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Dual Modulation of 5-fluorouracil Cytotoxicity Using Folinic Acid with a Dihydropyrimidine Dehydrogenase Inhibitor

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ABSTRACT. Dihydropyrimidine dehydrogenase (DPD) is the key enzyme of the fluorouracil (FU) catabolic pathway. We have shown that tumor cells expressing a high DPD activity are resistant to FU (*Eur J Cancer 30: 1517, 1994*), and that 5-ethynyluracil (776C), a very potent DPD inactivator, markedly enhances the FU cytotoxic effect (*Clin Cancer Res 1: 991, 1995*). Both experimental background and clinical experience have demonstrated the role of folinic acid (FA) in increasing FU efficacy. The aim of the present study was to investigate the dual FU pharmacomodulation based on the combination of FU with 776C and/or FA on 7 human cancer cell lines (2 head and neck, 3 breast, 1 colon, 1 duodenum) expressing a spontaneous FU sensitivity. These cell lines were chosen according to their ability to respond to FU modulation by FA and/or 776C. The potency of FU modulation was evaluated by the ratio between FU IC_{50} and FU IC_{50} in the presence of the tested biomodulator(s), defined as factor F. In cell lines sensitive to FU modulation by 776C only (median F value with 1 μM of 776C = 2.5), the addition of FA did not enhance FU-776C cytotoxicity. In contrast, for cell lines resistant to FU modulation by 776C, the FU-FA-776C combination led to a significant cytotoxicity enhancement as compared to FU-FA (median F values were 3.6 and 2.6 respectively). In cell lines responsive to FU modulation by FA and 776C, the median F values were 2.3 and 1.8 with FA and 776C, respectively. Interestingly, the dual modulation by FA + 776C led to a median F value at 6.3, suggesting more than the additive effects of FA and 776C. This synergistic interaction was statistically confirmed by multivariate ANOVA (FA × 776C interaction). The present study points out that FA plus 776C could prove to be a very attractive combination for future strategies in FU biomodulation. BIOCHEM PHARMACOL 53;11:1703–1709, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 5-fluorouracil; folinic acid; dihydropyrimidine dehydrogenase; modulation; drug interactions; tumor cell lines

In attempts to elucidate 5-fluorouracil (FU)† resistance, most attention has been paid to FU activation pathways without considering the possible role of FU catabolism at the tumoral level. Up to now, overproduction of thymidylate synthase has been the main mechanism of resistance identified in patients [1–3]. In eukaryote cells, the first step in the catabolism of the pyrimidine bases, thymine and uracil, is a hydrogenation by the enzyme uracil reductase or dihydropyrimidine dehydrogenase (DPD). The pyrimidine analogue FU is catabolized by DPD into dihydrofluorouracil (FUH2). Naguib *et al.* reported that DPD activity varies considerably in human tumor xenografts [4]. We have previously shown *in vitro* that tumoral DPD activity was an independent factor significantly related to FU sensitivity: the lower the DPD activity, the greater the FU sensitivity [5]. We further confirmed these experimental observations in head and neck cancer patients receiving FU-based chemotherapy, with complete responding patients exhibiting the lowest tumoral DPD activities [6]. Together, these data provide a strong pharmacological rationale for opening the spectrum of FU modulation with the possibility of inhibiting DPD activity. We recently tested the cytotoxic effect of FU combined with 5-ethynyluracil (776C), a very potent mechanism-based irreversible DPD inactivator [7, 8]. Twelve human cancer cell lines of various origins were investigated [9]. Enhancement of FU cytotoxicity by 776C occurred in the 6 cell lines expressing the greatest basal DPD activity, whereas 776C did not modify FU cytotoxic-

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† *Abbreviations:* DPD, (dihydropyrimidine dehydrogenase); 776C, (5-ethynyluracil); FBS, (fetal bovine serum); FU, (5-fluorouracil); FdUMP, (5-fluoro-deoxyuridinemonophosphate); FUH2, (dihydro 5-fluorouracil); FUPA, (α-fluoro β-ureidopropionic acid); FBAL, (α-fluoro β-alanine); FA, (folinic acid); ND, (non-detectable); DMEM, (Dulbecco's modified Eagle's medium); IC 50, (concentration inducing a 50% inhibition of the growth).

