Genetic and biochemical modulation of 5-fluorouracil through the overexpression of thymidine kinase: an in-vitro study

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The pro-drug 5-fluorouracil (5-FU) exerts its anti-proliferative action after conversion into cytotoxic metabolites. We previously demonstrated that the anti-cancer action of 5-FU could be enhanced by boosting thymidine phosphorylase (TP) activity in cancer cells, the first step of the DNA pathway, that yields the critical anti-thymidylate synthase (TS) fluorodeoxyuridine monophosphate (FdUMP) metabolite. In the present study, we further studied to what extent 5-FU activity could be optimized by overexpressing cancer cell thymidine kinase (TK), the second step of the DNA pathway, for which controversial data have been published so far. Additionally, screening of biochemical modulators likely to contribute to 5-FU activation was also carried out. TK-overexpressing colorectal cells were obtained after designing vectors harboring viral and human cDNA, and performing stable transfection in the human HT29 cell line. Anti-proliferative assays were subsequently performed so as to evaluate change in cell sensitivity to 5-FU, and metabolism monitoring was carried out to follow drug activation and FdUMP formation after cellular uptake. Finally, TS inhibition was assessed as a pharmacological endpoint. Results showed that overexpression of TK led to a marked desensitization of our model. A negative correlation ($r^2 = 0.87$) was found between the level of TK activity and 5-FU anti-proliferative action – the higher the activity, the lower the sensitivity. Of the various drugs screened as putative modulators, only those involved in TP activity proved to enhance 5-FU efficacy via optimized FdUMP formation. Conversely, genetically increasing TK activity did not modify 5-FU activation pathway nor subsequent TS inhibition in our model. Therefore, our results indicate that TK is not a limiting step in the production of anti-TS FdUMP and that tumor cells overexpressing TK are likely to resist 5-FU-based chemotherapies. *Anti-Cancer Drugs* 17:463–470 © 2006 Lippincott Williams & Wilkins.

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Introduction

Although 50 years old, 5-fluorouracil (5-FU) remains the most widely prescribed anti-cancer agent worldwide, despite limited response rates in patients. Therefore, optimizing its efficacy is still a major concern in experimental oncology. As 5-FU is a complex, multiple-step pro-drug, numerous studies have focused on the role played by the different tumoral enzymes responsible for the conversion of this drug towards active metabolites, as it has been shown that activating enzymes were downregulated in 5-FU-resistant cell lines [1]. The different pathways 5-FU is likely to undertake after cellular uptake have been extensively described and it is well acknowledged now that the drug is preferentially activated via the RNA pathway to form anti-RNA fluorouridine triphosphate (FUTP), with possible indirect formation of anti-thymidylate synthase (TS) fluorodeoxyuridine monophosphate (FdUMP) and, finally, fluorodeoxyuridine triphosphate (FdUTP) [2–5]. Our group is specialized in both genetic and biochemical modulation of fluoropyrimidine drugs (5-FU, capecitabine) via thymidine phosphorylase (TP), the first enzyme of the alternative cascade known as the DNA pathway that nearly directly yields the key FdUMP metabolite (Fig. 1). We have previously demonstrated that genetic [6], biochemical [7,8] or combined [9] strategies aimed at boosting tumoral TP activity led to a sharp increase of cell sensitivity, with enhanced FdUMP formation, TS inhibition and subsequent apoptosis induction, both in-vitro and in xenografted mice models. Additionally, a similar response was also observed with capecitabine in transfected cancer cells displaying high levels of TP activity [10]. Thymidine kinase (TK) is the second step of the DNA pathway, and, in this respect, should

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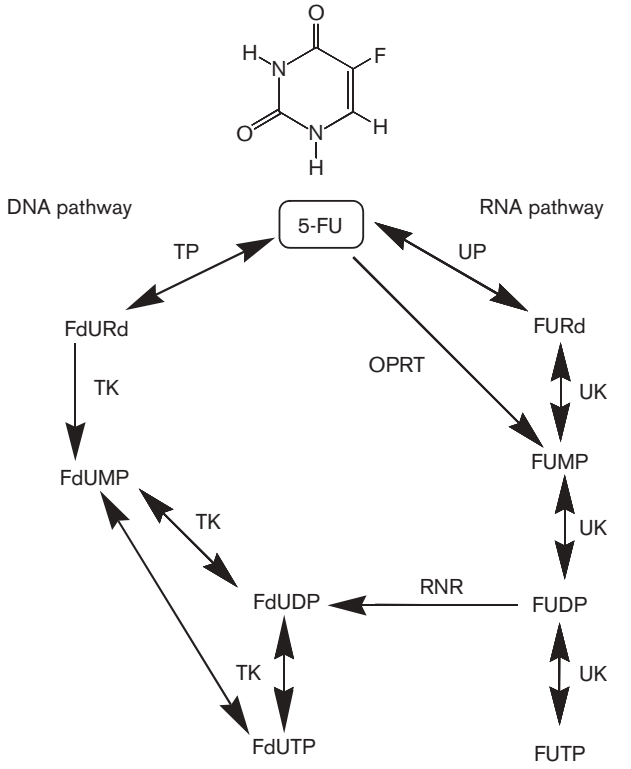
Fig. 1



Activation patterns of pro-drug 5-FU after cellular uptake towards fluoro-nucleosides and fluoro-nucleotides. UP: uridine phosphorylase; UK: uridine kinase; OPRT: orotate phosphoribosyl transferase; RNR: ribonucleotide reductase; TP: thymidine phosphorylase; TK: thymidine kinase. FdUMP, FdUTP and FUTP are the active metabolites interfering with TS, DNA and RNA, respectively.

