Combination of Thymidine Phosphorylase Gene Transfer and Deoxyinosine Treatment Greatly Enhances 5-Fluorouracil Antitumor Activity in Vitro and in Vivo

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Abstract

We reported previously that 5-fluorouracil (FUra) efficacy could be enhanced by increasing tumoral thymidine phosphorylase (TP) activity. Potentiated TP yield was achieved by either transfecting cells with human TP gene (A. Evrard et al., Br. J. Cancer, 80: 1726–1733, 1999) or associating FUra with 2′-deoxyinosine (d-Ino), a modulator providing the tumors with TP cofactor deoxyribose 1-phosphate (J. Ciccolini et al., Clin. Cancer Res., 6: 1529–1535, 2000). The purpose of the present work was to study the effects of a combined modulation (TP gene transfer + use of d-Ino) on the sensitivity to FUra of the LS174T human colorectal cell line. Results showed a near 4000 times increase of cell sensitivity in vitro after double (genetic + biochemical) modulation. This potentiation of tumor response was accompanied by a total change in the FUra anabolic pathway with a 5000% increase of cytosolic fluorodeoxyuridine monophosphate, a stronger and longer inhibition of thymidylate synthase, and 300% augmentation of DNA damage. Besides, whereas thymidine failed to inhibit FUra cytotoxicity in LS174T wild-type cells, the potentiation of the antitumor activity observed in the modulating regimen was partly reversed by thymidine, indicative of thymidylate synthase as the main drug target. The impact of this double modulation was next investigated in xenograft-bearing nude mice. Results showed that whereas FUra alone was completely ineffective on wild-type tumor growth, the size of TP-transfected tumors in animals treated with the FUra/d-Ino combination was reduced by 80% (P < 0.05). Our results suggest that FUra exhibits stronger antiproliferative activity when activated via TP through the DNA pathway and that high tumoral TP activity therefore leads to enhanced sensitivity to fluoropyrimidines.

Introduction

It is critical for a better understanding of the mechanisms of action of FUra to have a reasonable knowledge of the activation pathway the drug will be activated through after tumoral uptake.

Most data from the literature showed that FUra is mainly activated following the RNA pathway (1–3). Mechanisms underlying the formation of active FdUMP then require multi-step reactions toward synthesis through UP or orotate phosphoribosyltransferase of FUDP, followed by reduction into FdUDP and backwards dephosphorylation to FdUMP (3, 4).

Such a complex and indirect anabolism makes the predominance of FdUMP (and, subsequently, of TS inhibition) questionable in the pharmacological activity of FUra.

Indeed, although interference with de novo synthesis of dThd is admittedly the key mechanism of FUra antiproliferative action, some studies have reported that, in normal tissues and in some tumor models as well, TS may not always be the main site of action of this drug (5–7).

Besides, recent studies have suggested that direct FUra anabolism to active FdUMP through the DNA pathway could result in potentiated TS inhibition and higher drug efficacy (8–10). Schwartz et al. (8, 9) demonstrated that TP was the limiting step of FUra tumoral activation following the DNA pathway. Yielding tumoral TP activity could therefore enhance drug response by augmenting the direct formation of the active metabolite FdUMP.

Increasing TP activity can be achieved either by providing the cells with TP cofactors (11–13), associating FUra with drugs such as IFN (8), or transfecting tumors with TP cDNA to act as a “suicide gene” (9, 14, 15). Potentiated TP yield resulted in an increased formation of cytosolic FdUMP, with subsequent enhanced TS inhibition. In the present study, we assessed to what extent a double modulation strategy (TP-transfected cells exposed to FUra combined with d-Ino, a modulator increasing TP activity) could enhance tumor response in vitro and in xenograft-bearing mice.
Materials and Methods

Cell Lines. Human colorectal carcinoma LS174T and TP-overexpressing LS174T-c2 cells were obtained and fully characterized as described previously (15). The cells were maintained in DMEM supplemented with 10% FCS, 1% glutamine, 110 IU penicillin/ml, 100 µg streptomycin/ml, and 50 µg/ml kanamycin in a humidified CO2 incubator at 37°C.

Drugs and Chemicals. [3H]FUra (12.6 Ci/mmol) came from DuPont New England Nuclear (Les Ulis, France), [3H]thymine (40 Ci/mmol) was provided by Isotopochim (Gagnagobie, France), and [3H]dUMP (16 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). All other chemicals were purchased from Sigma Chemical Co. (St Quentin Fallavier, France).