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TABLE 1. Cell line characteristics

Cell lines	Localization	FU IC_{50} (mean + SD) FA = 0 776C = 0	Basal DPD activity (pmol/min/mg prot) (mean ± SD)	FU enhancement factor with 1 μM 776C (mean ± SD)	Maximal FU enhancement factor by FA* (mean ± SD)
CAL 27	Head and neck	143 ± 11	320 ± 75	1.8 ± 0.2	4.8 ± 1.2
CAL 33	Head and neck	214 ± 132	244 ± 2	2.4 ± 1.0	2.4 ± 0.2
CAL 85-2	Breast	245 ± 64	68 ± 1	1.6 ± 0.4	2.8 ± 0.4
CAL 51	Breast	534 ± 73	184 ± 2	1.9 ± 0.3	1.0 ± 0.2
HUTU 80	Duodenum	831 ± 126	251 ± 17	3.0 ± 0.5	1.0 ± 0.4
MCF 7	Breast	329 ± 118	ND	1.3 ± 0.7	3.9 ± 0.5
COLO 205	Colon	324 ± 100	6 ± 1	1.2 ± 0.3	5.8 ± 2.3

CAL 27, CAL 33, CAL 85-2 and CAL 51 were obtained from our institute. HUTU 80 (ref HTB 40) and COLO 205 (ref CCL 222) were obtained from the American Type Culture Center. The FU enhancement factor (factor F) is defined as the ratio of FU IC_{50} (FU alone) divided by FU IC_{50} in the presence of 776C and/or FA.

* Plateau value of the FU enhancement factor as a function of the FA concentration.

ity in the remaining cell lines expressing the lowest DPD activity [9].

FU modulation *via* DPD inhibition takes place at an early stage in the FU metabolic pathway. As a consequence, multiple pharmacomodulation based on both DPD inhibition and already known biochemical approaches would result in at least additive modulatory effects. The aim of the present study was thus to test such an approach on 7 human cancer cell lines by combining FU with 776C and folinic acid (FA), which has been shown to enhance FU cytotoxicity by optimizing thymidylate synthase inhibition. Moreover, this study was justified in the current context of clinical trials testing the efficacy of the FU-776C-FA association.

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. ^{14}C-FU labeled at position 6 (55 Ci/mol) was obtained from Amersham (Buckinghamshire, UK). ℓ folinic acid (ℓ FA) was purchased from Wyeth-Lederle (Puteaux, France). 776C was kindly provided by Glaxo-Wellcome (Research Triangle Park, North Carolina, USA). Folic acid-free Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO (Paisley, Scotland). DMEM medium and glutamine were obtained from Whittaker (Verviers, Belgium) and fetal bovine serum (FBS) was obtained from Dutscher (Brumath, France). Penicillin and streptomycin were obtained from Merieux (Lyon, France).

Cell Lines

Seven cancer cell lines of human origin were used: 2 head and neck, 3 breast, 1 colon and 1 duodenum (Table 1). Based on previous experiments at our laboratory [9, 10], these cell lines were chosen and classified into 3 groups according to the ability of FA and/or 776C to modulate FU cytotoxicity. Group I (CAL 27, CAL 33, CAL 85-2) comprised cell lines in which FU cytotoxicity was enhanced both by FA and 776C; group II (CAL 51, HUTU 80) comprised cell lines in which FU cytotoxicity was only potentiated by 776C; and group III (MCF 7, COLO 1) cell lines in which FU cytotoxicity was only potentiated by FA.

Cells were routinely cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 50000 units/liter penicillin and 80 μM streptomycin in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO_2. One week before starting the experiments, the cells were grown in a folate-controlled medium in order to simulate the physiological situation encountered in humans as closely as possible (folic acid-free DMEM medium supplemented with 40 nM of dℓ,5-methyltetrahydrofolate and 0.1 mM of ℓ,ascorbic acid, instead of the usual DMEM medium). The above folate-controlled medium was used until the end of the experiments.

