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The effect of fluoropyrimidines with or without thymidine phosphorylase inhibitor on the expression of thymidine phosphorylase

Michiel De Bruin[a], Toni Van Capel[a], Kees Smid[a], Masakazu Fukushima[b], Klaas Hoekman[a], Herbert M. Pinedo[a], Godefridus J. Peters[a,*]

[a] *Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, P.O. Box 7057, 1081 HV Amsterdam, The Netherlands*
[b] *Taiho Pharmaceutical Co. Ltd., 1-27 Misugidai, Hanno, Saitama 357-8527, Japan*

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Abstract

Thymidine phosphorylase (platelet-derived-endothelial-cell-growth-factor) catalyzes the reversible phosphorolysis of thymidine to thymine and 2-deoxyribose-1-phosphate, activates 5′ -deoxy-5-fluorouridine (5′ DFUR) and inactivates trifluorothymidine (TFT). The effect of 5′ DFUR and TFT with or without a specific thymidine phosphorylase inhibitor (TPI) on thymidine phosphorylase mRNA, protein expression and activity was studied, in three human colon cancer cell lines, WiDR, HT29 and Lovo exposed for 72 h at IC_{50} concentrations. In Lovo cells TFT plus TPI only increased thymidine phosphorylase-protein expression 1.7-fold; 5′ DFUR and TFT treatment increased thymidine phosphorylase mRNA levels 5- and 1.4-fold, respectively. In WiDR cells, 5′ DFUR plus TPI significantly decreased thymidine phosphorylase-protein. TFT and TFT plus TPI increased thymidine phosphorylase-protein 2- and 3-fold, respectively. TPI and 5′ DFUR decreased thymidine phosphorylase-mRNA levels significantly. In HT29 cells, 5′ DFUR and 5′ DFUR plus TPI decreased both thymidine phosphorylase-protein and thymidine phosphorylase-mRNA. In all cell lines 5′ DFUR and TFT did not affect thymidine phosphorylase activity, but treatment with TPI (alone or in combination) eliminated thymidine phosphorylase activity. This demonstrated that regulation is drug and cell line dependent.

Keywords: Thymidine phosphorylase; 5′-Deoxyfluorouridine; Trifluorothymidine; Thymidine phosphorylase inhibitor

1. Introduction

Thymidine phosphorylase is a pyrimidine nucleoside phosphorylase, catalyzing phosphorolytic cleavage of thymidine to thymine and 2-deoxy-ribose-1-phosphate. In addition, several fluoropyrimidines such as 5-fluorouracil (5FU), 5′ deoxy-5-fluorouridine (5′ DFUR) and trifluorothymidine (TFT) can act as substrate for thymidine phosphorylase (Ackland and Peters, 1999; Fukushima et al., 2000). Angiogenic properties have been ascribed to thymidine phosphorylase, since the pro-angiogenic platelet derived endothelial cell growth factor (Miyazono et al., 1987) showed a strong sequence homology to thymidine phosphorylase (Furukawa et al., 1992) and can phosphorolytically cleave thymidine (Usuki et al., 1992; Moghaddam and Bicknell, 1992). Thymidine phosphorylase is expressed in a wide range of normal tissues and cells. In a multitude of solid tumor types thymidine phosphorylase was found to be upregulated compared to normal tissues, including breast, bladder, gastric, colorectal and lung cancer (Ackland and Peters, 1999). Thymidine phosphorylase expression correlates with microvessel density in various studies of solid tumors (Reynolds et al., 1994; Takebayashi et al., 1996; van Triest et al., 2000). High thymidine phosphorylase was prognostic for a poor survival rate (Takebayashi et al., 1996).

In contrast, thymidine phosphorylase overexpression provided a survival benefit in node positive breast carcinomas treated in an adjuvant setting with cyclophosphamide, 5FU and methotrexate (Fox et al., 1997; Gasparini et al., 1999). This could possibly be ascribed to thymidine phosphorylase mediated enhanced 5FU activation (Evrard et al., 1999; De Bruin et al., 2003) or by an enhanced effect of methotrexate via a thymidine phosphorylase mediated depletion of the thymidine pool (Patterson et al., 1998).

* Corresponding author. Tel.: +31-20-4442633; fax: +31-20-4443844.
E-mail address: gj.peters@vumc.nl (G.J. Peters).

