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Inhibition of dihydropyrimidine dehydrogenase by α-interferon: experimental data on human tumor cell lines

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Abstract. Interferons (IFNs) are very promising fluorouracil (FU) biochemical modulators. The pharmacological origin sustaining the FU-IFN synergistic interaction is not clearly understood. It was recently shown that α-IFN was associated with a dose-dependent decrease in FU clearance in treated patients. Dihydropyrimidine dehydrogenase (DPD) is the key regulating enzyme for FU catabolism. The effects on DPD exerted by both the IFN dose and the duration of exposure were evaluated in a panel of five human cancer cell lines. All cell lines investigated exhibited quantifiable DPD activity with inter-cell-line variability (0.118–0.318 nmol min^{-1} mg $protein^{-1}$). A prolonged exposure to IFN (up to 5 days) was necessary to obtain a significant inhibition of DPD activity. A concentration-dependent significant decrease in DPD activity, reaching 50% of the initial activity determined for the highest IFN concentration (10^5 IU/ml), was demonstrated in all cell lines tested (5-day IFN exposure). For three cell lines, IFN potentiated the FU-induced growth inhibition in a concentration-dependent manner. Considering all cell lines and all IFN concentrations, it appears that globally, the greater the inhibition of DPD activity, the greater the FU potentiation (Spearman rank correlation on all cell lines, $P = 0.011$).

Introduction

Fluorouracil (FU) is increasingly used in the chemotherapeutic treatment of various types of malignant diseases, particularly for advanced colorectal carcinoma, head and neck cancer, and breast cancer. The recent renewal of interest in FU can be explained to a great extent by the coadministration of so-called FU biochemical modulators, which potentiate FU through its different routes of intracellular activation [15, 16]. A typical example is the successful association between FU and folinic acid, the latter increasing the intracellular pool of reduced folates necessary for an optimal blockage of thymidilate synthase [11, 18].

Other promising FU biochemical modulators are interferons (IFNs). After conducting an interesting initial clinical study [24], Wadler et al. [25] confirmed the promising antitumor activity triggered by the FU-IFN combination. Although it is clear that IFNs may exert their own antiproliferative activity [8], numerous experimental investigations have been undertaken to elucidate the pharmacological origin sustaining the FU-IFN synergistic interaction [4, 19, 20, 26]. It thus appears that IFN can inhibit the activity of thymidine kinase and, hence, reduce the utilization of exogenous thymidine by cells [7]. Other data suggest that α-IFN can sensitize cells to FU action by altering the nucleotide pools and causing an increase in DNA double-strand breaks [26]. Also, γ-IFN and α-IFN can inhibit the overexpression of thymidilate synthase that follows FU exposure [4, 20]. More recently, it was reported that α-IFN induced a marked increase in pyrimidine nucleoside phosphorylase activity and thus modulated the conversion of FU into its active forms [19]. In a clinical pilot study, Grem and colleagues [9] made the interesting and original observation that the administration of α-IFN was associated with a dose-dependent decrease in FU clearance. Since then, some authors have confirmed the reduction in FU clearance produced by α-IFN treatment [5], whereas other investigators have not found such an effect [17, 22, 23].

Our group has recently shown the existence of a significant correlation between FU clearance and the activity of dihydropyrimidine dehydrogenase (DPD), the key regulating enzyme for FU catabolism, as measured in peripheral blood mononuclear cells (PBMCs) of cancer patients [6]. It could thus be proposed that the underlying mechanism explaining the effect of IFN on FU pharmacokinetics could be a decrease in DPD activity. This hy-

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pothesis was recently confirmed by the observation of a decrease in the DPD activity measured in PBMCs of patients under α2a-IFN treatment [28]. However, it was not established whether these inhibitory effects of IFN on DPD are due to IFN itself or are mediated by IFN-induced host factors.

The objective of the present study was to examine the effects of IFN itself on DPD activity in a panel of human cancer cell lines in culture. It was also of interest to analyze whether the degree of DPD inhibition induced by IFN might correlate with the extent to which this cytokine potentiated FU cytotoxicity.