Measurement of DPD Activity

The cells were grown in the folate-controlled medium (75 cm^2 plates) and harvested when reaching 75–85% of confluence. DPD activity was measured according to the method described by Harris *et al.* [11] and under experimental conditions previously described by us [9]. DPD activity determination consisted in the quantification of ^{14}C-dihydro 5-fluorouracil (^{14}C-FUH2), ^{14}C-α-fluoro β-alanine (^{14}C-FBAL), and ^{14}C-α-fluoro β-ureidopropionic acid (^{14}C-FUPA), using a previously reported high-pressure liquid chromatographic method [12]. DPD activity was calculated by taking into account the sum of FUH2, FBAL and FUPA peaks. DPD activity was expressed as pmol of ^{14}C-FU catabolized per min and per mg of 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 inter-assay reproducibility (pooled cell suspension) gave a coefficient of variation of 12% (N = 8).

Evaluation of the Effects of 776C and FA on FU Cytotoxicity

FU was tested at 11 concentrations ranging from 10^{-7} to 10^{-2} M. FA was tested at 6 concentrations from 5×10^{-9} to 5×10^{-4} M final concentration. Since 10^{-6} M of 776C almost completely suppressed DPD activity, 776C was tested at 10^{-6} M. To mimic the clinical situation with respect to drug availability relative to the tumor mass, FU-776C-FA combinations were tested under culture conditions inspired by Pizao *et al.* [13] and previously investigated by us [9]: cells were concentrated and the volume of the culture medium was minimal so as to increase the cell:medium ratio. Exponentially growing cells were thus seeded in 96-well microtitration plates (V-shaped wells, 50 μL per well). The initial cell density was 12000–30000 cells/well, depending on the cell line. As compared to experimental conditions where flat-bottom wells are used, the initial cellular concentration was higher with the V-shaped wells. Despite this difference in initial cellular concentration we noted comparable OD value (MTT test) at the end of the 5-day experiments for controls. This means that cell growth was less marked in the V-shaped wells. First, FA and/or 776C was added and incubated for 30 min (100 μL total volume per well). FU was then added and the plates were centrifuged (5 min at 1000 rpm) in order to concentrate the cells in the bottom of the V-shaped wells. The excess of medium was carefully removed by aspiration and a minimal (10 μL) volume of medium was left to cover the cell pellet. Plates were covered with a freezer film to avoid evaporation. After 4.5 hr incubation, 100 μL/well of culture medium was added and incubation was continued for 115 hr. The growth inhibition was assessed by the MTT test [14]. The medium was removed and 100 μL/well fresh medium plus 50 μL/well MTT solution were added and incubated for 2 hr. The MTT-containing medium was carefully removed and formazan blue crystals were dissolved with dimethyl sulfoxide. The final staining was read on a Titertek spectrophotometer (Labsystems France, Les Ulis). 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, USA). The FU concentrations causing a 50% growth inhibition (IC_{50}) as compared to controls were computed. In addition, we calculated the FU cytotoxicity enhancement factor (F) defined as the ratio of FU IC_{50} (FU alone) divided by FU IC_{50} in the presence of 776C and/or FA. Each experimental point was performed in quintuplicate (coefficient of variation less than 10%) and 3 independent experiments were performed.

Statistics

Statistics were done on triplicate values of factor F. The influence of 776C and/or FA on the modulation of FU cytotoxicity (factor F) was analyzed according to a multivariate ANOVA in which the possible interaction between 776C and FA was included. The multivariate ANOVA was performed for each cell line separately as well as on the whole cell line panel. Statistics were performed on Statgraphics Software (Uniware, Paris, France).

RESULTS

The influence of FA and/or 776C on the dose-effect curves of FU are illustrated in Fig. 1. For CAL 27 cells, both FA and 776C increased the FU cytotoxic effect. For HUTU 80, only 776C increased the efficacy of FU. For MCF7, FU cytotoxicity was modulated by FA whereas 776C did not influence FU efficacy.

