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ORIGINAL ARTICLE

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Impact of different fluorouracil biochemical modulators on cellular dihydropyrimidine dehydrogenase

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Abstract In attempts to increase fluorouracil (FU) activity by pharmacomodulation, most attention has been paid to FU activation pathways without consideration of the presence and possible role of FU catabolism in the target tumoral cell itself. The first step in the catabolism of FU is hydrogenation by the enzyme dihydropyrimidine dehydrogenase (DPD). The purpose of the present study was to test the DPD-inhibitory effects of several agents whose use as FU biomodulators has been clinically established: cisplatin, hydroxyurea, dipyridamole, and allopurinol. Five cancer cell lines of human origin were used. Dipyridamole and hydroxyurea were the only modulators for which an augmentation in FU cell-growth inhibition (MTT test) was clearly evident for the whole panel of cell lines investigated ($P < 1.10^{-4}$ and $P = 0.005$, respectively). With dipyridamole the efficacy of FU was multiplied by a factor of around 5. Allopurinol and cisplatin had no obvious effect on cellular DPD activity (biochemical method). For dipyridamole and hydroxyurea, DPD activity showed a more or less marked concentration-related inhibition according to the cell line tested. Only dipyridamole produced reductions in FU IC_{50} values (50% growth-inhibitory concentrations), i.e., potentiation of FU cytotoxicity, that were significantly related to inhibition of cellular DPD activity. This DPD-mediated interaction between dipyridamole and FU is a new finding that could be important for a better understanding of FU-dipyridamole combination chemotherapy.

Key words Fluorouracil · Dihydropyrimidine dehydrogenase · Cisplatin · Hydroxyurea · Dipyridamole · Allopurinol

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Introduction

The mechanism of action of fluorouracil (FU) is relatively complex and involves several well-characterized biochemical pathways [5]. Consequently, FU targets for cytotoxicity are multiple; among them, DNA, RNA, and thymidylate synthase have been clearly identified [19]. The main objective of the so-called FU pharmacomodulators is to act upon the biochemical network of FU metabolism [23]. Numerous pharmacological agents have been shown to modulate FU cytotoxic activity and often find their way quickly from the laboratory to the patient [18]. Compounds such as uridine or allopurinol have shown promising activity in reducing FU toxicity [7], but most FU modulators are used to enhance the cytocidal effects of the antimetabolite [4]. The capacity of the FU modulators to enhance FU activity has been well established on an experimental basis. However, in contrast, the underlying mechanisms that control FU cytotoxicity enhancement have not been fully determined. This is especially true for α-interferon (α-IFN) [9] and platinum derivatives [20].

Overall, in attempts to increase FU activity by pharmacomodulation, most attention has been paid to FU activation pathways without consideration of the presence and possible role of FU catabolism in the target tumoral cell itself. In eukaryote cells the first step in the catabolism of the pyrimidine bases thymine and uracil is hydrogenation by the enzyme dihydropyrimidine dehydrogenase (DPD). Since FU is a pyrimidine analog, it is metabolized by DPD. We recently reported in vitro data showing that cellular DPD activity is very variable between tumors and, interestingly, is an independent factor significantly related to FU sensitivity [1]. These findings were further confirmed at the clinical level in a group of 62 patients with advanced head and neck cancer treated by FU, among whom the complete responders were those exhibiting the lowest tumoral DPD activity [15]. These data provided

a pharmacological basis for a new opening in the spectrum of FU modulation involving the ability to inhibit DPD activity. α-IFN can modulate FU activity through more or less well-established biochemical pathways [9]. We recently reported data on a time- and dose-dependent inhibition of DPD by α-IFN [14].