Determination of dTTP Nuclear Incorporation. Monitoring of the nuclear incorporation of thymine as dTTP was performed as described previously (13). Cells in exponential phase were exposed to 0.5 µM [3H]thymine (50 µCi) ± 1 mM d-Ino for 24 h. The cells were then harvested and lysed in 60% methanol. After centrifugation at 18,000 × g for 30 min, the supernatant was discarded, and the pellet was resuspended in 60% methanol, and radioactivity was assessed by liquid scintillation counting (Beckman).

Antiproliferative Assay. Exponentially growing cells were exposed in 96-well plates for 72 h to increasing concentrations of FUra ± 1 mM d-Ino ± 20 µM dThd. The IC50 was defined as the FUra concentration inhibiting 50% of cell growth. Control cells were incubated with the modulators when the latter were used in the experiments. Cell viability was evaluated using the classic colorimetric 3-(4,5-dimethyl-2-yli)-2,5-diphenyltetrazolium bromide assay (16).

Determination of [3H]FUra Cytosolic Metabolites. Separation and detection of the [3H]FUra metabolites were performed as described previously (13), with minor modifications (13). Exponentially growing cells were exposed for 4, 24, and 48 h to 2 µM [3H]FUra (100 µCi) ± 1 mM d-Ino. The cells were then harvested, and cytosol was isolated for HPLC analysis. The HPLC system consisted of a HP 1090 (Hewlett Packard) system coupled to a A200 radioactive flow detector (Packard). Separation of [3H]-labeled metabolites was achieved using a Lichrospher 100 RP18 5-µm column (Hewlett Packard) eluted by 50 mM KH2PO4 (pH 6.8) containing 5 mM tetrabutyl ammonium nitrate and 5–32% gradient methanol.

Determination of TS Inhibition Duration. TS activity was monitored as described previously (13, 17). Briefly, exponentially growing cells were exposed to 10 µM FUra ± 800 µM d-Ino for 24 h. FUra was removed, TS activity was assayed, and the cells were then allowed to grow in FUra-free medium for 48 h. Restoration of TS activity was then evaluated.

Determination of DNA Damage by Comet Assay. The DNA-damaging effects of FUra and d-Ino were studied as described previously (18), with minor modifications, using a single cell gel electrophoresis test also known as the Comet assay. The cells were incubated for 24 h with 1 µM FUra ± 1 mM d-Ino. A positive control was performed by incubating the cells for 30 min in the presence of hydrogen peroxide. Thereafter, lysed cells embedded in agarose were electrophoresed under alkaline conditions, dyed with a SYBR Gold fluorogen (Interchim), and analyzed by Komet 4.0.2 software (Optilas Systèmes). The mean tail moment was used to quantify the DNA damage by videomicroscopic measurements of 50 individual cells. Results are expressed after weighing the data by tail moment of cells exposed to d-Ino alone to minimize the threshold.

Animal Studies. The antitumor effect of FUra alone or combined with d-Ino was investigated in xenograft-bearing Swiss nude mice. Each animal was s.c. transplanted with 2 × 106 of both LS174T wt (right leg) and TP-transfected LS174T-c2 (left leg) cells. Treatments started after the tumor became measurable (15 days after tumor graft). Animals (n = 8 animals/group) were given either saline (i.p.), d-Ino (1.6 mg/kg, twice daily i.p.), FUra (35 mg/kg, daily i.p.), or a combination of both d-Ino and FUra for 5 days, as described previously (17). Tumor size was measured twice a week using calipers, and tumor weight was calculated using the standard formula: weight (mg) = [length (mm) × thickness (mm)]/2. In vivo studies were carried out in agreement with local animal welfare guidelines.

Statistical Analysis. Differences between treatments were analyzed using either one-way ANOVA or one-way ANOVA on ranks with multiple comparison Newman-Keuls tests to determine which groups were different from each other. A P of 0.05 was regarded as significant. All analyses were performed using Sigma Chemical Co. Stat 2.03 software (Spss Science, Erkrath, Germany).

