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Wide Range for Optimal Concentration of Folinic Acid in Fluorouracil Modulation—Experimental Data on Human Tumour Cell Lines

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The clinical use of the fluorouracil (FU)–folinic acid (FA) combination is hampered by the still open choice of the optimal schedule, with marked controversy as concerns the optimal FA dose. This *in vitro* study on FU–FA combinations in 17 human cancer cell lines, representative of tumour types responding to FU–FA treatment, reassesses the notion of the optimal FA concentration. Cells were exposed for 5 days to various FU–FA concentrations (0.07–77 μM, 14 concentrations, for FU; and 0.0025–100 μM for FA). The growth inhibition was assessed by the MTT test. The investigated cell lines exhibited FU IC_{50} ranging from 0.4 to 38.9 μM (median 3.7 μM). In six out of 17 cell lines investigated, the addition of FA did not result in a substantial enhancement of FU cytotoxicity (group 1). For the remaining 11 cell lines responding to FA supplementation (group 2), the maximal enhancement factor ranged from 3 to 8, meaning that in the presence of optimal FA concentration, the efficient FU concentration (IC_{50}) was reduced by between 3 and 8 as compared to the efficient FU concentration without FA supplementation. For cell lines responding to FA supplementation, the optimal FA concentrations ranged from 10^{-7} to 4×10^{-4} M (4000-fold range) with a median value at 9.6×10^{-7} M. Distribution of cell doubling time was not significantly different between group 1 and group 2. In contrast, the FU IC_{50} were significantly different ($P = 0.02$) between group 1 (median 7.4 μM) and group 2 (median 2.2 μM), thus indicating that cell lines with the greatest FU cytotoxicity enhancement by FA were those intrinsically sensitive to FU and *vice versa*.
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INTRODUCTION

ENHANCEMENT OF fluorouracil (FU) cytotoxicity by folinic acid (FA) is based on an optimal inhibition of thymidylate synthase (TS), due to an increase of the intracellular pool of 5-10-methylenetetrahydrofolate which, in turn, stabilises the inactive complex formed between TS and fluorodeoxyuridine monophosphate (FdUMP) [1, 2]. So far, FU–FA protocols have proven their clinical interest both in terms of improvement of response rate [3] and overall survival [4]. Interestingly, recent clinical trials have demonstrated an advantage for FU–FA as

compared to FU alone in terms of disease-free survival for colorectal cancer patients given adjuvant treatment [5]. However, the clinical use of FU–FA is hampered by the still open choice of the optimal schedule, with marked controversy as concerns the optimal FA dose. The question of the optimal FA dose is justified by the toxicity of FU–FA treatment, which was shown to be linked to the FA dose in both preclinical studies on laboratory animals [6] and in the clinical situation [7]. In addition, the cost of folinic acid is far from negligible [8]. Experimental studies from different groups concur as regards the view that 1–10 μM FA are necessary for obtaining the best efficacy when using the FU–FA combination [9,10]. Such concentrations are clinically achievable with high FA doses, i.e. 500 mg/m^2/day in continuous infusion over 5 days [11]. So far, results of clinical trials do not give a consensus regarding the need for high FA doses in order to optimise FU–FA treatment. For instance, the GISG Group [12] has reported a significant improvement in response rate with FU–FA protocol in patients receiving 500 mg/m^2 FA (2-h infusion), but not in patients receiving 25 mg/m^2 FA (10-min infusion). In contrast, other investigators found an equivalent response rate and a superior quality of life with a low FA dose [20 mg/m^2/day, intravenous (i.v.) bolus] as compared to a high one (200 mg/m^2/day, i.v. bolus) in the FU–FA protocol [13]. FA pharmacokinetic knowledge shows that at 25 mg/m^2(i.v. bolus), folate blood concentrations fall rapidly far below the micromolar range [14]. The FU–FA preclinical studies conducted so far, exploring the effect of FA concentration, were performed on one or two tumour cell lines [9,10] thus rendering it difficult to extrapolate the results from the bench to the bedside.

The aim of the present study was to undertake an *in vitro* study on FU–FA combinations by covering a large panel of 17 human cancer cell lines, representative of the tumour types responding to FU–FA treatment. The main objective of the study was to reassess the notion of the optimal FA concentration, with particular attention being paid to the cell-to-cell variability in the sensitivity to FU–FA treatment.