The purpose of the present study was to test the DPD-inhibitory effects of several agents whose use as FU biomodulators has been clinically established. We included cisplatin because previous investigators have suggested that this drug may decrease FU catabolism, thus leading to suspicion of an interaction with DPD activity [12]. Hydroxyurea is a ribonucleotide reductase inhibitor. This drug acts through scavenging of the tyrosyl free radical of the enzyme, which along with an iron center, is essential to ribonucleotide reductase activity [24]. Because DPD is a reductase containing an appreciable percentage of tyrosyl residues and an iron-sulfur [25], one could suspect a direct effect of hydroxyurea on DPD activity. Previous data have indicated that uridine is a strong inhibitor of DPD activity [22]. Allopurinol is converted to oxypurinol-5′-monophosphate, a potent inhibitor of orotidylate decarboxylase. This blockage may result in increased levels of orotate that could compete with uracil for the available 5-phosphoribosyl-1 pyrophosphate (PRPP) pool for activation in dioxyuridine monophosphate (dUMP) [21]. Thus, allopurinol (after intracellular activation steps) and dipyridamole (by blocking bidirectional transport of nucleosides [10]) may modify the cellular uridine pool. These two latter compounds can thus be suspected of indirectly affecting cellular DPD activity. On the basis of the above-mentioned arguments, we thus selected cisplatin, hydroxyurea, allopurinol, and dipyridamole. This was done to shed more light on the mechanism of their interaction with FU and to learn more about the potential role of DPD in FU pharmacomodulation.

Materials and methods

Chemicals

All chemicals, including MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], were obtained from Sigma Chemical Co. (St. Quentin Fallavier, France). FU, dihydrofluorouracil (FUH_2), α-fluoro-β-alanine (FBAL), and α-fluoro-β-ureidopropionic acid (FUPA) were kindly provided by Roche Laboratories (Neuilly, France). Cisplatin was obtained from Dakota; dipyridamole, from Boehringer Ingelheim (Paris); and hydroxyurea, from Sigma. 6-[^{14}C] -FU (55 Ci/mol) was obtained from Amersham (UK). Dulbecco's modified Eagle's medium (DMEM), glutamine were supplied by GIBCO (Paisley, UK). Fetal bovine serum (FBS) was obtained from Dutscher (Brumath, France). Penicillin and streptomycin were obtained from Merieux (Lyons, France).

Cell lines

Five cancer cell lines of human origin were used: three from head and neck carcinomas—CAL 27 [8], CAL 33 [8], and ORL 1 (HEP-2 ref ATCC CCL 23; American Type Culture Collection, Rockville, Md.); 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). 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/1, 86 μM streptomycin, and 2 m*M* glutamine. The doubling times for CAL 27, CAL 33, ORL 1, CAL 51, and PANC 3 were 4.5, 2.8, 3.2, 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 investigated compounds 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). FU modulators were tested separately for their own cell-growth effects (5-day exposure to the different cell lines). The IC_{10} value (the concentration causing a 10% growth inhibition relative to controls) was thus determined for each FU modulator and for each cell line tested.

Cells were then exposed for 5 days to the different combinations involving FU and the tested biochemical modulators. For a given cell line, the maximal concentration used in FU combination for each modulator was its own IC_{10} value for the cell line concerned. Two other concentrations were tested, $IC_{10}/3$ and $IC_{10}/10$. The growth-inhibitory effects were assessed by the MTT test [3] at 1 day after the end of the exposure to the drug combination. 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%). The FU concentration-effect curves were analyzed on GraphPad Software (ISI, USA). Two independent experiments were performed on each cell line.

Effects of investigated compounds on DPD activity

The whole panel of cell lines was examined for the effects of each FU modulator on cellular DPD activity. 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 the same modulator concentrations as those used in combination with FU, i.e., $IC_{10}/10$, $IC_{10}/3$, and IC_{10}. For basal DPD determination, cells were grown in parallel for 5 days without drug. After the incubation period (5 days) 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 plus supernatant).

DPD activity

DPD activity was measured using the method described in Harris et al. (11). 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 μM), reduced nicotinamide adenine dinucleotide phosphate (NADPH; final concentration, 250 μM), 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]. In addition to DPD activity being determined in cell lines after exposure to the FU modulators in the culture medium, the direct effect of these modulators on DPD activity was checked in the test tube. This was done for all cell lines and all drugs tested at the following concentrations in the incubation

mixture: $[IC_{10}]/10$, $[IC_{10}]/3$, IC_{10}, and $[IC_{10}] \times 5$. For a given cell line, IC_{10} was the concentration of FU modulator causing a 10% growth inhibition relative to controls. In all conditions, 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. FUH_2 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 FUH_2. Thus, the supernatant was analyzed for the presence of $[^{14}C]$-FUH_2, $[^{14}C]$-FBAL, and $[^{14}C]$ -FUPA as previously reported [1]. Detection was performed using a radioactive flow monitor (LD 506 Berthold, Wildbad, Germany).