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Capecitabine (Xeloda) is an example of a drug exploiting enhanced thymidine phosphorylase expression at tumor sites. Capecitabine is converted to 5FU in three distinct steps. The final step is the conversion of 5′ DFUR to 5FU and is mediated by thymidine phosphorylase. Enhanced thymidine phosphorylase expression at tumor sites therefore would selectively increase 5FU levels in situ (Miwa et al., 1998).

TFT was previously evaluated for cancer therapy (Ansfield and Ramirez, 1971) and has been suggested to have efficacy in 5FU resistant cell lines (Murakami et al., 2000). TFT is currently being developed as an oral fluoropyrimidine in combination with a specific potent inhibitor of thymidine phosphorylase, TPI ($K_i = 2 \times 10^{-8}$ M) as TAS-102 (TFT: TPI; 1: 0.5 molar ratio). TPI in this combination prevents TFT degradation, and increases bioavailability in vivo (Fukushima et al., 2000). Besides potentiation of TFT, TAS-102 has an additional role of decreasing the potential angiogenic porperties of thymidine phosphorylase by inhibition of its activity.

In summary, thymidine phosphorylase provides two potential therapeutic targets. One is inhibition, thereby lowering its potential angiogenic effect. The other is utilization of the high thymidine phosphorylase expression found in tumors to activate capecitabine (Focher and Spadari, 2001; Marchetti et al., 2001). It is known that enzymes from the pyrimidine pathway are highly regulated by either substrate or product (Peters and Veerkamp, 1983; Weckbecker, 1991). Therefore, the aim of this study was to determine the effect of clinically used oral fluoropyrimidines on thymidine phosphorylase expression. For that purpose we investigated whether 5′ DFUR and TFT with or without an inhibitor, had an influence on the expression and activity of thymidine phosphorylase in three colon cancer cell lines.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from Gibco BRL (Life Technology, Breda, The Netherlands). 5′ DFUR was purchased from Sigma (St. Louis, MO, USA), TFT and TPI were provided by Taiho Pharmaceuticals (Hanno, Japan). Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes, Hyperfilm ECL and ECL (plus) detection kit were obtained from Amersham International (Buckinghamshire, UK). The primary polyclonal antibody was goat anti-human platelet derived endothelial cell growth factor PD-ECGF (R&D systems, Abingdon, UK), the secondary antibody was peroxidase-conjugated rabbit anti goat (Dako, Glostrup, Danmark). RNAzol was obtained from Campro Scientific (Veenendaal, The Netherlands); Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) from Promega (Madison, WI, USA); deoxynucleotides (dNTPs), random hexamers and Taq polymerase from Pharmacia Biotech (Roosendaal, The Netherlands). All other chemicals were commercially available in analytical grades.

2.2. Cell lines

The origins of the human colon carcinoma cell lines, Lovo, WiDR and HT29, have been described previously (van Triest et al., 1999). Each cell line is known to differ in their thymidine phosphorylase activity (De Bruin et al., 2003). All colon cancer cell lines were maintained in DMEM supplemented with 10% fetal calf serum. All cells were cultured at 37 °C in a 5% CO_2 fully humidified atmosphere. Cell lines were growing exponentially as monolayers during the course of all experiments. In order to measure thymidine phosphorylase mRNA, protein and activity, cells were harvested after treatment, counted, snap frozen in liquid nitrogen, and stored at −80 °C until use.

2.3. Growth inhibition experiments

To determine the IC_{50}s (drug concentrations resulting in 50% growth inhibition), the sulforhodamine B (Sigma) staining method was used (Skehan et al., 1990; Keepers et al., 1991). Briefly, cells were seeded with or without 10 μM TPI at a density of 5000 cells/well, ensuring exponential growth during the experiment. Drugs were added after 24 h at various concentrations and cells were incubated for 72 h. After treatment cells were fixed with trichloro-acetic acid and stained with sulforhodamine B. Optical densities were measured on an automated spectrophotometric microplate reader (Spectra Fluor Tecan, Austria) at an absorbance of 540 nm. The IC_{50}s are represented as means of at least three values. The dose modifying factor (DMF) is used to express the effect of TPI and is calculated as: $(IC_{50} + TPI)/IC_{50}$.