optimize FdUMP formation within tumor cells. Still, this enzyme is also involved in the pyrimidine salvage pathway of cancer cells treated with anti-TS drugs, aiming at repleting thymidine pools [11]. TK is a critical pyrimidine metabolic pathway enzyme, which catalyzes the phosphorylation of thymidine to deoxythymidine monophosphate using either exogenous or endogenous metabolic thymidine as a substrate [12,13]. This salvage pathway is the only one by which phosphorylated thymidine can be introduced in DNA synthesis in cells with abolished TS and it has been hypothesized that high TK levels observed in human Hep2 cells treated with 5-FU could indeed explain enhanced nucleoside salvage in response to TS inhibition [3] and observations consistent with other studies showing that elevated pools of dUMP were responsible for recovery from TS inhibition after 5-FU treatment [3,14]. TK could therefore rescue cancer cells exposed to anti-TS drugs either by providing extra-substrate dUMP that can compete with cytotoxic FdUMP or by directly producing deoxythymidine monophosphate from exogenous thymidine when *de novo* synthesis through TS is abolished. Any increase in TK levels is normally S-phase dependent [15,16], but a rise in transcription of TK and other genes involved in DNA precursor metabolism has been described in response to 5-FU in various subsequent studies [17,18]. However, downregulation of TK protein has also been associated with resistance to 5-FU [1], thus highlighting the dual role this enzyme plays in the efficacy/inefficacy of fluoropyrimidine drugs as anticancer agents. Precisely because controversial data have been published on the role of TK in the anti-proliferative activity of 5-FU [17–22], we have investigated the impact of increasing levels of this enzyme in the canonical HT29 human colorectal model.

Material and methods

Cell lines

HT29 cells were kindly provided by Professor Yves Barra (FRE-CNRS 2737, Marseille, France). Cells were maintained in DMEM supplemented with 10% FCS, 1% glutamine, 110 IU penicillin/ml, 100 μg streptomycin/ml and 50 μg kanamycin/ml in a humidified CO_2 incubator at 37°C. Stock cultures were passaged weekly when confluence was approached. Cells were screened for mycoplasma contamination.

Chemicals

All chemicals came from Sigma (St Quentin Fallavier, France). Tritiated 5-FU (12 Ci/mmol), and thymidine (40 Ci/mmol) was purchased from NEN-Dupont (Les Ullis, France), and tritiated deoxyuridine monophosphate (dUMP, 16 Ci/mmol) came from Moravek Biochemicals (Brea, California, USA).

Human and viral TK cDNA subcloning

HSV-TK cDNA coding for the isoform 1 of this enzyme was kindly provided by Professor Christopher Barker (National Gene Vector Laboratory, Michigan, USA). The full-length viral TK cDNA was obtained by digesting the parental pNGVL1-TK vector with *Sal*I and *Bgl*II, and the insert was ligated into the same sites of the mammalian expression vector pBK-CMV to produce the pBK-CMV-TK phagemid. This vector was then transformed into cells (*Escherichia coli*, DH5α) and the phagemid cDNA purified using a Qiagen kit (Qiagen, Courtaboeuf, France).

Ready-to-use mammalian expression vector pEGFP-TK2 harboring the human TK cDNA was kindly provided by Dr Magnus Johanson (Karolinska Institute, Stockholm, Sweden).

Transfection of human and viral TK cDNA in HT29 cells

HT29 cells were cultured for 24 h until 75% confluence was reached. Cells were then stably transfected with p-BK-CMV-TK or pEGFP-TK2 using Lipofectamine reagent (Life Technologies, Cergy Pontoise, France),

following the manufacturer's recommendations. After a 48-h incubation, stable HT29/pEGFP-TK2 or HT29/pBK-CMV-TK transfectants were selected with geneticin (G418; Life Technologies) resistance (800 then 400 μg/ml culture medium). Twenty clones were randomly selected, and tested for TK activity, mRNA expression and 5-FU sensitivity.

TK mRNA expression

mRNA expression of TK after gene transfer was checked by RT-PCR as described elsewhere [23]. The 24mer primers 5′-GCGTTCGTGGCCCTCATCCCGCCG-3′ and 5′-GGCCCAGGCAAACACGTTATACAG-3′ were synthesized for amplification of a 369-bp sequence from HSV-1 TK. RT-PCR was carried out using AMV reverse transcriptase, DNA polymerase and the ACCESS RT-PCR kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's recommendations. Amplification products were separated by electrophoresis on an agarose gel (1.5%) and visualized after ethidium bromide staining. A 123-bp DNA ladder (Gibco/BRL, Gaithersburg, Maryland, USA) was used for reference and the plasmid bearing the HSV-1 TK gene (pBK-CMV-TK) was used as standard.

Determination of TK activity

Exponentially growing cells were trypsinized, isolated and lysed through multiple freezing/thawing cycles. Cytosols were obtained after centrifugation at 20 000 *g* for 30 min at low temperature and kept at –80°C until further analysis. Cytosols (50 μg) were then mixed with a pH 7.5 solution containing NaH_2PO_4 (35 mmol/l), ATP (2.5 mmol/l) and $MgCl_2$ (2.5 mmol/l). A 20 μl mix of thymidine (1 mmol/l) and [^{3}H]thymidine (20 μCi) was finally added before a 30-min incubation at 37°C. Reaction was stopped by adding 800 μl of ice-cold methanol. Samples were centrifuged at −20°C, 15 000g, and supernatant was isolated and stored at −80°C until HPLC analysis. Tritiated-thymidine monophosphate was monitored and quantified by reverse-phase radio-HPLC (A500; Packard, Rungis, France). Analytes were chromatographed on a Lichrospher, RP_8 5-μm column (Hewlett-Packard, Les Ullis, France) with a 10 mmol/l, pH 4 KH_2PO_4:methanol mobile phase pumped at a flow rate of 0.75 ml/min. TK activity was expressed as the quantity of thymidine monophosphate formed/μg protein/min and normalized to the activity observed in parental (untransfected) HT29 cells. TK activities were assessed in triplicate.

