Enhanced Antitumor Activity of 5-Fluorouracil in Combination with 2’-Deoxyinosine in Human Colorectal Cell Lines and Human Colon Tumor Xenografts

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ABSTRACT

We investigated the effects of 2’-deoxyinosine (d-Ino), a modulator yielding thymidine phosphorylase activity, on cellular pharmacology of 5-fluorouracil (FUra) in various human colorectal cell lines and its antitumoral activity when combined with FUra in human xenografts. Associating d-Ino with FUra increased by 38 up to 700 times the sensitivity of HT29 and FUra-resistant SW620 lines, respectively, but not of CaCo2 cells, although high levels of intracellular FdUMP and subsequent higher thymidylate synthase inhibition were observed. Cell death studies confirmed the ability of d-Ino to enhance both early and late apoptosis induced by FUra in HT29 and SW620 but not in CaCo2. Similarly, we showed that associating d-Ino increased by 68 up to 101% the Fas overexpression induced by FUra in HT29 and SW620 but not in CaCo2 cells. Anti-Fas and anti-FasL antibodies both partly reversed this increase of cell sensitivity, thus confirming the role Fas plays in the modulation of FUra toxicity by d-Ino. This Fas component could explain the discrepancy between the lines because CaCo2 has been described as insensitive to Fas-mediated apoptosis. Antitumor activity of the combination was next investigated in nude mice transplanted with SW620. Results showed that although FUra alone has little effect on SW620 xenografts (P > 0.05), associating d-Ino significantly reduced the tumor growth by 57% (P < 0.05). This study suggests that it is possible to reduce both in vitro and in vivo resistance to FUra by modulating the way the drug is converted after cellular uptake.

INTRODUCTION

Forty years after its synthesis (1), FUra remains a mainstay in the treatment of colorectal cancer. However, because the overall response rate does not exceed 20% (2), improving FUra efficacy is still a major concern of today’s chemotherapy. Because FUra antiproliferative activity depends on its intracellular conversion to FdUMP, fluorodeoxyuridine triphosphate, and FUTP interfering with TS, DNA, and RNA, respectively (3–5), attempts to control and predict FUra activation have been described extensively (6). Recent studies underlined the key role TP could play in FUra metabolism. Increasing TP activity includes the use of IFN-α and IFN-γ (7–9), pyrimidine analogues (10), or direct transfer of the TP gene (11–14). This new trend in FUra modulation led us to investigate the effects of d-Ino, a deoxyribose 1-phosphate precursor, the ability of which to potentiate FUra cytotoxicity was reported in the late 1960s and 1970s (7–9). The purpose of this present work is to further study to what extent d-Ino can affect FUra cellular pharmacology in various human colorectal cell lines, to elucidate the mechanism by which cell death is augmented by this association, and finally to assess the possible use of this combination in human xenografts models.

MATERIALS AND METHODS

Cell Lines. HT29, SW620, and CaCo2 human carcinoma cell lines were kindly provided by Prof. Barra (UPRES A CNRS 6032, Marseille, France). Cells were maintained in DMEM supplemented with 10% FCS (CaCo2: 12%), 1% glutamine (CaCo2: 2%), 110 IU penicillin/ml, 100 μg streptomycin/ml, and 50 μg kanamycin/ml in a humidified CO2 incubator at 37°C.

Drugs and Chemicals. [6-3H]-FUra (12.6 Ci/mmol) came from DuPont NEN (Les Ulis, France), [3H]thymine (40 Ci/mmol) was provided by Isotopchim (Ganagobie, France), and [3H]FdUMP (16 Ci/mmol) was from Moravek Biochemicals (Brea, CA, USA). All other chemicals were purchased from

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3 The abbreviations used are: FUra, 5-fluorouracil; FuUrd, 5-fluoro-2’-deoxyuridine; FdUMP, fluorodeoxyuridine monophosphate; FUDP, fluorouridine diphosphate; FUTP, fluorothymidine triphosphate; d-Ino, 2’-deoxyinosine; MoAb, monoclonal antibody; TP, thymidine phosphorylase; TS, thymidylate synthase; dR1P, deoxyribose 1-phosphate; HPLC, high-performance liquid chromatography; FACS, fluorescence-activated cell sorter.
Sigma Chemical Co. (St. Quentin Fallavier, France). Anti-Fas ZB4 and CH11 and anti-FasL 4A5 MoAbs were provided by Immunotech (Marseille, France).