Materials and methods

Chemicals. All chemicals, including MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Histopaque (polysucrose and sodium diatrizoate) were obtained from Sigma Chemical Co. (St. Quentin Fallavier, France). FU, dihydrofluorouracil (FUH2), α-fluoro-β-alanine (FBAL), α-fluoro-β-ureidopropionic acid (FUPA), and α2a-IFN (Roferon) were kindly provided by Roche Laboratories (Neuilly, France). 6-[^{14}C]-FU (55 Ci/mol) was obtained from Amersham (UK). Dulbecco's modified Eagle's medium (DMEM), glutamine, and fetal bovine serum (FBS) were supplied by Gibco (Paisley, UK). Penicillin and streptomycin were obtained from Merieux (Lyons, France).

Cell lines. Five cancer cell lines of human origin were used: two from head and neck carcinoma, CAL 27 and CAL 33 [7]; one from a malignant melanoma, CAL 7; one from a breast carcinoma, CAL 51; and one from a pancreatic carcinoma, PANC 3 (HS 766 T, ref ATCC HTB 134; American Type Culture Collection, Rockville, Md.). CAL 7, CAL 27, CAL 33, and CAL 51 came from our institute. All the cell lines were grown in DMEM supplemented with 10% FBS, 50,000 IU penicillin/l, 86 μ*M* streptomycin, and 2 m*M* glutamine. The doubling times for CAL 27, CAL 33, CAL 7, CAL 51, and PANC 3 were 4.5, 2.8, 6.7, 1.3, and 6.3 days, respectively. Cells were routinely cultured at 37° C in a humidified incubator (Sanyo) with an atmosphere containing 8% CO_2.

Effects of IFN on FU growth inhibition. Cells in the exponential growth phase were grown in 96-well microtitration plates (initial cell density, 5,000–7,000 cells/well according to the cell line tested). At 24 h after plating, cells were exposed for 5 days to various IFN-FU combinations (10, 10^2, 10^3, 10^4, and 10^5 IU IFN/ml; 0.08–80 μ*M* FU, 14 concentrations). The growth-inhibitory effects were assessed by the MTT test [3] 1 day after the end of the drug exposure. Absorbance was set at 540 nm and measured on a Titertek Twinreader. Each experimental determination was done in sextuplicate (coefficients of variation, less than 8%). Results were expressed as the relative percentage of absorbance detected in treated cells as compared with untreated control cells. The concentration-effect curves were analyzed on GraphPad software (ISI, USA), and the FU concentrations causing a 50% growth inhibition relative to the control values (IC_{50}) were calculated. Two independent experiments were performed on each cell line.

Effects of IFN on DPD activity. The effects of both IFN concentration and exposure time were examined. For dose-effect experiments, the whole panel of cell lines was investigated. Cells were grown in 25-cm^2 flasks (initial cell density, 200,000–500,000 cells/flask). At 24 h after plating, cells were exposed for 5 days to various concentrations of IFN (10, 10^2, 10^3, 10^4, and 10^5 IU/ml). The effects of IFN exposure times on DPD activity were evaluated in the CAL 33 cell line at 10^5 IU IFN/ml. Five different exposure times were tested: 24, 48, 72, 96, and 120 h. For all experimental conditions tested, after the incubation period the cells were rinsed three times with phosphate-buffered saline (PBS) trypsinized, and centrifuged. A cell suspension (5×10^6–10×10^6 cells/flask) was prepared in 35 m*M* sodium phosphate buffer (pH 7.5) containing 10% glycerol. After 5 min of centrifugation (250 *g*), the cell pellets were stored at –80° C (cell pellet + supernatant).

It must be emphasized that the investigations concerning the effect of IFN concentrations and those concerning the effect of exposure time were done at different times. Thus, the CAL 33 cells used to test the effect of IFN concentration were not at the same passage level as those used to investigate the effect of exposure time.

DPD activity. DPD activity was measured according to the method described by Harris et al. [10]. 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 (final concentration, 20 μ*M*), reduced nicotinamide adenine dinucleotide phosphate (NADPH; final concentration, 250 μ*M*), and $MgCl_2$ (final concentration, 2.5 m*M*). The total volume was 125 μl [in 35 m*M* sodium phosphate buffer (pH 7.5) containing NaN_3]. The duration of incubation was 30 min at 37° C. The reaction was stopped by the addition of 125 μl of ice-cold ethanol followed by 30 min of storage at –20° C. The samples were then centrifuged (400 *g*, 5 min) to remove proteins. As previously observed by other investigators [21], FUH2 was not the only product formed when DPD activity was determined in crude cytosolic extracts of tumor cells; the metabolites FBAL and FUPA were present in addition to FUH2. Thus, the supernatant was analyzed for the presence of [^{14}C]-FUH_2, [^{14}C]-FBAL, and [^{14}C]-FUPA using a previously reported high-pressure liquid chromatography method [22]. 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. Cytosolic proteins were quantified according to the Bradford assay (Bio-Rad, Ivry/Seine, France) using bovine γ-globulin as the standard. DPD activity was expressed as the quantity (in nanomoles) of [^{14}C]-FU catabolized per minute and per milligram 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^{-1} mg $protein^{-1}$. The stability of DPD activity during storage as evaluated by the interassay reproducibility in a pooled cell suspension gave a coefficient of variation of 10% (n = 10).