DPD activity was calculated by taking into account the sum of FUH_2, FBAL, and FUPA peaks. Cytosolic proteins were quantified by means of the Bradford assay (BioRad, 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 in 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 Spearman rank nonparametric test was used to test the changes in FU IC_{50} values and the evolution of cellular DPD activity as a function of the modulator concentration applied. Statistical analyses were performed on Statgraphics Software (Uniware, Paris, France).

Results

Figure 1 is a typical example of the transformation of tumor cell sensitivity to FU as a function of the added concentration of the modulator. Figure 2 describes the data obtained with all cell lines tested and all FU modulators investigated. A close analysis reveals that depending on the cell line, the FU modulator can potentiate FU-mediated cell-growth inhibition (% FU IC_{50} lowered) or, inversely, can be detrimental to FU activity (% FU IC_{50} increased). This is specifically the case for cisplatin, whose FU effects were increased for CAL 27, CAL 33, CAL 51, and PANC 3 and were inhibited for ORL 1. As expected, allopurinol produced detrimental effects upon FU activity. This was evident for all cell lines, CAL 33 cells excepted. Dipyridamole and hydroxyurea are the only modulators for which an augmentation in FU cell-growth inhibition is clearly evident for the whole panel of cell lines investigated ($P < 1.10^{-4}$ and $P = 0.005$, respectively). Table 1 allows a close examination of modulator effects on FU cytotoxicity, all cell lines and all experiments being considered. With dipyridamole the efficacy of FU is multiplied by a factor of around 5: 13–25% for the ratio of FU IC_{50} with dipyridamole/FU IC_{50} control (ORL 1, PANC 3).

The effects of the various FU modulators on cellular DPD activity are shown in Fig. 3. Certain compounds

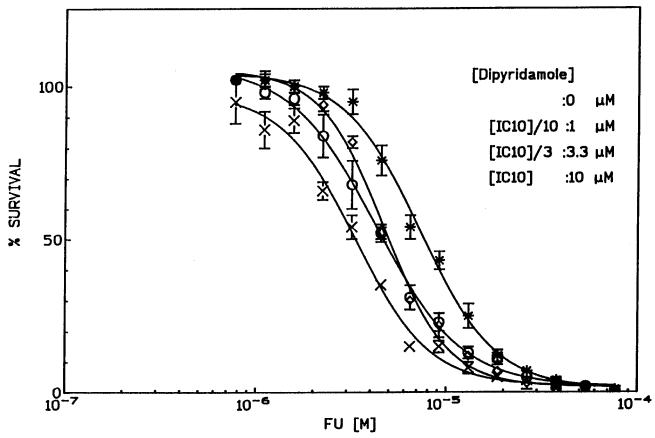

Fig. 1 FU concentration-cytotoxic effect curves as a function of increased concentration (µM) of dipyridamole (*asterisks* 0, *diamonds* 1, *circles* 3.16, *X* 10) on CAL 51 cells

have no obvious effect on cellular DPD activity. This is the case for allopurinol and cisplatin. For the two other FU modulators tested, DPD activity shows a more or less marked concentration-related inhibition according to the cell line tested ($P = 0.03$ and $P = 0.007$ for dipyridamole and hydroxyurea, respectively). Numeric data concerning the effect of FU modulators on cellular DPD activity are given in Table 1. When tested directly in the incubation mixture at concentrations ranging from $[IC_{10}]$ /10 up to $[IC_{10}] \times 5$, there was no direct effect of the FU modulators on DPD activity (around 10% variation in DPD activity, i.e., within the coefficient of variation of the method).

Figure 4 shows the relationship for all cell lines, all experiments, and all concentrations tested, between the change in FU IC_{50} value and the effect on DPD for each FU modulator included in the present study. Only dipyridamole produced reductions in FU IC_{50} values (potentiation of FU cytotoxicity) that were significantly related to inhibition of DPD activity.

