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A Role for Dihydropyrimidine Dehydrogenase and Thymidylate Synthase in Tumour Sensitivity to Fluorouracil

A. Beck, M.C. Etienne, S. Chéradame, J.L. Fischel, P. Formento, N. Renée and G. Milano

Despite being one of the oldest anti-cancer drugs, fluorouracil (FU) is still being increasingly used in cancer chemotherapy. The source of variability for FU sensitivity in patients may be complex, although an overproduction of thymidylate synthase (TS) was the only mechanism of resistance identified in tumours from FU-resistant patients. Dihydropyrimidine dehydrogenase (DPD) is the first and rate-limiting enzyme of FU catabolism. Thus, DPD activity may be a potential factor for controlling FU responsiveness. A panel of 19 human tumour cell lines, including digestive tract, breast and head and neck cancer cells, were investigated. Both TS and DPD activities were measured in parallel to FU responsiveness. None of the cell lines had been previously exposed to FU, and thus expressed a spontaneous sensitivity to FU. Sensitivity between cell lines showed marked differences, with IC_{50} values ranging from 45 ng/ml (colon cell line) to 5063 ng/ml (head and neck cell line). TS activity was measurable in all cell lines and varied within a 46-fold range. DPD activity was detected in all but four cell lines, showing a 100-fold range of variation. Cell lines most sensitive to FU exhibited the lowest DPD and TS activities and vice versa. Simple linear regression analysis showed that both TS ($r^2 = 0.22$, $P = 0.042$) and DPD ($r^2 = 0.27$, $P = 0.022$) activities were significantly correlated to FU effectiveness (log 10 IC_{50}): the greater the enzyme activities, the higher the FU IC_{50}. TS and DPD were demonstrated to be independent variables. A multiple regression analysis showed that the combination of TS and DPD activities explained 36% of the variability in FU IC_{50} ($r^2 = 0.36$, $P = 0.01$). Two groups of cell lines could be identified, one group with both low TS and low DPD activities (G1), and the other with either high TS and/or high DPD activities (G2). Mean FU IC_{50} values were 193 and 930 ng/ml in G1 and G2, respectively, and this difference in FU sensitivity was highly significant ($P = 0.009$). The present study shows, for the first time, that DPD activity in tumour cells is an independent factor significantly related to FU sensitivity. These results should encourage DPD and TS coupled measurements in tumours of patients before FU treatment in order to establish their prognostic relevance. DPD and TS measurements could also be used during the treatment course to determine the implication of these enzymes in the development of tumour resistance to FU.
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INTRODUCTION

DESPITE BEING one of the oldest anti-cancer drugs, fluorouracil (FU) is still increasingly used in cancer chemotherapy. FU is not only considered the standard drug for the treatment of advanced colorectal cancer, but is also one of the major drugs in the treatment of carcinoma of the oral cavity [1] and breast [2]. Most of the current clinical protocols including FU comprise one or more of the so-called FU biomodulators [3], of which folinic acid is the most frequently used [4]. The overall clinical response rate to FU, even in association with biomodulators, remains rather low [5]. Moreover, biomodulation is not particularly specific to the tumour and, in general, host toxicity increases with the improvement of response rate [6]. There is thus a potential interest in early individual identification of FU-responsive tumours.

FU cellular activation includes several enzyme pathways [7, 8] leading to at least three well-identified cellular targets which are thymidylate synthase (TS), RNA and DNA. On an experimental basis, resistance to FU has been extensively studied, and occurs at different levels [9–13]. Thus, the source of variability for FU sensitivity in the patients may be complex. Several studies have suggested or demonstrated that overproduction of TS was related to FU resistance in treated patients [2, 14–16], although elevation in the amount of TS is not unanimously recognised as a determinant factor for FU resistance [17–19]. Moreover, in attempts to explain resistance, most attention has been paid to the FU activation routes. Surprisingly, the role of FU catabolism at the cellular target level has been so far neglected.