Statistical analysis. The evolution of DPD activity (percentage of control values, all cell lines) as a function of IFN concentration was tested by Spearman rank correlation. The evolution of DPD activity (percentage of control values, CAL 33 cell line) as a function of IFN incubation time was tested by the Kruskal-Wallis test. For each cell line, the evolution of the FU IC_{50} ($\log_{10}$ IC_{50}) as a function of IFN concentration ($\log_{10}$ concentration) was tested by linear regression analysis. The link between the ratio of the FU IC_{50} value determined in the absence of IFN to the FU IC_{50} value obtained in the presence of IFN and the ratio of the basal DPD activity to the DPD activity determined in the presence of IFN was tested using Spearman rank correlation. All statistical analyses were performed on Statgraphics software (Uniware, Paris).

Results

All cell lines investigated exhibited initial measurable DPD activity with marked intertumor variability; the DPD activity ranged from 0.118 (CAL 27) to 0.318 (CAL 51) nmol min^{-1} mg $protein^{-1}$. Figure 1 shows the curves generated for the effect of IFN concentration on DPD activity as measured in the whole panel of cell lines. In all cell lines tested there was a concentration-dependent decrease in DPD activity after 5 days of IFN exposure (Spearman rank correlation for all cell lines, $P < 10^{-4}$). At the highest IFN concentration tested (10^5 IU/ml) there was a 50% decrease

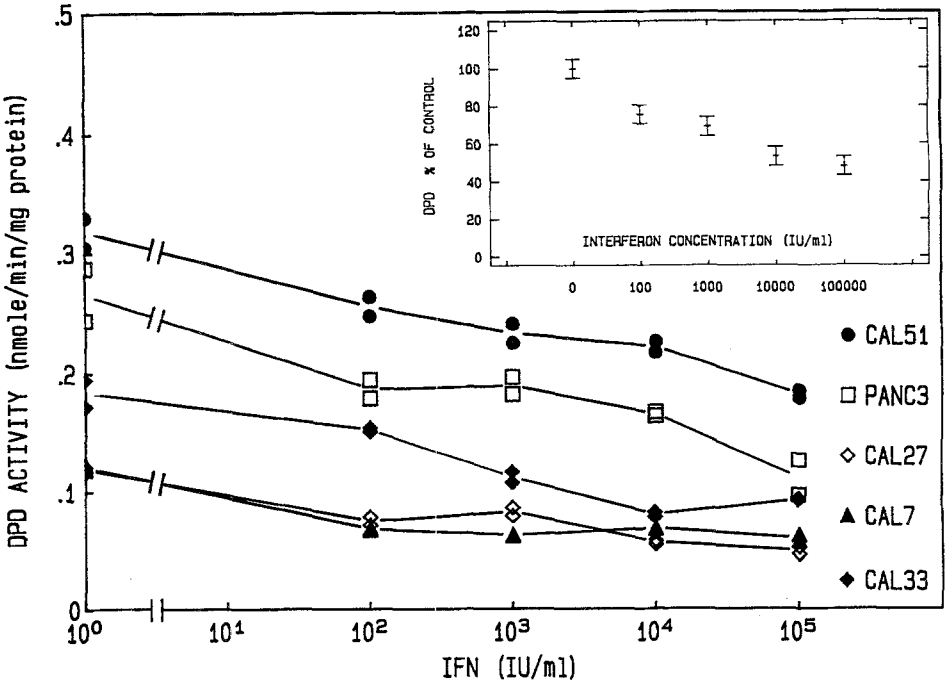

Fig. 1. Effect of the α-2a-IFN concentration on DPD activity as measured in five human cancer cell lines. The DPD values at the x-axis origin refer to initial DPD activities determined in the absence of IFN. *Insert:* Illustration of the significant decrease in DPD activity as a function of IFN concentration (all cell lines; Spearman rank correlation, $P < 10^{-4}$). *Control*, DPD activity measured in cells without exposure to IFN