Results

dTTP Intranuclear Incorporation. We monitored the incorporation of thymine in the nucleus after cytosolic conversion into dTTP as marker of TP activity (8). Little but significant dTTP incorporation was found in wt cells incubated with [3H]thymine, suggesting a weak basal TP activity. Conversely, the dTTP incorporation was potentiated by more than 55 times when wt cells were incubated with [3H]thymine + d-Ino or in transfected LS174T-c2 cells exposed to [3H]thymine alone. Combining both approaches resulted in a 2000-fold increase of dTTP nuclear incorporation from tritiated thymine (Fig. 1).

Antiproliferative Assays. FUra always exhibited a higher cytotoxicity in cells displaying increased TP activity, regard-
Table 1  Potentiation of FUra cytotoxicity by biochemical, genetic, and combined modulation

<table>
<thead>
<tr>
<th>d-Ino (1 mM)</th>
<th>FUra IC50 (µM) and fold change versus FUra alone on wt</th>
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<tr>
<td>LS174T wt cells</td>
<td>LS174T-c2 cells</td>
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<td></td>
<td>66 ± 13 (&lt;1)</td>
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<td>0.057 ± 0.01² (&lt;1158)</td>
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* P < 0.05, significantly different from LS174T wt cells exposed to FUra alone (one-way ANOVA on ranks with Newman-Keuls multiple comparison test).

Results are the mean of three separate experiments.

Evaluation of DNA Damage. We used the Comet assay to assess the single-strand breaks induced by FUra. We found that DNA damage was potentiated by 20% in LS174T-c2 cells and by 51% in wt cells when d-Ino was associated with FUra. A more than additive effect (+303%) was observed when both approaches were combined (Fig. 6).

Animal Studies. Effect of double modulation was then investigated in xenograft-bearing nude mice. The animals were treated with either saline, FUra, high-dose d-Ino, or a combination of both drugs. Preliminary studies showed that high doses of d-Ino were well tolerated by the mice and did not affect tumor growth (17). No difference has been observed in the animal weights at study conclusion (data not shown). No difference was found between the growth of wt and TP-transfected xenografts in control groups or in animals treated with d-Ino alone. Results showed that FUra had little but no effect on LS174T wt growth, whereas a 43% decrease was observed in LS174T-c2 tumors (P > 0.05). Similarly, combining FUra with d-Ino induced a 62% decrease of LS174T wt tumors (P < 0.05). An 80% decrease was achieved in the LS174T-c2 group treated with FUra/d-Ino (P < 0.05), indicating that the combined modulation regimen had the greatest antitumor effect (Fig. 7).

Discussion

Studying the biochemical determinants of FUra efficacy is still a major concern of today’s chemotherapy (19, 20). Among the numerous factors likely to improve cell sensitivity, the role of tumoral enzymes involved in the conversion of prodrug FUra into active metabolites has been studied extensively. Because tumor response to fluoropyrimidines is multifactorial, determining a single marker of FUra efficiency remains hazardous and is often prone to controversy, even in vitro. For example, the activity of TK has been presented as a key determinant of response to FUra (21–23), whereas other studies have correlated high TK activity with resistance to fluoropyrimidine (24, 25), a fact that we experienced as well after transfecting the viral HSV1-TK gene into HT29

less of the manner in which potentiated TP yield was obtained: gene transfer (LS174T-c2 cells); use of d-Ino as a TP cofactor; or a combination of both. The increase in cytotoxicity ranged from 550 (TP-transfected cells) to 1158 times (FUra + 1 mM d-Ino in wt cells). The highest increase in FUra efficacy was achieved by exposing TP-transfected LS174T-c2 cells to FUra + d-Ino. This combination led to a more than additive 3882-fold potentiation of the drug efficacy (Table 1).

We then used dThd to determine the part of TS inhibition in the pharmacological activity of FUra. We found that dThd enhanced FUra potency by 3 times in LS174T wt cells. On the other hand, when FUra was associated with d-Ino in wt cells or after double modulation, the increase in FUra cytotoxicity described above in LS174T-c2 cells was partly reversed by dThd (Fig. 2).

Monitoring of Trinitiated FUra Activation. HPLC analysis performed on the cytosols of wt cells exposed to 2 µM [3H]FUra showed that the main activation pattern of the drug was the RNA pathway, leading to the formation of tritiated nucleoside and nucleotides throughout the time period studied. Monitoring of FUra metabolism after association with 1 mM d-Ino on LS174T-c2 cells or a combination of both showed a complete change of the metabolites detected, indicating that modulated FUra was then activated mainly via TP through the DNA pathway (Fig. 3). As a result, the active metabolite FdUMP level showed an up to 5700% increase at 4 h after combined modulation (Fig. 4).