Antiproliferative Assays. Exponentially growing cells were exposed in 96-well plates to increasing concentrations of FUra, alone or combined with 1 mM d-Ino. Anti-CD95 ZB4 (500 ng/ml) and anti-FasL 4A5 (1000 ng/ml) MoAbs were added when indicated. The IC50 was defined as the FUra concentration inhibiting 50% of the cell growth. Control cells were incubated with d-Ino or MoAbs when the latter were used in the experiments. After 72 h of continuous exposure, cell growth was evaluated using the classic colorimetric 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide assay (15).

Determination of [3H]FUra Intracellular Metabolites. Separation and detection of [3H]FUra metabolites were performed as described previously (14). Exponentially growing cells were exposed to various combination of 100 μCi of tritiated FUra (final concentration 2 μM) alone or associated with 600 μM d-Ino for time intervals ranging from 0.5 up to 72 h. Cells were harvested, and cytosols were isolated for HPLC analysis. The HPLC consisted of a HP 1090 (Hewlett Packard) system coupled to a A200 radioactive flow detector (Packard). Separation of tritiated metabolites was achieved using a Lichrospher 100 RP18 5 μm column (Hewlett Packard) eluted by 50 mM K2HPO4 (pH 6.8) containing 5 mM tetrabutyl ammonium nitrate and 12% (0–9 min) to 16% (9–60 min) methanol.

Determination of dTTP DNA Incorporation. Cells were exposed to 50 μCi of tritiated thymine (final concentration 0.5 μM) with or without 1 mM d-Ino for 1, 24, and 48 h. Cells were then treated as described in “Determination of [3H]FUra Metabolites.” After centrifugation at 18,000 × g for 30 min, supernatant was discarded, and pellet was resuspended in 60% methanol and counted for radioactivity by liquid scintillation counting (Beckman).

TS Activity. TS activity was assessed as described previously (16). Briefly, exponentially growing cells were exposed to various combinations of FUra alone or associated with 1 mM d-Ino. Inhibition of TS activity was evaluated after two exposition schedules: a short exposition time (5 h) to high-dose FUra (10 times the respective IC50) and a long exposition (72 h) with a normal dose (IC50). Cells were then harvested, and pellet was stored at −80°C until analysis. TS activity was assayed following the standard Roberts method based on tritiated H2O.

Table 1 Modulation of 5-FUra antiproliferative activity by d-Ino +/- anti-Fas and anti-FasL, MoAbs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FUra (μM)</th>
<th>FUra + d-Ino</th>
<th>FUra + d-Ino + ZB4</th>
<th>FUra + d-Ino + 4A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>0.95 ± 0.1d</td>
<td>0.025 ± 0.007e</td>
<td>0.12 ± 0.04f</td>
<td>1.35 ± 0.07</td>
</tr>
<tr>
<td>SW620</td>
<td>73 ± 7</td>
<td>0.1 ± 0.03c</td>
<td>3.5 ± 1.4c</td>
<td>8.5 ± 0.7f</td>
</tr>
<tr>
<td>CaCo2</td>
<td>6.5 ± 0.7</td>
<td>5.5 ± 0.7</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

a d-Ino was used at a concentration of 1 mM.
b Anti-Fas ZB4 MoAb was used at a concentration of 500 ng/ml.
c Anti-FasL 4A5 MoAb was used at a concentration of 1000 ng/ml.
d Mean ± SD of three separate experiments.
e P < 0.05, one-way ANOVA with Tukey test.
f NE, not evaluated.

Fig. 1 Monitoring of [3H]FUra conversion to nucleosides and nucleotide monophosphate in SW620 cells. Cells were exposed to 2 μM tritiated FUra alone (A) or combined with 600 μM d-Ino (B) for 1–72 h. Cytosols were analyzed by HPLC, as described in “Materials and Methods.” Values are from one representative experiment.
release from [3H]dUMP in the presence of excess of methylene tetrahydrofolate (17).

**Apoptosis Studies.** Cells in exponential phase were exposed to d-Ino (1 mM), FUra (0.5 μM), or a combination of both for 48 h. Cells were then harvested; early apoptotic changes and late apoptosis were detected by simultaneous staining with Annexin V and propidium iodide using Annexin V FITC staining kit (Euromedex, Souffelweyersheim, France). Cells were treated following the manufacturer’s guideline. FACS analysis was carried out in a FACScan flow cytometer (Becton Dickinson) using Cell Quest Software. Apoptosis measured in untreated cells was defined as 100%.