The evolution of the FU potentiation factor (factor F) as a function of FA concentration in the culture medium, with or without 776C supplementation (1 μM), is shown in Fig. 2 for characteristic cell lines. For CAL 27 cells, there is a constant increase in F values according to FA concentrations. When 776C is added to FA, the evolution of F values is more accentuated, reaching $F = 13.2 \pm 3.1$ (Fig. 2A). Increasing FA concentrations up to 5×10^{-4} M did not modify FU cytotoxicity for HUTU 80 cells; in contrast, 776C at 10^{-6} M led to factor F values comprised between 2.5 and 3.1 (Fig. 2B). For MCF 7 cells, the presence of 776C did not significantly influence FU cytotoxicity ($F = 1.3 \pm 0.7$), whereas the addition of FA led to a factor F comprised between 1.8 and 5.2 (Fig. 2C). When considering all cell lines, it clearly appears that FA induced a constant increase in F values, this augmentation being amplified when 776C was associated with FA (Fig. 2D).

Table 2 summarizes for each cell line the median F values obtained when FU was modulated by FA at 5.10^{-6} M, 776C, and FA + 776C. This table also presents the statistics on the influence of FA and 776C on FU modulation, as well as on the possible interaction between 776C and FA on FU modulation. Interestingly, 776C enhanced FU-FA cytotoxicity even in the cells where it could not increase cytotoxicity of FU alone (MCF 7 and COLO 205). A significant interaction between 776C and FA on FU potentiation was demonstrated in cell lines in which FU was sensitive to the individual modulation by FA and 776C (CAL 27, CAL 33 and CAL 85-2) as well as on the whole panel of investigated lines. As shown by the median F values, this interaction reflected more than additive effects of 776C and FA when tested in combination.

DISCUSSION

The importance of FU catabolism in tumoral cells has been recently underlined [5, 6, 15] and strengthens the notion that DPD inhibition could be a promising and complementary target for FU pharmacomodulation. 776C has previously been demonstrated to be a very potent, mechanism-based, irreversible DPD inactivator *in vitro* [7] and *in vivo* [8]. Interestingly, previous studies have shown that 776C improves FU efficacy, FU therapeutic index, and oral FU