The clone exhibiting the highest TK activity was subsequently selected and tested for further experiments (e.g. 5-FU metabolism and TS inhibition studies).

Anti-proliferative assays

Parental HT29 cells were plated in 96-wells plate (7000 cells/well) in standard culture medium. After allowing cell attachment to proceed overnight, cells were exposed to increasing concentrations of 5-FU, alone or combined with various agents [TP cofactors, TK cofactors, uridine phosphorylase (UP) cofactors, dihydropyrimidine dehydrogenase (DPD) inhibitors, efflux inhibitor, etc.] tested at different concentrations. After 72 h of continuous exposure, cell viability was quantified using the classic colorimetric MTT assay. IC_{50} was defined as the concentration inhibiting 50% of cell growth. An increase of sensitivity to 5-FU was defined as the ratio between the IC_{50} of 5-FU associated with a modulator and the IC_{50} of 5-FU alone. Cytotoxicity was assessed from three separate experiments.

Determination of 5-FU intracellular metabolites

Monitoring of tritiated 5-FU activation was performed in wild-type HT29 cells alone, combined with 800 μmol/l 2′-deoxyinosine (dIno), and in the clone with the highest TK activity alone or combined with 800 μmol/l dIno. Based upon our previous expertise in exploring 5-FU metabolism in human colorectal cell lines, a 4-h incubation time was chosen to identify the pathways the prodrug was activated through [6–8]. Approximately 3×10^6 exponentially growing cells were plated in T75 flask and maintained overnight in standard culture conditions. The following day, cells were exposed to various combination of 20 μCi of tritiated 5-FU (12.6 Ci/mmol, final concentration 2 μmol/l) alone or associated to modulators for 4 h. Cells were then washed twice with PBS, trypsinized and centrifuged at 800 *g* for 5 min. The cell pellet was resuspended in 500 μl of 60% methanol and vortexed for 30 min. The suspension was stored at –80°C overnight and then centrifuged at 18 000 *g* for 30 min. The supernatant was isolated, dried under nitrogen, reconstituted with 100 μl of mobile phase and directly injected onto the HPLC system. Detection and semi-quantification of 5-FU and its metabolites (FUH2, FURd, FdURd, FUMP, FdUMP, FUDP, FdUDP, FUTP, FdUTP) was achieved by ion-pair reverse-phase chromatography, as described previously, with minor modifications [2–4]. The HPLC consisted of a HP 1090 (Hewlett Packard) system coupled to an A500 radioactive flow detector. Separation was achieved using a Lichrospher-100 RP_{18} 5-μm column eluted by 50 mmol/l K_2HPO_4, pH 6.8 containing 5 mmol/l tetrabutyl ammonium nitrate and a 6–35% gradient of methanol.

TS inhibition study

The effect of 5-FU on TS activity was studied on wild-type HT29 cells alone, combined with dIno, and in the clone with the highest TK activity alone or combined with dIno. Approximately 3×10^6 exponentially growing cells were plated in T75 flasks and maintained overnight in standard culture conditions. The following day, cells were exposed to various combinations of 1 μmol/l 5-FU alone or associated with 800 μmol/l dIno. Inhibition of TS activity was evaluated after 24 h exposure. Cells were

then washed twice with PBS, trypsinized and centrifuged at 800 *g* for 5 min. The cell pellet was stored at −80°C until analysis. TS activity was assayed following a slightly modified Roberts method based on tritiated H_2O release from [5-^{3}H]dUMP in the presence of methylenetetrahydrofolate (MTHF) as described previously [7,8]. Briefly, 25 µl of cell extract was added to a Tris–HCl dithiothreitol solution containing 60 µmol/l of MTHF and 1 µmol/l of tritiated dUMP. After incubation at 37°C for 10, 20 and 30 min (triplicates), reactions were stopped by adding 300 µl of 15% acid charcoal solution. Samples were centrifuged at 14 000 *g* for 15 min at 0°C and a 100-µl aliquot of the supernatant was assayed for radioactivity using a liquid scintillation counter.

Statistical analysis

One-way ANOVA analysis with Tukey multiple comparison tests, Kruskal–Wallis one-way ANOVA on ranks with Dunn's method and the *t*-test were used to evaluate differences between mean values recorded for the different experiments. $P < 0.05$ was regarded as statistically significant. Statistical analyses were carried out using Sigma Stat software (Jandel Scientific, Erkrath, Germany).

Results

TK expression and activity in HT29 cells

Human HT29 cells were transfected with either pEGFP-TK2 or pBK-CMV-TK and stable transfectants selected for geneticin resistance. The growth rate of transfected cells did not differ from that of wild-type HT29 (data not shown). Twenty clones were randomly selected and assayed for TK expression and activity. Semi-quantitative RT-PCR assay showed a wide range of TK mRNA overexpression as compared with the parental cell line (data not shown). Accordingly, stable transfectants showed TK activities increased by 27–737% (HT29/pBK-CMV-TK clones) and 4–73% (HT29/pEGFP-TK2) as compared with wild-type cells (Fig. 2a and b). The HT29 clone displaying the highest level of TK activity was the HT29/pBK-CMV-TK A4 (+ 737% overexpression as compared with parental HT29, $P < 0.05$, one-way ANOVA with multiple comparison testing). This clone was subsequently tested for 5-FU metabolism and TS inhibition studies.