2.4. Treatment

In order to study the effects TFT and 5′ DFUR on thymidine phosphorylase expression and activity cells were exposed the to their respective IC_{50} concentrations found in the presence of thymidine phosphorylase inhibitor, regardless of whether TPI was actually present or not. For TFT the IC_{50} values did not change in the absence of TPI. For 5′ DFUR the absence of TPI slightly increased the drug cytotoxicity resulting in an IC_{60} for WiDR and HT29. The Lovo cell line showed a larger increase in cytotoxicity giving an IC_{75}. However, we preferred to keep the drug concentration constant. In all treatments TPI was used at a concentration of 10 μM which is non-toxic to the cells and results in complete thymidine phosphorylase inhibition (De Bruin et al., 2003).

2.5. Western blot analysis

Western blotting conditions for the cell lines under investigation have been described previously (De Bruin et al., 2003). Briefly, logarithmic growing cells were harvested and cell pellets were lysed, sonificated and centrifuged. Protein content of each sample was assayed using the BioRad assay (BioRad Laboratories, Richmond, CA). Thirty-microgram protein of each sample was loaded, separated on gel and electroblotted onto a nitrocellulose membrane. Detection was performed using the primary antibody goat anti-human PD-ECGF, followed by horseradish peroxidase conjugated rabbit anti-goat antibody combined with ECL plus. Protein expression was quantified by densitometric scanning (model GS-690 and Molecular Analist, BioRad Laboratories).

2.6. Competitive template reverse transcriptase polymerase chain reaction (RT-PCR) to determine thymidine phosphorylase mRNA expression levels

The quantitative RT-PCR technique is based on the co-amplification of a competitive template, functioning as an internal standard designed specifically for each different target. The principles have been described in detail elsewhere (Willey et al., 1998).

RNA was extracted from 5×10^6 cells by the RNAzol™ method. Each extract was checked for DNA contamination and reverse transcribed by M-MLV-RT using random hexamers as described by the manufacturer (Promega). competitive templates were designed for β-actin (Rots et al., 2000) and thymidine phosphorylase, using primers previously described (De Bruin et al., 2003). Competitive templates were dissolved to a known concentration. The polymerase chain reaction (PCR) was used for co-amplification of the cDNA samples with competitive templates to ensure accurate quantification of the native template. In order to normalize thymidine phosphorylase expression to that of β-actin, one master mix was prepared and aliquoted. To each aliquot either the primer pair for β-actin or thymidine phosphorylase together with cDNA sample was added. PCR products (native template, heteroduplex and competitive template) were separated by electrophoresis and measured by densitometry. The intensity of the native template and competitive template bands was quantified by digital image analysis using Scion Image software (NIH, Bethesda, DC, USA). Concentrations of native template molecules of thymidine phosphorylase and β-actin in the cDNA samples were calculated by the ratio of native template/competitive template after amplification. The concentration of the competitive template mixture was used as described previously (Rots et al., 2000). The relative expression of thymidine phosphorylase mRNA was given as the ratio of the concentration native template of thymidine phosphorylase versus native template of β-actin.

2.7. Thymidine phosphorylase activity

The thymidine phosphorylase activity was determined using thymidine as a substrate, where the conversion to thymine is directly proportional to the activity of thymidine phosphorylase (Laurensse et al., 1988). Twenty million cells were resuspended in 1 ml 50 mM Tris/1 mM EDTA (pH 7.4), sonicated and centrifuged at $21{,}000 \times g$ at 4 °C. Linearity of the reaction was assured in protein and time. Aliquots of the $21{,}000 \times g$ supernatant (HT29; 25 μl: WiDR; 25 μl: Lovo; 10 μl) were mixed with 10 μl 0.8 M K_2HPO_4 and 10 μl 56 μM [2-^{14}C]-thymidine (specific activity: 60 μCi/μmol). Tris/EDTA (50/1 mM, pH 7.4) buffer was added to achieve a final volume of 80 μl. Each sample was incubated for 15 or 30 min at 37 °C. The reaction was terminated by heating to 95 °C for 3 min. After termination, 20 μl thymine (5 mM) and thymidine (5 mM) were added to visualize the products of the reaction on thin layer chromatography (TLC) sheets. Five-microliter reaction mixture was spotted onto polyethyleneimine-cellulose TLC sheets, which were then developed in H_2O. Separated substrate and products were cut from the TLC sheets and radioactivity content determined using a liquid scintillation counter after addition of Optima Gold liquid scintillation counting fluid (Packard Instrument, Chemical Operations, Groningen, The Netherlands).