Discussion

In the present study the so-called FU modulators were combined with FU at relatively modest concentrations (no more than their own IC_{10} value). This was done to avoid the confusing factor of metabolic stress involved in the use of higher ranges of modulator concentrations in the culture medium. This methodologic option guarantees that, if shown, the effects of a modulator on cellular DPD activity are mostly due to their interaction with the DPD or the DPD-related biochemical pathway. On the other hand, this methodologic option may lessen the FU-potentiation effects that might have been seen with higher concentrations of the modulators. Nevertheless, the underlying aim of the present study was not to focus on the analysis of FU cytotoxicity modification in the presence of the

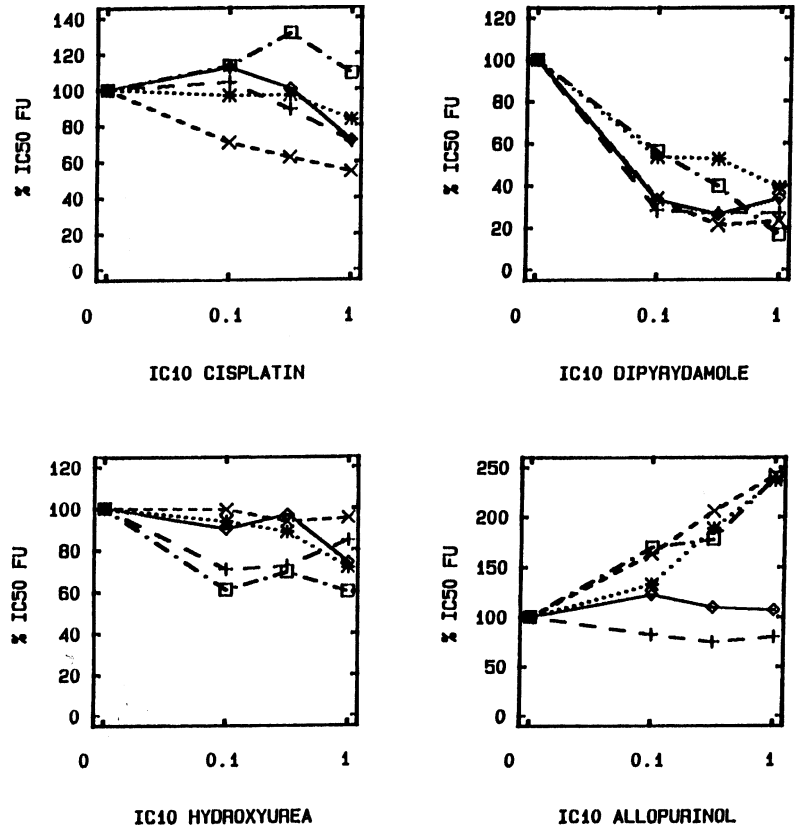

Fig. 2 Changes in FU IC_{50} values as a function of all FU modulators tested. % IC_{50} FU = ratio of IC_{50} FU with modulator/IC_{50} FU without modulator × 100. Points represent means of two separate experiments as in Table 1 (*squares* ORL 1, *X* PANC 3, *asterisks* CAL 51, + CAL 33, *diamonds* CAL 27). Statistics (Spearman rank nonparametric test): cisplatin, $P = 0.11$; dipyridamole, $P < 1.10^{-4}$; hydroxyurea, $P = 0.005$; allopurinol, $P = 0.04$

Table 1 Maximal effects of FU modulators on FU cytotoxicity and cellular DPD activity. A and B are the data for the two separate experiments IC_{10}. The concentration of the modulator alone that inhibits 10% of cell growth as compared with controls, % *FU* IC_{50} the change in FU IC_{50} value as compared with controls when FU is combined with the highest concentration of the modulator [IC_{10}], % *DPD* the change in cellular DPD activity when cells are incubated with the highest concentration of the modulator [IC_{10}])

