Dihydropyrimidine Dehydrogenase: A Tumoral Target for Fluorouracil Modulation

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme of 5-fluorouracil (FU) catabolism. Ethynyluracil (776C) is a very potent, mechanism-based irreversible DPD inhibitor that improves the antitumor efficacy and the therapeutic index of FU in laboratory animals. We tested the cytotoxic effects of the FU-776C combination on a panel of 12 human cancer cell lines (4 breast, 4 head and neck, 3 colon, and 1 duodenum). Basal DPD activity (radioenzymatic assay) and FU sensitivity [FU 50% inhibitory concentration (IC50), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloctazolium bromide test] were determined. The FU potentiation by 776C was calculated from the ratio (F) of FU IC50 without 776C divided by FU IC50 with 776C. 776C was not cytotoxic to any of the cell lines tested. On CAL51 cell line expressing a high basal DPD activity, FU enhancement by 776C was a saturable phenomenon related to the 776C concentration; the inhibition of DPD increased between 10−12 to 10−6 M of 776C. For the following studies, 776C was tested at 10−6 M. FU IC50 varied from 15 to 7770 μM among cell lines (median, 390 μM). Basal DPD activity ranged from not detectable (<1 pmol/min/mg protein) to 320 pmol/min/mg protein among cell lines (median, 53 pmol/min/mg protein). For the 12 cell lines tested, the mean F ranged from 0.7 (no enhancement of FU cytotoxicity by 776C) up to 5.2 and was significantly related to the basal DPD activity: the greater the DPD activity, the greater the FU enhancement factor (Spearman rank correlation, P = 0.019). Enhancement of FU cytotoxicity by 776C occurred only in the six cell lines expressing the greatest basal DPD activity (>50 pmol/min/mg protein, F ranging between 1.7 and 5.2), whereas 776C did not modify FU cytotoxicity in the remaining cell lines expressing the lowest DPD activity (<50 pmol/min/mg protein, F ranging between 0.7 and 1.4). F was significantly different between these two groups of cell lines (P = 0.005). These results point out that DPD is an interesting target for FU pharmacomodulation.

INTRODUCTION

Extensive studies indicate that resistance to FU2 may take place at different cellular loci (1–4). As a consequence, the source of variability for FU sensitivity in treated tumors is multiple and complex. Up to now, overproduction of thymidylic synthase has been the main mechanism of resistance identified in patients (5, 6). However, elevation of thymidylic synthase is not unanimously recognized as a determinant factor for FU resistance (7, 8). Overall, in attempts to elucidate FU resistance, most attention has been paid to FU activation pathways without considering the presence and the possible role of FU catabolism in the target tumor cell itself. In eukaryote cells, the first step in the catabolism of the pyrimidine bases, thymine and uracil, is a hydrogenation by DPD. FU is a pyrimidine analogue that it is metabolized by DPD. Naguib et al. (9) reported that the level of DPD activity varies considerably in human tumor xenografts. It follows that FU cellular catabolism mediated by DPD could be variable, and could thus modulate FU engagement in anabolic pathways. Thus, DPD activity could be a possible source of variability in FU sensitivity at the target tumoral level. We recently investigated a panel of 19 human cancer cell lines, and reported that cellular DPD activity was very different between tumors and, interestingly, was an independent factor significantly related to FU sensitivity (10). Based on these experimental findings, we measured DPD activity in tumor biopsies of 62 head and neck cancer patients before neoadjuvant chemotherapy including FU (11). Our previous experimental observations were confirmed at the clinical level, with complete responding patients exhibiting the lowest tumoral DPD activities. Together, these data provide a pharmacological basis for a new opening in the spectrum of FU modulation with the possibility of inhibiting DPD activity.

776C is a very potent, mechanism-based, irreversible inhibitor of DPD in vitro (12) and in vivo (13) that improves the efficacy, therapeutic index, and p.o. bioavailability of FU in laboratory animals (14–16). In this study, we tested the combination of FU and 776C on a panel of 12 human tumor cell lines representative of the spectrum of tumor localizations with a recognized clinical efficacy of FU chemotherapy. Basal DPD activity and intrinsic FU sensitivity were determined. The effects of 776C on FU cytotoxic action were investigated, and the degree of FU cytotoxicity enhancement by 776C was related to intrinsic DPD cellular activity.