**Detection of the CD95 (APO1-fas) Receptor.** Exponentially growing cells were exposed to 0.5 μM of FUra alone or combined with 1 mM d-Ino for 72 h. Cells were then trypsinized, washed, and exposed to 4 ng/μl of CH11 anti-Fas MoAb for 45 min at 4°C. After two washing steps, cells were resuspended in DMEM containing 1:200 (v/v) goat antimouse IgM (Immuno-tech, Marseille, France) and incubated for an additional 30 min at 4°C. Cells were then washed twice, and cell surface expression of the CD95 was assessed by FACScan. Analysis was carried out in a FACScan flow cytometer using Cell Quest Software. Cells exposed to goat antimouse IgM only served as negative FITC control. Relative Fas expression was defined as the ratio of fluorescence CH11 Fas MoAb:isotype-matched negative control MoAb. Relative Fas expression in untreated cells was considered as 100%.

**In Vivo Studies.** The antitumor effect of FUra combined with d-Ino was investigated in the SW620 mouse xenograft model. Swiss nude mice (Iffa Credo, L’Arbresles, France) were s.c. transplanted with fragments of SW620 harvested from s.c. growing tumors in nude mice. Mice care was in agreement with animal welfare guidelines. To avoid unnecessary use of animals, a prior experiment (saline versus d-Ino) was first carried out to assess the tolerance of high-dose d-Ino alone in SW620-bearing mice and its possible effect on tumor growth. The second part of the experiment (saline versus FUra versus FUra + d-Ino) started only after checking the absence of tumoral effect of d-Ino alone versus control in the prior experiment. All analyses were performed using Sigma Stat software (Jandel Scientific).

**RESULTS**

**Modulation of Antiproliferative Activity**

Results of cytotoxic studies are summarized in Table 1. Associating high dose d-Ino to FUra significantly increased HT29 and SW620 sensitivity by 38 and 700 times, respectively. Conversely, using d-Ino did not improve FUra cytotoxic activity in CaCo2. The increase of FUra toxicity in HT29 cells by d-Ino was reduced to eight times by ZB4 MoAb, and totally reversed when 4A5 MoAb was associated with the drugs. Similarly, ZB4 and 4A5 MoAbs reduced from 730 down to 21 and 9 times, respectively, when 4A5 MoAb was associated with the drugs. Similarly, ZB4 and 4A5 MoAbs reduced from 730 down to 21 and 9 times, respectively, when 4A5 MoAb was associated with the drugs.

**Modulation of Intracellular Activation**

HPLC analysis performed on cytosols of HT29, SW620, and CaCo2 cells exposed to [3H]FUra for various incubation times showed that the main activation pathway of the drug led to the formation of 5-fluorouridine, fluorouridine monophosphate, FUDP, and FUTP metabolites. Using d-Ino induced a switch of the FUra activation from the RNA to the DNA pathway, with FdUrd, FdUMP, fluoroxyuridine diphosphate,
and fluorodeoxyuridine triphosphate metabolites being then detected in the different lines we tested. This complete reversal in the activation pathway was constant throughout time up to 72 h, although FdUrd and FdUMP peaked for the shorter incubation times only (Fig. 1).

**[3 H]dTTP DNA Incorporation**

We monitored [3 H]thymine DNA incorporation as a marker of TP activity in whole cells. Incubating the cells with d-Ino induced a greater dTTP incorporation throughout time in all of the lines we tested. dTTP nuclear incorporation was potentiated by 13, 207, and 126 times (after 1, 24, and 48 h, respectively) in HT29 cells (P < 0.05; one-way ANOVA on ranks with Student-Newman-Keuls testing); 15, 22, and 8 times in SW620 cells (P < 0.05); and 11, 3, and 6 times in CaCo2 cells (P < 0.05).

**TS Inhibition**

FUra alone showed a marked effect on TS activity. However, associating d-Ino augmented TS inhibition in the different cell lines according to the exposition schedule. TS activity was inhibited by 78% (HT29), 95% (SW620), and 96% (CaCo2) after exposition to high-dose FUra for 5 h, whereas a total inhibition (100%) was observed in the different lines when d-Ino was associated to the drug. TS inhibition after 72-h exposure to lower doses of FUra was 48% (HT29), 75% (SW620), and 58% (CaCo2); combining d-Ino improved up to 81% (HT29), 99% (SW620), and 86% (CaCo2) the TS-directed action of the fluoropyrimidine (Fig. 2).