Cell lines	FU IC_{50} μ*M* (SD)	Basal DPD activity nmol/min^{-1} mg protein^{-1} (SD)		Cisplatin % DPD	Cisplatin % FU IC_{50}	Cisplatin IC_{10} μ*M*	Hydroxyurea % DPD	Hydroxyurea % FU IC_{50}	Hydroxyurea IC_{10} μ*M*	Dipyridamole % DPD	Dipyridamole % FU IC_{50}	Dipyridamole IC_{10} μ*M*	Allopurinol % DPD	Allopurinol % FU IC_{50}	Allopurinol IC_{10} μ*M*
CAL 27	2	0.049	**A**	93	89	0.33	85	63	39.4	64	37	19.8	85	116	500
	(0.41)	(0.009)	**B**	106	54	0.33	103	95	39.4	80	30	19.8	76	89	500
CAL 33	0.3	0.088	**A**	112	82	0.33	70	102	26.2	47	41	10.3	112	77	3
	(0.36)	(0.034)	**B**	127	60	0.50	70	67	26.2	49	23	9.9	103	83	3
CAL 51	4.5	0.221	**A**	66	86	0.33	43	70	32.9	63	45	9.9	78	235	150
	(0.2)	(0.054)	**B**	51	80	0.33	60	73	39.4	50	33	9.9	77	238	90
ORL 1	23.6	0.056	**A**	75	80	0.33	65	53	26.3	70	13	7.1	68	263	100
	(0.6)	(0.015)	**B**	95	137	0.33	59	66	26.3	49	22	5.9	61	117	100
PANC 3	8.2	0.292	**A**	80	46	0.10	43	103	60.5	95	25	6.9	151	201	200
	(0.26)	0.056)	**B**	60	62	0.16	41	88	65.7	87	22	8.9	140	184	220

clinically relevant FU modulators. However, the results obtained justify several comments.

On analysis of the data in a global way for the whole cell-line panel, only dipyridamole and hydroxyurea demonstrated a statistically significant potentiation of FU cytotoxicity. However, when tumor cell lines are taken individually in considering all tested compounds, it appears that FU cytotoxicity was modified more or less radically (Fig. 2). Globally, FU potentiation was related to the modulator concentration and, at the higher FU-modulator concentrations tested (IC_{10}), the FU IC_{50} values were reduced by a factor ranging from 0.8 (with cisplatin and hydroxyurea) to 0.2 (with dipyridamole). In certain cases and according to the