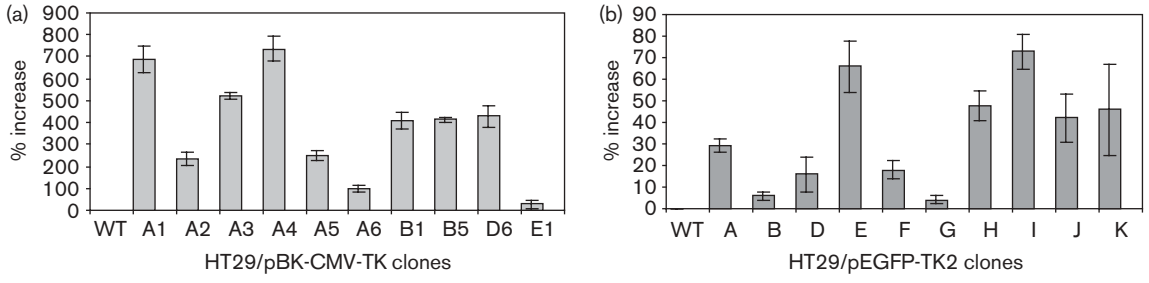
Anti-proliferative assay

Evolution of parental HT29 cell sensitivity to 5-FU after association with various modulators is reported in Table 1. Of the different drugs tested, only DPD inhibitor bromovinyluracil and precursor of TP cofactors dIno led to a significant improvement in 5-FU anti-proliferative activity (sensitivity increased by 5 and 23 times, respectively). Conversely, cofactors implicated in TK activity such as ATP and $MgCl_2$ led to a sharp decrease in 5-FU cytotoxicity, whereas no significant change was observed when associating 5-FU with UP cofactors. The impact of TK gene transfer on HT29 sensitivity to 5-FU is displayed in Fig. 3. A significant correlation was observed between levels of TK activity and corresponding IC_{50}s; the higher the activity, the higher the resistance ($r^2 = 0.87$). Although the association 5-FU + dIno was highly active in wild-type cells as compared with 5-FU alone ($IC_{50} = 0.035 \pm 0.07$ compared with 0.85 ± 0.1 µmol/l, $P < 0.05$, *t*-test), this very combination failed to show a similar efficacy when tested in the HT29/pBK-CMV-TK A4 clone displaying the highest level of TK activity ($IC_{50} = 0.67 \pm 0.3$ µmol/l $P > 0.05$, *t*-test).

Determination of 5-FU metabolites

The various metabolic patterns obtained in wild-type or TK-transfected clone A4 cells exposed to tritiated 5-FU alone or modulated with dIno are displayed in Fig. 4. In parental HT29, 5-FU was mainly activated through UP following the RNA pathway to yield principally FURd and FUMP. A similar metabolic profile with a predominant RNA pathway was observed in the HT29/pBK-CMV-TK A4 clone treated with 5-FU alone; no anti-TS FdUMP metabolite was detectable in these cells. Only dIno, as a TP modulator, led to a complete change in the activation

Fig. 2



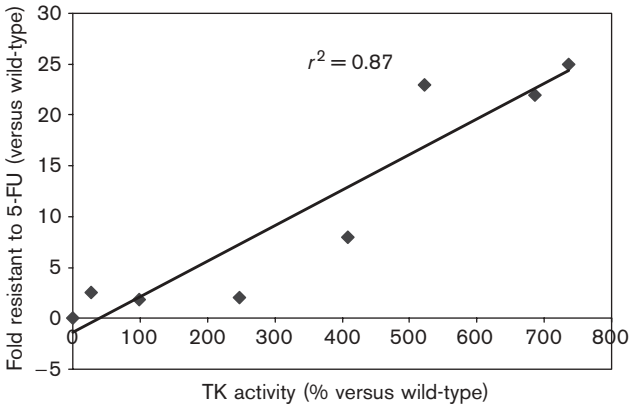
Increase in TK activity in HT29 cells stably transfected with pBK-CMV-TK (a) and pEGFP-TK2 (b). Up to 734% increase was observed as compared with TK activity in wild-type cells. Values are means ± SD of three separate experiments. Clone HT29/pBK-CMV-TK A4 displayed the highest increase in TK activity ($P < 0.05$, one-way ANOVA with multiple comparison testing).

Table 1 Screening of modulators: impact on 5-FU cytotoxicity in wild-type HT29 cells

Speculated action	Modulator	Concentration (μmol/l)	5-FU IC (μmol/l) ± SD	Fold increase in sensitivity[a]
–	–	–	0.85 ± 0.1	1
DPD inhibitor	bromovinyluracil	100	0.4 ± 0.08	2
		500	0.15 ± 0.1	5.3
TK cofactors	ATP	200	2 ± 0.9	0.4
		2000	3 ± 0.8	0.3
		5000	8.5 ± 2.6	0.094
		10 000	10 ± 2.9	0.08
		30 000	62 ± 18.7	0.013
	$MgCl_2$	100	4 ± 1.1	0.2
		1000	38 ± 6.9	0.02
		10 000	>50	0.016
UP cofactors	D-ribose	100	0.85 ± 0.09	0.9
		500	0.8 ± 0.2	1
	D-ribose 1-P	100	1 ± 0.2	0.8
		500	0.9 ± 0.1	0.88
TP cofactors	deoxy-D-ribose	100	0.5 ± 0.08	1.6
		500	0.6 ± 0.1	1.3
	deoxy-D-ribose 1-P	100	0.4 ± 0.1	2
		500	1.3 ± 0.3	0.6
		150	0.9 ± 0.09	0.9
	dIno	300	0.35 ± 0.1	2.3
		600	0.08 ± 0.05	10
		1200	0.035 ± 0.07	22.9
	ethyldeoxyinosine	10	0.6 ± 0.08	1.3
		300	5 ± 1.1	0.16
Others	azidothymidine (AZT)	5	0.95 ± 0.2	0.8
		10	0.75 ± 0.09	1.1
	dideoxyinosine (DDI)	10	1 ± 0.2	0.8
	folinic acid	500	0.9 ± 0.1	0.9
	dipyridamole	10	0.8 ± 0.2	1

[a]Ratio between IC measured with 5-FU alone and combined with a modulator.