MATERIALS AND METHODS

Chemicals

All chemicals including MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] and Dl-5-methyltetrahydrofolate were obtained from Sigma Chemical Co. (St Quentin Fallavier, France). FU was kindly provided by Roche Laboratories (Neuilly, France). FA (racemic form) was obtained from Lederle Laboratories (Rungis, France). Dulbecco's modified Eagle's medium (DMEM) folic acid-free (ref. 041-90648 M), glutamine and fetal bovine serum (FBS) were from Gibco (Paisley, U.K.). Penicillin and streptomycin were from Merieux (Lyon, France).

Cell lines

Seventeen cancer cell lines of human origin were used: four head and heck, six breast and seven various digestive tract carcinomas (origin and growth characteristics are given in Table 1). In order to remain as close as possible to the physiological folate concentration in humans, cells were routinely grown in a folate-free DMEM medium supplemented with 40 nM of dl-5-methyltetrahydrofolate (plus 0.1 mM ascorbic acid for folate stabilisation), 10% FBS, 2 mM glutamine, 50 000 U/l penicillin and 80 μM streptomycin. All experiments were performed in the same medium at least 4 weeks after routine culture in this medium. Cells were grown in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO_2.

Evaluation of the FU-induced growth inhibition

Cells were grown in 96-wells microtitration plates (0.32 cm^2/well). The initial cell density was 5000–7000 cells per well depending on the cell line, in order to allow an exponential growth for the duration of the whole experiment. Twenty-four hours after plating, cells were exposed for 5 days to various FU–FA concentrations (0.07–77 μM, 14 concentrations, for FU; and 0.0025–100 μM for FA). Preliminary investigations (unshown data) were carried out with four FA concentrations for targeting the optimal FA concentration range; definitive experiments were then undertaken with seven FA concentrations. Each experimental condition was performed in sextuplicate. The growth inhibition was assessed by the MTT test [15], 1–2 days after the end of FU–FA exposure. Results were expressed as the relative percentage of absorbance compared to controls without drug. The growth of cells in the presence of FA alone was identical to that of cells without FA. The dose–effect curves were analysed on GraphPad software (ISI, U.S.A.), and the FU concentrations causing a 50% growth inhibition as compared to controls (IC_{50}) were calculated. FU IC_{50} were determined during three independent experiments. For each FA concentration tested, we calculated the factor of FU cytotoxicity enhancement (factor F) defined as FU IC_{50} without FA divided by FU IC_{50} with FA. Plots of the evolution of F as a function of the FA concentration were made according to sigmoid curves. The maximal enhancement of FU cytotoxicity by FA was defined as the plateau of the sigmoid curve (F_{max}). In order to get an accurate evaluation of the optimal FA concentration required for maximal FU modulation, we defined the optimal FA concentrations as those corresponding to 90% of the plateau of the sigmoid curves. FA concentrations were expressed as the racemic form. All calculations were performed on GraphPad software.

Statistics

Statistics were performed on Statgraphics software (Uniware, Paris). Distribution of tested variables (FU IC_{50} and doubling time) were compared between FA responsive tumours ($F_{max} > 2$) and FA non-responsive tumours ($F_{max} < 2$) using the non-parametric Mann–Whitney test. The influence of the tumour origin on F_{max} was analysed according to the non-parametric Kruskal Wallis test.

RESULTS

Table 1 summarises all the results regarding intrinsic FU sensitivity (FU IC_{50}), cell doubling times, F_{max} and optimal FA concentrations, on the whole panel of tested cell lines. Cell doubling times ranged from 1.3 to 6.3 days (mean 2.8 days). The investigated cell line panel exhibited a wide cell-to-cell variability as concerns spontaneous sensitivity to FU, with IC_{50} values ranging from 0.4 to 38.9 μM (median = 3.7 μM). In six of the 17 cell lines investigated, the addition of FA did not result in a substantial enhancement of FU cytotoxicity ($F_{max} < 2$ ranging between 1.0 and 1.6, group 1). For the remaining 11 cell lines responding to FA supplementation, the maximal enhancement factor ranged from 3.0 to 8.1 (group 2), meaning

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Table 1.