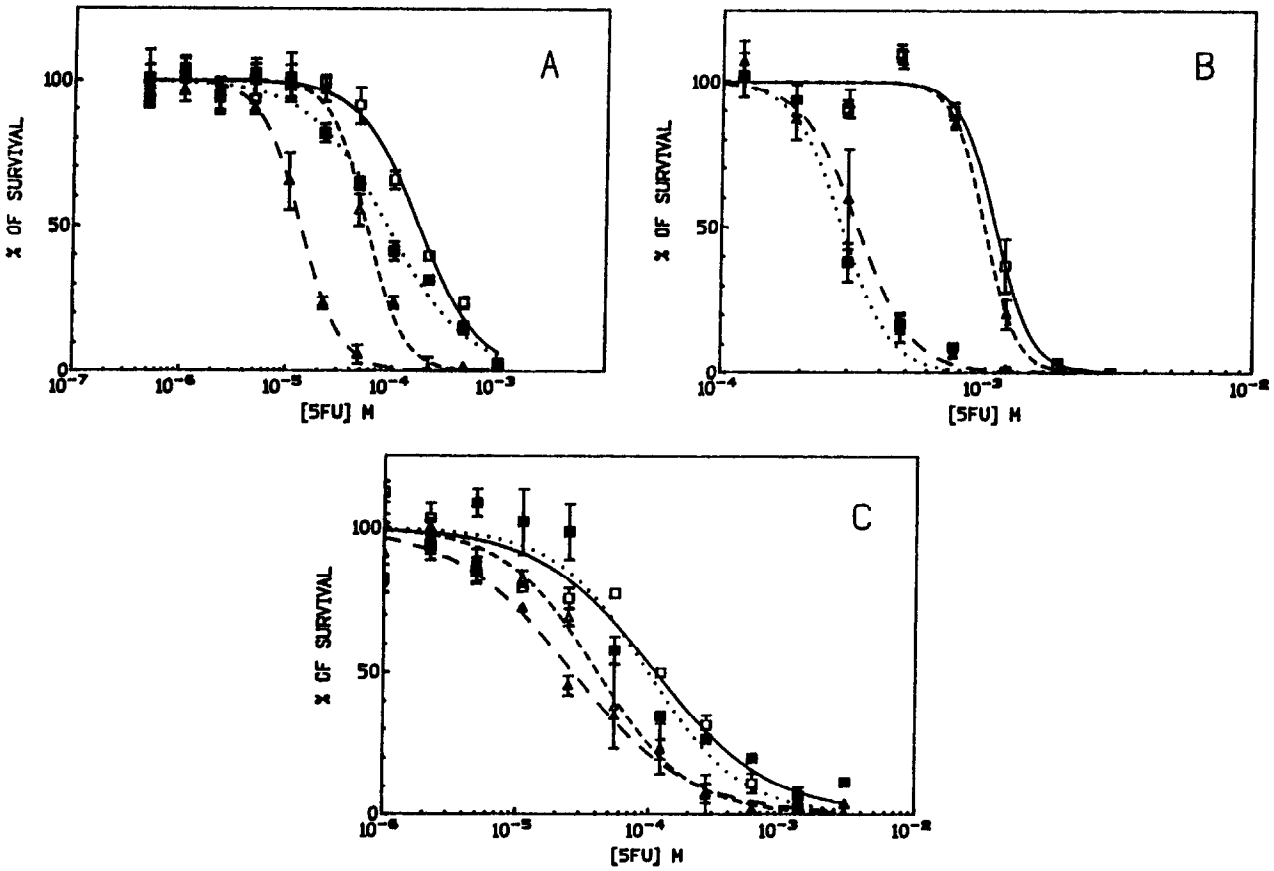

FIG. 1. Dose-effect curves of FU and FU plus FA and/or 776C for characteristic cell lines: A = CAL 27 for which FU is modulated by FA and 776C; B = HUTU 80 for which FU is modulated by 776C only; and C = MCF 7 for which FU is modulated by FA only. □ = FU alone; ■ = FU + 1 μM 776C; △ = FU + 5 μM FA; ▲ = FU + 1 μM 776C + 5 μM FA.

availability in laboratory animals [16–18]. Enhancement of FU cytotoxicity by 776C *via* DPD inhibition could be explained, at first view, by an increased activation of FU *via* its anabolic pathways. Another complementary explanation could lie in the role of FU catabolites. Spector *et al.* [19] recently reported that co-administration of FUH2 in FU-776C treated rats bearing colorectal carcinoma decreased the antitumor activity of FU-776C compared to that produced by FU alone. Thus, FUH2 or other downstream FU catabolites impaired FU cytotoxicity. In line with this observation, 776C could improve FU efficacy by decreasing the formation of FU catabolites.

We recently confirmed on a panel of 12 human cancer cell lines [9] that DPD activity measured on intact cells was suppressed in the presence of 776C. The degree of DPD inhibition was 776C-concentration-dependent and approximately 100% inhibition was achieved with a micromolar concentration of 776C. For this reason, 776C was tested at 1 μM in the present study. In addition, we demonstrated that the level of tumoral DPD activity was a significant predictor of the FU cytotoxicity enhancement by 776C [9]. In fact, FU cytotoxicity was not enhanced in cell lines expressing a low DPD activity. In contrast, FU sensitivity was markedly increased, up to a factor of 5, in cell lines expressing the highest DPD activity [9]. The present study confirms this finding, since cell lines resistant to 776C modulation were those expressing the lowest DPD activity (non-detectable (ND) and 6 pmol/min/mg prot respectively in MCF7 and COLO 205, Table 1). However, elevated DPD activities in tumor cells are apparently not a prerequisite for benefit from 776C in animals [16, 17].

From the clinical point of view, benefit from FU biochemical modulation is rare, a noticeable exception being FU modulation by leucovorin [20]. In colorectal cancers, with the exception of the study from the Southern Oncology Group demonstrating that leucovorin did not improve the FU response rate [21], randomized trials have demonstrated the efficacy of FU modulation by leucovorin in the treatment of metastatic disease [20, 22] as well as in the adjuvant situation [23, 24]. Interestingly, FU biomodulation *via* DPD inhibition takes place at an early stage in the FU metabolic pathway. Thus, at least additive effects could be expected from a multiple pharmacomodulation, including a DPD inhibitor and other biochemical modulators acting on the anabolic pathway. Such a strategy was recently explored by Cao *et al.* [25] on rats bearing chemically induced colorectal carcinoma. These authors used UFT (mixture of ftorafur, a FU prodrug, and uracil, a competitive DPD inhibitor) and studied the effects of the combination of UFT with FA and/or PALA (which in-