2 The abbreviations used are: FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase (EC 1.3.1.2); 776C, 5-ethynyluracil (776C85); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloctazolium bromide; FUH2, dihydrofluorouracil; FUPA, α-fluoro β-ureidopropionic acid; FBAL, α-fluoro β-alanine; IC50, 50% inhibitory concentration.
MATERIALS AND METHODS

Chemicals. All chemicals including MTT and dl-5-methyltetrahydrofolate were obtained from Sigma Chemical Co. (St. Quentin Fallavier, France) and were of the highest purity available. FU, FUH₂, FUPA, and FBAL were kindly provided by Roche Laboratories (Neuilly, France). 776C was kindly provided by Burroughs Wellcome (Research Triangle Park, NC). [14C]FU was labeled at position 6 (55 Ci/mol) was obtained from Amersham (Buckinghamshire, United Kingdom). DMEM medium and glutamine were obtained from Whittaker (Verviers, Belgium), and FBS was obtained from Dutscher (Brumath, France). Penicillin and streptomycin were obtained from Merieux (Lyon, France).

Cell Lines. Twelve cancer cell lines of human origin were used: four head and neck, four breast, three colon, and one duodenum (Tables 1 and 2). Cells were routinely cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 50,000 units/liter penicillin, and 80 μg/mL streptomycin in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO₂.

Determination of DPD Activity. For all cell lines, DPD activity was measured in cells cultured in the previously described medium (75-cm² plates). The cells were harvested when reaching 75~85% of confluence. DPD was assayed in CALS1 cells that were incubated for 5 h with 776C at 10⁻¹² to 10⁻⁶ M (75-cm² plates), and were then washed and incubated in the culture medium without 776C for an additional 24 h before trypsinization. DPD activity was measured according to the method described by Harris et al. (17). After trypsinization, the cells were washed three times with PBS, then a cell suspension (10⁷ cells/mL) was prepared in 35 mM sodium phosphate buffer (pH 7.5) containing 10% glycerol. The cell suspension was then centrifuged (5 min, 250 × g), and the cells were stored at −80°C (cell pellet plus supernatant) without loss 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 supernatant (i.e., 5 × 10⁵ cells) with [14C]FU (20 μM final), NADPH (250 μM final), and MgCl₂ (2.5 mM final). Total volume was 125 μL (in 35 mM sodium phosphate buffer, pH 7.5, containing NaCl). The mixtures were incubated for 30 min at 37°C. The reactions were 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 analyzed for the presence of [14C]FUH₂, [14C]FBAL, and [14C]FUPA using a previously reported high-pressure liquid chromatographic method (18). Detection was performed using a radioactive flow monitor (LD506 Berthold; Wildbad, Germany). DPD activity was calculated by taking into account the sum of the FUH₂, FBAL, and FUPA peaks. DPD activity was expressed as pmol [14C]FU catabolized/min/mg protein. Each sample was assayed in duplicate, and DPD activity was measured during three independent experiments. The sensitivity limit was 1 pmol/min/mg protein. The interassay reproducibility (pooled cell suspension) gave a coefficient of variation of 12% (n = 8).

The panel of cell lines was divided according to their basal DPD activity. DPD median value was 53 pmol/min/mg protein. Group 1 comprised cell lines with DPD activity above the median value (CAL51, CAL85-2, CAL27, CAL34, HEP2, HUTU80, Table 1), and Group 2 comprised cell lines with DPD activity below the median value (MCF7, ZR75, KB, WIDR, SW620, COLO205, Table 2).

Evaluation of the Effects of 776C on FU Cytotoxicity. Experimentally growing cells were seeded in 96-well microtitration plates (V-shaped wells). The initial cell density was 12,000~30,000 cells/well, depending on the cell line. For the CAL51 cell line expressing a high basal DPD activity, we studied 776C ranging from 10⁻¹² to 10⁻⁶ M (eight concentrations tested). On CAL51 cells, 10⁻⁶ M 776C almost completely suppressed DPD activity, thus we tested 776C at 10⁻⁶ M in the other cell lines. To mimic the clinical situation with respect to drug availability relative to the tumor mass, FU-776C combinations were tested under culture conditions inspired by Pizzo et al. (19); as described below, the cells were concentrated, and the volume of the culture medium was minimal so as to increase the cell:medium ratio. After addition of 776C, cells were incubated for 30 min (100 μL/well). Then, FU was added (11 different concentrations ranging from 10⁻⁷ to 10⁻¹ M), and the plates were centrifuged (5 min at 1000 rpm) to concentrate the cells in the bottom of the V-shaped wells. The excess of medium was then removed carefully by aspiration and a minimal (10 μL) volume of medium was left for covering the cell pellet. Plates were then covered with a freezer film to avoid evaporation. After 4.5-h incubation, 100 μL culture medium were added to each well, and incubation was continued for 115 h. The medium was then carefully removed and 100 μL/well fresh medium plus 50 μL/well MTT solution were added and incubated for 2 h. The MTT-containing medium was then carefully removed, and formazan blue crystals were dissolved with DMSO. The final coloration was read on a Titertek Spectrophotometer. Results were expressed as the relative percentage of absorbance compared to controls without drug. The dose-effect curves were analyzed on GraphPad Software (ISI, Philadelphia, PA), and the FU concentrations causing a 50% growth inhibition (IC₅₀) as compared to controls were computed. In addition, we calculated the FU cytotoxicity enhancement factor (F) defined as the ratio of FU IC₅₀ without 776C divided by FU IC₅₀ with 776C. Each experimental point was performed in quintuplicate (coefficient of variation less than 10%), and two independent experiments were performed.