Cell line (origin*)	Tumour type	Doubling time (days) Mean ± S.D.	FU IC_{50} (μM) Mean ± S.D.	F_{max}	Optimal FA concentration (M)
MCF 7 (Pr Rochefort)	Breast	1.7 ± 0.2	2.5 ± 1.1	5.9	3.8×10^{-4}
T 47 D (Pr Rochefort)	Breast	4.7 ± 0.6	1.0 ± 0.6	3.6	0.9×10^{-7}
CAL 51	Breast	1.3 ± 0.2	5.1 ± 1.6	1.0	N.E.
ZR 75 (Pr Rochefort)	Breast	2.6 ± 0.8	0.4 ± 0.1	8.1	3.5×10^{-7}
CAL 85–2	Breast	1.6 ± 0.1	2.2 ± 0.6	5.1	1.3×10^{-5}
CAL 120	Breast	2.8 ± 0.0	7.3 ± 2.4	5.6	4.8×10^{-6}
CAL 14	Colon	2.4 ± 0.7	13.4 ± 4.4	1.2	N.E.
WIDR (EORTC)	Colon	1.7 ± 0.8	4.0 ± 0.1	5.2	1.1×10^{-6}
COLO 205 (ATCC) ref CCL 222	Colon	1.9 ± 0.5	0.4 ± 0.2	5.1	2.0×10^{-6}
SW 620 (ATCC) ref CCL 227	Colon	3.7 ± 1.1	3.7 ± 1.4	1.6	7.8×10^{-5}
SW 403 (ATCC) ref CCL 230	Colon	1.9 ± 0.4	0.4 ± 0.2	3.0	1.2×10^{-7}
HSC 66T (ATCC) ref HTB 134	Pancreas	6.3 ± 2.3	31.1 ± 12.9	1.0	N.E.
HUTU 80 (ATCC) ref HTB 40	Intestine	4.1 ± 0.0	9.1 ± 0.9	1.3	N.E.
CAL 33	Head and neck	2.8 ± 0.8	2.7 ± 0.9	5.3	1.4×10^{-7}
CAL 27	Head and neck	4.5 ± 1.4	1.6 ± 0.2	6.0	8.1×10^{-7}
HEP 2 (ATCC) ref CCL 23	Head and neck	2.2 ± 0.3	38.9 ± 2.1	3.9	1.2×10^{-7}
KB (ATCC) ref CCL 17	Head and neck	1.6 ± 0.2	5.8 ± 1.7	1.2	N.E.

*CAL cell lines come from our institute. Pr Rochefort is from INSERM U 148, Montpellier. EORTC, European Organization for Research and Treatment of Cancer; ATCC, American Type Culture Collection (Rockville, MD); N.E., no effect. Mean ± S.D. for FU IC_{50} and doubling time were calculated from three independent experiments. See Material and Methods section for definition of F_{max} (maximal enhancement factor) and optimal folinic acid (FA) concentrations.

that in the presence of optimal FA concentration, the efficient FU concentration (IC_{50}) was reduced by between 3 and 8 as compared to the efficient FU concentration without FA supplementation. The tumoral origin of the investigated cell lines did not significantly influence the FU cytotoxicity enhancement by FA (breast versus head and neck versus digestive tract, $P = 0.22$).

For cell lines responding to FA supplementation, the optimal FA concentrations ranged from 10^{-7} to 4×10^{-4} M (4000-fold range) with a median value at 9.6×10^{-7} M. Figure 1 illustrates in detail typical sigmoid curves showing the evolution of the enhancement factor (F) as a function of FA concentrations applied in the presence of FU. For CAL 51 cells, whatever the FA concentrations tested, FU cytotoxicity was never enhanced by FA. For SW 403 and WIDR cells, the curves significantly fitted a sigmoid, as for all cell lines responding to FA supplementation. The SW 403 cell line is a typical example of FU modulation at low FA concentrations (optimal FA concentration 1.2×10^{-7} M). In contrast, the optimal FA concentration required for WIDR cells is 10 times higher than that required for SW 403 cells.

