are known to enhance strongly the stability of the ternary complex formed between TS, FdUMP and CH_2FH_4 and thus increase 5-FU efficacy compared to intermediate or short polyglutamate chains [20]. So far, two experimental studies have suggested the importance of FPGS with regard to 5-FU sensitivity [22, 32]. The present study demonstrates, for the first time and on a large panel of cancer cell lines, that intrinsic 5-FU sensitivity (without ℓ-FA supplementation) is significantly linked to the FPGS activity in conditions with (Figure 2b) and withouth FA supplementation (Figure 2a). However, intrinsic 5-FU sensitivity (without ℓ-FA supplementation) was not linked to the basal concentration of reduced folates. Also, in cells grown in the presence of [ℓ-FA]90, 5-FU sensitivity was independent of the $[CH_2FH_4 + FH_4]90$. Thus, the fact that 5-FU sensitivity was linked to FPGS activity but not to the intracellular concentration of reduced folates suggests that the importance of folate polyglutamylation in 5-FU sensitivity is probably due to stabilisation of the ternary complex more than to its effect on folate retention. In order to understand 5-FU sensitivity at the target cell level at least in part, it is suggested that the quality of the folates, i.e. the distribution of polyglutamate chain length, could be more relevant than the global reduced folate concentration.

From the present data, it appears that the pattern of folate accumulation in the two FA-resistant cell lines (one duodenum and one breast) was the same as that observed in the 12 FA-sensitive cell lines. This means that whatever the FA sensitivity of the cell line or its basal FPGS activity, intracellular reduced folate concentrations continuously increased without any evidence of saturation up to 300 μM of ℓ-FA added in the culture medium. Based on a large panel of human cancer cell lines, our observation confirms previous data obtained from a limited number of preclinical models [33–36]. It thus appears that the intracellular reduced folate concentration achieved inside the cells is not a limiting factor for 5-FU potentiation by FA. Therefore, increasing the intracellular folate concentrations above a certain threshold will not further improve the 5-FU cytotoxicity probably because maximal TS inhibition has already been reached. We further examined the possible factors which could explain the variability of 5-FU potentiation under FA supplementation between cell lines. Only two cell lines out of 14 were resistant to FA supplementation and the 5-FU IC_{50} values of these two FA-resistant cell lines (4.1 and 9.7 μM) were close to the median 5-FU IC_{50} value of the whole panel of cell lines (5.2 μM). It can thus be hypothesised that the group of FA-resistant cell lines presently investigated was too small to demonstrate the previously shown relationship between F_{max} (maximal 5-FU cytotoxicity enhancement factor) and intrinsic 5-FU sensitivity [23]. However, it is noteworthy that among the whole panel of investigated cell lines, these two FA-resistant lines were the two expressing the lowest FPGS activity (2.5 and 2.8 pmol/min/mg protein). Therefore, although the correlation between FPGS activity and F_{max} on the whole panel of cells was not significant ($P = 0.11$), the role of FPGS activity for FA responsiveness under 5-FU therapy merits further analysis. The use of an RT-PCR method requiring a small quantity of tumoral tissue would be well suited to this purpose [37].

In our previous work performed on 17 cell lines [23], a tremendous variability was shown in [ℓ-FA]90 values (range 0.05–200 μM). Present data confirm the variability in [ℓ-FA]90 values (range 0.7–108 μM). It must be emphasised that these concentrations are markedly higher than the physiological concentrations of reduced folates encountered in human plasma [29]. ℓ-FA concentrations in the range 1–10 μM are observed in patients receiving high-dose FA [27], thus the wide variability in [ℓ-FA]90 is in favour of high-dose FA administration in order to achieve high folate concentrations in plasma and counteract this pharmacodynamic variability, as well as the pharmacokinetic variability. In support of this, a recent study performed on head and neck cancer patients receiving cisplatin–5-FU–FA therapy has shown that non-responding patients exhibited significantly lower plasma concentrations of ℓ-FA + ℓ-5-methyltetrahydrofolate than partial and complete-responding patients [27]. In contrast to the 154-fold range variability found here for [ℓ-FA]90, the levels of $[CH_2FH_4 + FH_4]90$ required for 5-FU modulation were quite close between cell lines, showing a 5-fold range variability (7.6–38.3 pmol/mg protein). This discrepancy between extracellular and intracellular data could be due to differences in the folate transport capacity between cell lines. In fact, previous studies have reported the complexity of the folate transport system, which involves a high-affinity cellular folate transport system [38], as well as a membrane-binding protein [39] and a cytosolic folate-binding protein [40].

In the literature, data on reduced folate measurement in human tumours are very scarce [41–43]. The present study provides new data on a set of 96 tumour samples covering localisations usually treated 5-FU: head and neck carcinoma ($n = 50$), colorectal cancer ($n = 16$) and liver metastases from colorectal cancer ($n = 30$). In contrast to our *in vitro* data showing no significant difference in reduced folates between localisations (Table 1), the distribution of basal reduced folates in tumoral biopsies from patients was significantly different according to the tumour localisation. Lower folate concentrations were found in head and neck tumours (median value of the sum = 3.8 pmol/mg protein). In this set of head and neck cancer patients, we did not demonstrate any difference in the folate distribution between normal and tumoral tissues. Since head and neck

cancer patients are known to suffer from denutrition and folate deficiency due to alcoholism [44], one could suspect a systemic folate deficiency to be the cause of the low folate levels found. We did not have the opportunity to measure folate concentrations in serum and red blood cells. However, it would be of interest to investigate further the link between systemic and tumoral folate levels in the different 5-FU-treated localisations. The highest tumoral folate concentrations were observed in liver metastases. This observation could suggest the influence of the local environment, since the liver contains high folate levels. If we consider the median $[CH_2FH_4 + FH_4]90$ value (25 pmol/mg protein), present clinical data show that all head and neck tumours and primary colorectal tumours were located below this optimum *in vitro* concentration. This information explains and reinforces the need for FA supplementation in order to optimise 5-FU efficacy in the clinical setting.

In conclusion, these data strengthen the rationale of 5-FU modulation by FA and demonstrate the importance of the FPGS enzyme for 5-FU responsiveness. This potentially promising determination is currently under investigation in our institute in an attempt to identify 5-FU ± FA responding patients [45].

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