Dihydropyrimidine dehydrogenase (DPD) is the first and rate-limiting enzyme of the chain of reactions which regulate FU

Correspondence to G. Milano.
The authors are at the Centre Antoine Lacassagne, 36 Voie Romaine, 06050 Nice cedex 1, France.
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catabolism. Naguib and colleagues [20] studied DPD activity in various human tissues and in human tumour xenografts. They found that the enzyme activity was high in the human liver and, interestingly, very variable in the tumours. These underlying differences in tumoral DPD activity induce a variable FU degradation prior to FU engagement in the anabolic pathway. Thus, DPD activity may be a potential factor for controlling FU sensitivity. It was our goal to test this hypothesis *in vitro*. On a panel of 19 different human tumour cell lines, we measured both TS and DPD activities in parallel to FU responsiveness. All the cell lines had never been previously exposed to FU, and thus exhibited a spontaneous sensitivity. The investigated cell lines were representative of the spectrum of FU-treated human malignancies and comprised digestive tract cancer, breast cancer and head and neck cancer.

MATERIALS AND METHODS

Chemicals

All chemicals including MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide], dl-tetrahydrofolic acid and dl-5-methyltetrahydrofolate were obtained from Sigma Chemical Co (St Quentin Fallavier, France) and were of the highest purity available. FU, dihydrofluorouracil (FUH2), α-fluoro β-ureidopropionic acid (FUPA), and α-fluoro β-alanine (FBAL) were kindly provided by Roche Laboratories (Neuilly, France). [^{14}C]FU labelled at position 6 (55 Ci/mol) was obtained from Amersham (U.K.). [^{3}H]dUMP labelled at position 5 (16 Ci/mmol) was from Moravek Biochemicals (Brea, California, U.S.A.). DMEM medium (folic acid free, Ref 041-90648 M), glutamine and foetal bovine serum (FBS) were from Gibco (Paisley, U.K.). Penicillin and streptomycin were from Merieux (Lyon, France). The 5–10 methylenetetrahydrofolate (mTHF) was prepared from tetrahydrofolic acid according to the method initially described by Moran and colleagues [21].

Cell lines

Nineteen cancer cell lines of human origin were used: five head and neck, six breast and eight various digestive tract carcinoma (origin and growth characteristics are given in Table 1). All experiments were performed in a folate-controlled medium, i.e. folate-free DMEM 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. Cells were routinely cultured in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO_2.

Enzymatic activities

For determination of TS and DPD activities, cells were grown in 75-cm^2 flasks (initial density 3×10^5–2×10^6 cells per flask) for 3–4 days and trypsinised when reaching 75% of confluence. In each case, cytosolic proteins were quantified according to the Bradford assay (Bio-Rad SA, Ivry/Seine, France) using bovine γ globulin as standard.

DPD activity. DPD activity was measured according to the method described by Harris and colleagues [22]. A cell suspension (5×10^6–10×10^6 cells/ml) was prepared in 35 mM sodium phosphate buffer pH 7.5 containing 10% glycerol. This cell suspension was then centrifuged (5 min, 250 *g*) and the cells were stored at −80°C (cell pellet plus supernatant) without impairment of DPD activity. On the day of the assay, the cell suspension was freeze–thawed three times and centrifuged for 30 min at 28 000 *g* (4°C). The supernatant was kept on ice until assayed (within 15 min). The assay consisted of incubating 50 μl of the supernatant (i.e. 250 000–500 000 cells) with [^{14}C]FU (20 μM final), NADPH (250 μM final) and $MgCl_2$ (2.5 mM final). Total volume was 125 μl (in 35 mM sodium phosphate buffer pH 7.5 containing NaN_3). The duration of incubation was 30 min at 37°C. The reaction was stopped by addition of 125 μl ice-cold ethanol followed by 30 min storage at −20°C. The samples were centrifuged (400 *g*, 5 min) to remove proteins and the supernatant was analysed for the presence of [^{14}C]FUH_2, [^{14}C]FBAL and [^{14}C]FUPA, using a previously-reported high-pressure liquid chromatography method [23]. Detection was performed using a radioactive flow monitor (LD 506 Berthold, Wildbad, Germany). DPD activity was calculated by taking into account the sum of FUH2, FBAL and FUPA peaks. DPD activity was expressed as nmol of [^{14}C]FU catabolised per min and per mg of protein. Each sample was assayed in duplicate and DPD activity was measured during two independent experiments. The sensitivity limit was 0.002 nmol/min/mg protein. The stability of DPD activity during storage, evaluated by the inter-assay reproducibility (pooled cell suspension) gave a coefficient of variation of 12% ($n = 8$).