Fig. 3



Effect of increased TK activities on sensitivity to 5-FU. A significant correlation ($r^2 = 0.87$) was found between TK levels and resistance. The resistance factor is defined as the ratio between the respective IC_{50}s (the higher the ratio, the higher the resistance).

pattern of 5-FU, with a switch to the alternative DNA pathway and subsequent formation of FdUMP via deoxynucleoside FdURd. No qualitative or semi-quantitative differences were observed between parental and TK-transfected cells exposed to the 5-FU + dIno combination.

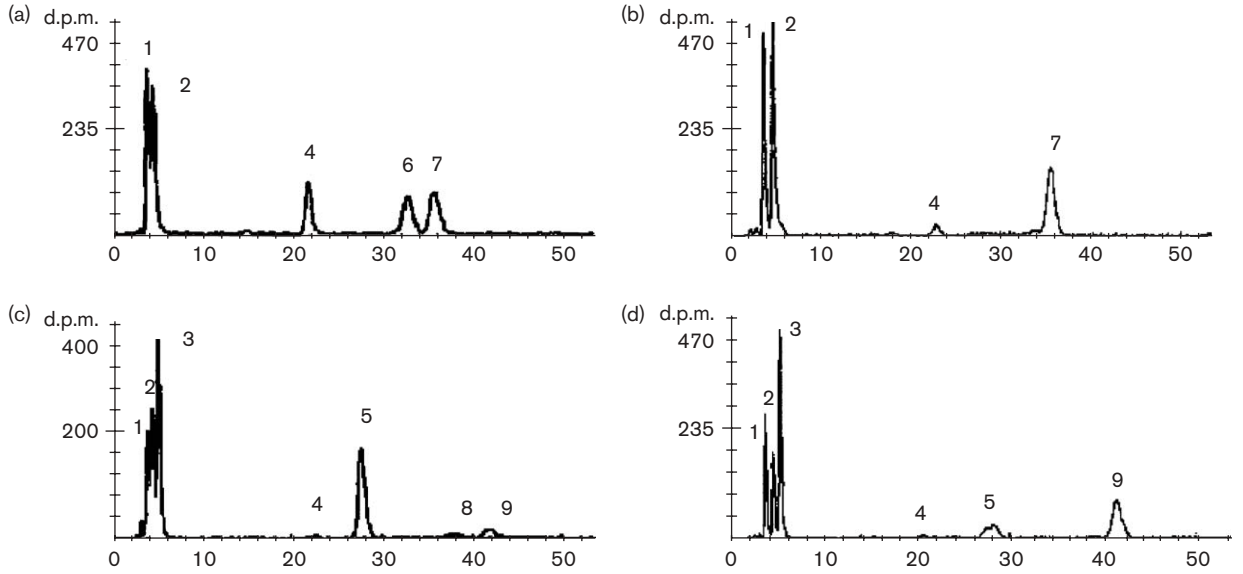
TS inhibition

Levels of TS inhibition in parental or TK-transfected cells exposed to 5-FU alone or combined with dIno are displayed in Fig. 5. Only limited inhibition (35%) of TS activity was observed in wild-type HT29 cells treated with 5-FU alone. Conversely, the combination of 5-FU with dIno led to an optimized, 80% inhibition of TS activity ($P < 0.05$, one-way ANOVA with multiple comparison testing). Using 5-FU alone on the HT29/pBK-CMV-TK A4 clone overexpressing TK failed to significantly enhance any further TS inhibition. Optimized TS inhibition was achieved in these cells overexpressing TK only when combining 5-FU with dIno ($P < 0.05$, one-way ANOVA with multiple comparison testing). No significant difference was observed however, in the extent of TS inhibition between wild-type and TK-transfected cells exposed to the 5-FU + dIno association ($P < 0.05$, *t*-test).

Discussion

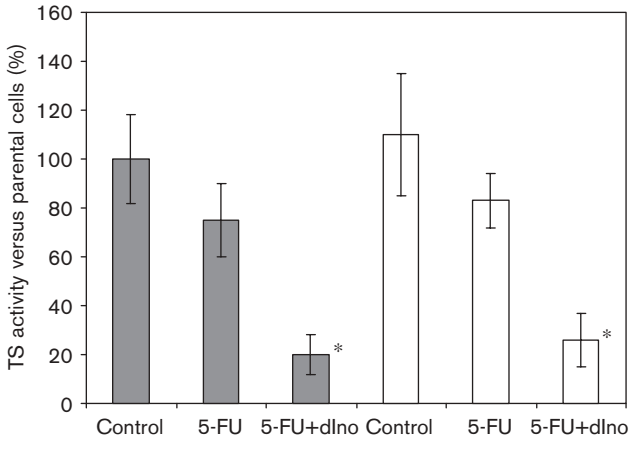
Modulation of 5-FU is probably as old as 5-FU itself. Given the complex activation pattern of this pro-drug, extensive studies have been undertaken for decades to optimize intra-tumoral formation of active metabolites, the key anti-TS FdUMP admittedly being the most important one. Controversial data have been collected so far on the respective roles of UP, orotate phosphoribosyl transferase (OPRT) and TP as the first, critical steps in 5-FU activation patterns. It is well established now that due to the lack of TP cofactor dR1P, the DNA pathway is

Fig. 4



Monitoring of 5-FU activation in HT29 cells. Cells were exposed for 4 h to 2 μmol/l 5-FU (20 μCi) associated or not with 800 μmol/l dIno. In wild-type (a) and HT29/pBK-CMV-TK clone A4 cells transfected with TK (b), 5-FU alone was mainly metabolized through the RNA pathway. Switch to the alternative DNA pathway, with subsequent formation of anti-TS FdUMP, was only achieved when combining 5-FU with dIno in wild-type (c) and TK^+ (d) cells. Chromatograms are from one representative experiment: 1, 5-FU; 2, FURd; 3, FdURd; 4, FUMP; 5, FdUMP; 6, FUDP; 7, FUTP; 8, FdUDP; 9, FdUTP.