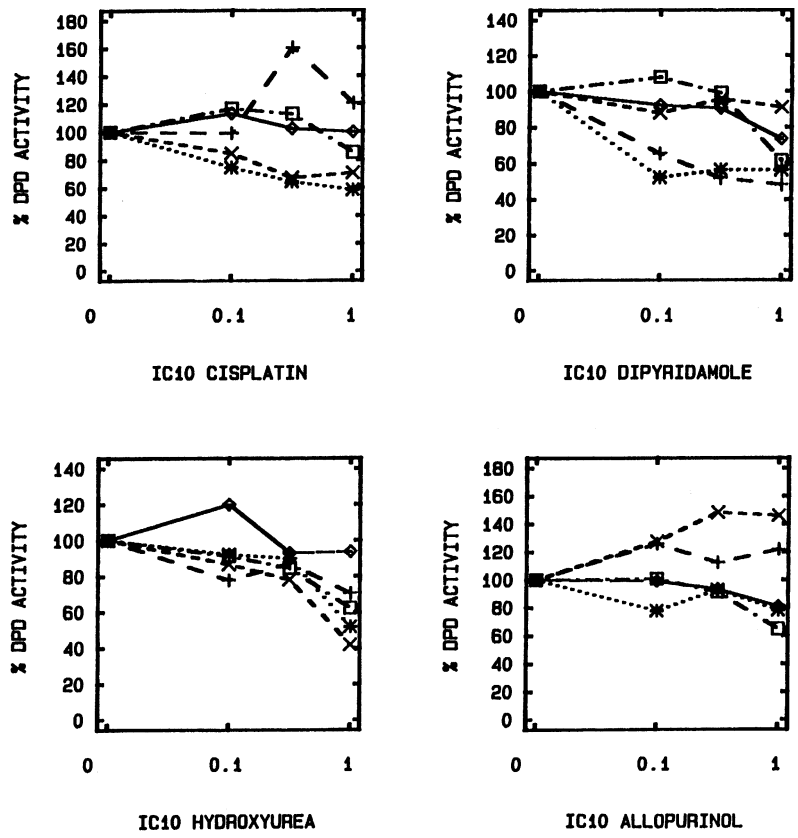

Fig. 3 Changes in cellular DPD activity as a function of all FU modulators tested. % DPD activity = ratio of cellular DPD activity with modulator cellular/basal DPD activity × 100. Points represent means of two separate experiments as in Table 1 (*squares* ORL 1, *X* PANC 3, *asterisks* CAL 51, + CAL 33, *diamonds* CAL 27). Statistics (Spearman rank nonparametric test): cisplatin, $P = 0.39$; dipyridamole, $P = 0.03$; hydroxyurea, $P = 0.007$; allopurinol, $P = 0.58$

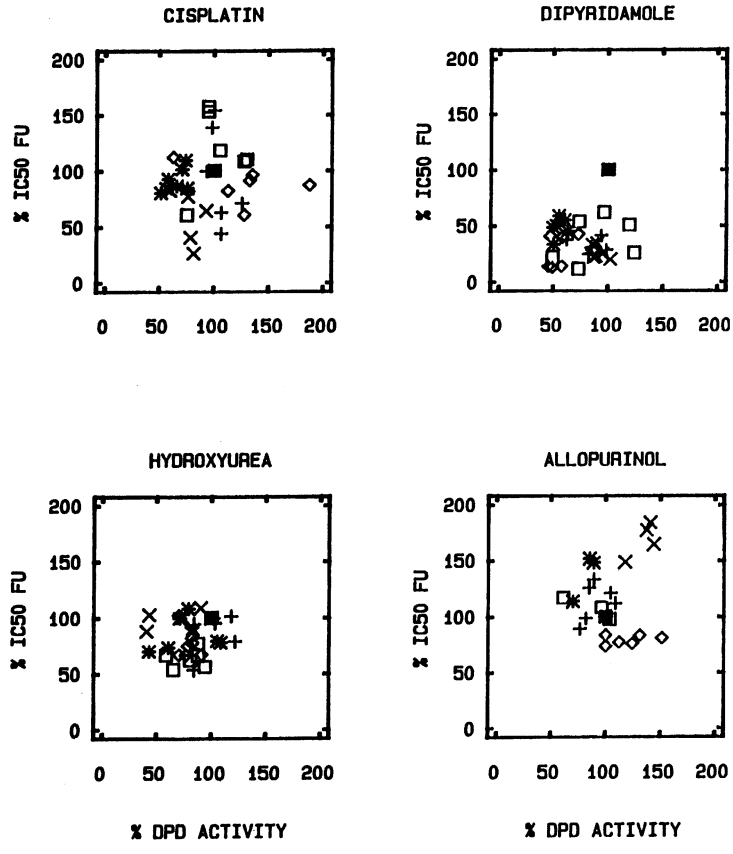

Fig. 4 Link between the changes in cellular DPD activity and the evolution of IC_{50} FU. % IC_{50} FU as defined in Fig. 2; % DPD activity as defined in Fig. 3 (*squares* ORL 1, *X* PANC 3, *asterisks* CAL 51, + CAL 33, *diamonds* CAL 27). Statistics (Spearman rank-correlation test): cisplatin, $P = 0.66$; dipyridamole, $P = 0.004$; hydroxyurea, $P = 0.09$; allopurinol, $P = 0.6$

cell line considered, FU cytotoxicity was enhanced as expected (reduction in % IC_{50} FU, Fig. 2). On the other hand, however, FU effects could be weakened. This was apparent for cisplatin. For the latter drug, this dual effect and the possible antagonistic effect upon FU must be borne in mind for a better understanding of the clinical results of using FU pharmacomodulation. Allopurinol was included as a negative control in the panel of FU modulators. As expected and in agreement with previous data [7], there was marked and detrimental interference between allopurinol and FU. This phenomenon was clearly related to the allopurinol concentration (Fig. 2). The cell lines ORL 1, CAL 51, and PANC 3 led to more than a 2-fold reduction in FU cytotoxicity as shown by the % IC_{50} FU values, which ranged from 200% to 250% (Fig. 2). In contrast, CAL 27 and CAL 33 cells did not manifest such a marked change in their FU sensitivity in the presence of allopurinol. Cellular differences in the capacity to transform allopurinol into species interfering with FU activity [21] could explain these differences between cell lines.