TS activity. TS activity was measured according to the tritium-release assay described by Spears and Gustavsson [24] with a modification concerning the concentration of substrate ([^{3}H]dUMP). Analysis of the different protocols described in the literature showed that final concentrations of dUMP ranged between 40 nM [24] and 100 000 nM [25]. The results we obtained when performing the assay at these different concentrations revealed that TS activity continuously increases as a function of the dUMP concentration, and thus a plateau in TS activity was not reached even at 100 000 nM. The K_m values determined on CAL33, CAL51 and CAL27 were 0.46, 0.53 and 0.52 μM, respectively. In addition, a very strong correlation was demonstrated between TS activities measured at 100 and 1000 nM dUMP, respectively (nine cell lines investigated, TS measured at 1000 nM = 5.8 × TS measured at 100 nM, $r = 0.99$, $P < 0.0001$). On this basis, and despite being below the K_m value, TS measurement was performed at the suboptimal concentration of 110 nM [^{3}H]dUMP instead of 40 nM as previously described by Spears and Gustavsson [24]. Linearity with proteins was assessed in CAL 85-2 and PANC 3 cells in the range of 0.1–0.94 mg/ml. Based on this observation, TS measurements were performed with a fixed cell concentration of 4×10^6 cells/ml in all cell lines. The cell suspension (4×10^6 cells/ml) was made in 50 mM Tris–HCl buffer pH 7.3 containing 2 mM dithiothreitol. After sonication on an ice bed (three times at 10 s intervals), the cell suspension was immediately centrifuged at 100 000 *g* for 30 min (4°C). At this step, cytosols were stored in liquid nitrogen, without impairment of TS activity. The assay consisted of incubating 25 μl of cytosol (i.e. 10^5 cells) with [^{3}H]dUMP (110 nM final concentration) and mTHF (0.62 mM final concentration) in a total volume of 55 μl (in the previous buffer). After 0, 5, 10, 20 and 30 min of incubation at 37°C, the reaction was stopped on ice. The excess of [^{3}H]dUMP was removed by adding 300 μl of activated charcoal (15%) containing 4% trichloracetic acid (5 min centrifugation at 14 000 *g*, room temperature). The $^{3}H_2O$ formed during the incubation was then counted in an aliquot of 150 μl of the above supernatant. Results were expressed as nmoles of $^{3}H_2O$ formed per min per mg of protein, based on the linear regression obtained from the incubation times. Each incubation time was performed in quad-

ruplicate and TS determinations were at least duplicated (two or three independent experiments). The sensitivity limit was 100 fmol/min/mg protein. The stability of TS activity during storage, evaluated through the interassay reproducibility over 5 weeks (single-use aliquots of a pooled cytosol) gave a coefficient of variation of 7% (n = 5).

Evaluation of the FU-induced growth inhibition

Cells were grown in 96-well microtitration plates (0.32 cm^2/well). The initial cell density was 5000–7000 cells per well in order to allow an exponential growth for the duration of the whole experiment. Twenty-four hours after plating, cells were exposed to various FU concentrations (10–10 000 ng/ml, 14 concentrations) for 5 days. Each concentration was performed in sextuplicate. The growth inhibition was assessed by the MTT test [26] 1–2 days after the end of FU exposure. Results were expressed as the relative percentage of absorbance compared to controls without drug. 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.

Statistics

Linear regression, multiple regression and Student's tests were performed on Statgraphics software (Uniware, Paris, France). Analysed variables were log 10 IC_{50}, TS activity, DPD activity and doubling time.

RESULTS

Table 1 shows FU sensitivity and enzyme activities for the whole cell line panel. The cell doubling times varied from 1.3 days (CAL 51) to 6.3 days (PANC 3). FU sensitivity between cell lines showed marked differences, with IC_{50} values ranging from 45 ng/ml (COLO 4) to 5063 ng/ml (ORL 1). No correlation was demonstrated between IC_{50} values and cell doubling times. TS activity was measurable in all cell lines and varied within a 46-fold range. DPD activity was detected in all but four cell lines within a 100-fold range of variation. The variability in FU IC_{50}, TS and DPD activities encountered within cell lines was explained by the fact that experiments were repeated at distance in time. It must be emphasised that, globally, the variability within cell lines was much lower than the variability between cell lines.