Fig. 5



TS inhibition in wild-type (grey bars) and TK^+ (HT29/pBK-CMV-TK clone A4) cells exposed for 24 h to 2 μmol/l 5-FU alone or combined with 800 μmol/l dIno. Results are means ± SD of three separate experiments. $*P < 0.05$, one-way ANOVA with multiple comparison testing. No statistical difference was observed between the levels of TS inhibition in wild-type and TK^+ cells treated with the 5-FU + dIno combination ($P > 0.05$, *t*-test).

not predominant in the activation of 5-FU, which is, therefore, mainly activated through the RNA pathway via UP or OPRT, with possible reduction of FUDP to FdUDP and backwards dephosphorylation to produce indirectly FdUMP [4,5]. Consequently, in human cancer cells, RNA-directed effects of 5-FU seem to be predominant as compared with anti-TS and anti-DNA actions observed in mice models [2]. This hypothesis is supported by the fact that adding exogenous thymidine did not protect human cells from 5-FU cytotoxicity, as it did in mice cancer models [3]. As the RNA pathway thus seemed to be critical, making tumor cells overexpress UP after gene transfer has been proposed to further enhance 5-FU efficacy [5], although it is noteworthy that this very strategy failed to render human breast cancer MCF-7 cells more sensitive to 5-FU in our laboratory [24]. Even more controversial is the role TP, the first step of the alternative DNA pathway, plays in the efficacy of fluoropyrimidine drugs. Many studies have shown a significant improvement in 5-FU efficacy, both *in vitro* and *in vivo*, after either biochemical or genetic modulation aimed at increasing TP activity in tumor cells [6–10, 25–27]. Still, because TP promotes neo-angiogenesis [28], the actual impact of high TP levels on the clinical outcome of patients treated with 5-FU remains to be elucidated; more than contradictory results having been published so far [29]. To a lesser extent, TK plays a dual role as well – this enzyme being implicated in both fluoropyrimidine activation through the DNA route and in salvage pathways in cells with decreased thymidine pools [30]. As a result, actual impact of TK levels on 5-FU response remains controversial. Low TK activities have been associated with a poor response after 5-FU

treatment [1,17–19], whereas other studies have shown that, conversely, high TK levels contributed to rescuing the cells subjected to TS inhibition by repleting the folate pools [20,21,31,32]. More recently, genetic overexpression of TK showed little or no effect on 5-FU sensitivity [22], thus adding even more controversy to this issue. In this respect, we studied to what extent making HT29 cells overexpress TK would change, or not, their sensitivity to 5-FU. HT29 cells were chosen in this study since they are a widely used colorectal model for studying fluoropyrimidine drugs [33,34], for which our laboratory has expertise in the exploration of 5-FU metabolism [7,8]. TK overexpression was first obtained after stable transfection of either viral or human isoforms of this gene. Both human and viral TK were tested so as to overcome putative differences in substrate specificity. It is noteworthy, however, that resulting TK levels were markedly higher after transfecting viral cDNA than in cells stably transfected with pEGFP-TK2, probably due to the extremely potent CMV promoter in the p-BK-CMV-TK vector harboring the HSV-TK gene. Up to 730% increase in TK activity was achieved with the p-BK-CMV-TK phagemid and subsequent evaluation of 5-FU cytotoxicity showed a significant decrease in cell sensitivity when high levels of TK were reached – a result fully in line with the observations by Look *et al.*, Findenig *et al.* and Chung *et al.* [20,21,32], but contradictory to those made by Inaba *et al.* and Zhang *et al.* [17–19]. The negative impact of high TK levels in the cytotoxicity of 5-FU was subsequently confirmed by boosting cellular TK activities with cofactors, which similarly led to a striking desensitization of our HT29 model. Radio-HPLC monitoring of 5-FU metabolization confirmed that the RNA route was predominant in our parental model, the drug being activated mainly to FURd and the subsequent fluororibonucleotides – an observation consistent with previous studies performed in our laboratory [6–9] and fully in line with data from the literature [4]. Surprisingly, increasing TK activity as in the HT29/p-BK-CMV-TK A4 clone did not permit us to modify drug metabolism, the RNA route being still the main one 5-FU was activated through after cellular uptake. Only the increase of TP activity with dIno triggered the alternative DNA pathway in parental HT29 cells, with formation of anti-TS FdUMP metabolite and subsequent improvement in TS inhibition. Although one would have expected that combining dIno treatment in clones with high TK levels would have yielded even more FdUMP metabolite, no such difference was observed between wild-type and TK-transfected cells treated with the 5-FU + dIno combination. This strongly suggests that TK is not the limiting step in the production of anti-TS FdUMP, at least in our in-vitro model. Consequently, and in full agreement with the previous observation, no improvement in TS inhibition was seen in cells transfected with TK, as compared with the extent of TS inhibition measured in parental HT29 cells with basal TK levels, and no significant difference was observed either in TS inhibition between wild-type and TK-transfected cells treated with the 5-FU + dIno combination. Still, because a marked decrease in sensitivity was observed in cells with high TK activities despite equal levels of TS inhibition, one can speculate that the resistance observed is not based upon a loss of pharmacological efficacy towards TS linked to extra-dUMP formation likely to compete with FdUMP [3,14], but probably to an increased rescue through the TK-driven pyrimidine salvage pathway – a hypothesis strongly supported by data from the literature [20,32]. As previously demonstrated by our group [7–9,35], only TP cofactors and, to a lesser extent, DPD inhibitors led to improved 5-FU efficacy with a striking change in the drug activation pathway and subsequent enhanced TS inhibition. Interestingly, modulation of the RNA pathway with the appropriate cofactors did not result in increasing efficacy in 5-FU anti-proliferative action, thus confirming that this pattern is already predominant in tumor cells exposed to fluoropyrimidines – an observation fully consistent with our previous experiments performed in UP-transfected cancer cells [24] and data from the literature [36]. Other putative modulators we tested such as the efflux inhibitor dipyridamole [37] or azidothymidine likely to interfere with nucleoside metabolism [21,38,39] did not change sensitivity to 5-FU either, at least under our experimental conditions. Taken together, our present study suggests that TK is not a limiting step in the activation of 5-FU towards the critical FdUMP metabolite and that, conversely, high TK levels in tumor cells are a factor of diminished sensitivity to 5-FU-based treatment, probably by triggering the pyrimidine salvage pathway in response to TS inhibition.