TS Inhibition Study. The extent of TS inhibition after 24 h of treatment, along with the restoration of activity 48 h after removal of the drugs, is displayed in Fig. 5.

TS activity was inhibited by 88% in LS174T wt cells after a 24-h exposure to FUra alone. TS inhibition was potentiated up to 99% when d-Ino was used as a modulator of FUra activation. FUra alone induced a 91% inhibition of the enzyme in LS174T-c2 cells, whereas a total inhibition (100%) of activity was observed in the double-modulated regimen. The duration of the inhibition was assessed 48 h later. Only 3% of TS was still inhibited in LS174T wt cells treated previously with FUra alone (P > 0.05). Conversely, TS activity was still significantly inhibited by 52%, 33%, and up to 68% in LS174T wt cells exposed to FUra + d-Ino, LS174T-c2cells treated with FUra alone, and LS174T-c2 cells treated with FUra + d-Ino, respectively.
Similarly, although UP has been described as an important enzyme responsible for FUrA anabolism in several models (26), an attempt to modulate drug response after human UP transfection into MCF7 cells in our laboratory failed to increase FUrA cytotoxicity (27). Because TP is the first step of the DNA activation pathway leading to the direct formation of FdUMP (4), several studies have focused on the role this enzyme plays in FUrA efficacy. Schwartz et al. (8, 9, 12) first showed that TP-overexpressing colorectal tumor cells were more sensitive to fluoropyrimidine therapy, although the same authors could not confirm it clinically next. Similarly, we reported in previous studies that various murine and human cell lines exhibited higher sensitivity to FUrA when TP activity was potentiated. Higher TP yield was obtained either by gene transfer (14, 15) or by the use of d-Ino (13, 18), a TP cofactor precursor (28) that can be used as a convenient biochemical tool to orientate the FUrA anabolism through the DNA pathway (8, 29, 30).

Because intracellular levels of TP cofactor dR1P have been described as negligible in cells or tissues (31), we assumed that providing the tumors with dR1P precursor would be critical for optimizing the enzyme yield in cells transfected with human TP cDNA.

In the present work, we therefore examined to what extent increasing TP activity by double [genetic (TP gene transfer) + biochemical (d-Ino administration)] modulation would help enhance tumor response to FUrA in vitro and in vivo. Our experiments were carried out in LS174T human adenocarcinoma cells because these cells display little but no basal TP activity (15). They were therefore the most suitable model in which to study the effects of any increase of this enzyme expression and/or activity in terms of sensitivity to fluoropyrimidine drugs. In this respect, the various other cell lines we used previously to explore FUrA molecular pharmacology such as HT29, CaCo2, or SW620 cells (13, 17) were not selected here.

HPLC analysis showed that FUrA is essentially activated through the RNA pathway in the LS174T cells. As a result, the main metabolites found in these tumor cells were ribonucleoside fluorouridine and ribonucleotides. Either biochemical, genetic, or double modulation triggers the DNA pathway with subsequent accumulation of cytosolic deoxy derivatives, including active FdUMP. This change in the anabolic pathway was accompanied by a strong potentiation of FUrA activity.
The combined modulation strategy led to a striking 4000 times increase in cell sensitivity, a drug response enhancement never reported in previous attempts to modulate FUra efficacy. This dramatic synergistic effect confirms our hypothesis that although a strong TP overexpression could be achieved by gene transfer, providing the cells with cofactor dR1P as well was indeed critical to optimize the enzymatic yield that will ensure proper FUra activation through the DNA pathway.

Analysis of TS activity suggested that a stronger and prolonged inhibition observed in cells displaying higher TP activity and exposed to FUra was probably at the origin of this increase in cytotoxicity.