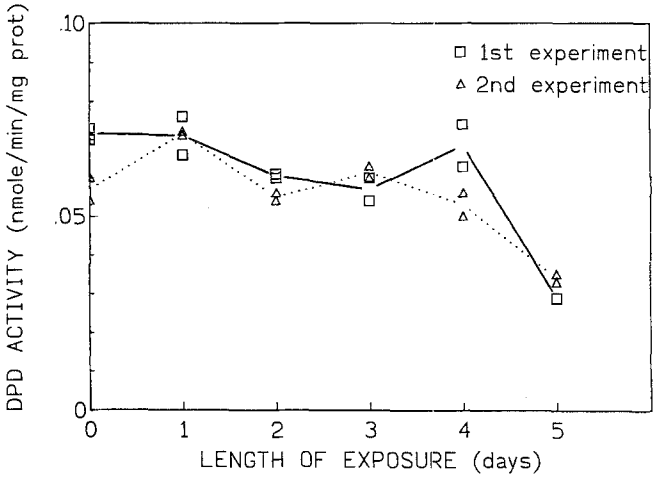

Fig. 2. Effect of α-2a IFN exposure times on DPD activity as measured in CAL 33 cells. The IFN concentration applied was 10^5 IU/ml. The experiment was duplicated. The duration of exposure was significantly linked to DPD activity (Kruskal-Wallis test, 6 levels, $P = 0.025$). This difference comes from the last time point (5-day exposure)

in DPD activity as compared with the initial level. Figure 2 shows the time dependency of the effects of IFN on DPD activity; a prolonged exposure to IFN (up to 5 days) was necessary to obtain a significant inhibition of DPD activity (Kruskal-Wallis test, $p = 0.025$).

Figure 3 shows the evolution of FU concentration-response curves generated for CAL 7 cells in the presence of IFN. It appears that cell exposure to IFN alone leads to growth-inhibitory effects (cell survival values on the x-axis origin). There was a constant shift to the left for the FU concentration-response curves obtained in the presence of increasing concentrations of IFN. It is noteworthy that for the first IFN concentration tested (10^2 IU/ml), which was not cytostatic on its own, there was a marked decrease in the FU IC_{50} value as compared with the experimental condition without IFN. Table 1 summarizes the results, obtained for all cell lines investigated. With the exception of CAL 51 cells, a specific and independent effect of IFN on cellular growth was demonstrated in all cell lines. For CAL 27, CAL 33, and CAL 7, IFN potentiated the FU growth inhibition (decreased FU IC_{50}) in a concentration-dependent manner (Table 1). For CAL 51 cells, IFN had both no growth-inhibitory effect by itself and no action on FU growth inhibition. Another situation was observed for PANC 3 cells, in which IFN inhibited cell proliferation by itself but did not enhance FU growth inhibition. An analysis of the relationship between FU sensitivity (control FU IC_{50}) and initial DPD activity showed that the cells most resistant to FU (PANC 3 and CAL 51) exhibited the highest initial DPD activities (0.265 and 0.318 nmol min^{-1} mg $protein^{-1}$, respectively).

Finally, considering all cell lines and all IFN concentrations, it appears that globally, the greater the inhibition of DPD activity, the greater the FU potentiation (Spearman rank correlation on all cell lines, $P = 0.011$). The significance of this correlation was highly improved the analysis was performed on CAL 27, CAL 33, and CAL 7 cells only (Spearman rank correlation on these three cell lines, $P = 0.003$). In fact, as noted above, no FU potentiation was observed for PANC 3 cells or for CAL 51 cells, although some inhibition of DPD activity occurred in these two cell lines during IFN treatment.