2.8. Statistics

The one-tailed paired Student's *t*-test was used to study the effect of TPI on IC_{50}s of the different fluoropyrimidines. In order to study the effect of the different treatments on mRNA, protein expression and thymidine phosphorylase activity, the two-tailed paired *t*-test was used.

3. Results

3.1. Growth inhibition experiments

The results of the growth inhibition experiments are shown in Table 1. In all three cell lines, TPI decreased the

Table 1
IC_{50}s of the fluoropyrimidines (expressed in μM) in the presence or absence of TPI, for the three colon cancer cell lines

Cell line	5′ DFUR	5′ DFUR + TPI	DMF[a]	TFT	TFT + TPI	DMF
Lovo	24.9 ± 5.1	103.5 ± 17.3	4.2[b]	0.5 ± 0.1	0.4 ± .0.1	1.0
WiDR	90.1 ± 12.0	222.5 ± 25.0	2.5[c]	2.5 ± 0.8	3.5 ± 1.0	1.4
HT29	176.9 ± 27.3	275.8 ± 15.9	1.6	3.9 ± 1.0	3.7 ± 0.7	0.9

Modified from De Bruin et al. (2003).

[a] DMF: Dose modifying factor.

[b] Significant differences between drug and drug with TPI (Student *t*-test): $P < 0.05$.

[c] Significant differences between drug and drug with TPI (Student *t*-test): $P < 0.01$.

sensitivity to 5′ DFUR, but to a differing extent. Modulation was most pronounced in the Lovo cells. Surprisingly there was no effect of TPI on the IC_{50}s of TFT, despite the fact that TFT is a good substrate for thymidine phosphorylase, as described previously (De Bruin et al., 2003).

3.2. mRNA

The effects of drug treatment on the mRNA expression of the three colon cancer cell lines are shown in Fig. 1. In Lovo cells both TFT and 5′ DFUR increased thymidine phosphorylase mRNA, 1.4- and 5-fold, respectively. WiDR and HT29 cells showed different effects for 5′ DFUR treatment with or without thymidine phosphorylase inhibitor, both of which lowered the mRNA expression. TFT had little or no effect on thymidine phosphorylase mRNA expression in WiDR and HT29 cells. In TPI treated cells, a variable effect on mRNA was found; no effect was found in either Lovo or HT29 cells, whereas in WiDR cells TPI alone decreased thymidine phosphorylase mRNA.

3.3. Protein expression

The effect of drugs on protein expression was different for Lovo and WiDR cells (Fig. 2) compared to the effect observed for mRNA expression. However, in HT29 a more consistent pattern was found in relation to the observed mRNA levels. In the Lovo cells, a small increase of thymidine phosphorylase protein was found in TFT plus TPI treated cells. A more pronounced effect of TFT plus TPI was observed in the WiDR cells. In both WiDR and HT29 cells, a decrease of thymidine phosphorylase protein levels was detected in the 5′ DFUR treated cells, reflecting the decreased thymidine phosphorylase mRNA in these cells.