Statistics. Analyzed variables were FU IC₅₀, factor F, and DPD activity. Data were expressed as the mean values of duplicate (FU IC₅₀, factor F) or triplicate (basal DPD activity) data. The relationship between F and DPD was analyzed according to the Spearman rank correlation test. Two-sample comparison was done according to the Mann-Whitney nonparametric U test. Statistical tests were performed on Statgraphics Software (Unixware, Paris, France).

RESULTS

776C itself (10⁻⁶ M) had no cytotoxic effect on any of the cell lines tested. The enhancement of FU cytotoxicity by 776C in CAL51 cells is shown in Fig. 1A. A marked shift of FU dose-response curves to the left is observed when increasing 776C concentrations, thus revealing a 776C concentration-
related enhancement of FU cytotoxicity. The plot of the FU cytotoxicity enhancement factor (F) as a function of 776C concentration shows that FU cytotoxicity enhancement by 776C is a saturable phenomenon related to the applied 776C concentration (Fig. 1B); maximum enhancement occurred with $10^{-6}$ M 776C and led to an enhancement factor close to 3. Fig. 2A illustrates the modulation of DPD activity as a function of the applied 776C concentration (8 concentrations tested ranging from $10^{-12}$ to $10^{-6}$ M) on CAL51 cells for the two independent experiments performed ( ■ , first experiment; ○ , second experiment).

Tables 1 and 2 summarize data for all tested cell lines. For the whole panel of cells, mean FU IC$_{50}$ varied from 15 $\mu$M (ZR75) up to 7770 $\mu$M (WIDR), thus reflecting a wide range in spontaneous FU sensitivity (median, 390 $\mu$M); basal DPD activity ranged from not detectable (<1 pmol/min/mg protein) to 320 pmol/min/mg protein (median, 53 pmol/min/mg protein). For the 12 cell lines tested, the mean F ranged from 0.7 (no enhancement) up to 5.2 (CAL33) and was significantly related to the basal DPD activity: the greater the DPD activity, the greater the FU enhancement factor (Spearman rank correlation, $P = 0.019$, Fig. 3). Fig. 3 also reveals that enhancement of FU cytotoxicity by 776C was observed only in the six cell lines expressing the greatest basal DPD activity (median, 214, Group 1) with F ranging between 1.7 and 5.2 (median, 3.2). In contrast, 776C did not modify FU cytotoxicity of the remaining cell lines expressing the lowest DPD activity (median, 5, Group 2; F between 0.7 and 1.4, median 1.0). F were significantly different between Group 1 and Group 2 ($P = 0.005$).
DISCUSSION

The numerous biochemical approaches for FU pharmacomodulation developed so far were focused at different levels of the complex pyrimidine and/or folate metabolic pathways (20, 21). From the clinical point of view, benefit from FU biochemical modulation is rare, a noticeable exception being for leucovorin supplementation of FU. Yet, in colorectal cancer, results from randomized trials have demonstrated the efficacy of FU modulation by leucovorin for treating metastatic disease (22) or as adjuvant chemotherapy (23). For some drugs like cisplatin or bevamisole, the exact mechanisms of FU biomodulation have not yet been clearly established. In fact, the starting point for FU metabolism is the crossroads between anabolic and catabolic pathways, with DPD-mediated catabolism controlling the amount of drug available for anabolism. As recently highlighted by Kamm et al. (24) using $^{19}F$ nuclear magnetic resonance spectroscopy, FU catabolism within colon tumors in mice is preponderant over anabolism (the percentage of tumor anabolites and catabolites to total $^{19}F$ containing compounds was 26 and 51%, respectively). We have previously shown in vitro on a large panel of 19 human cancer cell lines that FU sensitivity was significantly related to tumoral DPD activity, the more sensitive cell lines expressing the lowest DPD activity (10). Interestingly, we confirmed this finding in the clinical setting on 62 head and neck cancer patients. Complete responder patients exhibited significantly lower tumoral DPD activity than non-complete responder patients (11). Thus, the importance of Hi catabolism in tumor cells (10, 11) strengthens the concept that inhibition of DPD represents a promising and complementary target for FU pharmacomodulation. Present data are in line with our previous investigations and confirm the presence of intratumoral DPD activity with a wide variability between tumor sources (10) and from tumor to tumor within the same cancer localization (11). Interestingly, in a global way, the distribution
range of cellular DPD activity presently found (4–320 pmol/min/mg protein) matched very well with what we previously determined in tumors from head and neck cancer patients (13–193 pmol/min/mg protein; Ref. 11).