Conclusion

In this study, increased TK activity was obtained in colorectal HT29 cells both after gene transfer and after providing tumors with appropriate cofactors. Resulting sensitivity to widely used 5-FU was markedly decreased, thus suggesting that high TK levels are associated with drug resistance. Further studies will be undertaken to elucidate whether repleting pyrimidine pools after TS inhibition is at the origin of this loss of sensitivity. Enhancement of 5-FU activation towards the cytotoxic FdUMP metabolite and, subsequently, of TS inhibition with markedly increased sensitivity was only achieved through modulation of TP activity, thus confirming that this enzyme is the critical, limiting step in the optimization of 5-FU activation and, subsequently, its efficacy as an anti-cancer agent.

References

1 Wang W, Cassidy J, O'Brien V, Ryan KM, Collie-Duguid E. Mechanistic and predictive profiling of 5-Fluorouracil resistance in human cancer cells. *Cancer Res* 2004; **64**:8167–8176.

2 Laskin JD, Evans RM, Slocum HK, Burke D, Hakala MT. Basis for natural variation in sensitivity to 5-fluorouracil in mouse and human cells in culture. *Cancer Res* 1979; **39**:383–390.
3 Berger SH, Hakala MT. Relationship of dUMP and free FdUMP pools to inhibition of thymidylate synthase by 5-fluorouracil. *Mol Pharmacol* 1984; **25**:303–309.
4 Peters GJ, van Groeningen CJ, Laurensse EJ, Pinedo HM. A comparison of 5-fluorouracil metabolism in human colorectal cancer and colon mucosa. *Cancer* 1991; **8**:1903–1909.
5 Cao D, Pizzorno G. Uridine phosphorylase: an important enzyme in pyrimidine metabolism and fluoropyrimidine activation. *Drugs Today (Barc)* 2004; **40**:431–443.
6 Evrard A, Cuq P, Ciccolini J, Vian L, Cano JP. Increased cytotoxicity and bystander effect of 5-fluorouracil and 5-deoxy-5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase. *Br J Cancer* 1999; **80**:1726–1733.
7 Ciccolini J, Peillard L, Aubert C, Formento P, Milano G, Catalin J. Monitoring of the intracellular activation of 5-fluorouracil to deoxyribonucleotides in HT29 human colon cell line: application to modulation of metabolism and cytotoxicity study. *Fundam Clin Pharmacol* 2000; **14**:147–154.
8 Ciccolini J, Peillard L, Evrard A, Cuq P, Aubert C, Pelegrin A, *et al.* Enhanced antitumor activity of 5-fluorouracil in combination with 2′-deoxyinosine in human colorectal cell lines and human colon tumor xenografts. *Clin Cancer Res* 2000; **6**:1529–1535.
9 Ciccolini J, Cuq P, Evrard A, Giacometti S, Pelegrin A, Aubert C, *et al.* Combination of thymidine phosphorylase gene transfer and deoxyinosine treatment greatly enhances 5-fluorouracil antitumor activity *in vitro* and *in vivo*. *Mol Cancer Ther* 2001; **1**:133–139.
10 Ciccolini J, Fina F, Bezulier K, Giacometti S, Roussel M, Evrard A, *et al.* Transmission of apoptosis in human colorectal tumor cells exposed to capecitabine, Xeloda, is mediated via Fas. *Mol Cancer Ther* 2002; **1**:923–927.
11 Broet P, Romain S, Daver A, Ricolleau G, Quillien V, Rallet A, *et al.* Thymidine kinase as a proliferative marker: clinical relevance in 1,692 primary breast cancer patients. *J Clin Oncol* 2001; **19**:2778–2787.
12 Taylor AT, Stafford MA, Jones OW. Properties of thymidine kinase partially purified from human fetal and adult tissue. *J Biol Chem* 1972; **247**: 1930–1935.
13 Fujiwaki R, Hata K, Nakayama K, Moriyama M, Iwanari O, Katabuchi H, *et al.* Thymidine kinase in epithelial ovarian cancer: relationship with the other pyrimidine pathway enzymes. *Int J Cancer* 2002; **99**:328–335.
14 Myers CE, Young RC, Johns DG, Chabner BA. Assay of 5′-fluorodeoxyuridine 5′-monophosphate deoxyuridine 5-monophosphate pools following 5-fluorouracil. *Cancer Res* 1974; **34**:2682–2688.
15 Kim YK, Lee AS. Identification of a protein-binding site in the promoter of the human thymidine kinase gene required for the G_1–S-regulated transcription. *J Biol Chem* 1992; **267**:2723–2727.
16 Mikulits W, Hengstschlager M, Sauer T, Wintersberger E, Mullner EW. Overexpression of thymidine kinase mRNA eliminates cell cycle regulation of thymidine kinase enzyme activity. *J Biol Chem* 1996; **271**:853–860.
17 Inaba M, Naoe Y, Mitsuhashi J. Mechanisms for 5-fluorouracil resistance in human colon cancer DLD-1 cells. *Biol Pharm Bull* 1998; **21**:569–573.
18 Zhang ZG, Harstrick A, Rustum YM. Mechanisms of resistance to fluoropyrimidines. *Semin Oncol* 1992; **19** (**Suppl**):S4–S9.