Cell lines most sensitive to FU exhibited the lowest DPD and TS activities and vice versa. Simple linear regression analysis showed that both TS (r^2 = 0.22, P = 0.042) and DPD (r^2 = 0.27, P = 0.022) activities were significantly correlated to FU effectiveness (log 10 IC_{50}): the greater the enzyme activities, the higher the FU IC_{50} (Figure 1). TS and DPD were demonstrated to be independent variables (linear regression, P = 0.55). For example, COLO1, COLO2 and PANC 3 exhibited comparable TS activities whereas they expressed different FU sensitivities (IC_{50} between 50 and 4051 ng/ml); a close look at their DPD activities explains this apparent discrepancy: the most resistant cell line (PANC 3) exhibited the greater DPD activity and the most responsive cell line (COLO1) had very low DPD activity. A multiple regression analysis showed that the combination of TS and DPD activities explained 36% of the variability in FU IC_{50} (r^2 = 0.36, P = 0.01); Figure 2 illustrates the fitting between predicted and experimental IC_{50}. The overall variability in observed FU IC_{50} showed a 100-fold range (1.6–3.6 in the log scale) whereas, considering predicted FU IC_{50}, the variability was reduced to a factor of 10 maximum.

Table 1. Fluorouracil sensitivity and enzyme activities for different cell lines

Cell line	Origin	Doubling time Mean ± S.D. (days)	TS (10^{-5} nmole/min/mg protein)	DPD (nmole/min/mg protein)	FU IC_{50} Mean ± S.D. (ng/ml)
Breast					
MCF7	Pr. Rochefort	1.7 ± 0.2	48 (11/46/88)	N.D. (N.D./N.D.)	321 ± 147
T47D	Pr. Rochefort	4.7 ± 0.6	102 (43/181/84)	0.004 (0.004/0.003)	125 ± 71
CAL51	C.A.L.	1.3 ± 0.2	568 (479/658)	0.202 (0.107/0.298)	657 ± 209
ZR75	Pr. Rochefort	2.6 ± 0.8	170 (200/140)	0.005 (0.005/0.005)	56 ± 17
CAL85-2	C.A.L.	1.6 ± 0.1	480 (438/521)	0.135 (0.137/0.132)	280 ± 72
CAL120	C.A.L.	2.8 ± 0.0	405 (424/386)	0.014 (0.013/0.016)	953 ± 314
Digestive tract					
CAL 14	C.A.L.	2.4 ± 0.7	1054 (1236/871)	N.D. (N.D./N.D.)	1741 ± 570
WIDR	E.O.R.T.C.	1.7 ± 0.8	108 (90/126)	0.003 (0.003/0.004)	525 ± 17
CAL 124	C.A.L.	1.6 ± 0.6	243 (235/251)	0.014 (0.014)	103 ± 75
COLO1	A.T.C.C. ref CCL222	1.9 ± 0.5	68 (82/54)	0.004 (0.004/0.005)	50 ± 21
COLO2	A.T.C.C. ref CCL227	3.7 ± 1.1	38 (62/15)	N.D. (N.D./N.D.)	477 ± 182
COLO4	A.T.C.C. ref CCL230	1.9 ± 0.4	147 (218/77)	N.D. (N.D./N.D.)	45 ± 20
INT1	A.T.C.C. ref HTB40	4.1 ± 0.0	650 (533/767)	0.095 (0.094/0.095)	1178 ± 119
PANC3	A.T.C.C. ref HTB134	6.3 ± 2.3	23 (12/34)	0.378 (0.374/0.382)	4051 ± 1673
Head and neck					
CAL33	C.A.L.	2.8 ± 0.8	839 (777/902)	0.126 (0.125/0.128)	352 ± 122
CAL27	C.A.L.	4.5 ± 1.4	565 (605/524)	0.116 (0.115/0.117)	207 ± 22
ORL1	A.T.C.C. ref CCL23	2.2 ± 0.3	821 (820/822)	0.119 (0.116/0.122)	5063 ± 271
ORL2	A.T.C.C. ref CCL17	1.6 ± 0.2	367 (303/431)	0.030 (0.033/0.027)	756 ± 222
ORL3	A.T.C.C. ref CCL138	1.8 ± 0.3	184 (201/167)	0.057 (0.055/0.058)	149 ± 131

N.D., non-detectable (below sensitivity limit); S.D., standard deviation for at least three separate experiments.
Cell line origins: Pr Rochefort (INSERM U 148) is at Montpellier, France; CAL is our Institute; ATCC is the American Type Culture Collection (Rockville, Maryland, U.S.A.).