**Apoptosis Studies**

Results of detection of apoptosis are summarized in Fig. 3. A greater induction of both early and late apoptosis was observed in HT29 and SW620 exposed to FUra combined with d-Ino, compared with FUra alone. Enhancement of FUra-induced apoptosis when d-Ino was associated ranged from 19 to 130% (late/early), respectively, in HT29 cells and from 210 to 497% in SW620. Conversely, no increase of apoptosis was detected in CaCo2 (1.7% to 9%) exposed to the association compared with cell death caused by FUra alone.

**Expression of Fas CD95 Protein**

Exposing the cells to FUra alone induced an overexpression of Fas in HT29 (79%) but not in SW620 cells (1%). Associating d-Ino enhances Fas overexpression in HT29 (147%) and triggers Fas in SW620 (103%). On the contrary, we could not detect CD95 protein on the CaCo2 cell surface, whether the cells were exposed to FUra alone or combined with d-Ino because no difference in fluorescence was observed between cells exposed to anti-Fas CH11 MoAb and cells exposed to isotype-matched negative control MoAb only (Fig. 4).

**In Vivo Studies**

**Effects of d-Ino Alone on Tumor Growth.** The final tumor weights of mice treated with NaCl (mean, 1520 ± 695 mg) or d-Ino (mean, 1805 ± 580 mg) was not statistically different (P = 0.459; Student’s t test). Moreover, no difference was either observed in animal weights before (NaCl: 21.7 ± 0.7 g, d-Ino: 21.4 ± 1 g; P = 0.663; Student’s t test) and by the end of the experiment (NaCl: 23.8 ± 1.4 g, d-Ino: 23.5 ± 1.7 g; P = 0.765; Student’s t test) between the two groups, suggesting that the d-Ino doses used had been well tolerated.

**Effects of Combined d-Ino and FUra on Tumor Growth.** ANOVA analysis performed on final tumor weights at day 23 showed that there was a statistical difference between the treatments (P = 0.005). Further multiple comparison testing indicated that combining d-Ino to FUra significantly reduced...
tumor growth by 57% ($P < 0.05$; Tukey), whereas FUra alone had little effect ($P > 0.05$). Evolution of tumor weights is displayed in Fig. 5.

**DISCUSSION**

Several decades after the first use of FUra in colorectal cancer, its metabolism and mechanisms of action are still under scrutiny. According to the predominance of the active metabolites FdUMP, fluorodeoxyuridine diphosphate, and FUTP, FUra can exert its cytotoxic activity by inhibiting TS, interfering with DNA or RNA, respectively (3, 18). However, FdUMP is admittedly the main key active metabolite (19, 20), although some authors reported that TS may not be always the main locus of action of FUra, essentially in normal tissues (21, 22), but in some tumor models as well (23, 24). FdUMP formation is under the dependence of various enzymatic activities (25–27), but most data suggest its synthesis from FUDP through ribonucleotide reductase (26). Alternatively, FdUMP could be directly formed from FdUrd after activation of FUra by TP, but this pathway does not seem to be predominant in cells normally exposed to FUra, probably because of the lack of TP cofactor dR1P (28). However, several reports on FUra modulation by IFN recently highlighted the key role TP could play in FUra activation, suggesting that FUra cytotoxic activity could be enhanced when FdUMP is directly formed through the DNA pathway (11, 29, 30). In a previous work, we showed that transfecting the TP gene to human LS174T colon carcinoma led to an accumulation of intracellular FdUMP. This change of anabolism was accompanied with an 80 times increase in FUra toxicity in transfected cells compared with the parent line (14). Schwartz et al. (10) screened several pyrimidine analogues for their ability to increase TP activity and to enhance FUra efficacy both in vitro and in vivo, thus suggesting that providing the cells with precursors of dR1P could be a convenient alternative for augmenting TP activity and subsequent FdUMP accumulation. In this present work, we studied the effects when combined with FUra of d-Ino, a purine analogue providing dR1P after phosphorolytic cleavage (31). Although d-Ino is almost as old as FUra itself (7, 8, 29, 32), the extent of its effect on FUra cellular pharmacology and induction of apoptosis has not been fully reported yet, nor its possible in vivo use. Using high-dose d-Ino proved to greatly increase TP activity, FdUMP formation, and subsequent TS inhibition in the various cell lines we tested. Yet, although a significant increase of cell sensitization and apopto-
sis induction was observed in HT29 and SW620 cells, the CaCo2 line appeared to be resistant to the modulation. To understand this disparity, we then studied the cell death signaling occurring after exposition of the cells to the association. We showed an increase of Fas expression in HT29 and SW620 cells exposed to the combination, whereas both anti-Fas and anti-FasL MoAbs reversed, in part, the increase of cell sensitivity. These data suggest strongly a Fas component in the enhancement of FUra apoptosis by d-Ino. This implication of Fas signaling could explain the resistance of CaCo2 to this association, because this line has been described as insensitive to Fas-mediated apoptosis (33, 34).