19 Zhang ZG, Malmberg M, Yin MB, Slocum HK, Rustum YM. Isolation and characterization of a human ileocecal carcinoma cell line (HCT-8) subclone resistant to fluorodeoxyuridine. *Biochem Pharmacol* 1993; **45**:1157–1164.
20 Look KY, Moore DH, Sutton GP, Prajda N, Abonyi M, Weber G. Increased thymidine kinase and thymidylate synthase activities in human epithelial ovarian carcinoma. *Anticancer Res* 1997; **17**:2353–2356.
21 Findenig G, Mader RM, Fritzer-Szekeres M, Steger GG, Jaeger W, Szekeres T. Modulation of 5-fluorouracil resistance in human colon tumor cell lines by azidothymidine. *Oncol Res* 1996; **8**:189–196.
22 Van Dillen IJ, Mulder NH, Meijer C, Dam WA, Kamstra E, De Vries L, *et al.* Antagonism of HSV-tk transfection and ganciclovir treatment on chemotherapeutic drug sensitivity. *J Chemother* 2005; **17**:289–296.
23 Milanesi G, Barbanti-Brodano G, Negrini M, Lee D, Corallini A, Caputo A, *et al.* BK virus-plasmid expression vector that persists episomally in human cells and shuttles into Escherichia coli. *Mol Cell Biol* 1984; **4**:1551–1560.
24 Cuq P, Rouquet C, Evrard A, Ciccolini J, Vian L, Cano JP. Fluoropyrimidine sensitivity of human MCF-7 breast cancer cells stably transfected with human uridine phosphorylase. *Br J Cancer* 2001; **84**:1677–1680.
25 Schwartz EL, Baptiste N, Megati S, Wadler S, Otter BA. 5-Ethoxy-2′-deoxyuridine, a novel substrate for thymidine phosphorylase, potentiates the antitumor activity of 5-fluorouracil when used in combination with interferon, an inducer of thymidine phosphorylase expression. *Cancer Res* 1995; **55**:3543–3550.
26 Schwartz EL, Baptiste N, Wadler S, Makower D. Thymidine phosphorylase mediates the sensitivity of human colon carcinoma cells to 5-fluorouracil. *J Biol Chem* 1995; **270**:19073–19077.
27 Braybrooke JP, Propper DJ, O'Byrne KJ, Koukourakis MI, Patterson AV, Houlbrook S, *et al.* Induction of thymidine phosphorylase as a pharmacodynamic end-point in patients with advanced carcinoma treated with 5-fluorouracil, folinic acid and interferon alpha. *Br J Cancer* 2000; **83**:219–224.
28 Hotchkiss KA, Ashton AW, Klein RS, Lenzi ML, Zhu GH, Schwartz EL. Mechanisms by which tumor cells and monocytes expressing the angiogenic factor thymidine phosphorylase mediate human endothelial cell migration. *Cancer Res* 2003; **63**:527–533.
29 Ciccolini J, Evrard A, Cuq P. Thymidine phosphorylase and fluoropyrimidines efficacy: a Jekyll and Hyde story. *Curr Med Chem Anticancer Agents* 2004; **4**:71–81.
30 Romain S, Martin PM, Klijn JG, van Putten WL, Look MP, Guirou O, *et al.* DNA-synthesis enzyme activity: a biological tool useful for predicting anti-metabolic drug sensitivity in breast cancer? *Int J Cancer* 1997; **74**:156–161.
31 van der Wilt CL, Backus HH, Smid K, Comijn L, Veerman G, Wouters D, *et al.* Modulation of both endogenous folates and thymidine enhance the therapeutic efficacy of thymidylate synthase inhibitors. *Cancer Res* 2001; **61**:3675–3681.
32 Chung YM, Park S, Park JK, Kim Y, Kang Y, Yoo YD. Establishment and characterization of 5-fluorouracil-resistant gastric cancer cells. *Cancer Lett* 2000; **159**:95–101.
33 Gmeiner WH, Trump E, Wei C. Enhanced DNA-directed effects of FdUMP[10] compared to 5FU. *Nucleosides Nucleotides Nucleic Acids* 2004; **23**:401–410.
34 Leteurtre E, Gouyer V, Rousseau K, Moreau O, Barbat A, Swallow D, *et al.* Differential mucin expression in colon carcinoma HT-29 clones with variable resistance to 5-fluorouracil and methotrexate. *Biol Cell* 2004; **96**:145–251.
35 Fanciullino R, Giacometti S, Aubert C, Fina F, Martin PM, Piccerelle P, *et al.* Development of stealth liposome formulation of 2′-deoxyinosine as 5-fluorouracil modulator: *in vitro* and *in vivo* study. *Pharm Res* 2005; **22**:2051–2057.
36 Ackland SP, Peters GJ. Thymidine phosphorylase: its role in sensitivity and resistance to anticancer drugs. *Drug Resist Updat* 1999; **2**:205–214.
37 Tsavaris N, Kosmas C, Polyzos A, Genatas K, Vadiaka M, Paliaros P, *et al.* Leucovorin + 5-fluorouracil plus dipyridamole in leucovorin + 5-fluorouracil-pretreated patients with advanced colorectal cancer: a pilot study of three different dipyridamole regimens. *Tumori* 2001; **87**:303–307.
38 Yasuda C, Kato M, Kuroda D, Ohyanagi H. Experimental studies on potentiation of the antitumor activity of 5-fluorouracil with 3′-azido-3′-deoxythymidine for the gastric cancer cell line MKN28 *in vivo*. *Jpn J Cancer Res* 1997; **88**:97–102.
39 Li YX, Coucke PA, Paschoud N, Mirimanoff RO. Cytotoxic interactions of 5-fluorouracil and nucleoside analogues *in vitro*. *Anticancer Res* 1997; **17**:21–27.