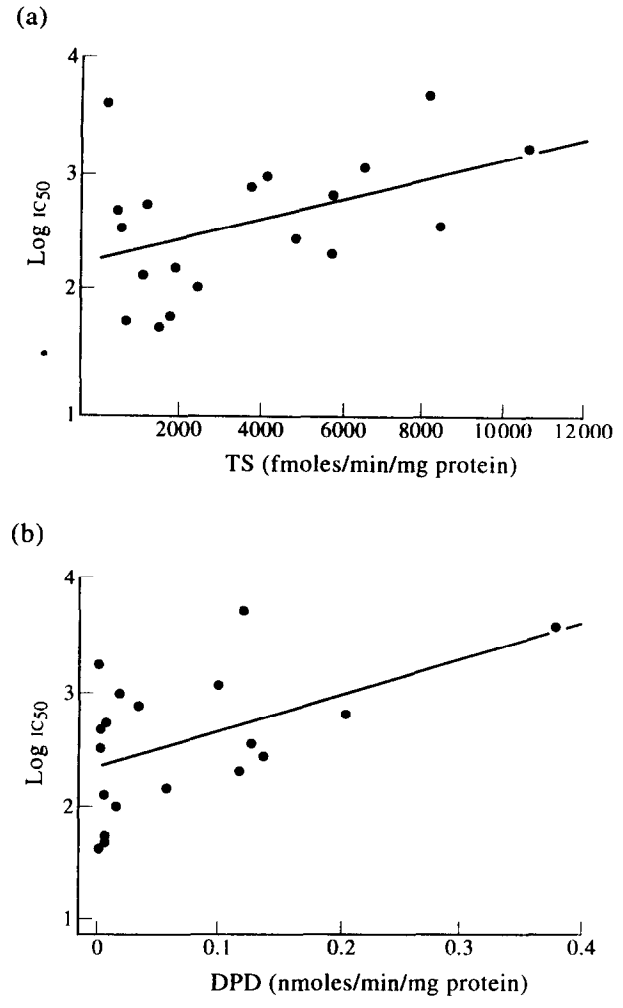

Figure 1. (a) Linear regression for log IC_{50} as a function of TS activity (log IC_{50} = 2.24 + 0.0009 TS, r = 0.47, P = 0.042, n = 19). (b) Linear regression for log IC_{50} as a function of DPD activity (log IC_{50} = 2.35 + 3.22 DPD, r = 0.53, P = 0.022, n = 19).

Figure 3 illustrates that TS and DPD were independent variables. Based on this plot, two groups of cell lines could be identified: one group (G1) with both low TS and low DPD ($<400 \times 10^{-5}$ and <0.1 nmol/min/mg protein, respectively), and the other (G2) with either high TS or high DPD or both. Mean FU IC_{50} were 193 and 930 ng/ml in G1 and G2 groups, respectively (Figure 4). This difference in FU sensitivity was highly significant between G1 and G2 groups (Student's test, P = 0.009).

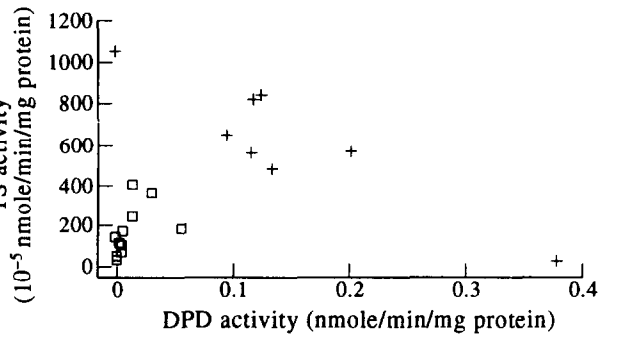

Figure 3. Plot of TS activity versus DPD activity. Open squares correspond to G1 group and pluses correspond to G2 group.

DISCUSSION

The experimental conditions underpinning the present study were established in order to minimise the unavoidable gap existing between *in vitro* investigations and the clinical setting. The presence of folates in the culture medium was controlled (20 nM 1,5-methyltetrahydrofolate), and thus reflected the physiological situation in patients [27]. The spectrum of human cell lines investigated was representative of FU clinically-responsive cancers. Contrary to other investigators studying FU resistance on experimental tumours obtained by selection pressure during more or less prolonged FU exposure [10, 12, 19], 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.