The present study shows that DPD activity measured on intact cells can be suppressed by the mechanism-based irreversible DPD inhibitor 776C. This observation represents a complementary argument concerning the pharmacological activity of 776C. Yet, previous investigators had established the inhibitory efficacy of 776C against DPD from tissue extracts of the rat, human liver (12, 15). The degree of DPD inhibition herein observed was 776C concentration related, and approximately 100% inhibition was achieved with a micromolar concentration of 776C (Fig. 2A). In 6 of the 12 cell lines investigated, 776C significantly enhanced FU cytotoxicity. The key result of the present study is that the level of tumoral DPD activity is a significant predictor for the level of FU cytotoxicity enhancement by 776C in cell culture (Fig. 3). Clearly, FU sensitivity in cells expressing a low DPD activity was unchanged when applying 776C in the culture medium (Table 2). In contrast, FU sensitivity was markedly increased, up to a factor 5, in cells exhibiting a high DPD activity (Table 1). It is interesting to underline that this factor of 5 is close to that obtained in vitro when modulating FU by folinic acid (25). The enhancement of FU cytotoxicity by 776C via DPD inhibition could be explained, in the first analysis, by an increased activation of FU via its anabolic pathways. Another complementary explanation could lie on the role of FU catabolites. Spector et al. (26) recently found that coadministration of FUH₂ in FU-776C-treated rats bearing colorectal carcinoma decreased the antitumor activity to the level produced by FU alone. Thus, FUH₂ or other downstream catabolites of FU, impaired the antitumor activity of FU. Accordingly, 776C appeared to improve the efficacy of FU by preventing the formation of FU catabolites.

Interestingly, FU biomodulation via DPD inhibition takes place at an early stage in the FU metabolic pathway; it follows that multiple pharmacomodulation based on both DPD inhibition and already known biochemical approaches would result in additive modulatory effects. We tested such an approach on the CAL33 cell line by combining FU with 776C and folinic acid (data not shown): both 776C (10⁻⁶ M) and folinic acid (5.10⁻⁶ M) gave a similar enhancement of FU cytotoxicity (FU enhancement factor close to 3), whereas the coadministration of FU with folinic acid plus 776C led to a FU enhancement factor of 11. This suggests an even more than additive effect resulting from this multiple pharmacomodulation of FU, and supports the results obtained from tumor-bearing animal studies (15).

Future clinical use of DPD inhibitors is potentially complex, and other considerations must be presently discussed. These considerations concern the DPD-FU interrelationships in the whole organism leading to question the tumoral specificity and the pharmacokinetic consequences when targeting DPD. Interestingly, as compared to tumors showing high DPD activity (up to 200 pmol/min/mg protein; Ref. 11), both normal intestinal mucosa (7 ± 7 pmol/min/mg protein; Ref. 9) and normal bone marrow (5 ± 1 pmol/min/mg protein; recalculated from Ref. 27) exhibit lower levels in this enzymatic activity. This can lead to anticipation of an improved FU therapeutic index when using DPD inhibitors in FU-treated patients. Considering pharmacokinetic consequences of DPD inhibition, it must be borne in mind that liver is the organ exhibiting the highest DPD activity (9). Consequently, FU is cleared from the organism chiefly by DPD-mediated hepatic catabolism (21, 28). Recent results from ourselves (29) and from other investigators (17) have demonstrated a significant relationship between FU total-body clearance and DPD activity measured in peripheral blood mononuclear cells. Moreover, other studies (30, 31) have reported patients developing severe FU toxicities (lethal for certain) associated with a marked DPD deficiency shown in peripheral blood mononuclear cells. Thus, from this point of view, the clinical use of DPD inhibitors must be considered with care, and special attention must be paid to the choice of the FU dose. For instance, it is known that IFN-α can modulate FU activity at more or less well-identified biochemical levels. Among these, we have recently shown a time- and dose-dependent inhibition of DPD (32). Accordingly, patients treated with the FU-IFN combination exhibit an increased total-body exposure to FU (up to 50%) as compared to patients treated with FU alone (33). It is thus not surprising that the preclinical use of specific DPD inhibitors like 5-benzyloxybenzyluracil (34) or 776C (14) produced in laboratory animals a marked increase of FU terminal half-life and a subsequent increase in the area under the plasma concentration time curve of FU. Accordingly, in the first cohort of patients treated with 776C, the FU dose was 10 mg/m² and the terminal half-life of FU was markedly increased (4.7 ± 0.8 h) as compared to patients receiving FU alone (10 min; Ref. 35).

The present results should encourage the ongoing clinical trials of FU pharmacomodulation including DPD inhibitors.

REFERENCES


Dihydropyrimidine dehydrogenase: a tumoral target for fluorouracil modulation.

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