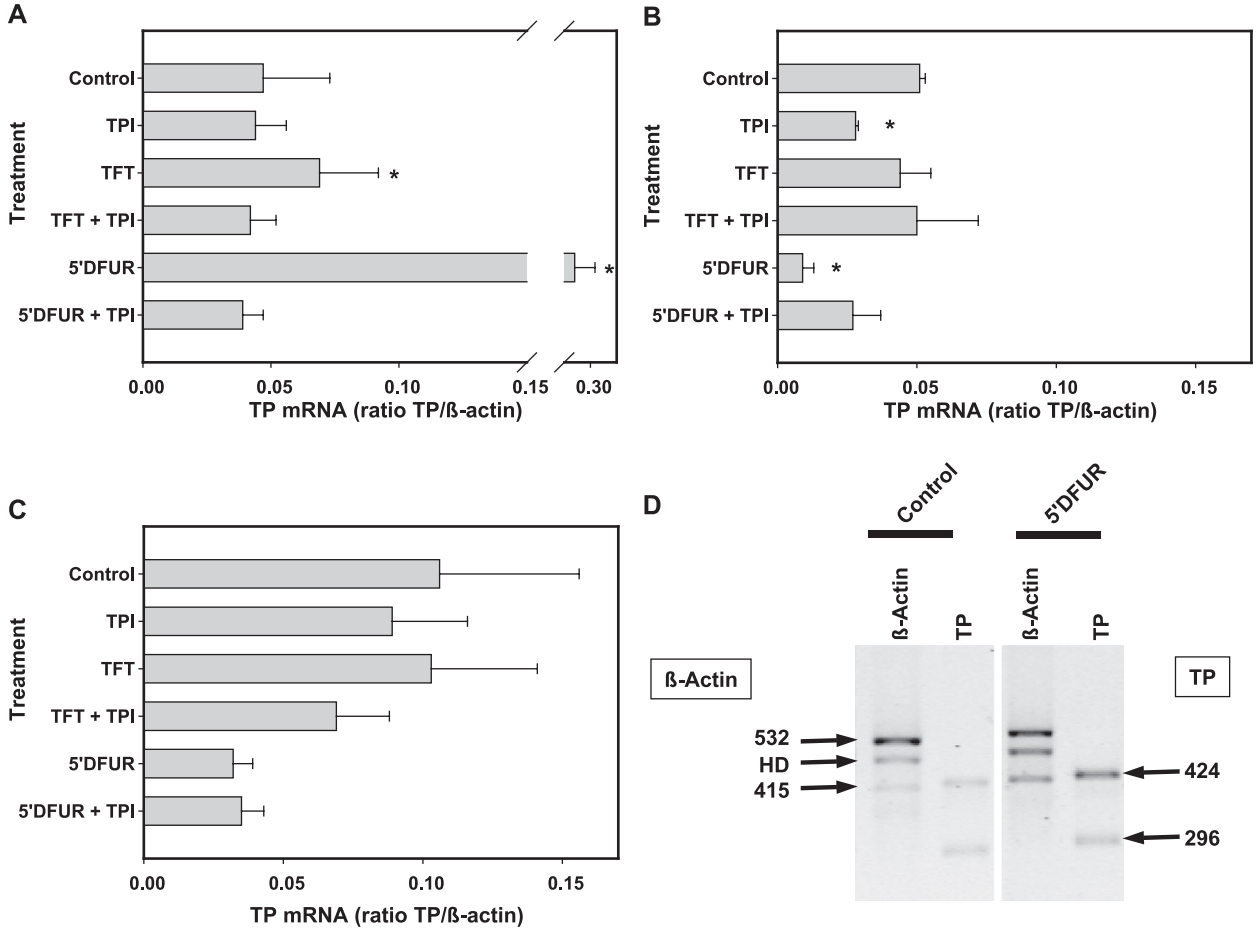

Fig. 1. Thymidine phosphorylase mRNA expression as measured with competitive template-RT-PCR in three colon cancer cell lines after 72 h exposure to IC_{50}s of the different fluoropyrimidines with or without 10 μM thymidine phosphorylase inhibitor. (A) Lovo, (B) WiDR and (C) HT29. Significant differences ($P < 0.05$) compared to controls are marked by an asterisk. Values are means ± S.E. of three independent experiments. (D) Representative example of an agarose gel on which PCR products are separated according to their expected sizes. The gels show three bands for β-actin: bands of 532 and 415 base pairs are encoded by the forward and reverse primer for the native cDNA and competitive template, respectively. The third band is the heteroduplex (HD) consisting of native cDNA and competitive template. For TP only two bands are visible: the native cDNA of 424 base pairs and 294 base pairs for the competitive template; the heteroduplex was formed occasionally. The bands were scanned and the OD was used to calculate a ratio between the native cDNA and competitive template. The contribution of the heteroduplex was calculated as described previously (Rots et al., 2000; De Bruin et al., 2003).

3.4. Thymidine phosphorylase activity

In order to determine whether the effect on TP mRNA and protein was reflected in an effect on activity we measured TP activity in drug treated cells (Fig. 3). In