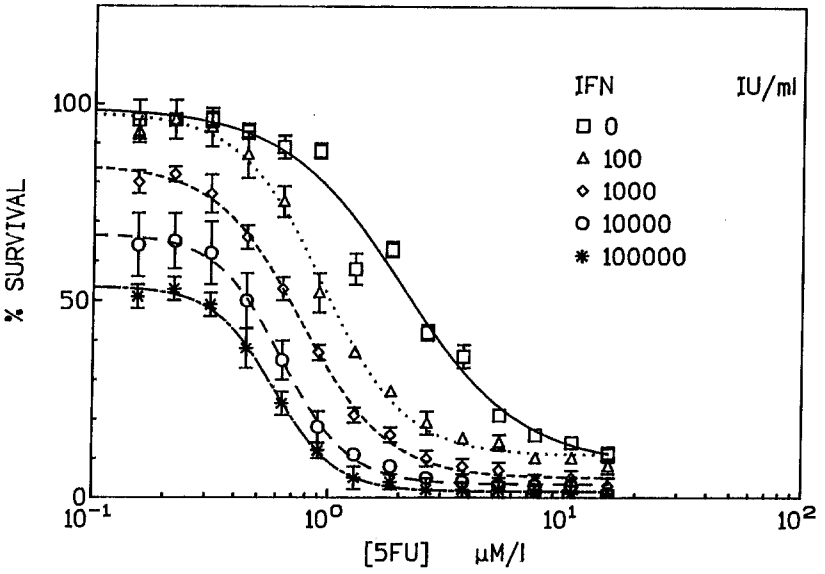

Fig. 3. Effect of α-2a-IFN on FU growth inhibition in CAL 7 cells (experiment A, Table 1). *Vertical bars* indicate standard deviations calculated from six determinations

Table 1. Effect of α-2a-IFN on FU cytotoxicity

Cell line	DPD activity (nmol min^{-1} mg $protein^{-1}$) mean ±SD	FU IC_{50} (µ*M*/l) as a function of IFN concentration IFN (IU/ml)					Statistics[a]
		0	10^2	10^3	10^4	10^5	
CAL 27	0.118±0.002	(A) 0.65	0.40 (97)	0.31 (93)	0.24 (76)	0.28 (65)	$r = 0.98$
		(B) 1.38	0.92 (93)	0.58 (82)	0.42 (69)	0.32 (63)	$p < 0.01$
CAL 33	0.184±0.011	(A) 0.40	0.12 (56)	0.06 (18)	0.06 (15)	0.05 (13)	$r = 0.99$
		(B) 0.45	0.18 (78)	0.12 (62)	0.09 (45)	0.07 (40)	$p < 0.01$
CAL 7	0.122±0.002	(A) 2.07	0.95 (96)	0.71 (87)	0.61 (69)	0.55 (58)	$r = 0.98$
		(B) 1.43	0.77 (101)	0.55 (94)	0.45 (85)	0.47 (68)	$p < 0.01$
CAL 51	0.318±0.012	(A) 6.54	6.24 (102)	5.97 (104)	6.10 (98)	5.83 (97)	$r = 0.158$
		(B) 7.77	7.70 (100)	8.14 (100)	8.00 (100)	7.95 (100)	NS
PANC 3	0.265±0.010	(A) 47.07	70.11 (86)	88.26 (75)	88.25 (70)	89.94 (60)	$r = 0.66$
		(B) 44.50	50.75 (105)	70.30 (90)	45.70 (69)	67.13 (50)	NS

A and B refer to two separate experiments. Data given in parentheses represent the percentage of survival of treated cells as compared with control cells after a 5-day exposure to α-2a-IFN only. NS, Not significant

[a] Linear regression analysis between the $\log_{10}$ FU IC_{50} and the $\log_{10}$ IFN concentration

Discussion

A recent study by Yee et al. [28] showed that the DPD activity measured in patient PBMCs was diminished following a short period of treatment with IFN (1–4 days). For three patients under Roferon treatment, we examined the effects of IFN treatment on the DPD activity measured in PBMCs (data not shown). For these three patients we observed an initial decrease in DPD activity (1st week of treatment) as compared with the DPD activity measured before treatment. However, during prolonged treatment this decrease was not followed by a maintenance of these depressed levels of enzyme activity. However, it could not be established from these data whether the inhibitory effects of IFN on DPD activity were attributable to IFN itself or were mediated by IFN-induced host factors. This consideration prompted Yee et al. [28] to study the effect of IFN treatment on FU catabolism using intact PBMCs. Adding INF-α-2a directly to cytosolic preparations or incubating intact PMBCs for 4 h with INF-α-2a did not affect FU catabolism.