This was then confirmed by adding dThd to the medium to fully address the part of TS inhibition in the pharmacological effects of FUra following the different regimen tested (32). When used in combination with FUra alone on wt cells, dThd increases the drug efficacy by 3 times, a property that is admittedly associated with enhanced FUra incorporation into mRNA (33–35). On the other hand, dThd proved to partially reverse the increase in FUra cytotoxicity obtained after either single or combined modulation. dThd thus acts as a rescue agent, most probably by repleting the dTTP pool (36). The spectacular switch of dThd effects from enhancement to rescue toward FUra cytotoxicity strongly suggests that although it is significantly inhibited in LS174T cells, TS is not normally the main locus of action of FUra in this cell line, an observation that has been reported previously in some other models (6, 7). Taken together with our analytical data, this reversal of the effects of dThd according to the pattern of FUra activation clearly demonstrates that the drug’s mechanism of action depends on its early activation pathway. Thus, increasing tumoral TP yield to trigger the DNA pathway appears to be necessary to induce a TS-directed effect that will be reversed by the use of dThd.

We next carried out Comet assay studies to determine the importance of DNA damage as the end point of FUra activation through the DNA pathway. In concordance with the above-mentioned observations, we found that the highest increase of single-strand breaks occurred in TP-transfected cells exposed to FUra + d-Ino.

Finally, we studied to what extent transposing the double modulation strategy to xenograft-bearing nude mice would potentiate FUra efficacy in vivo. The double modulation regimen proved to improve tumor size reduction by FUra at study conclusion from 0% to 80%. Interestingly, the single modulation regimen led to intermediary decreases in tumor size (43% and 62%, respectively), an observation fully consistent with the data we obtained in vitro.

However, although it was significantly improved, the FUra antitumor activity in vivo in the modulating regimen was not as spectacular as the in vitro enhancement would have suggested. Such a loss of efficacy between in vitro and animal studies is fairly common in experimental therapeutics. This is due mostly to the lack of control of experimental conditions possibly affecting the tumor response to FUra in vivo, e.g., the dThd levels in the xenografts. Besides, d-Ino, as a purine derivative, is likely to be catabolized by erythrocytic nucleoside phosphorylases (37), thus preventing the modulator to be as efficient as it proved to be in vitro.

Additional studies will be required to precisely identify the pharmacological effects of double-modulated FUra in vivo, although our in vitro background provides strong support for the hypothesis that increased TP activity is the key mechanism by which FUra efficacy is optimized.

The relationships between TP activity and chemosensitivity are still debated. Because TP has been reported to promote neoangiogenesis (38, 39), some studies, including those in fluoropyrimidine-based therapies, have associated high tumoral TP activity with poor clinical response (40, 41). However, no clear association was observed between response status and TP expression in other studies (42), and conversely, some clinical data demonstrated that higher tumoral TP activity was correlated with better survival in FUra-treated patients (43, 44).

This discrepancy indicates that TP status alone cannot predict chemosensitivity as a single molecular marker be-
cause of the numerous factors involved in the sensitivity to fluoropyrimidines (i.e., downstream apoptosis signaling pathways) and because of the ambivalent and probably tumor-dependent effects of this enzyme (42, 45). Saito et al. (44) showed in patients with advanced gastric carcinoma that TP expression was correlated with both microvessel density and favorable prognosis in FUra-treated groups, an unexpected finding that perfectly highlights the dual role played by TP in cancer treatment with fluoropyrimidines. Similarly, our results clearly indicate that higher TP activity will lead to dramatically increased sensitivity to FUra, both in vitro and in animals. Besides, immunohistochemical analysis of vimentin and BNH9 proteins as angiogenesis markers in slices of TP-overexpressing LS174T-c2 xenografts did not show any increase of endothelial protein expression compared with wt xenograft (data not shown). These data are in agreement with the fact that untreated LS174T-c2 tumors did not grow any faster than untreated wt ones, an observation reported in previous studies carried out with other murine and human TP-overexpressing tumors (14, 17).

Although xenograft models in nude mice are certainly not the most suitable model to address the issue of neoangiogenesis, our results indicate that the combined modulating regimen selectively increases cancerous tissue sensitivity to FUra without promoting tumor growth in untreated animals. Thus, increasing TP activity in tumors appears to be an interesting and very efficient way to optimize FUra efficacy both in vitro and in vivo, provided that the cells are sensitive to Fas-mediated apoptosis (17, 46).

Despite the potential tumor growth enhancement that could result from neoangiogenesis induction by TP, we believe that because TP-overexpressing cells are selectively killed by fluoropyrimidine therapy, they should not be able to promote angiogenesis.

In conclusion, our data shed light on the utterly controversial issue of TP in cancer treatment with fluoropyrimidines because our results clearly indicate that potentiated TP activity is critical for optimizing FUra efficacy in vitro and in vivo.

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References


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