Other investigators and the present authors have provided data suggesting that DPD determination in cancer patients is potentially useful for improving FU treatment at two levels: first, in measuring DPD in circulating lymphocytes for phenotyping DPD deficiency and establishing the risk of FU-clearance abnormalities and drug-related toxicity [6, 13] ; and second, in determining DPD in the tumor itself so as to predict intrinsic tumor sensitivity to FU [15]. Specific DPD inhibitors have been designed, and tested compounds such as ethynyluracil (EU) or benzyloxibenzyluracil have been shown to enhance FU-induced tumor regression significantly as recently shown in tumor-bearing animals [2, 17]. We recently studied the effects of EU on a panel of several human cancer cell lines expressing spontaneous and variable sensitivity to FU [16]. Interestingly, for the cell lines expressing the highest basal DPD activity, EU enhanced FU cytotoxicity with a potentiation factor ranging between 1.3 and 5.6. In contrast, EU had no effect on FU activity for cell lines presenting low DPD activity. DPD can thus be considered a new pivotal target for FU modulation.

The main objective of the present study was to screen a series of clinically used FU modulators for their possible interference with cellular DPD activity. The choice of these FU modulators was dictated by their possible direct or indirect effect on DPD activity (see Introduction). As judged from the present data, cisplatin, which was tested at pharmacologically compatible concentrations, exhibited no evident effect on cellular DPD activity (Fig. 3). This information could be of practical importance since cisplatin is frequently used in combination with FU to treat several types of cancer [18]. In these cases, there is a highly improbable possibility of an FU-pharmacokinetics alteration due to an inhibition of hepatic DPD activity by cisplatin derivatives. The same conclusion holds true for chemotherapy protocols associating allopurinol with FU.

In contrast, dipyridamole and hydroxyurea demonstrated a statistically significant concentration-related inhibition of DPD activity (Fig. 3). It should be noted that neither dipyridamole nor hydroxyurea manifested a DPD-inhibitory effect when incubated directly in the cellular cytosol preparations at the same concentrations as those used in culture medium. Hydroxyurea's free radical selectively scavenges the tyrosyl free radical of ribonucleotide reductase [24]. Both an iron center and the tyrosyl residue are essential to ribonucleotide reductase activity [24]. On the basis of the presence of iron-sulfur and an appreciable percentage of tyrosyl residues in the DPD structure [25], it would be tempting to deduce the presence of a similar interaction between hydroxyurea and DPD to explain the present observations. However, the lack of a direct effect of hydroxyurea on DPD activity (hydroxyurea directly incubated in the cellular cytosol) precludes such a hypothesis. Clearly, further investigations are necessary to confirm and to improve our understanding of the hydroxyurea-DPD interaction demonstrated above.

A biochemical basis explaining the DPD-inhibitory effects of dypiridamole is less difficult to figure out. Yet, Tuchman et al. (22) have provided convincing data showing that uridine and, to a lesser degree, thymidine do inhibit DPD activity in vitro. Dipyridamole acts as an inhibitor of bidirectional transport of nucleosides through the cellular membrane [10]. It follows that the presence of dipyridamole may result in an intracellular accumulation of uridine and thymidine, resulting in a dipyridamole concentration-related inhibition of DPD activity as presently shown. Complementary investigations on intact cells aimed at quantifying variations in cellular concentrations of uridine and thymine would be required for validation of this hypothesis. To date, the rationale for the combined use of dipyridamole and FU has been strictly based on the inhibitory effects of dipyridamole vis à vis nucleoside salvage and efflux of FU nucleosides. The participation of these effects in explaining the enhancement of FU activity by dipyridamole certainly remains valid.

However, the present data provide a new and original hypothesis based on DPD inhibition. The statistically significant correlation between FU potentiation and DPD inhibition by dipyridamole (Fig. 4) is another strong experimental argument reinforcing this hypothesis. The underlying interactions between DPD and dipyridamole are important for a better understanding of the FU-dipyridamole association and also carry potential clinical repercussions; FU activity can be modified not only by the impact of dipyridamole on the target cells themselves but also by a possible FU-pharmacokinetics modification.

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