The present study shows for the first time a concentration- and time-dependent inhibition of DPD activity by IFN itself. This is the central finding of the present work. This concentration-dependent inhibition was demonstrated in all five cell lines (of different human tumor origin) investigated. The IFN concentration range tested was selected to cover the IFN serum concentrations previously reported in patients under IFN treatment [27]. A 30% fall in DPD activity was shown at the concentration range observed in patients (100–500 IU/ml, Fig. 1). DPD inhibition was close to 50% at the highest IFN concentration tested. We recently established that the systemic clearance of FU in cancer patients is significantly correlated with the DPD activity measured in PBMCs [6]. Interestingly, Grem et al. [9] showed that the administration of IFN was associated on

average with a 50% reduction in FU clearance. Also, Danhauser et al. [5] demonstrated that FU clearance was reduced by 20%–35% after IFN administration.

Thus, the present results can lead to the conclusion that the variations observed in the DPD activity in PBMCs of patients receiving IFN are due to the action of IFN itself at the cellular level. However, the participation in DPD inhibition of additional IFN-induced host factors cannot be totally excluded. A time dependency of the effects of IFN on DPD activity was observed in the present study (Fig. 2). Interestingly, in all paired samples obtained from IFN-treated patients, Yee et al. [28] noted a modest decrease in FU catabolism as measured in PBMCs after 2 days of IFN treatment; in contrast, the decrease observed on day 4 was pronounced and significant as compared with the baseline DPD activity. This finding is fully in agreement with the present data and strengthens the relevance of the in vitro model used herein. The observation that a refractory period is necessary before the effects of IFN on DPD activity become apparent can have practical clinical consequences for the optimization of IFN-FU protocols. This experimental observation suggests that IFN does not interfere with DPD activity by a direct signal transfer mediated by the IFN cell-membrane receptor. A modification in the regulation of DPD synthesis would seem likely. This lag time is in agreement with previous data showing that α-IFN induces the synthesis of a specific 2.2-kb mRNA coding for a 42,000-Da polypeptide in vitro, the induction of this mRNA necessitating the presence of newly synthesized protein mediator(s) [2]. It must be borne in mind that previous reports have shown that IFNs can alter hepatic drug metabolism (cytochrome P-450 activities) in treated mice [1].

The present observations also raise significant and original arguments in favor of a biochemical modulation of FU activity by IFN. As concerns the biochemical enhancement of FU cytotoxicity, most efforts have focused on the modulation of FU activation routes [15–18]. To date, the catabolic route as a potential tool for modulating FU activity has been neglected, with the exception of the proposed use of thymidine, which is known to inhibit FU catabolism [14]. However, thymidine is delicate to handle in association with FU because it also provides a rescue for FU-induced cytotoxicity. It must be stressed that it was not within the scope of the present study to examine in detail the cellular profile of FU catabolites. Nevertheless, these results may provide a strong basis for discussing the critical role played by the FU catabolic route in FU sensitivity.

First, it appears that measurable DPD activity was found in all the cell lines investigated. These activities were far from negligible since they approached those found in PBMCs [6]. Some investigators have studied the pyrimidine base catabolism in normal tissue and in human tumor cell lines [13]. They found measurable DPD activity in several human extrahepatic tissues and in all tumor cell lines; these activities were generally lower than those found in the present study, and it must be pointed out that the cell line with the highest DPD activity was a cancer cell line of pancreatic origin. In the present study it was also shown that among the panel of cell lines investigated, the cell lines most resistant to FU (PANC 3, CAL 51) were also those exhibiting the highest initial DPD activities. In future clinical studies it would be interesting to evaluate prospectively the tumoral DPD activity as a factor of resistance to FU treatment. In the context of the present study, mention must be made of the investigations by Naguib and Hao [12], who recently identified 5-benzyloxybenzyluracil (BBU) as a strong inhibitor of DPD activity. Interestingly, BBU enhanced the efficacy of FU against human colon tumor cells both in culture and implanted as xenografts in immunodepressed mice.

From the present study, an unavoidable question was to determine whether IFN, by inhibiting DPD activity, would be capable of enhancing FU sensitivity in tumors exhibiting high levels of DPD activity. Such a conclusion could not be reached since although it was shown that IFN induced a decrease in DPD activity associated with a potentiation of FU activity in most of the cell lines tested, this was not found to be the case in CAL 51 cells, in which IFN inhibited DPD activity without increasing the growth-inhibitory effects of FU. It is hoped that the proven modulation of DPD activity by IFN will stimulate further studies aimed at providing a better understanding and a better clinical management of the FU-IFN association.

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