Distribution of cell doubling time was not significantly different between groups 1 and 2 ($P = 0.84$). In contrast, the FU IC_{50} were significantly ($P = 0.02$) higher in group 1 (median 7.4 μM)

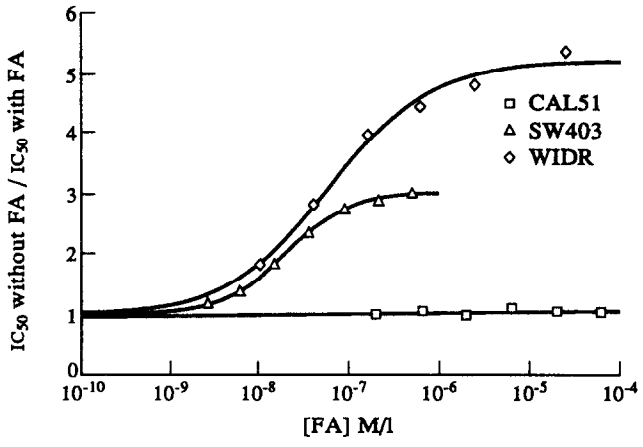

Figure 1. Evolution of the enhancement factor (FU IC_{50} without FA/ FU IC_{50} with FA) as a function of the applied FA concentration, in CAL 51, SW 403 and WIDR cell lines.

than in group 2 (median 2.2 μM), indicating that cell lines with the greatest FU cytotoxicity enhancement by FA were those intrinsically sensitive to FU and vice versa (Figure 2).

DISCUSSION

The experimental design of the present study was chosen in order to minimise the unavoidable gap existing between *in vitro* investigations and the clinical setting. The concentration of reduced folates in the culture medium (20 nM 1,5-methyltetrahydrofolate) reflected the physiological situation in patients [16]. The drug exposure schedule (5-day continuous exposure to FU and FA) was based both on a strong pharmacological rationale, showing that increased duration of exposure to FA enhances the *in vitro* effects of FU due to an accumulation of intracellular polyglutamated reduced folates [9, 17], and on protocols used in the clinic [4, 18]. The spectrum of human cell lines investigated was representative of FU-FA clinically responsive cancers [3]. It must be stressed that the human cell lines used in the present study had never been manipulated for inducing artificial resistance to FU, and thus expressed a spontaneous sensitivity to FU. The median FU IC_{50} value was 3.67 μM (477 ng/ml), which is a concentration in the range of

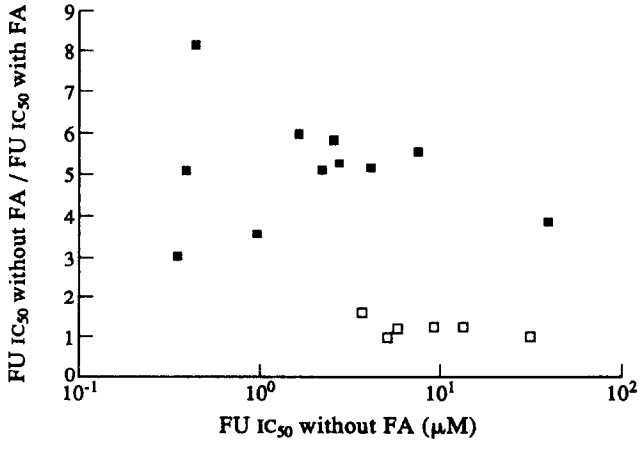

Figure 2. Plot of intrinsic FU sensitivity (FU IC_{50}) versus enhancement factor in FA non-responsive cell lines (group 1, open square) and FA responsive cell lines (group 2, filled square).

those measured at steady state in plasma of patients receiving a 5-day continuous infusion [19].

Three major conclusions can be drawn from the present study. First, the human derived tumour cell lines exhibited a very large spectrum of sensitivity to FA supplementation, with a enhancement factor ranging from 1 (no FU modulation) up to 8. Park and colleagues [20] conducted a similar *in vitro* FU–FA study focused on 11 human colorectal carcinoma cell lines. As compared to the present data, the authors observed a more modest effect of FA on FU modulation. However, their experimental design (culture medium containing high folate concentration) precluded firm conclusions to be drawn from the FA effects. From a practical point of view, the present results lead to the following question: are there pertinent tumour indicators allowing FU–FA-responsive tumours to be identified? Basal TS activity was analysed (unpublished data) and was not significantly different between FA responsive cell lines (group 2) and FA non-responsive cell lines (group 1). The cell capacity to proliferate, estimated by the doubling time, was not correlated to F_{max}. Since it was demonstrated that the longer the reduced folate polyglutamate forms, the more effective the FU cytotoxicity [21], the folylpolyglutamate synthetase (FPGS) activity could thus be a potential predicting factor for identifying FU–FA responsive tumours. This hypothesis was tested by Houghton and colleagues [22] on human colon adenocarcinoma transplanted in immune depressed mice. These authors observed that non-responsive tumours to FU–FA combination were those unable to expand the intracellular pool of polyglutamate forms. Nevertheless, no relationship was found between FPGS activity and the intracellular pattern of reduced folate polyglutamate forms. However, a clear-cut role of FPGS activity as an indicator for FU-FA sensitivity cannot be ruled out, since the authors based their conclusion on four tumours only. From the present study, comparison of FA responsive cell lines (group 2) with FA non-responsive cell lines (group 1) demonstrated a significant link with intrinsic FU sensitivity (FU IC_{50}). This suggests that the FU sensitive tumours are the best candidates for FA supplementation. This finding concurs with the results of a recent clinical trial conducted in recurrent or metastatic colorectal cancer patients previously treated by FU, and whose progressing disease was treated in second line by an FU–FA combination [23]; no evidence of FU–FA efficacy was observed in these patients.