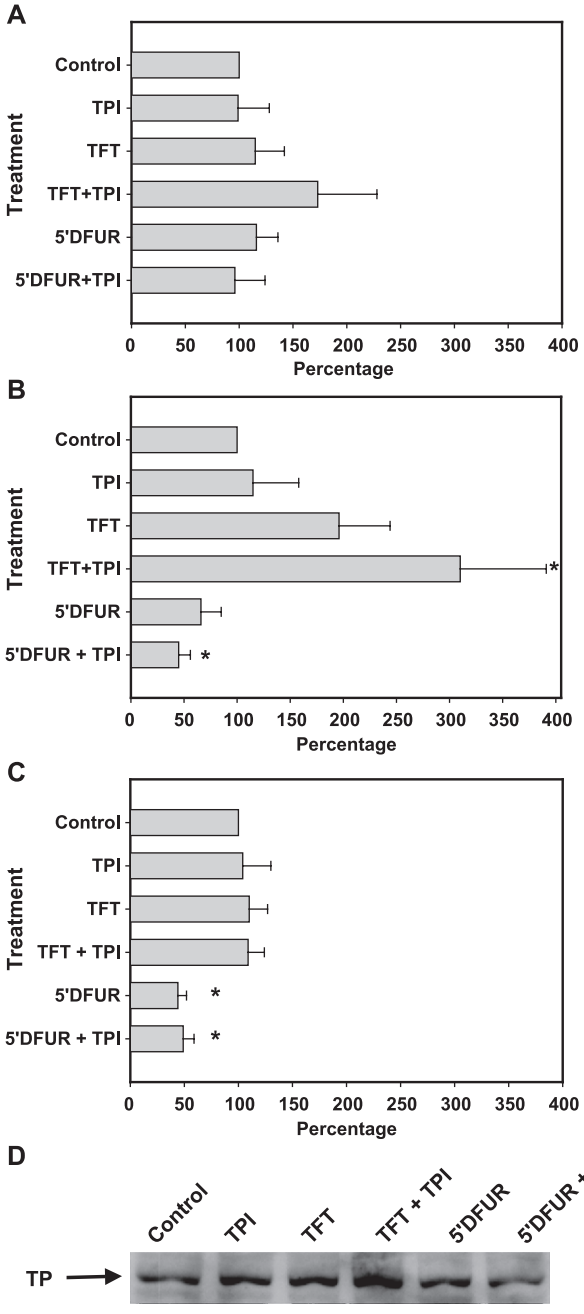

Fig. 2. Thymidine phosphorylase protein expression in three colon cancer cell lines after 72 h exposure to IC_{50}s of the different fluoropyrimidines with or without 10 μM TPI. Protein levels in untreated cells were 0.143, 0.070, 0.093 ng thymidine phosphorylase/μg protein for Lovo, WiDR and HT29, respectively. (A) Lovo, (B) WiDR and (C) HT29. Significant differences ($P<0.05$) compared to controls are marked by an asterisk. Controls of each experiment were set at 100% and used to calculate means ± S.E. (three independent experiments). (D) Representative example of a Western blot of Lovo cells.

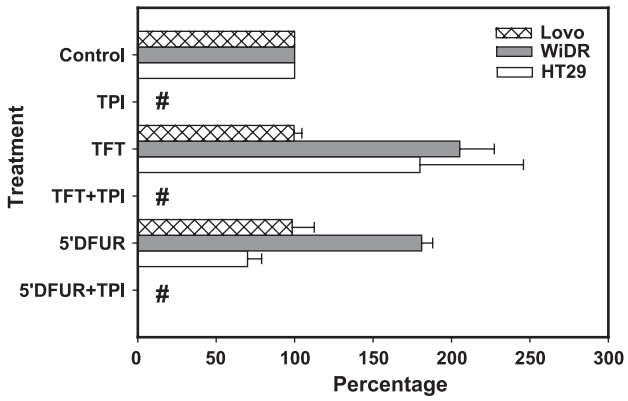

Fig. 3. Thymidine phosphorylase activity in fluoropyrimidine treated Lovo, WiDR and HT29 cells. Cells were treated with IC_{50} concentrations of the drugs, with or without 10 μM TPI. Activities in untreated cells were 5858, 1029 and 1862 pmol/h/mg protein for Lovo, WiDR and HT29 cells, respectively. #: Activity was below 10% of the control or just around the detection limit, precluding calculation of a meaningful mean. Controls of each experiment were set at 100% and used to calculate means ± S.E. (three independent experiments).

Lovo and HT29 cells, both 5′ DFUR and TFT did not affect thymidine phosphorylase activity but in WiDR cells, TFT increased thymidine phosphorylase activity almost 2-fold.