This study underlines the potential clinical importance of TS in tumour resistance to FU. This was suggested by earlier *in vitro* studies showing that either overexpression of TS protein (FdUMP binding assay or western blot analysis) [28, 29] or TS activity [28] were associated with FU resistance. However, until now, the relationship between TS expression and FU sensitivity has not been demonstrated on such a large number of clinically-relevant human tumour cell lines. Interestingly, recent clinical studies confirm the potential interest of tumoral TS determination in relation to FU responsiveness and/or survival in the patients [15, 16, 30].

The present data concur with the findings of Naguib and colleagues [20] who reported measurable and variable DPD activities in human tumour xenografts. These authors found a

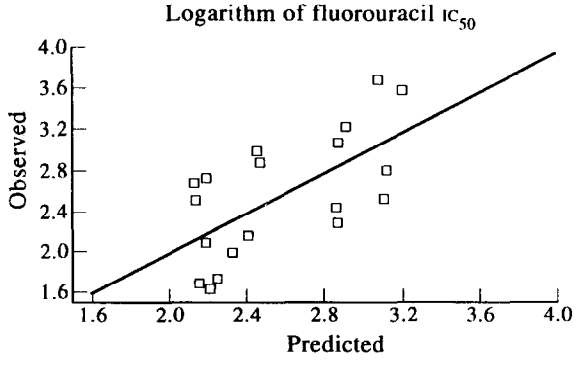

Figure 2. Predicted FU IC_{50} versus observed FU IC_{50} from the multiple regression model: log IC_{50} = 2.09 + 0.0008 TS + 2.86 DPD (F ratio = 6.04, r = 0.60, P = 0.01, n = 19).

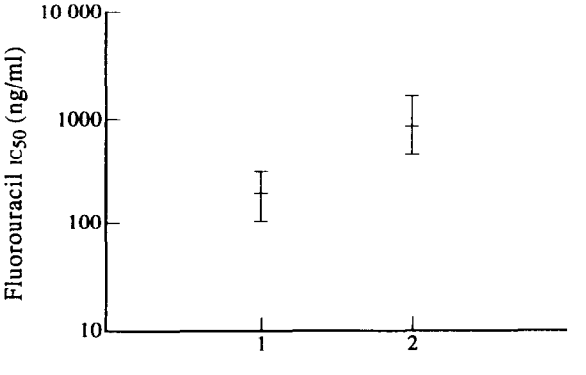

Figure 4. Plot of the 95% confidence interval for FU IC_{50} in G1 (1) and G2 (2) groups respectively (Student's test on log 10 IC_{50}: G1 versus G2, P = 0.009).

25-fold range of variation in DPD activity between tumours; here a 100-fold range was observed and, for the first time to our knowledge, it was established that a relationship does exist between DPD activity in tumour cells and FU sensitivity. Thus, FU catabolism has a relevant place at the target cellular level, with marked variability, which may account for the variability in tumoral response. It was beyond the scope of the present study to determine the FU metabolite profile in the cells. Likewise, it was not the aim of the study to characterise the DPD isozymic forms suggested by Naguib and colleagues [20].

From the present data, it appears that DPD and TS are independent and complementary markers for FU sensitivity. Consideration of both DPD and TS activities distinguished between FU-sensitive cells (low DPD and TS activities) and FU-resistant cells (high TS or DPD activities, or both). The group of sensitive cell lines exhibited a mean FU IC_{50} at 200 ng/ml as compared to approximately 1000 ng/ml in the group of resistant cell lines. Interestingly, FU concentrations encountered in plasma of patients receiving a 5-day infusion of FU (1 g/m^2/day) are in the range of 137–359 ng/ml (mean ± S.D.) [31]. Thus, the fitting between the plasma concentrations achieved in patients and the efficient concentrations for cytotoxicity in FU sensitive cells strengthens the validation of the chosen *in vitro* model.