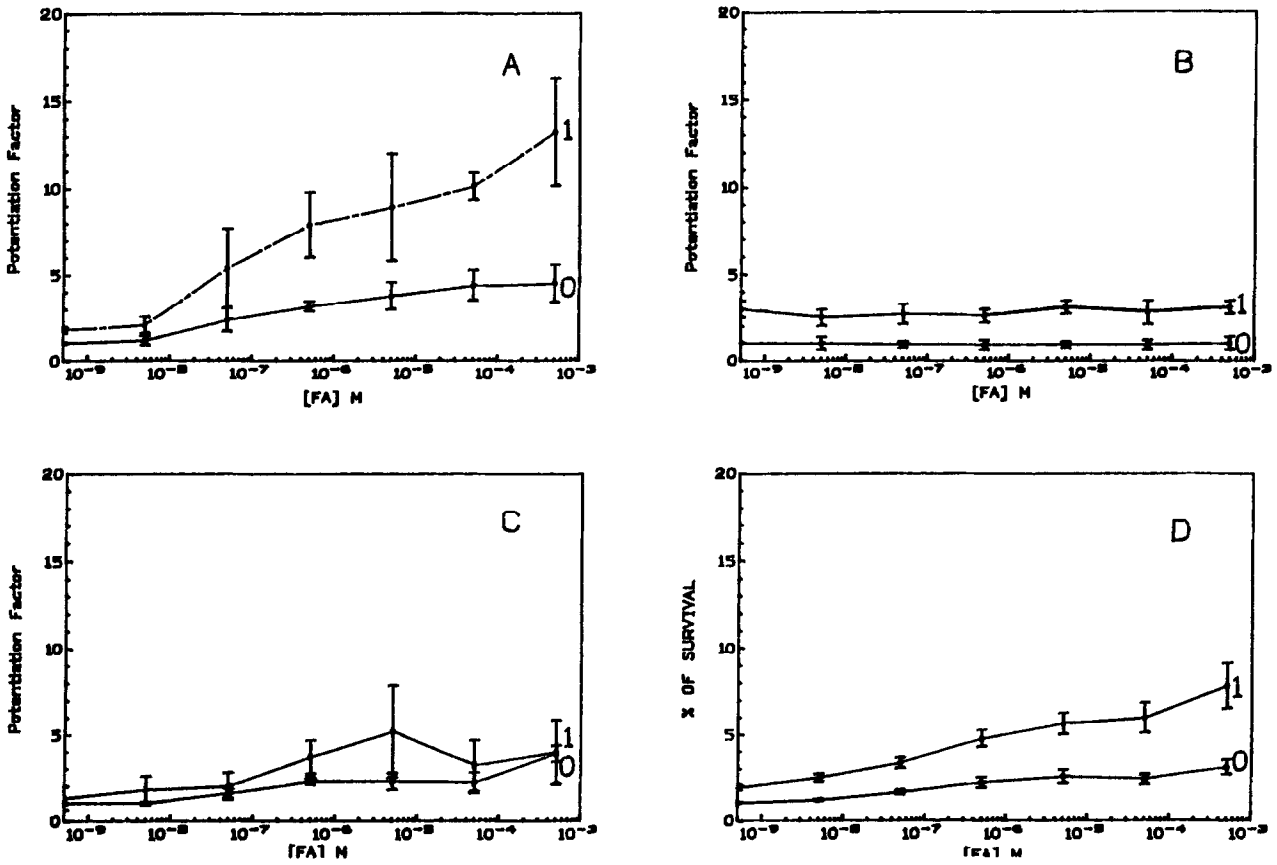

FIG. 2. Plot of the evolution of the FU potentiation factor (F) according to the FA concentration applied in the culture medium in the presence (1) or absence (0) of 1 μM 776 C for characteristic cell lines and for the whole panel of cell lines. A = CAL 27; B = HUTU 80; C = MCF 7; and D = whole panel. Vertical bars indicate the confidence interval of factor F for each FA concentration tested.

creases FU incorporation into RNA). Interestingly, the combination of UFT-FA-PALA produced the highest antitumor efficacy, with 100% of the treated animals achieving complete and sustained tumor regression [25]. These results, based on a single tumoral model, provide good prospects in the clinical setting for a multiple FU modulation including a DPD inhibitor together with more conventional biomodulators. In another study performed on the same animal model [26], 100% of complete response was also obtained with 776C administered in association with ftorafur.