The most striking observation was the marked reduction of thymidine phosphorylase activity in cells exposed to TPI alone or in combination with TFT or 5′ DFUR. In order to determine whether this effect was due to remaining TPI levels inhibiting thymidine phosphorylase activity several approaches were used to remove TPI from the extracts: (1) Cells were extensively washed before snap freezing. This would theoretically reduce TPI levels below concentrations, which would not inhibit thymidine phosphorylase: (2) Removal of TPI by binding to charcoal based on the method of removal of nucleosides from cell extracts (van Triest et al., 1999). However, interference of the separation of substrate and product on TLC was observed: (3) Filtration of cell extracts using protein-binding filters. None of the clean up methods restored the enzyme activity to a measurable level. Therefore, it was concluded that TPI was bound to the enzyme, exerting its inhibitory effects.

4. Discussion

In this study, it was observed that two thymidine phosphorylase substrates affect thymidine phosphorylase mRNA and protein expression. However, these effects did not result in major changes in thymidine phosphorylase activity. Mader et al. (1997) also found no change in thymidine phosphorylase mRNA after a 7-day exposure to 5FU, although expression of ribonucleotide reductase and thymidylate synthase were increased. However, it must be noted that Mader et al. did not determine enzyme activity in that study.

Expression of thymidine phosphorylase is known to be induced by cytokines, e.g. tumor necrosis factor-α, interferon-α, β, γ and interleukin-1α (Eda et al., 1993; Schwartz et al., 1992; Sawada et al., 1998). Combinations of 5FU, capecitabine and 5′ DFUR with these cytokines have been studied extensively (Morita et al., 2001; Braybrooke et al., 2000; Makower and Wadler, 1999). Besides cytokines, other anticancer drugs which are not thymidine phosphorylase substrates, like taxanes, cyclophosphamide, mitomycin C (Sawada et al., 1998; Endo et al., 1999) and radiation (Sawada et al., 1999) have been shown to increase thymidine phosphorylase expression. In several experiments (Sawada et al., 1998, 1999), a concomitant increase of tumor necrosis factor-α was observed, and provided a possible explanation for the increased thymidine phosphorylase expression. It appeared that under natural (unexposed) conditions thymidine phosphorylase mRNA is related to thymidine phosphorylase enzyme activity (De Bruin et al., 2003). However, under stress conditions such as substrate and possibly cytokine exposure this relationship is more complex. Binding of transcription factors like those induced by interferons (Goto et al., 2001) might be affected under stress conditions. The differences in post-translational regulation might be related to changes in the binding of substrates and inhibitors to the protein, thus affecting enzyme activity.

The three cell lines show a different response to the fluoropyrimidines regarding thymidine phosphorylase mRNA and protein expression. This indicated a difference in regulation at the level of transcription and translation. Although drug treatment resulted in these fluctuations, it did not result in significant changes in thymidine phosphorylase activity. This indicates that regulation of thymidine phosphorylase activity in drug treated cells is probably not at the mRNA or protein synthesis level but at the enzyme itself. This is most likely an interaction of the analog substrate on the enzyme itself resulting in some enzyme induction, but dependent on the cell line. This suggested that the clinical efficacy of both 5′ DFUR and TFT is possibly not affected by an effect of the drug on enzyme activity.

The observed results suggest that TPI is bound to thymidine phosphorylase, making it a very effective inhibitor of thymidine phosphorylase. Fukushima et al. (2000) did not investigate the nature of the binding of TPI to the protein and reported a low K_i of 2×10^{-8} M with competitive inhibition. The data did not exclude a potential binding to the enzyme. Our data indicate that TPI is bound to thymidine phosphorylase, but it was beyond the scope of this investigation to determine the kinetic properties of this binding. However, this finding is consistent with other enzymes in pyrimidine metabolism such as dihydroorotic acid dehydrogenase (Peters et al., 1990), or thymidylate synthase (van Triest et al., 1999), which can be inactivated by an inhibitor. The prolonged decrease of thymidine phosphorylase activity by TPI may be of major interest in view of the preclinical observations that thymidine phosphorylase inhibition decreased tumor angiogenesis and suppressed tumor growth (Matsushita et al., 1999) in thymidine phosphorylase transfected human epidermoid KB cells. Therefore administration of TPI to patients as in the TAS-102 combination may serve two purposes, inhibition of TFT degradation, but also inhibition of angiogenesis. Considering the long retention of thymidine phosphorylase inhibition this might be a major effect. No inhibition by 5′ DFUR of thymidine phosphorylase activity was observed in this investigation; while TPI completely inhibited thymidine phosphorylase activity. Both results are desirable for their use in the clinic.

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