The second conclusion is that FU cytotoxicity enhancement by FA, when existing, is a saturable phenomenon related to the applied FA concentration. Houghton and coworkers [6] examined a series of factors that may influence the antitumour efficacy of FU–FA combinations in four human derived colon adenocarcinoma xenografts transplanted in the mouse. The authors paid particular attention to the FA doses administered, which ranged from 50 to 1000 mg/m^2 (i.v. bolus). The growth inhibitory effects of FU were enhanced by the highest FA doses in three tumours and by a low FA dose in one. In a similar study, Wright and colleagues [24] treated EMT-6 mammary adenocarcinoma bearing mice with FU and FA at three different FA doses (45, 90 and 180 mg/kg/injection × 8 over 48 h). Likewise, tumour growth rates were significantly lower from controls receiving FU alone for the highest FA doses only. One could argue from these two studies that the chosen experimental designs, with only three tested FA doses, did not permit a saturable FA effect to be demonstrated. From the present study, it clearly appears that above a FA concentration threshold depending on each cell line and leading to a maximal FU

modulation, further increases in FA concentration do not result in a further enhancement of FU cytotoxicity. On HCT-8 cells exposed to increased FA concentrations, Zhang and Rustum [10] measured the intra-cellular amount of short- and long-chain reduced folate polyglutamates, since long-chain polyglutamates are preferentially retained within the cells and lead to an optimal TS inhibition. They found that above an FA concentration threshold of 1 μM, there was a down-shift from long- to short-chain reduced folate polyglutamate forms in the cells. This observation corroborated a saturable effect in the modulation of fluorodeoxyuridine by FA [10]. Although their data were based on a single cell line, it can be suggested that the saturation of FA effects observed in the present study could be due, at least in part, to a saturation in the formation of intracellular long-chain reduced folate polyglutamates.

The third conclusion concerns the very wide cell-to-cell variability for the optimal FA concentrations required for maximal FU modulation. In fact, for the 11 cell lines responding to FA supplementation, a 4000-fold range in optimal FA concentrations was observed. This result makes the recommended 10 μM optimal FA concentration rather peremptory. Moreover, this recommendation was based on two experimental studies [9,10], each performed on one cell line only, thus making the extrapolation of these observations hazardous. The present data confirm that for some cell lines (CAL 85-2, SW 620, MCF 7), FA concentrations in the range of 10–380 μM are necessary for an optimal FU cytotoxicity enhancement. On the other hand, it is evident that for other cell lines (T47D, SW 403, CAL 33, HEP2), optimal FA concentrations are at least 100 times less (0.1 μM). It must be stressed that plasma concentrations in the range of 0.1 μM can be achieved after i.v. administration of low FA doses (25 mg/m^2) [14]. It is thus not surprising that in the clinical situation some authors have claimed that response rates are not different between low and high FA doses associated with FU [13], whereas others have claimed that high FA doses are necessary for an optimal modulation of FU [12]. In line with the present experimental data, one explanation for this discrepancy could lie in intrinsic tumour characteristics (intra-cellular reduced folates, FPGS activity . . .), making certain tumours sensitive to low FA supplementation when, for others, higher FA concentrations are necessary.

To conclude, it is undeniable that FU–FA protocols have proven their antitumour efficacy in different human cancers. It is hoped the present data may contribute to resolving the controversy between high FA doses and low FA doses defenders, and may shed light on the notion of the right optimal FA dose for a given tumour. In this sense, further complementary investigations remain to be carried out in order to select reliable tumour markers for FU–FA sensitivity.

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