The mechanisms by which FUra causes apoptosis are still not understood completely. It is much likely that the mechanisms underlying cell death induced by fluoropyrimidines are cell type specific and administration schedule dependent. The down-regulation of Bcl2 is associated with an up-regulation of Bax (35); augmentation of the p53 protein level (36, 37) has already been related to FUra-induced apoptosis. Other studies showed that FUra could cause apoptosis via a Fas-dependent pathway but without correlating this Fas overexpression with a particular mechanism of action of the drug (38). Houghton et al. (34) and Tillman et al. (39) demonstrated recently that thymineless death in colon cancer cells was mediated via Fas. The same isolated later a Fas component in cells treated with FUra modulated by leucovorin and/or IFN-γ (34, 39). Our results are consistent with these latest findings, because we showed that associating d-Ino to FUra resulted in an accumulation of FdUMP with increased TS inhibition.

Because cancer now is more considered as a matter of deficiency of apoptosis rather than proliferative cells, involvement of anticancer drugs in apoptosis is a key issue in today’s chemotherapy (40). Our data highlight the apoptosis-inducing potential of FUra via its active FdUMP metabolite through the Fas system. In addition, the insensitivity of CaCo2 cells to the modulation of FUra by d-Ino underlines the critical role cell death signaling plays in the response to fluoropyrimidine treatment. Although FdUMP accumulation and near total TS inhibition was achieved by modulating FUra conversion, the absence of Fas CD95 protein on the CaCo2 surface prevents the drug from exhibiting stronger antiproliferative action.

The feasibility of using d-Ino combined to FUra in vivo was next investigated in SW620-bearing nude mice. Because high levels of erythrocytic purine nucleoside phosphorylase could catabolize d-Ino before it reaches the tumor tissue (41), elevated but well-tolerated doses of d-Ino were administered to the animals. Although doses and treatment schedule were chosen empirically, our data showed that combining d-Ino to FUra significantly reduced tumor growth, whereas FUra alone has little but no effect.

Our results indicate that FUra efficacy can be improved both in vitro and in vivo by modulating the way the drug is converted after cellular uptake, and that direct formation of FdUMP via TP leads to increased cytotoxicity as long as tumor cells are sensitive to Fas-mediated apoptosis. Several studies revealed the role of TP in neoangiogenesis (42–44), underlining the dual role TP plays in treatment of colorectal cancer by fluoropyrimidines (45–47). Because it has been reported that TP exerts its angiogenic properties by catalytic action on thymidine to release chemoattractant and angiogenesis-inducing factors (48), increasing TP activity in its phosphorylase way only with d-Ino should not promote the development of angiogenesis.

It is commonly acknowledged that the main origin of TS-directed FdUMP metabolite comes from the reduction of FUDP by ribonucleotide reductase after prior conversion of FUra to FUDP via uridine phosphorylase or orotate phosphorylase transferase. Because several studies suggest that FUra could exert an higher cytotoxic activity when converted directly to FdUMP from FdUrd via TP, we assessed in this work to what extent d-Ino, a dRIP donor increasing TP activity, could affect FUra cellular pharmacology. We showed that d-Ino induces FdUMP accumulation with enhancement of both early and late apoptosis in a Fas-dependent manner, and that resistance of the SW620 line could be overcome in vitro and in vivo by combining the two drugs. Although the mechanism by which cell death is mediated in cells exposed to FUra is still not fully understood, our data show that there is a strong correlation between FdUMP accumulation and Fas expression. Complementary studies will have to be carried out to optimize treatment schedule and to fully confirm the mechanism of action of d-Ino when combined to FUra in vivo.

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