We have evaluated herein the cytotoxic activity of FU associated with 776C and FA on a panel of 7 human cancer cell lines expressing spontaneous sensitivity to FU. These cell lines were chosen according to their previously demonstrated ability to respond to FU modulation by FA and/or 776C [9, 10]. The concentrations of FU, 776C and ℓ FA tested covered those encountered in patients [27, 28, 29], and particular attention was paid to the folate concentration in the culture medium (cells grown at the physiological concentration of the physiological folate, i.e. 40 nM of dℓ 5 methyltetrahydrofolate). The criterion chosen to evaluate FU modulation was the ratio between FU IC_{50} and FU IC_{50} in the presence of the tested biomodulator(s), defined

TABLE 2. Median FU enhancement factor (extremes) obtained with the different tested combinations (FA concentration or 5 μM)

Tested combination	CAL 27	CAL 33	CAL 85-2	CAL 51	HUTU 80	MCF 7	COLO 205	All cells
FU-FA	3.9 (2.9–4.5)	1.3 (1.2–3)	2.6 (2–3.6)	1 (0.9–1.4)	1 (0.7–1.1)	2.3 (1.8–2.9)	3.5 (2.7–9.1)	2.3 (0.7–9.1)
FU-776C	1.8 (1.5–1.9)	2.9 (1.2–3.2)	1.8 (1.2–1.8)	1.9 (1.6–2.2)	2.7 (2.7–3.6)	1.2 (0.6–2)	1.1 (0.9–1.5)	1.8 (0.6–3.6)
FU-FA-776C	8.4 (6.1–12.1)	5.3 (5.3–10.5)	8 (5.5–10)	2.4 (1.7–2.5)	3.2 (2.7–3.4)	4.2 (3.2–8.3)	5 (4.6–6.3)	5.3 (1.7–12.1)
Statistics *P* values								
776C effect	0.012	0.009	0.004	0.0005	$<1.10^{-4}$	NS	NS	$<1.10^{-4}$
FA effect	0.0006	0.02	0.0005	NS	NS	0.01	0.004	$<1.10^{-4}$
776C × FA interaction	0.04	0.05	0.01	NS	NS	NS	NS	0.005

The FU enhancement factor is defined as the ratio of FU IC_{50} (FU alone) divided by FU IC_{50} in the presence of 776C and/or FA.

as factor F. Interestingly, in cell lines responsive to FU modulation by FA and 776C, the dual modulation by FA + 776C led to F values much higher than those observed with FA or 776 C, suggesting more than additive effects of FA and 776C. This synergistic interaction was confirmed by the significant P value of the FA × 776C interaction demonstrated by the multivariate ANOVA ($P < 0.0001$). The FA × 776C interaction was also significant when the whole cell line panel was considered ($P = 0.0065$, Table 2). Due to inhibition of the catabolic pathway, FU modulation by 776C is supposed to enhance the formation of all FU anabolites including 5-fluorouridine thriphosphate (FUTP) which is incorporated into RNA, and 5-fluoro deoxyuridine (FdUMP) which inhibits thymidylate synthase. Interestingly, this significant interaction between 776C and FA strongly suggests a dual interaction of FA and 776C resulting from an optimal inhibition of thymidylate synthase due to an increase in FdUMP and 5–10 methylenetetrahydrofolate concentrations. Above all, the present study points out that combining FA and 776C is a very attractive modulation strategy for FU chemotherapy. Even though these *in vitro* data may not reflect *in vivo* results, particularly as regards a modification of the therapeutic index, they may be useful in the context of the ongoing phase II trials on FU combined with 776C and FA.

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