The most insensitive cell lines were PANC 3 (IC_{50} = 4051 ng/ml) and ORL 1 (IC_{50} = 5063 ng/ml). PANC 3 exhibited the highest DPD activity (0.378 nmol/min/mg protein), while its TS activity was among one of the lowest. Alternatively, ORL 1 exhibited an average value for DPD activity, but the highest TS activity of the whole cell panel. Noteworthy, among the human tumour xenografts explored by Naguib and colleagues [20], the highest DPD activity was expressed in a tumour of pancreatic origin. In the clinic, pancreatic cancer shows a very low response rate to FU. One explanation for this FU resistance could lie in the high capacity of pancreatic tumour cells to catabolise FU. This suggests a potential interest in using DPD inhibitors as FU biomodulators. Such an approach has already been tested by using thymidine, which inhibits DPD activity [32], but the use of thymidine was hampered by the fact that this nucleoside may resume FU cytotoxicity at the DNA level. Yee and colleagues have recently shown that interferon α-2a was able to inhibit DPD activity in leucocytes of patients under interferon α-2a treatment [33]. Moreover, in human tumour cell lines, we previously observed a dose- and time-dependent inhibition of DPD activity by interferon α (unpublished data). Naguib and colleagues [34] have recently identified 5-benzyloxybenzyluracil as a strong inhibitor of DPD activity which enhanced the efficacy of FU against human colon tumour cells.

We conclude that DPD activity is a promising and independent marker of FU sensitivity in human tumours tested *in vitro*. The present data should encourage DPD- and TS-coupled measurements in tumours of patients before FU treatment in order to establish their prognostic relevance. Preliminary TS investigations in patients appear interesting [15, 16, 30]. Longitudinal DPD and TS measurements (if possible at all) could provide an insight into the relevance of these enzymes in the development of tumour resistance. Finally, an active search for DPD inhibitors may provide an attractive approach for FU biomodulation.

1. Dreyfuss AI, Clark JR, Wright JE, *et al.* Continuous infusion high-dose leucovorin with 5-fluorouracil and cisplatin for untreated stage IV carcinoma of the head and neck. *Ann Int Med* 1990, **112**, 167–172.
2. Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989, **7**, 890–899.
3. Grem JL. Fluorinated pyrimidines. In Chabner BA, Collins JM, eds. *Cancer Chemotherapy, Principles and Practise*. Philadelphia, PA, Lippincott, 1990, 180–225.
4. Poon MA, O'Connell MJ, Wieand HS, *et al.* Biochemical modulation of fluorouracil with leucovorin. Confirmatory evidence of improved therapeutic efficacy in advanced colorectal cancer. *J Clin Oncol* 1991, **9**, 1967–1972.
5. Advanced colorectal cancer meta-analysis project. Modulation of fluorouracil in patients with advanced colorectal cancer. Evidence in terms of response rate. *J Clin Oncol* 1992, **10**, 896–903.
6. Johnson PWM, Thompson PI, Seymour MT, *et al.* A less toxic regimen of 5-fluorouracil and high-dose folinic acid for advanced gastrointestinal adenocarcinomas. *Br J Cancer* 1991, **64**, 603–605.
7. Diasio RB, Harris BE. Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokin* 1989, **16**, 215–237.
8. Parker WB, Cheng YC. Metabolism and mechanism of action of 5-fluorouracil. *Pharm Ther* 1990, **48**, 381–395.
9. Reichard P, Sköld O, Klein G, Revesz L, Magnusson PH. Studies on resistance against 5-fluorouracil. Enzymes of the uracil pathway during development of resistance. *Cancer Res* 1962, **22**, 235–243.
10. Ardalan B, Cooney DA, Jayaram HN, *et al.* Mechanisms of sensitivity and resistance of murine tumors to 5-fluorouracil. *Cancer Res* 1980, **40**, 1431–1437.
11. Houghton JA, Maroda SJ, Phillips JO, Houghton PJ. Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinoma in mice. *Cancer Res* 1981, **41**, 144–149.
12. Mulkins MA, Heidelberg C. Biochemical characterization of fluoropyrimidine-resistant murine leukemic cell lines. *Cancer Res* 1982, **42**, 965–972.
13. Wang FS, Aschele C, Sobrero A,Chang YM, Bertino JR. Decreased folylpolyglutamate synthetase expression: a novel mechanism of fluorouracil resistance. *Cancer Res* 1993, **53**, 3677–3680.
14. Clark JL, Berger SH, Mittelman A, Berger FG. Thymidilate synthase gene amplification in a colon tumor resistant to fluoropyrimidine chemotherapy. *Cancer Treat Rep* 1987, **71**, 261–265.
15. Johnston PG, Fischer E, Rockette HE, Fischer B, Wolmark N, Allegra CJ. Thymidylate synthase expression is an independent predictor of survival/disease free survival in patients with rectal cancer. *Proc ASCO* 1993, **202**, 599.
16. Lenz HJ, Leichman C, Danenberg P, *et al.* Thymidylate synthase gene expression predicts response of primary gastric cancer. *Proc ASCO* 1993, **199**, 585.
17. Peters GJ, Laurensse E, Leyva A, Lankelma J, Pinedo HM. Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5'-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res* 1986, **46**, 20–28.
18. Aiba K, Allegra C, Park J, Chabner B. Multifactorial resistance of 5-fluorouracil in human colon cancer cell lines. *Proc AACR* 1990, **31**, 424.
19. Aschele C, Sobrero A, Faderan MA, Bertino JR. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules. *Cancer Res* 1992, **52**, 1855–1864.
20. Naguib FNM, El Kouni AM, Cha S. Enzymes of uracil catabolism in normal and neoplastic human tissues. *Cancer Res* 1985, **45**, 5405–5412.
21. Moran RG, Spears CP, Heidelberger C. Biochemical determinants of tumor sensitivity to 5-fluoro-2' deoxyuridilate, 2' deoxyuridilate and thymidilate synthase. *Proc Natl Acad Sci USA* 1979, **76**, 1456–1460.
22. Harris BE, Song R, Soong S, Diasio RB. Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. *Cancer Res* 1990, **50**, 197–201.
23. Sommadossi JP, Gewirtz DA, Diasio RB, Aubert C, Cano JP, Goldman ID. Rapid catabolism of 5-fluorouracil in freshly isolated rat hepatocytes as analyzed by high-performance liquid chromatography. *J Biol Chem* 1982, **257**, 8171–8176.
24. Spears CP, Gustavsson BG. Methods for thymidylate synthase pharmacodynamics: serial biopsy, free and total TS, FdUMP and dUMP, and H_4 Pteglu and CH_2-H_4 Pteglu assays. In Rustum Y,

McGuire JJ, eds. *The Expanding Role of Folates and Fluoropyrimidines in Cancer Chemotherapy*. New York, Plenum Press, 1988, 97–104.
25. Armstrong RD, Diasio RB. Improved measurement of thymidylate synthetase activity by a modified tritium-release assay. *J Biochem Biophys Meth* 1982, **6**, 141–147.
26. Carmichael J, De Graff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semi-automated colorimetric assay in assessment of chemosensitivity testing. *Cancer Res* 1987, **47**, 936–942.
27. Kones R. Folic acid, 1991. An update with new recommended daily allowances. *Southern Med J* 1990, **83**, 1454–1458.
28. Van der Wilt CL, Pinedo HM, Smid K, Peters GJ. Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumors. *Cancer Res* 1992, **52**, 2922–2928.
29. Johnston PG, Drake JC, Trepel J, Allegra CJ. Immunological quantification of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and -resistant human cancer cell lines. *Cancer Res* 1992, **52**, 4306–4312.
30. Horikoshi T, Danenberg KD, Stadlbauer THW, *et al.* Quantification of thymidylate synthase, dihydrofolate reductase and D-T diaphorase gene expression in human tumors using the polymerase chain reaction. *Cancer Res* 1992, **52**, 108–116.
31. Fleming RA, Milano G, Etienne MC, *et al.* No effect of dose, hepatic function, or nutritional status on 5FU clearance following continuous (5-day), 5FU infusion. *Br J Cancer* 1992, **66**, 668–672.
32. Ho DH, Townsend L, Luna MA, Bodey GP. Distribution and inhibition of dihydrouracil dehydrogenase activities in human tissues using 5-fluorouracil as a substrate. *Anticancer Res* 1986, **6**, 781–784.
33. Yee LK, Allegra CJ, Steinberg SM, Grem JL. Decreased catabolism of fluorouracil in peripheral blood mononuclear cells during combination therapy with fluorouracil, leucovorin and interferon α-2a. *J Natl Cancer Inst* 1992, **84**, 1820–1825.
34. Naguib FNM, Hao SN. Potentiation of 5-fluorouracil efficacy by the dihydrouracil dehydrogenase inhibitor, 5 benzyloxybenzyluracil. *Proc AACR* 1993, **34**, 438.

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

A. Beck, M.C. Etienne, S. Chéradame, J.L. Fischel, P. Formento, T. Guillot and G. Milano

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